DESCRIBING THE IONOREGULATORY CHANGES THAT UNDERLIE COLD ACCLIMATION IN *DROSOPHILA MELANOGASTER*

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Abstract

At low temperatures *Drosophila* lose the ability to regulate ion and water balance across the gut leading to a lethal accumulation of K^+ in the haemolymph (hyperkalemia). Cold-acclimation can mitigate these ion imbalances, but the physiological mechanisms that facilitate this process are still not understood. Upon adult emergence, *D. melanogaster* females were subjected to seven days at 25°C (warm-acclimation) or 10°C (cold-acclimation). Cold-acclimation reduced the critical thermal minimum (CT_{min}), sped up recovery from chill coma, improved survival following cold stress, and mitigated cold-induced hyperkalemia. In parallel, cold-acclimated flies experienced increased Malpighian tubule fluid and K^+ secretion and reduced rectal K^+ reabsorption. These changes were independent of Na^+/K^+- or V-type H^+-ATPases. These results suggest that modification of Malpighian tubule and gut mitigate cold-induced hyperkalemia. Additionally, I present preliminary findings on the effects of acclimation, sex, and blood-feeding on the cold tolerance of the arboviral disease vector mosquito, *A. aegypti*.

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Statement of Contributions

Dr. Andrew Donini, Dr. Heath MacMillan, and Gil Yerushalmi conceived of the projects. Gil Yerushalmi conducted all experiments with the exception of the Ramsay assays which were conducted by Lidiya Misyura (excluding ISME). Lidiya Misyura also assisted with critical thermal minimum and chill coma recovery time measurements in both *D. melanogaster* and *A. aegypti* and the set up of fly gut preparations for SIET. Sima Jonusaite, Fargol Nowghani, and Andrea Durant provided training and assistance for enzyme activity assays. Gil Yerushalmi conducted all statistical analysis with the assistance of Dr. Heath MacMillan. Gil Yerushalmi prepared this thesis with input from Dr. Andrew Donini, Dr. Heath MacMillan, and Dr. Jean-Paul Paluzzi.

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1. **Yerushalmi, G. Y.**, Misyura, L., MacMillan, H. A. and Donini, A. (2018). Functional plasticity of the gut and the Malpighian tubules underlies cold acclimation and mitigates cold-induced hyperkalemia in *Drosophila melanogaster*. *J. Exp. Biol.* **221**, jeb.174904.
2. MacMillan, H. A., **Yerushalmi, G. Y.**, Jonusaite, S., Kelly, S. P. and Donini, A. (2017). Thermal acclimation mitigates cold-induced paracellular leak from the *Drosophila* gut. *Sci. Rep.* **7**, 8807.

1. Introduction

1.1 Insect cold tolerance

Insects have a tremendous impact on modern societies. Many are beneficial as they perform billions of dollars worth in ecological services (Losey and Vaughn, 2006) and act as biocontrol agents (Pimentel et al., 2005). Conversely, many others act as vectors for a number of devastating diseases (Hemingway and Ranson, 2000) or cause billions of dollars in economic loss as agricultural pests (Pimentel et al., 2001). As arguably the most diverse animal group on earth, insects are found in nearly every terrestrial ecosystem ranging from dry deserts to the freezing poles (Chown and Terblanche, 2006). The geographical distribution (e.g. latitude and elevation) of insects is closely linked to species-specific limits to thermal tolerance and performance (Addo-Bediako et al., 2000; Kimura, 2004). Particularly in cold environments, the minimum tolerable temperature is closely related to minimum environmental temperatures (Hazell et al., 2010; Kellermann et al., 2012; Kimura, 2004). In addition to increases in mean global temperatures, recent climate change models are predicting a greater frequency of temperature extremes, both low and high, leading to more frequent crossing of thermal limits (Easterling, 2000; Williams et al., 2015). Accordingly, a considerable effort has been directed at understanding the physiological mechanisms that underlie the limits to insect performance and survival at low temperatures, as well as the mechanisms of cold tolerance adaptation and plasticity (Overgaard and MacMillan, 2017).

Traditionally, insect cold tolerance classification has largely focused on the ability (or lack thereof) of insects to survive or avoid freezing (Sinclair et al., 2003; Zachariassen, 1985). Freeze tolerant insects have unique adaptations that allow for their survival following the freezing of their extracellular bodily fluids (Ramløv, 2000; Zachariassen, 1985). For example,

the accumulation of ice-nucleating proteins (proteins that promote ice formation) in the haemolymph initiates freezing in the extracellular space and thus delays the onset of intracellular freezing, where ice formation is most lethal (Clark and Worland, 2008; Zachariassen, 1985). Freeze avoiding insects, by contrast, have adaptations that serve to suppress the supercooling point of their bodily fluids (the temperature at which spontaneous freezing occurs) and thereby avoid freezing altogether (Zachariassen, 1985). Such adaptations include the accumulation of polyols, carbohydrates, and antifreeze proteins, all of which reduce the supercooling point of the haemolymph (Zachariassen, 1985). Lastly, chill-susceptible insects are those that succumb to the effects of chilling at temperatures well above the freezing point of their body fluids (Bale, 1996; Overgaard and MacMillan, 2017). Although the majority of insect species are considered chill susceptible the physiology underlying chill susceptibility has been largely understudied until recently and is the focus of this thesis.

Three metrics are principally used to assess the cold tolerance of chill susceptible insects (Figure 1.1). First, the critical thermal minimum (CT_{min}) is the temperature at which insects lose coordination and subsequently enter a state of complete neuromuscular paralysis known as a chill coma (Block, 1990; Mellanby, 1939). In the case of mild and/or short cold exposures, chill susceptible insects may recover from a chill coma and regain full neuromuscular function (Macdonald et al., 2004; MacMillan and Sinclair, 2011a). The rate of this recovery, normally measured as time for the insect to stand following removal from cold exposure, is highly variable among and within insect species, and is thus used as another metric of chill-tolerance, termed chill coma recovery time (CCRT) (Gibert and Huey, 2001; Jean David et al., 1998).

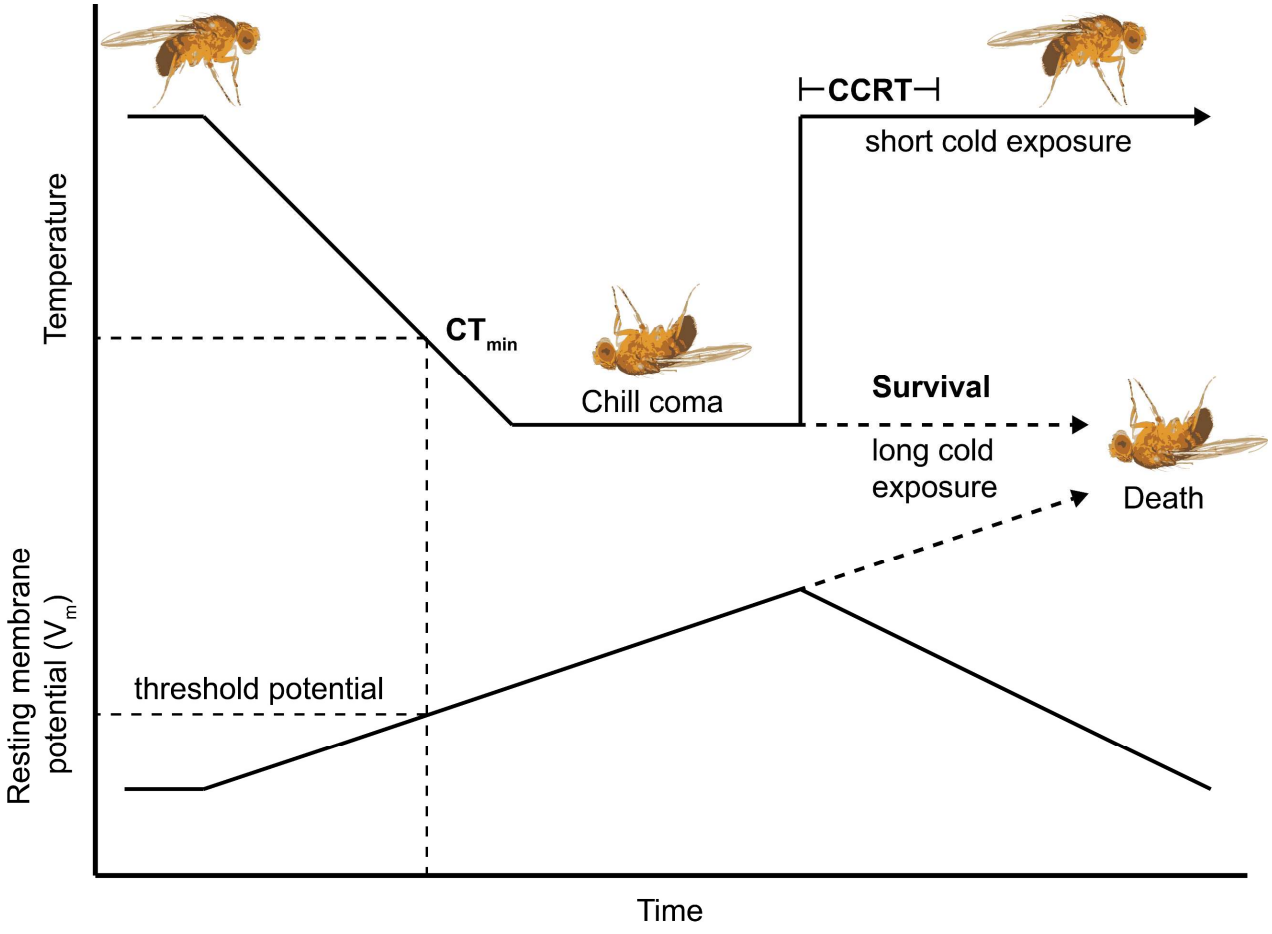

Figure 1.1. Low temperatures depolarize resting muscle membrane potential in chill-susceptible insects. Depolarization of resting membrane potential in the cold occurs in two phases. First, membrane potential is lost with the immediate onset of a cold exposure, and likely results from the decreased contribution of ion-motive pumps via the electrogenic effect. Secondly, after a chronic exposure to low temperatures, membrane depolarization continues despite no further change in temperature. This results from the gradual loss of K^+ gradients (due to increased haemolymph $[K^+]$) which normally sustain a low resting membrane potential via the diffusion potential of K^+ leak. The dotted lines illustrate the impact of a prolonged cold exposure: continued depolarization of resting membrane potential that ultimately lead to cold-induced injuries and death. Modified from MacMillan et al. (2012).

Alternatively, following an intense cold exposure (longer duration and/or lower temperature), chill-susceptible insects acquire irreversible injuries and eventually die (Koštál et al., 2004; Koštál et al., 2006; Rojas and Leopold, 1996). Since there is great variability with the rates of survival following cold exposure, both among and within species, chilling survival is also utilized as a measure of insect cold tolerance (Andersen et al., 2015a; MacMillan et al., 2015a). While there is often a high degree of covariance in these three metrics (CT_{min}, CCRT, and chilling survival), such that all tend to improve in more chill tolerant insects (whether resulting from adaptation or acclimation), the underlying physiological mechanisms that determine each of the metrics is different (see section 1.2), and uniquely informative, and thus all are used in the investigation of chill-susceptibility in insects (MacMillan and Sinclair, 2011a; MacMillan et al., 2015a).

The traditional classification of insects based on their tolerance to freezing is sometimes problematic in that it hides the large variation in cold tolerance that exists within chill susceptible species and results from evolutionary adaptation, seasonal variation, long-term acclimation, or even acute exposure to low temperatures (rapid cold-hardening) (Chown and Terblanche, 2006; Hoffmann et al., 2003; Kelty and Lee Jr, 2001; Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2015a). In this study, I examine the impact of long-term cold acclimation (i.e. days to weeks) on the physiology underlying cold tolerance. Cold acclimation influences all of the aforementioned metrics that are used to assess cold tolerance. For example, in a study conducted on several *Drosophila* species, CT_{min} was shown to vary widely within each studied species in response to developmental temperature, where generally, for every 4°C reduction in developmental temperature, CT_{min} decreased by ~1°C (Gibert and Huey, 2001). Similarly, the effect of cold acclimation on reducing CCRT is pronounced. For example, following exposure to

12.5°C for three weeks, the CCRT of the fall field cricket was reduced from 50 min to less than 20 min (Coello Alvarado et al., 2015). The relationship between developmental temperature and CCRT was also noted in natural populations of *Drosophila ananassae* that inhabit a wide range of thermal conditions, demonstrating the importance of thermal plasticity in the extension of a species' range (Sisodia and Singh, 2010). Lastly, cold-acclimation has been shown to greatly improve chilling survival in a number of insects including firebugs, cockroaches, fruit flies, and migratory locusts, among others (Andersen et al., 2017a; Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2015a). For example, a 6-day pre-exposure to 15°C altered the Lt_{50} (lethal time of exposure to -2°C that results in 50% survival) from less than 5 h to over 20 h, drastically improving the survival rates of the cold-acclimated flies when exposed to the cold (MacMillan et al., 2015a). While improvements in cold tolerance following cold-acclimation have been well described, the physiological mechanisms underlying these adjustments remain poorly understood.

1.2 Physiology of chill coma and injury

1.2.1 Cold-induced cellular depolarizations drive chill coma onset

The onset of a chill coma is characterized by the cessation of movement and the loss of neuromuscular activity at low temperatures (Goller et al., 1990; Hazell and Bale, 2011; Hosler et al., 2000). To resolve the mechanisms underlying this failure at the tissue-level, analyses of both muscle and nerve performance at low temperatures were conducted (Overgaard and MacMillan, 2017).

As temperature declines, the resting membrane potential (V_m) of insect muscle cells are progressively depolarized and action potentials decrease in amplitude and increase in duration

(Goller et al., 1990; Hosler et al., 2000). Species that can preserve muscle V_m in the cold also enter chill coma at lower temperatures, which suggests that the loss of muscle excitability in the cold may be responsible for chill-coma onset (Hosler et al., 2000; MacMillan et al., 2015a). The negative membrane potential in muscle cells is set primarily by Na^+/K^+-ATPase (Goller et al., 1990; Hosler et al., 2000; Sinclair et al., 2004). Na^+/K^+-ATPase actively transports 3 Na^+ ions out of the cell in exchange for 2 K^+ into the cell, and is thus responsible for the maintenance of negative membrane potential in two main ways. First, the net movement of positive charge out of the cell creates a negative potential through the electrogenic effect (V_e) – the unequal pumping of charged molecules across a membrane (Apell, 1989; Overgaard and MacMillan, 2017) – as calculated by the following equation:

$$V_e = i_p \times R_m$$

Where i_p represents the ion transport rate, and R_m represents membrane resistance. Second, the established electrochemical gradients also contribute to cellular polarization through the unequal distribution and passive diffusion of ions across the plasma membrane, known as diffusion potential (V_d) (Overgaard and MacMillan, 2017). V_d is described by the Goldman-Hodgkin-Katz equation:

$$V_d = \frac{RT}{F}\ln\left(\frac{P_{Na^+}[Na^+]_i + P_{K^+}[K^+]_i}{P_{Na^+}[Na^+]_o + P_{K^+}[K^+]_o} \ldots\right)$$

where R is the ideal gas constant, T is the temperature in Kelvin, F is Farraday's constant, P_{ion} is the permeability of the ion, and $[ion]_o$ and $[ion]_i$ are the extracellular and intracellular concentrations of the ion, respectively. Similarly to other animals, insect V_d is largely determined by the gradient of K^+ due to its relatively high permeability across biological membranes (Dawson et al., 1989; Hoyle, 1953). The following equation demonstrates the additive effects of

diffusion potential (V_d) and electrogenic potential (V_e) in the determination of resting membrane potential (V_m):

$$V_m = V_d + V_e$$

Similarly to other enzymes, the activity of ion-motive ATPases decreases at low temperatures (Ellory and Willis, 1982; MacMillan et al., 2015b). This effect slows rates of ion transport across the cell membrane and thus decreases the contribution of V_e to V_m in the cold. Since chill-susceptible insects can enter chill-coma prior to any substantial disturbances of ion homeostasis (see section 1.2.2), coma onset is likely unrelated to changes in V_d.

In addition to the failure of Na^+/K^+-ATPase, recent evidence also suggests that failure of L-type Ca^{2+} channels at low temperatures may also be involved in reducing muscle excitability in migratory locusts (Findsen et al., 2016). In insects, muscle action potentials activate voltage-gated Ca^{2+} channels (as opposed to voltage-gated Na^+ channels in vertebrate skeletal muscle), and it is the influx of extracellular Ca^{2+} which triggers further release of Ca^{2+} from the sarcoplasmic reticulum to initiate muscle contraction (Collet and Belzunces, 2007; Singh and Wu, 1999; Washio, 1972). At low temperatures, L-type calcium channels require greater cellular depolarizations to become activated and produce smaller calcium currents, and are thus less likely to initiate the rapid rise in intracellular calcium required for contraction (Findsen et al., 2016). Failure of these channels is therefore also likely to be involved in the reduced excitability of insect muscle cells at low temperatures (Findsen et al., 2014; Findsen et al., 2016). Together, both cold-induced depolarizations and failure of excitation contraction coupling inhibit muscle function in the cold and are likely both involved in the initiation of a chill-coma.

In addition to the failure of muscle function, there is also good evidence supporting a potential role for neuronal failure at low temperatures. Studies of the migratory locust and fruit flies demonstrate that neuronal silence coincides with coma onset, and results from a rapid surge in extracellular [K^+] in the brain (Armstrong et al., 2012; Rodgers et al., 2010). The small volume of extracellular fluid around the nerves is thought to allow for the rapid accumulation of K^+ due to the reduced cellular K^+ uptake at low temperatures, and may thus be responsible for elevating nerve V_m which also relies heavily on the K^+ equilibrium potential (MacMillan and Sinclair, 2011a; Rodgers et al., 2010). Another study, however, conducted on locusts demonstrated neuronal activity at temperatures at or below CT_{min}, suggesting that in these insects neuronal failure is not responsible for chill coma onset (Findsen et al., 2014). While the neuromuscular synapse is another potential target where failure may occur, a recent study demonstrated normal post synaptic function following exogeneous stimulations in locust muscles at low temperatures (Findsen et al., 2016). Further studies are required, however, to confirm normal post-synaptic potentials *in vivo*.

While there continues to be some conflicting evidence regarding the exact mechanism underlying the onset of chill coma, it is clear that both muscle and nervous tissues are ultimately inhibited at low temperatures, and likely both play some role in chill-coma onset (MacMillan and Sinclair, 2011a).

1.2.2 Chronic chilling causes a progressive loss of ion homeostasis

In contrast to the immediate effects of low temperatures, prolonged cold exposure (that may lead to injury or death) is often accompanied by large disruptions of ion and water homeostasis in the cold (Figure 1.1, Figure 1.2) (Andersen et al., 2013; Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b; MacMillan et al., 2015c). When chill

susceptible insects are exposed to low temperatures, Na^+ leaks down its concentration gradient, away from the haemolymph and into the gut (Koštál et al., 2004; MacMillan and Sinclair, 2011b).

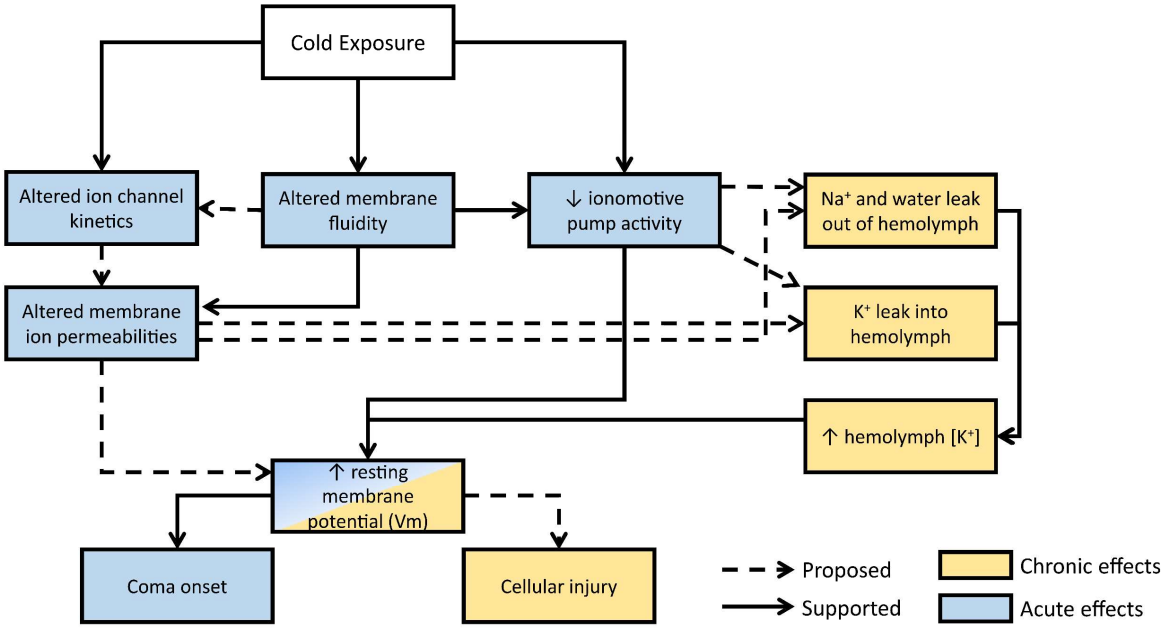

Figure 1.2. An integrative model of the effects of low temperatures on chill-susceptible insects. Directly at the onset of a cold exposure (acute; blue boxes), insects face changes to membrane fluidity, decreased ion-motive pump activity, and altered ion channel kinetics. In parallel, insect cells experience a rapid depolarization at the onset of a cold exposure, resulting in the onset of a chill coma – complete neuromuscular paralysis in the cold. However, following a prolonged cold exposure (chronic; yellow boxes), reduced active transport is no longer sufficient to restore passive cation leak, which leads to net cation leak. This ultimately leads to the increased concentration of haemolymph K^+, further elevating resting membrane potential. It is at this point that cellular injury begins to occur, and is proposed to be related to the additive acute and chronic effects on resting membrane potential. Dashed lines represent proposed but unexplored relationships, while solid lines represent tested and supported phenomenon. Adapted from Overgaard and MacMillan (Overgaard and MacMillan, 2017).

Since Na^+ is a major haemolymph osmolyte in many insects, water passively follows into the gut, leading to an overall reduction in haemolymph water content (Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2012). This reduction in haemolymph volume leads to the concentration of haemolymph K^+, a commonly reported consequence of low temperatures in chill-susceptible insects (Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2015c; Yerushalmi et al., 2016). Despite this, cold-induced haemolymph K^+ elevations are not fully explained by the loss of haemolymph Na^+ and water content alone in crickets (Des Marteaux and Sinclair, 2016), fruit flies (MacMillan et al., 2015a), and migratory locusts (Andersen et al., 2013; Findsen et al., 2013), suggesting that passive leak of K^+ from tissues and/or specifically the gut may also be involved, but the mechanism behind this leak remains unknown.

1.2.2.1 Biochemical mechanisms of cold-induced ion imbalances

While the nature of ion imbalances in chill-susceptible insects has been well described, little is known about the biochemical mechanisms underlying these imbalances. Two potential mechanisms include membrane phase transitions and an imbalance in passive and active ion transport in the cold (Overgaard and MacMillan, 2017). During severe cold exposure, the fluidity of the plasma membrane may change from a fluid-like phase to a gel-like phase, altering membrane function and leading to a potential loss of selective permeability to specific ions (Hazel, 1995). In response to low temperatures, *D. melanogaster* increase the ratio of unsaturated to saturated fatty acids in their membrane phospholipid tails, which presumably retains membrane fluidity and function at low temperatures (Overgaard et al., 2006). Fatty acid composition also vary in the same manner among *Drosophila* species originating from different climates but reared under common garden conditions (Ohtsu et al., 1998). In addition to membrane phospholipids, cholesterol is also an important regulator of membrane fluidity as it

acts to stabilize fluidity at both low and high temperatures, and thus acts as a buffer to changes in membrane fluidity during changing temperatures (Hazel and Williams, 1990). Consistent with the importance of membrane fluidity, it has previously been shown that cholesterol augmented diets improve the cold tolerance of *D. melanogaster* and act to buffer membrane fluidity in isolated cells (Lee et al., 2006; Shreve et al., 2007). Cumulatively, these adjustments suggest that maintenance of membrane fluidity is important for the development of cold tolerance in *Drosophila*, but a direct link between membrane fluidity alterations and cold-induced disruptions in ion homeostasis has yet to be clearly demonstrated. A second mechanism proposed to mediate ion leak in the cold involves unequal effects of temperature on active and passive ion transport (MacMillan and Sinclair, 2011a). Low temperatures slow enzymatic activity by affecting the tertiary structures of enzymes, slowing down the rates of active ion transporters such as Na^+/K^+-ATPase and V-type H^+-ATPase (Hochachka and Somero, 1984; MacMillan et al., 2015b). In contrast, the passive diffusion of ions through channels is minimally affected by temperature (assuming the integrity of the channel is not compromised) (Zachariassen et al., 2004). As Zachariassen et al. (2004) points out, a reduction of temperature from 20°C to -20°C, decreases passive leak of ions by about 15%, while active transport decreases by roughly 95%. The relative stability of passive ion leak in comparison to active ion-transport can therefore lead to a net movement of ions down their electrochemical gradients as suggested by the observed redistribution of Na^+ and K^+ in cold exposed insects. Ion-motive pumps including the V-type H^+ ATPase and Na^+/K^+-ATPase are heavily involved in active ion transport across key ionoregulatory organs such as the Malpighian tubules (MT) and the hindgut (HG) (see section 1.3), and their reduced activity at low temperatures may explain the high rate of water and Na^+ leak into the gut at low temperatures (Linton and O'Donnell, 1999; MacMillan and Sinclair,

2011b). Additionally, in *D. melanogaster*, cold acclimation results in large differences in whole-body activity of Na^+/K^+-ATPase, suggesting that its regulation may at least partly underlie cold tolerance plasticity (MacMillan et al., 2015b). It is important to note that while discussed separately, temperature effects on membrane fluidity and ion-motive pump activity are likely both involved in cold-induced loss of ion homeostasis and may be occurring in parallel and interacting to result in the observed phenotypes (Figure 1.2).

1.2.2.2 Cold-induced haemolymph [K^+] elevation predicts chill coma recovery time

Chill coma recovery time is slowed by longer or colder treatments (Gibert and Huey, 2001; Jean David et al., 1998; Koštál et al., 2004; Macdonald et al., 2004; MacMillan et al., 2012), and has been shown to directly correlate with the degree of cold-induced elevation of haemolymph [K^+] (MacMillan et al., 2012; Yerushalmi et al., 2016). Furthermore, during recovery from a chill-coma, crickets actively reuptake Na^+ and water from the gut into the haemolymph to restore low haemolymph [K^+] (MacMillan et al., 2012). Recovery itself is metabolically costly, as illustrated by a period of increased metabolic rate, which is prolonged with longer cold exposures, and closely matches the time required to restore ion homeostasis (MacMillan et al., 2012). Therefore, recovery from a chill coma involves the active restoration of ion and water homeostasis, and as such, the total time to recover (CCRT) is likely dependent on both the degree of ion balance disruption (MacMillan et al., 2012; Yerushalmi et al., 2016) and the capacity of the animal to re-establish homeostasis (MacMillan et al., 2015b). Furthermore, the restoration of haemolymph [K^+] correlates with the restoration of muscle V_m in locusts, suggesting that recovery of ion homeostasis is important for the recovery of normal neuromuscular function (MacMillan et al., 2014).

1.2.2.3 Mechanisms of chilling injuries

While the cause of cold-induced injury is not well understood, multiple independent studies have shown that chilling injury is closely associated with the degree of haemolymph $[K^+]$ elevation, such that the time of an approximate two-fold increase in haemolymph $[K^+]$ is roughly predictive of a species' median lethal temperature (LT_{50}) (Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b; MacMillan et al., 2014). The most probable link between haemolymph $[K^+]$ and chill-injury is the effect of extracellular K^+ on resting membrane potential (MacMillan et al., 2015c). Similarly to other animals, insects are greatly dependent on K^+ to maintain low V_m (Dawson et al., 1989; Fitzgerald et al., 1996; Rheuben, 1972). The cell membrane is highly permeable to K^+ due to the presence of K^+ leak channels (Alberts et al., 2002). This high permeability in concert with its high electrochemical gradient (maintained by Na^+/K^+-ATPase) leads to a constant leak of K^+ that maintains a negative V_m (Djamgoz, 1987; Hoyle, 1953). The disruption of normal K^+ gradients during chronic cold exposures, and thereby the equilibrium potential of K^+ (E_k) have been repeatedly correlated to chilling injury in a variety of insects including cockroaches, crickets, flies, and locusts (Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b; MacMillan et al., 2015c). To directly assess the impact of haemolymph $[K^+]$ on cell death, Macmillan et al. (2015c) exposed *in vitro* muscle tissue preparations of migratory locusts to high $[K^+]$, low temperatures, or a combination of the two. Interestingly, cellular injury was only found to occur when the tissues were exposed to both factors, illustrating that high $[K^+]$ is only damaging when combined with low temperatures (MacMillan et al., 2015c). This combined effect is thought to occur due to the additive depolarization of V_m as low temperatures alone reduce the enzymatic activity of Na^+/K^+-ATPase,

thus reducing V_e and the resulting disturbances in K^+ gradients reduce the V_d of K^+ by compromising the rate of K^+ leak (MacMillan et al., 2012; MacMillan et al., 2015c).

While no direct mechanistic link between cold-induced cell depolarization and cell death (chilling injury) has been demonstrated, several mechanisms have been proposed. Recently, it was shown that brief cold exposures cause small elevations in intracellular Ca^{2+}, triggering Ca^{2+}/Calmodulin kinases, that are thought to be responsible for the initiation of a cold-acclimation response (Teets et al., 2013). While normally responsible for Ca^{2+} removal, during prolonged cold exposures, the activity of Ca^{2+}-ATPases is reduced, leading to chronically high intracellular [Ca^{2+}], thought to activate Ca^{2+}-dependent proteases and lipases that ultimately destroy cellular integrity (Boutilier, 2001; Hochachka, 1986; Yi et al., 2007). This is different from the accumulation of Ca^{2+} in excitation-contraction coupling in muscle cells as cold-induced intracellular Ca^{2+} elevations tend to be more chronic (Koštál et al., 2004; Zachariassen et al., 2004). Related studies have also illustrated a clear connection between apoptosis and cold-induced cell death by linking continuous plasma membrane depolarizations (as seen with chill-susceptible insects at low temperatures) to be predictive of apoptotic cell death (Bortner et al., 2001) and by demonstrating that *D. melanogaster* muscle injury in the cold is dependent on apoptotic signalling (Yi et al., 2007). Furthermore, cold-acclimated insects suppress apoptotic signalling pathways (Goto, 2000; Teets et al., 2013; Yi et al., 2007). Ultimately, both necrotic and apoptotic cell death are thought to underlie chilling injury and organismal death and thus acquired cold tolerance partly depends on the avoidance and inhibition of necrosis and apoptosis, respectively (Overgaard and MacMillan, 2017).

1.3 Ion and water regulation in insects

The regulation and preservation of ion and water homeostasis is critical to the survival of insects, particularly during processes such as desiccation, feeding, and osmotic influx of water in freshwater environments (Larsen et al., 2014). At the organismal level, ion and water homeostasis are maintained by the regulated permeability and transport of ions and water across organs, many of which are associated with the alimentary canal. Specifically, the midgut, Malpighian tubules, and the hindgut of insects play a key role in ion and water homeostasis.

The midgut is the largest segment of the *D. melanogaster* gut and is responsible for carrying out the vital functions of nutrient digestion, absorption, and defence against ingested pathogens (Overend et al., 2016). In *D. melanogaster*, the midgut is separated into three functionally unique regions based on luminal pH: a narrow anterior neutral zone, a narrow acid secreting middle zone, and a wide basic posterior zone (Shanbhag and Tripathi, 2009). Great variation is thought to exist in ion transport across the midgut based on epithelial cell morphology, such that the anterior midgut is theorized to be absorptive, the middle midgut to be both absorptive and secretory, and the posterior midgut to reabsorb water (Shanbhag and Tripathi, 2009). It has also been shown recently in larval *D. melanogaster* that transepithelial ion movement is energized by both V-type H^+-ATPase and Na^+/K^+-ATPase, but the mechanism underlying these transport pathways, particularly in adults, remains poorly understood (D'Silva et al., 2017).

Unlike the midgut, a more developed understanding exists for the role and function of the Malpighian tubules – diverticula of the gut composed of two cell types (principal and stellate cells) that function as the main site of ion and water secretion from the haemolymph into the gut in insects. The tubules actively transport ions (K^+, Na^+, and Cl^-) from the haemolymph leading to

passive water flux into the lumen thereby producing the primary urine (Larsen et al., 2014). The movement of ions across the tubules is mainly energized by the electrical and chemical gradients formed by apically located (lumen-facing) V-type H^+-ATPase on the larger principal cells which pumps protons into the tubule lumen (Beyenbach, 2001). Secondary transport pathways then utilize the H^+ gradient to exchange cations (Na^+, K^+) for protons while passive Cl^- transport is facilitated across small intercalating stellate cells and via the paracellular pathway (Beyenbach et al., 2010). In addition, in *D. melanogaster*, basally located (haemolymph-facing) Na^+/K^+-ATPases also contributes to Malpighian tubule fluid secretion and modulates K^+ secretion rate by facilitating its preferential entry over Na^+ from the haemolymph into principal cells (Linton and O'Donnell, 1999). Malpighian tubule function is carefully controlled by diuretic hormones (DHs), which precisely regulate rates of MT fluid and ion secretion and the ratio of secreted Na^+:K^+ to activate or suppress diuresis as required by the animal (Coast, 2007).

The primary urine travels the length of the Malpighian tubules where it enters the gut lumen at the junction of the midgut and hindgut, mixing with contents from the midgut before passing posteriorly to the hindgut where the reabsorption of water, ions, and metabolites takes place prior to the excretion of wastes (Phillips et al., 1987; Wigglesworth, 1932). The hindgut of *Drosophila* is composed of the ileum and rectum. Most ion and water reabsorption occurs at specialized areas of thickened rectal epithelia called rectal pads that actively absorb ions to create local osmotic gradients for the reabsorption of water (Larsen et al., 2014). The degree of fluid reabsorption varies greatly among and within insect species. For example, while up to 80-95% of the water is reabsorbed in the stick insect (Ramsay, 1955), negligible fluid reabsorption takes place in *Rhodnius prolixus* during the rapid diuresis that ensues upon blood feeding, when copious water is being ingested (Maddrell and Phillips, 1975). The mechanisms underlying this

reabsorption have yet to be assessed in *Drosophila*, and have thus far been studied in cockroaches, locusts and adult blowflies, all of which also possess rectal pads (Larsen et al., 2014). In these insects, the movement of fluid is driven by the active uptake of Na^+, K^+, and Cl^- into the epithelial cells of the pads and then into intercellular spaces. The osmolarity of the intercellular space is highest near the lumen and lowest near the haemocoel and it is thought that the absorption of ions back into epithelial cells, along with decreased water in the downstream areas of the intercellular space, allows for the recycling of ions to maintain a continuous gradient for the reabsorption of water (Larsen et al., 2014). While the biochemical drivers of ion transport in the rectal pads remain poorly understood, both Na^+/K^+-ATPase and V-type H^+-ATPase were localized to the basolateral and apical membranes, respectively, of the rectal pads of female *A. aegypti* mosquitoes (Patrick et al., 2006).

Since cold exposure causes the leak of Na^+ and water into the alimentary canal in insects, an understanding of the physiological plasticity of the involved organs in response to cold-acclimation is critical to understand how hyperkalemia is mitigated following cold acclimation (MacMillan and Sinclair, 2011b).

1.4 Altered Malpighian tubule activity in cold tolerant insects assists in maintaining ion homeostasis at low temperatures

Perhaps most important to the prevention of perturbations to ion homeostasis in the cold is the ability of key ionoregulatory organs of insects, such as the gut and the Malpighian tubules, to prevent cold-induced ion leak or actively work to overcome it. While, to date, no assessment has been conducted on the ionoregulatory organs of cold acclimated insects, functional differences in ion transport have been described in the Malpighian tubules and rectums of cold tolerant *Drosophila* species (Andersen et al., 2017b; MacMillan et al., 2015d). Notably, while

the ratio of secreted Na^+:K^+ was unaltered in chill tolerant Malpighian tubules in the cold, chill susceptible tubules experienced elevations in the Na^+:K^+ ratio (MacMillan et al., 2015d). The stable Na^+:K^+ secretion ratio likely assists these chill-tolerant flies in preventing hyperkalemia by (1) maintaining excretion of K^+ in the cold which may otherwise prove to be lethal and (2) avoiding the loss of haemolymph Na^+, which is important for the maintenance of haemolymph volume. Recent work by Andersen et al. (2017b) supports the former point, directly showing that in contrast to chill susceptible species, cold tolerant *Drosophila* species retain Malpighian tubule K^+ secretion rates in the cold. In addition to the Malpighian tubules, the recta of more chill tolerant *Drosophila* species decreased K^+ reabsorption in the cold, while recta of chill susceptible species increased K^+ reabsorption in the cold (Andersen et al., 2017b). Together, the rectum and Malpighian tubules therefore work in concert to remove haemolymph K^+ in the cold and prevent cold induced hyperkalemia as species with improved ion regulation at low temperatures experienced no hyperkalemia in the cold and had better chill-survival (lower LT_{50}; the temperature of 50% mortality). While the underlying mechanisms that allow for Malpighian tubule and rectal function at low temperature remains unknown, ion and water transport in both organs is energized by ion-motive ATPases, and thus the regulation of these pumps is a possibility. For example, Na^+/K^+-ATPase activity is dramatically reduced in cold-acclimated *D. melanogaster* (MacMillan et al., 2015b). These organs are also carefully controlled by neuroendocrine factors and these factors are an additional mechanism by which cold tolerant insects may regulate Malpighian tubule and rectal function. For example, a recent study demonstrated that *D. melanogaster* (and a subset of other tested dipterans) exposed to 24 hours at low temperatures had a marked increase in the expression of the capa neuropeptide gene, whose downstream products, CAPA peptides, promote diuresis in the Malpighian tubule of *D.*

melanogaster (Terhzaz et al., 2015). Furthermore, injections of CAPA peptides led to improved CCRT, suggesting a potential role of this neuropeptide (and possibly others as well) in altering tubule function in a way that mitigates ion imbalances in the cold, and consequently improves cold tolerance (Terhzaz et al., 2015).

Lastly, the midgut is also an important ionoregulatory organ in insects (Coast, 2007), and may also contribute in mitigating disturbances to ion homeostasis in the cold; however, to the best of my knowledge, gut physiology in relation to insect cold tolerance has not been examined to date.

1.5 Experimental goals and hypotheses

To further our understanding of the physiological mechanisms underlying chill tolerance, I investigated the functional changes in the ionoregulatory organs of *D. melanogaster* that occur with cold acclimation. Specifically, I examined whether ion-motive ATPase activity and the functions of the gut and the Malpighian tubules differed in warm-and cold-acclimated flies. To do this, I firstly confirmed that cold-acclimation improves the cold tolerance of *D. melanogaster* and mitigates cold-induced hyperkalemia. Secondly, I quantified the activities of Na^+/K^+-ATPase and V-type H^+-ATPase in the primary ionoregulatory organs (midgut, Malpighian tubules, and hindgut) to test whether their activity is modified during cold acclimation. Lastly, I assessed Malpighian tubules and gut function directly to examine how physiological plasticity of these organs may aid in the maintenance of ion balance in the cold. I hypothesized that ion-motive ATPase activity will increase in the Malpighian tubules of cold-acclimated flies, and that these increases will enable a higher capacity for K^+ clearance at low temperatures (Figure 1.3). Conversely, I hypothesized that in the midgut and hindgut, decreases in ion-motive ATPase activity will reduce K^+ absorption and likewise mitigate hyperkalemia in the cold (Figure 1.3).

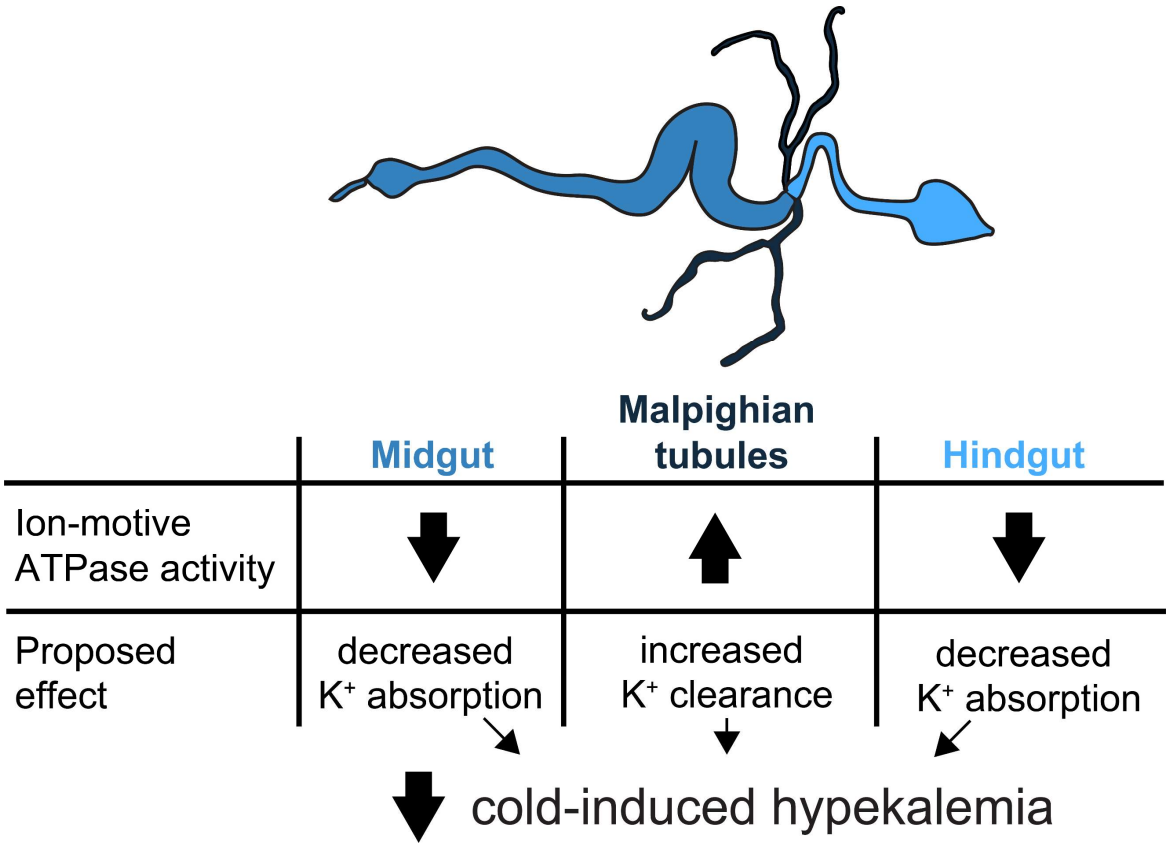

Figure 1.3. Summary of hypotheses regarding the ionoregulatory changes in the midgut, Malpighian tubules, and hindgut that prevent cold-induced hyperkalemia in cold-acclimated *D. melanogaster*. Ion-motive ATPases such at Na^+/K^+-ATPase and V-type H^+-ATPase are the main drivers of epithelial transport in the gut and the Malpighian tubules of insects, and their activity is proposed to alter gut and tubule function to reduce K^+ absorption and increase K^+ excretion. Decreased ion-motive ATPase activity in absorptive organs such as the midgut and the hindgut is therefore predicted to reduce K^+ absorption while increased ion-motive ATPase activity in the Malpighian tubules is proposed to increase K^+ clearance. Cumulatively, these changes are proposed to facilitate the net excretion of K^+ in the cold and aid in avoiding the lethal accumulations of haemolymph $[K^+]$ in the cold.

2. Methods

2.1 Animal husbandry and acclimation treatments

The population of *Drosophila melanogaster* used in this study was established in 2008 by combining 35 isofemale lines from London, Ontario (43°00'N, 81°15'W) and Niagara-on-the-Lake, Ontario (43°04'N, 79°04'W) (Marshall and Sinclair, 2010). Fly rearing was conducted as previously described (Yerushalmi et al., 2016) by transferring 100-150 mature adults (~1-3 weeks old) into 200 mL plastic bottles containing ~50 mL of a standard rearing diet (Bloomington *Drosophila* medium; Lakovaara, 1969) for 1-2 h, ensuring an approximate egg density of 100-150 eggs/bottle. The bottles were then stored at 25°C and a 14:10 h light:dark cycle. Filter paper was placed in each bottle to increase surface area for pupation. Newly-emerged adults were collected daily and transferred into 40 mL plastic vials containing 7-10 mL of the rearing diet. The vials were then randomly assigned to one of the two treatments: warm- or cold-acclimation. Warm-acclimated (WA) flies were maintained at 25°C with a 14:10 h light:dark cycle and cold-acclimated (CA) flies were maintained at 10°C with 10:14 h light:dark cycle (aimed to mimic summer and fall conditions, respectively). All experiments were conducted on non-virgin adult females following a one-week exposure to their acclimation treatment.

2.2 Chilling tolerance phenotypes

Three metrics of chilling tolerance were assessed in the present study including (1) critical thermal minimum (CT_{min}), (2) chill coma recovery time (CCRT) after 6 h at 0°C, and (3) chilling survival. CT_{min} and CCRT were measured as previously described (Yerushalmi et al., 2016).

For the assessment of CT_{min}, flies were individually placed in 4 mL glass screw-top vials. 36 vials were then attached to a custom-built rack and placed in a temperature-controlled bath (Model MX7LL, VWR International, Mississauga, Canada) containing a 1:1 mixture of ethylene glycol and water at room temperature (25°C). The bath temperature was then ramped down at a rate of 0.15°C min^{-1} and the temperature was monitored independently using a pair of type-K thermocouples connected to a computer running Picolog (version 5.24.8) via a Pico TC-08 interface (Pico Technology, St. Neots, UK). Flies were individually observed throughout the ramping period and the temperature at which no fly movement was observed following a disturbance of the vial with a plastic probe was recorded as its CT_{min}.

To measure CCRT, female flies were individually placed in 4 mL glass screw-top vials (as above). The vials were placed in a sealed plastic bag and submerged in an ice-water mixture (0°C) for 6 h. The vials were then removed from the ice-water mixture and placed at room temperature (25°C) where the flies were individually observed for movement. The time that it took an individual fly to stand on all six legs following its removal from the cold treatment was recorded as its CCRT.

To determine the chill-survival of each acclimation group, female flies were isolated in groups of 10 into empty 40 mL plastic vials and placed in an ice water mixture (0°C). Vials were then removed at pre-determined time points (based on preliminary trials) to ensure close to 100% and 0% survival at the minimal and maximal durations in the cold, respectively. The warm-acclimated flies were exposed to 0°C for 0 h, 6 h, 12 h, 18 h, 24 h, 30 h, 36 h, and 42 h, while the cold-acclimated flies were exposed to 0°C for 0 h, 6 h, 18 h, 36 h, 54 h, 72 h, 90 h, and 110 h. Upon removal from the cold treatment, flies were transferred into 40 mL vials with 7-10 mL of fresh food and maintained at their respective acclimation temperature for 24 h to recover. Fly

survival was assessed following this recovery period where flies that could stand on all limbs were scored as alive and those that could not were scored as dead.

2.3 Haemolymph [K^+] measurements

Haemolymph [K^+] was assessed in flies from both acclimation groups after various durations at 0°C using the ion-selective microelectrode technique (ISME) as previously described (Jonusaite et al., 2011). The cold-exposure durations used here mirrored those chosen for chill-survival analysis (see above), except for 36 h and 42 h for warm-acclimated flies, where haemolymph extraction was unsuccessful. Haemolymph droplets were collected by placing individual flies in 200 µL pipette tips and attaching the tips to a custom-made device (as previously described by MacMillan and Hughson (2014)), which allows for the careful adjustment of the air pressure passing through the tip. The pressure was carefully adjusted to expose the head through the pipette tip and an antenna was then carefully removed under a dissection microscope. Droplets of haemolymph that accumulated at the ablated antennas were immediately placed under paraffin oil for assessment using ISME.

Ion-selective microelectrodes were prepared from borosilicate glass capillaries (TW150-4; World Precision Instruments, Sarasota, USA) and pulled using the P-97 Flaming-Brown micropipette puller (Sutter Instruments Co., Novato, USA) to a tip diameter of ~5 µm. The microelectrodes were then salinized in vapours of N,N-dimethyltrimethylsilylamine (Fluka, Buchs, Switzerland) at 300°C for 1 hour. K^+-selective microelectrodes were backfilled with 100 mM KCl and front loaded with the K^+ Ionophore cocktail B (Fluka). Na^+-selective electrodes (used for assessment of [Na^+] in the Malpighian tubule secreted fluid, see below) were backfilled with 100 mM NaCl and front loaded with the Na^+ Ionophore II cocktail A (Fluka). The tips of the ion-selective microelectrodes were then dipped in polyvinyl chloride (PVC) to prevent the

leakage of ionophore into the paraffin oil. To complete the circuit, a conventional microelectrode was prepared from borosilicate glass capillaries (IB200F-2; WPI) and backfilled with 500 mM KCl. Both electrodes were connected to a PowerLab 4/30 data acquisition system (AD Instruments Inc., Colorado Springs, USA) through an ML 165 pH Amp and analyzed with LabChart 6 Pro software (AD Instruments Inc.). Once the set up of ISME was complete, 5 µL calibration droplets with known concentrations of the ion of interest and a 10-fold difference in its concentration were measured. To ensure ion-specificity, the lower of the two concentration droplets was corrected in ionic strength using LiCl. For example, for the measurement of haemolymph [K^+], 10 mM KCl/90 mM LiCl and 100 mM KCl were used for calibration. The final ion concentrations were then calculated with the following equation:

$$a^h = a^c \times 10^{\frac{\Delta V}{S}}$$

Where a^h is the haemolymph ion concentration, a^c is the concentration of one of the calibration droplets, ΔV is the difference in voltage between the haemolymph and the calibration solution and S is difference in voltage between two calibration droplets with a tenfold difference in ion activity.

2.4 Malpighian tubule fluid and ion secretion rates

To assess differences in Malpighian tubule activity, modified Ramsay assays (Ramsay, 1954) were conducted on tubules extracted from cold- and warm-acclimated flies and at 0°C, 5°C, 10°C, and 23°C. To isolate Malpighian tubules, individual flies are first dipped in 70% ethanol for 5-10 seconds for the removal of cuticular waxes and transferred into a dish lined with a silicone elastomer (Sylgard 184; Dow Corning Corp., Midland, USA) containing *Drosophila* saline (10 mM glutamine, 20 mM glucose, 15 mM MOPS, 4.2 mM NaH_2PO_4, 10.2 mM

$NaHCO_3$, 8.5 mM $MgCl_2$ (hexahydrate), 2 mM $CaCl_2$ (dihydrate), 20 mM KCl, 117.5 mM NaCl, pH 7.0). The anterior pair of Malpighian tubules was then isolated along with a part of the ureter by carefully excising the ureter near the ureter-gut junction. Upon their removal, the Malpighian tubules were transferred into a modified Sylgard-lined dish containing 35 µL droplets of a 1:1 mixture of *Drosophila* saline and Schneider's insect medium (Sigma-Aldrich, St. Louis, USA) placed in premade wells under paraffin oil. Using a glass probe, one tubule was placed in the saline droplet and the other was carefully wrapped around a metal pin adjacent to the droplet, ensuring exposure of the excised ureter to the paraffin oil where secreted fluid would accumulate and later be collected for analysis.

To assess Malpighian tubule function at 0°C, 5°C and 10°C, after the Ramsay assays were set up at room temperature, the Sylgard-lined dish housing the assay was placed in a glass dish containing ~1cm of water within a Precision™ Low Temperature BOD Refrigerated Incubator (Model PR205745R, ThermoFisher Scientific, Waltham, USA). The temperature of the water bath holding the Ramsay assay dishes was monitored independently using type-K thermocouples and maintained within 1°C of treatment temperature at all times. Because chilling slows rates of fluid transport (Anstee et al., 1979; MacMillan et al., 2015d; Maddrell, 1964), incubation times were adjusted depending on temperature to ensure a droplet of sufficient size for measurement and analysis by ISME; Ramsay assays were incubated for 30 min, 120 min, 150 min, or 180 min for assays running at 23°C, 10°C, or 5°C, or 0°C, respectively. Following this incubation period, a glass probe was used to isolate the primary urine droplet under paraffin oil. Droplets of the secreted fluid were then suspended in oil (to ensure droplets are roughly spherical in shape) and droplet diameter was measured using the ocular micrometer of a Motic® K-400L

Stereo Microscope (Motic North America, Richmond, Canada). The fluid secretion rate was then calculated using the following equation:

$$\text{secretion rate} = \frac{\frac{4}{3}\pi(\frac{d}{2})^3}{t}$$

Where d is the diameter of the secreted fluid droplet in mm, and t is the incubation period of the Ramsay assay in minutes.

Lastly, $[Na^+]$ and $[K^+]$ of the secreted fluid were assessed using ISME as described above (see section 2.3).

2.5 Midgut and hindgut K^+ flux

The scanning ion-selective microelectrode technique (SIET) was used to measure K^+ flux across the midgut and hindgut epithelium as previously described (Jonusaite et al., 2013; Rheault and O'Donnell, 2001). Briefly, a K^+-selective microelectrode was prepared as described above and mounted onto a headstage with an Ag/AgCl wire electrode (WPI). The headstage was connected to an ion polarographic amplifier (IPA-2, Applicable Electronics, Forestdale, USA). The circuit was completed using a reference electrode composed of 3% agar in 3 M KCl that solidified inside a glass microcapillary. One end of the electrode was placed in the bathing solution while the other end was connected to a headstage via an Ag/AgCl half-cell (WPI). Ion selective microelectrodes were calibrated in 5 mM KCl/45 mM LiCl and 50 mM KCl solutions.

For their assessment using SIET, individual whole guts were carefully isolated and bathed in fresh *Drosophila* saline in the lid of a 35 mm Petri dish. Individual measurements were conducted 5-10 µm from the gut epithelium and 100 µm away, for the assessment of concentration differences near and away from the preparation. To minimize potential gradient

disturbance effect of the electrode movement, a 4 s wait time was employed between the two positions of measurement, followed by a 1s recording period. For each measured position, this protocol was repeated four times, and the average voltage gradient between the two points was used for the calculation of K^+ flux. The Automated Scanning Electrode Technique (ASET) software (version 2.0, Science Wares, East Falmouth, USA) was used to automatically run the sampling protocol and calculate the average voltage gradient at each assessed site. A measurement of background noise was recorded for each preparation ~3mm away from the gut and was used in the calculation of K^+ flux to account for mechanical disturbances in the ion gradients that arise from the movement of the electrode during sampling.

Voltage gradients were converted into concentration gradients using the following equation as previously described by Donini and O'Donnell (2005):

$$\Delta C = C_B \times 10^{\frac{\Delta V}{S}} - C_B$$

Where ΔC is the concentration gradient between the two measured points, C_B is the background ion gradient measured away from the gut preparation, ΔV is the voltage gradient obtained from ASET, and S is the slope of the electrode. While in reality this technique measures ion activity, data can be expressed in concentrations if it is assumed that the ion activity coefficient in the experimental solution is the same as that of the calibration (Donini and O'Donnell, 2005).

Measurement sites across the gut included six equidistant sites across the midgut, three sites across the ileum (averaged and represented as a single flux), and 2-3 sites on the rectal pads (averaged and represented as a single flux). For each site, two or more measurements were taken and averaged. All measurements were conducted at room temperature (~23°C) and 6°C.

2.6 Quantification of organ specific temperature effects on K^+ balance

Temperature effects on K^+ transport were compared between 23°C and 6°C for the rectum and between 23°C and 5°C in the Malpighian tubules. This was done by calculating the temperature coefficient (Q_{10}), which represents the effect of a 10°C reduction in temperature on K^+ transport. Q_{10} values were calculated using the following equation (the Malpighian tubules are used as an example):

$$Q_{10} = \left(\frac{\text{ion transport rate at 23°C}}{\text{ion transport rate at 5°C}}\right)^{\left(\frac{10}{\Delta T}\right)}$$

Where ΔT is the difference in temperature (18°C). To estimate the relative effect of temperature on K^+ transport in the Malpighian tubules in comparison to the rectum, the following equation was used:

$$\Delta Q_{10} = \text{MT } K^+ \text{ secretion rate } Q_{10} - \text{Rectum } K^+ \text{ flux } Q_{10}$$

The further this metric deviates from zero, the greater imbalance in the overall circuit of K^+ transport between these two organs.

2.7 Na^+/K^+-ATPase and V-type H^+-ATPase enzyme activity

Tissue-specific Na^+/K^+-ATPase and V-type H^+-ATPase activities were measured as described by Jonusaite et al. (2011) by quantifying the ouabain- (Sigma-Aldrich Canada, Oakville, Canada) or bafilomycin-sensitive (LC Laboratories, Woburn, USA) hydrolysis of adenosine triphosphate (ATP; BioShop Canada, Burlington, Canada).

Midguts, Malpighian tubules, and hindguts were each collected from warm- and cold-acclimated flies. To isolate Malpighian tubules, individual flies are first dipped in 70% ethanol for 5-10 seconds for the removal of cuticular waxes (to prevent flies from floating on saline) and

transferred into a dish lined with a silicone elastomer (Sylgard 184; Dow Corning Corp., Midland, USA) containing *Drosophila* saline (10 mM glutamine, 20 mM glucose, 15 mM MOPS, 4.2 mM NaH_2PO_4, 10.2 mM $NaHCO_3$, 8.5 mM $MgCl_2$ (hexahydrate), 2 mM $CaCl_2$ (dihydrate), 20 mM KCl, 117.5 mM NaCl, pH 7.0). In an effort to minimize rapid thermal plasticity, dissections were conducted at temperatures approximating the acclimation treatments. Warm-acclimated flies were dissected at room temperature (~23°C) and cold-acclimated flies were dissected at ~10°C by placing the dissecting dish on a PE100 Inverted Peltier System connected to the PE95 controller (Linkam Scientific Instruments, Tadworth, England) in the view of the dissecting microscope. Following the dissection of each individual fly, isolated organs were transferred to 2 mL microcentrifuge tubes and immediately flash frozen using liquid nitrogen. Once 30 individual flies were dissected, the collected samples were then transferred to -80°C for later tissue processing.

To homogenize the organs, frozen samples were thawed on ice and 100 µL of homogenizing buffer was added to each tube (150 mM sucrose,10 mM Na_2EDTA, 50 mM imidazole, 0.1% deoxycholic acid; pH 7.3). The samples were homogenized on ice using a PRO 250 homogenizer with a 5 x 75 mm generator (PRO Scientific Inc., Oxford, USA) for 8-10 seconds and centrifuged at 10,000 × *g* for 10 minutes at 4°C using a 5810R centrifuge (Eppendorf Canada, Mississauga, Canada). The resulting supernatants were then collected into 2 mL tubes and stored at -80°C.

Three assay solutions (A, B, and C) containing the appropriate enzymes and reagents were prepared. Solution A was composed of 4 U/mL lactate dehydrogenase (LDH), 5 U/mL pyruvate kinase (PK), 2.8 mM phosphoenolpyruvate (PEP), 3.5 mM ATP, 0.22 mM NADH, 50 mM imidazole, and titrated to a pH of 7.5. Solutions B and C were similar in composition but

also contained 5 mM ouabain or 10 µM bafilomycin, respectively, for the inhibition of the ATPases under investigation. Following their preparation, each solution was mixed in a 3:1 ratio with a salt solution (189 mM NaCl, 10.5 mM $MgCl_2$, 42 mM KCl, 50 mM imidazole, pH 7.5).

Prior to running the assays, an adenosine diphosphate (ADP) standard curve was run to ensure that all reagents used are working normally. First, 0 nM, 5nM, 10 nM, 20 nM, 40 nM ADP standards were prepared by diluting stock ADP in imidazole buffer. Two technical replicates containing 10 µL of each ADP standard were then added to a 96-well polystyrene microplate (BD Falcon™, Franklin Lakes, USA) and 200 µL of the assay solution (solution A + salt solution) was added to each well. The plate was then placed in a Thermo Electron Multiskan™ Spectrum microplate spectrophotometer (Thermo Electron Co., Waltham, USA) set to 25°C and measuring absorbance at 340 nm (the peak absorbance of NADH). The recorded absorbance was analyzed using Skanlt version 2.2.237 (Thermo Electron Co.). The assay solution was approved if the optical density of the ADP standards were within 0.2 and 0.9 and if the slope of the curve was between -0.012 and -0.014 (Jonusaite et al., 2011).

To run both assays (Na^+/K^+-ATPase and V-type H^+-ATPase), experimental homogenates were thawed and added to a 96-well microplate that was kept on ice in six replicates of 10 µL each. Following this, 200 µL of the assay solutions (Salt solution mixed with solutions A, B, or C) were added to two replicates for each experimental sample, resulting in two technical replicates per sample. The plate was then inserted into the microplate reader and the disappearance of NADH (peak absorbance: 340 nm) was assessed over a 30 min period.

Upon assay completion, raw absorbance data was extracted from the Multiskan Spectrum data acquisition system and the rates of NADH disappearance were independently assessed in R version 3.3.1 (R Core Team, 2015) using the lmList() function available in the lme4 package

(Bates et al., 2016). Na^+/K^+-ATPase and V-type H^+-ATPase specific ATP consumption was determined by assessing the difference in activity between samples running with or without ouabain or bafilomycin, respectively. Enzyme activity was standardized to the protein content of each sample using a Bradford assay (Sigma-Aldrich Canada) according to the manufacturer's guidelines and using bovine serum albumin as a standard (Bioshop Canada Inc., Burlington, Canada). Final enzyme activities were calculated using the following equation:

$$\text{enzyme activity} = \frac{\Delta\text{Activity}}{\text{S} \times [\text{P}]}$$

Where ΔActivity is the difference in ATP hydrolysis in the absence and presence of ouabain or bafilomycin, S is the slope of the ADP standard curve, and [P] is the protein concentration of the sample.

2.8 Malpighian tubule size

Malpighian tubule size was assessed for flies of both acclimation groups. The anterior pair of tubules of individual flies were dissected out, ensuring that no direct contact was made with the Malpighian tubules themselves. Images of the tubules were then captured using an Olympus IX81 inverted microscope (Olympus Canada, Richmond Hill, Canada). Images were recorded and analyzed using Olympus cellSens digital imaging software version 1.12 (Olympus Canada). Malpighian tubule length was measured from the ureter-Malpighian tubule junction to the distal end of each tubule. Tubule width varied along its length and thus width measurements were always taken ~100 µm from the ureter-tubule junction.

2.9 Statistical analysis

CCRT and CT_{min} were analyzed using unpaired student's t-tests. Survival following cold stress was analyzed using a generalized linear model with a binomial error distribution. The Lt_{50} for each acclimation group was determined from the exposure time corresponding to 0.5 survival in the produced models and was extracted using the dose.p() function available in the mass package for R (Venables and Ripley, 2003). Linear regressions were used to regress haemolymph $[K^+]$ against chilling exposure duration and survival proportions against haemolymph $[K^+]$. Tissue-specific ATPase activity, Malpighian tubule protein content, and Malpighian tubule length and width were compared between the acclimation treatments using unpaired student's t-tests. Two-way ANOVAs were used to determine the independent and interacting effects of acclimation temperature and exposure temperature on Malpighian tubule fluid and ion secretion rates, ion concentrations in the secreted fluid, and the ratio of Na^+:K^+ in the secreted fluid. Holm-Sidak post-hoc tests were then conducted to compare differences in activity between the two acclimation treatments at each exposure temperature. All statistical tests were conducted on GraphPad Prism version 6.0.1 (GraphPad Software, La Jolla, USA) with the exception of the assessment of chilling survival, which was conducted in R version 3.3.1 (R Core Team, 2015).

3. Results

The detailed results of all statistical analyses (variables compared, statistical test, resultant statistics, degrees of freedom, and *P*-value) that are described are outlined in tables 3.1 (sections 3.1, 3.2, 3.4, and 3.6) and 3.2 (section 3.3).

3.1 Cold acclimation improved chill survival, critical thermal minimum, and chill coma recovery time

Cold-acclimated flies survived exposure to 0°C for significantly longer than warm-acclimated flies (Logistic Regression; $P < 0.0001$; n = 3-6 vials of 7-12 flies per time point per group; Figure 3.1A). The time at 0°C that resulted in 50% mortality (Lt_{50}) was 29.18 ± 0.64 h for warm-acclimated flies and 59.46 ± 1.94 h for cold-acclimated flies; a two-fold difference. In addition, the CT_{min} of cold-acclimated flies was significantly lower than that of warm-acclimated flies (unpaired t-test; $P < 0.0001$; Figure 3.1B); on average, cold-acclimated individuals entered a chill coma ~3.5°C below warm-acclimated flies. The chill coma recovery time following 6 h at 0°C was lower by approximately 50% in cold-acclimated flies (unpaired t-test; $P < 0.0001$; Figure 3.1C). While cold-acclimated flies recovered in 17.3 ± 0.9 min, warm-acclimated flies required 36.4 ± 2.9 min to recover from the same amount of time at 0°C.

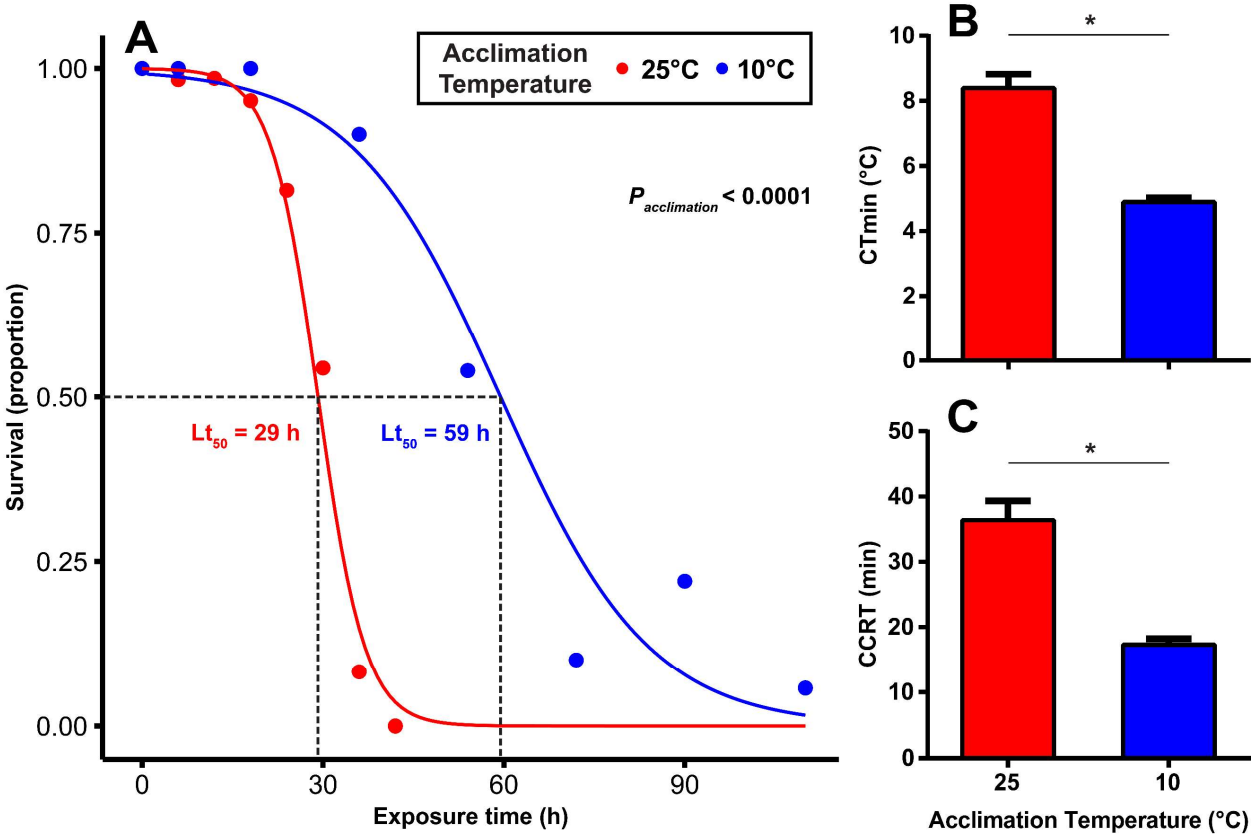

Figure 3.1. Cold acclimation improves the chill tolerance of adult *D. melanogaster* females. (A) Survival proportions of warm- (red) and cold-acclimated (blue) *D. melanogaster* adult females exposed to 0°C for varying time periods (n = 3-6 vials of 7-12 flies per time point per group). Survival following various durations at 0°C was higher in cold-acclimated flies. The dotted lines represent the median lethal time (Lt_{50}). McFadden pseudo R^2 were 0.56 for both acclimation treatments. Note: unlike R^2, a McFadden pseudo $R^2 > 0.4$ represents a good fit (McFadden, 1978). (B) Critical thermal minimum (CT_{min}) of warm- and cold acclimated flies (n = 18 flies per group). CT_{min} was lower in cold-acclimated flies. (C) Chill coma recovery time (CCRT) for warm- and cold- acclimated flies (n = 20 flies per group). CCRT was significantly lower in cold-acclimated flies. All bars represent mean ± SEM. Asterisks denote significant difference (unpaired t-test; $P < 0.001$).

3.2 Cold acclimation mitigated cold-induced hyperkalemia

Haemolymph [K^+] levels were assessed in warm- and cold-acclimated flies following exposures to 0°C that were similar in length to those used in the survival analysis (see methods). With increasing duration to cold exposure, haemolymph [K^+] significantly increased in both warm-acclimated flies (linear regression; $P = 0.0045$; $R^2 = 0.83$) and cold acclimated flies (linear

regressions; $P = 0.0117$; $R^2 = 0.77$; Figure 3.2A). The rate of $[K^+]$ accumulation, however, differed by a factor of 9 among the acclimation groups (ANCOVA; $P = 0.0004$). Whereas haemolymph $[K^+]$ increased at a rate of ~1.5 mM/hour in warm-acclimated flies, cold-acclimated flies accumulated $[K^+]$ at a rate of ~0.17 mM/hour (Figure 3.2A). Haemolymph $[K^+]$ in the cold predicted chill survival in both acclimation-groups (linear regression; $P = 0.012$ and $R^2 = 0.83$ for warm-acclimated flies and $P = 0.018$ and $R^2 = 0.63$ for cold-acclimated flies; Figure 3.2B). The slope of these predictive relationships, however, differed between the two acclimation treatments such that a similar level of mortality was associated with a much smaller change in haemolymph $[K^+]$ in cold-acclimated flies (ANCOVA, $P = 0.015$; Figure 3.2B).

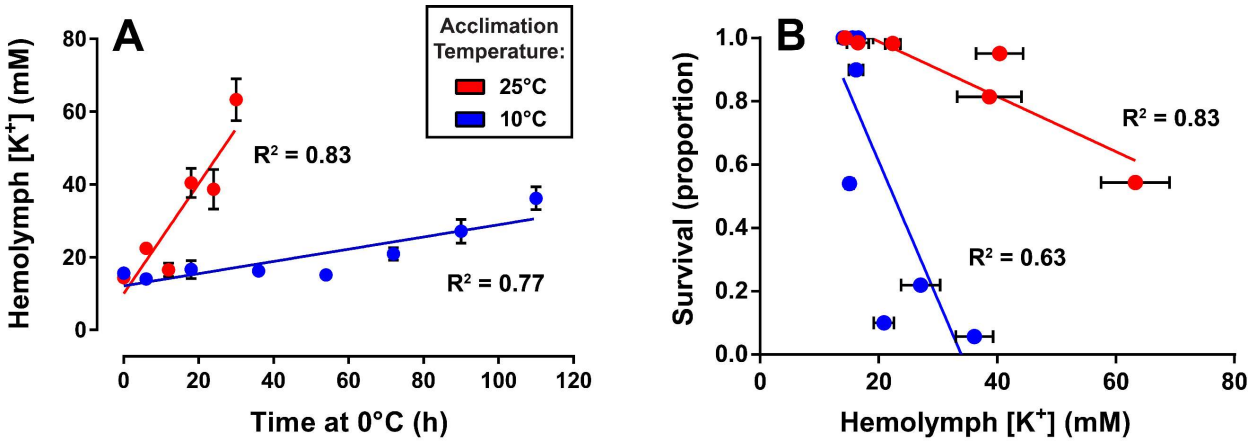

Figure 3.2. Cold acclimated flies can regulate haemolymph $[K^+]$ better than warm acclimated flies during chilling but succumb to cold induced injury at lower haemolymph $[K^+]$. (A) Haemolymph $[K^+]$ following chronic exposure of warm- (red) and cold-acclimated (blue) *D. melanogaster* females to 0°C. The rate of haemolymph $[K^+]$ increase was lower in cold-acclimated flies (ANCOVA; $P = 0.0004$). (B) The relationship between haemolymph $[K^+]$ and mortality following chilling. Chilling related death occurred at lower haemolymph $[K^+]$ in cold-acclimated flies in comparison to warm-acclimated flies (ANCOVA; $P = 0.0011$). Bars represent mean ± SEM. Bars that are not visible are obscured by the symbols.

3.3 Cold acclimation altered Malpighian tubule fluid and ion secretion across a range of temperatures

Malpighian tubule function was measured via Ramsay assays at 0°C, 5°C, 10°C, and 23°C (n = 7-16 individuals per temperature per acclimation group). Exposure temperature and acclimation temperatures interacted to affect all assessments of Malpighian tubule function, including fluid secretion rates, ion concentrations in the secreted fluid, ion secretion rates, and the ratio of Na^+:K^+ secretion ($P < 0.05$ in all cases; see Table 3.2 for all two-way ANOVA results). Notably, the fluid secretion rates at 5°C, 10°C, and 23°C were significantly higher in cold-acclimated flies (Holm-Sidak test; $P = 0.0015$, $P = 0.0002$, $P < 0.0001$, respectively; Figure 3.3A) where the most pronounced difference was an 826% higher secretion rate at 10°C, the same temperature as the cold-acclimation temperature (Figure 3.3A). Cold acclimation altered both [Na^+] and [K^+] in the secreted fluid. For warm-acclimated flies, [Na^+] was relatively stable in the secreted fluid between 5°C and 23°C (~75 mM), but was elevated to 147 ± 19 mM at 0°C (Figure 3.3B). Cold-acclimated flies secreted fluid with lower [Na^+] relative to warm-acclimated flies at every tested temperature (Holm-Sidak test; $P = 0.0032$, $P = 0.0442$, $P = 0.0002$, $P = 0.0128$ for 0°C, 5°C, 10°C, and 23°C; Figure 3.3B), and never exceeded the [Na^+] measured at 23°C. In parallel, while [K^+] was stable in cold-acclimated flies throughout all exposure temperatures, [K^+] in the secreted droplets of warm-acclimated flies was significantly reduced at 0°C and 10°C in comparison to cold-acclimated flies (Holm-Sidak test; $P = 0.0004$, and $P = 0.0073$, respectively; Figure 3.3C). Changes in Na^+ and K^+ concentrations of the fluid secreted by Malpighian tubules can result from changes to fluid or ion secretion rates, or both. Rates of Na^+ secretion at 23°C were 49% higher in cold-acclimated flies, a significant difference (Holm-Sidak test; $P = 0.0030$; Figure 3.3A). Similar trends were noted at 5°C and 10°C where Na^+

secretion was increased by 86% and 115%, respectively, but these differences were not significant. The secretion rate of K^+ decreased at lower temperatures in both cold- and warm-acclimated flies. The rate of this decrease was much slower in cold-acclimated flies as K^+ secretion was lower in warm-acclimated flies at every exposure temperature, allowing for the maintenance of stable [K^+] secretion at lower temperatures (Holm-Sidak test; $P = 0.0060$, $P = 0.0018$, $P = 0.0002$, $P = 0.0022$, for 0°C, 5°C, 10°C, and 23°C, respectively; Figure 3.3B). In cold-acclimated flies, the ratio of Na^+:K^+ in the secreted fluid was maintained between 0.18 ± 0.10 at 10°C and 0.56 ± 0.10 at 23°C. In contrast, this ratio was highly disturbed in warm-acclimated flies, rising from 0.59 ± 0.16 at 23°C to 1.87 ± 0.43 at 0°C (Figure 3.3C). This ratio of Na^+:K^+ was significantly higher in warm-acclimated flies in comparison to cold-acclimated flies at 0°C and 10°C (Holm-Sidak test; $P = 0.0080$, $P = 0.0093$, respectively; Figure 3.3C) but not at 5°C or 23°C.

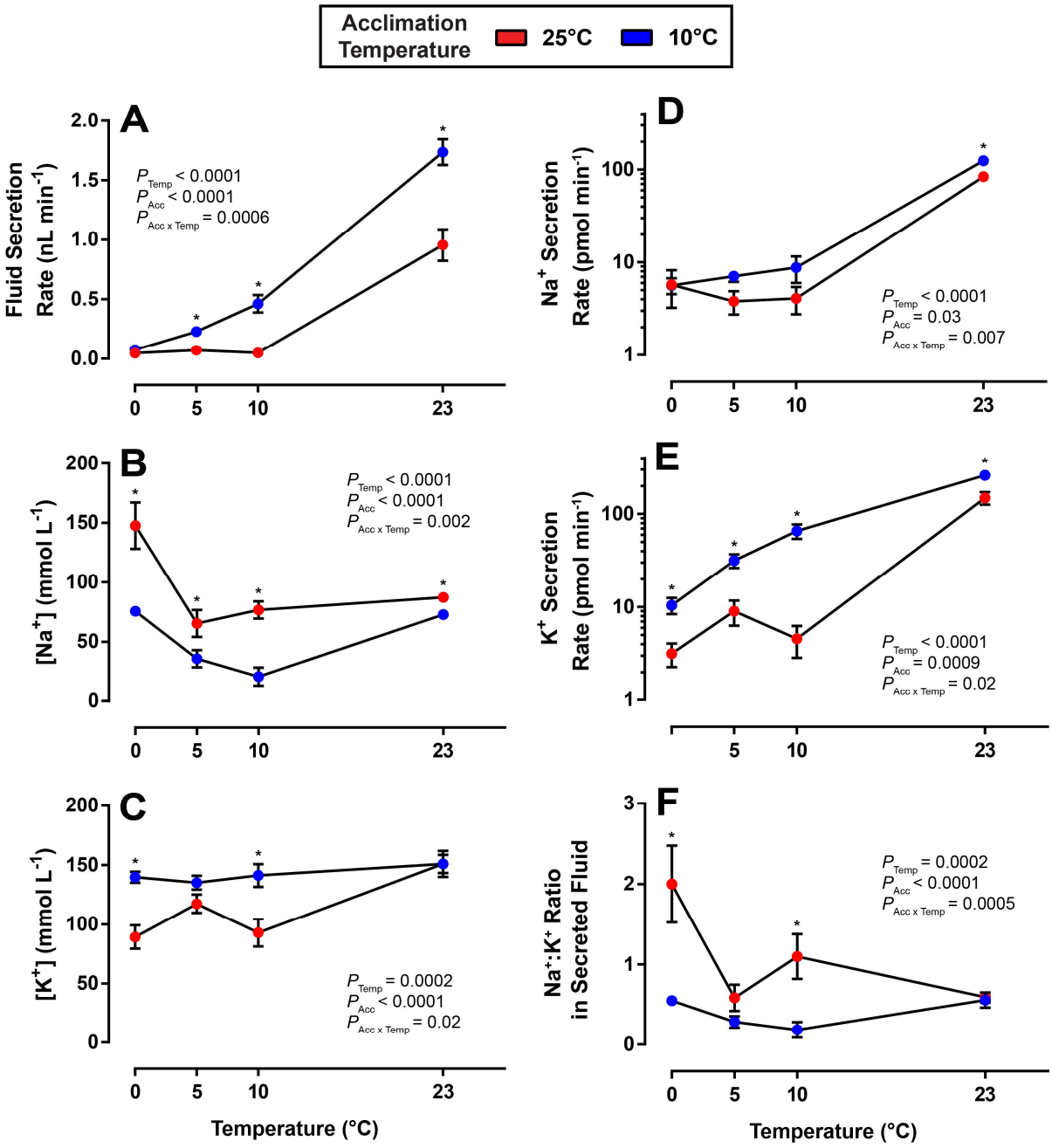

Figure 3.3. Cold acclimation altered Malpighian tubule function in female *D. melanogaster* across a variety of thermal conditions. (A) Malpighian tubule fluid secretion rate, (B) $[K^+]$ and (C) Na^+ in the secreted fluid, (D) Na^+ and (E) K^+ secretion rates, and (F) Na^+:K^+ secretion ratio assessed at 0°C, 5°C, 10°C, and 23°C in warm- (red) and cold-acclimated flies (blue). Bars represent mean ± SEM, and bars that are not clearly visible are obscured by the symbols. Asterisks denote significant differences between warm- and cold acclimation at the same exposure temperature (Holm-Sidak test; $P < 0.05$). Two-way ANOVAs were conducted on all three variables and the resulting *P*-values are embedded in each respective panel (see Table 3.1 for all two-way ANOVA results).

3.4 Cold-acclimation reduces rectal reabsorption of K^+

K^+ flux was assessed along the midgut and hindgut (ileum and rectum) of warm- and cold-acclimated flies using the scanning ion-selective electrode technique at 6°C and 23°C (Figure 3.4). No significant effect of acclimation temperature on K^+ flux was detected whether measurements from 6 sites along individual midguts were averaged (one-way ANOVA; P_{Acc} = 0.57; Figure 3.4A) or analyzed as individual sites (Holm-Sidak test; $P > 0.05$; n = 5-13 midguts per acclimation group per temperature; Figure 3.4A$_{1\text{-}2}$). A significant main effect of acclimation was seen in the ileum (P_{Acc} = 0.045; Figure 3.4B) as well as an interaction effect of acclimation and temperature (P = 0.016; Figure 3.4C). Lastly, in the rectum significant main effects were found for both temperature (P = 0.047; n =5-11 hindguts per acclimation group per temperature) and acclimation (P = 0.045), where K^+ flux was higher over the recta of warm-acclimated *D. melanogaster*.

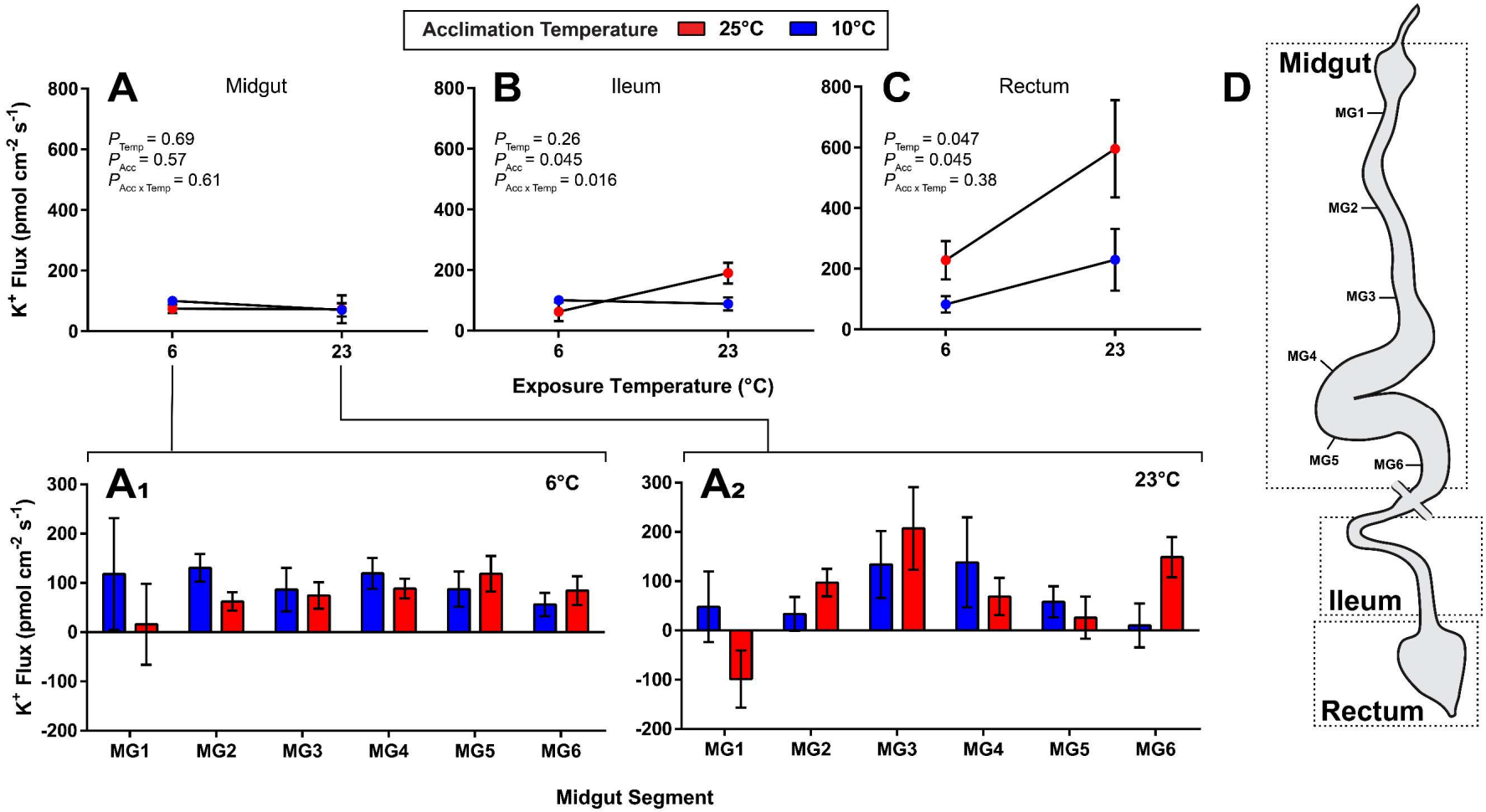

Figure 3.4. K^+ reabsorption is reduced in the rectums of cold-acclimated *D. melanogaster* females. (A) Mean K^+ flux in the midgut (average of six midgut sites; n = 5-13 flies per site per acclimation group per temperature), (B) ileum (n = 5-11 flies per acclimation group per temperature), and (C) rectum (rectal pads; n = 5-9 flies per acclimation group per temperature) of warm- (red) and cold-acclimated flies (blue). (D) Schematic diagram of alimentary canal illustrating sites of K^+ flux measurements. Midgut K^+ flux was measured at six equidistant sites along the midgut denoted by MG1 (anterior end) to MG6 (posterior end) at (A_1) 6°C and (A_2) 23°C. Bars represent means ± SEM.

3.5 Cold acclimation mitigated cold driven imbalances in K^+ transport

Temperature effects on K^+ transport in the Malpighian tubules and the recta were assessed via calculation of Q_{10} values in flies from both acclimation groups (see section 2.6). In warm acclimated flies, the Q_{10} of Malpighian tubule K^+ secretion was 4.7 and the Q_{10} of rectal K^+ flux was 1.8, resulting in a ΔQ_{10} of 2.7 (Figure 3.5). In contrast, in cold acclimated flies, the Q_{10} of Malpighian tubule K^+ secretion was 3.2 and the Q_{10} of rectal K^+ flux was 2.0, resulting in a ΔQ_{10} of 1.2 (Figure 3.5).

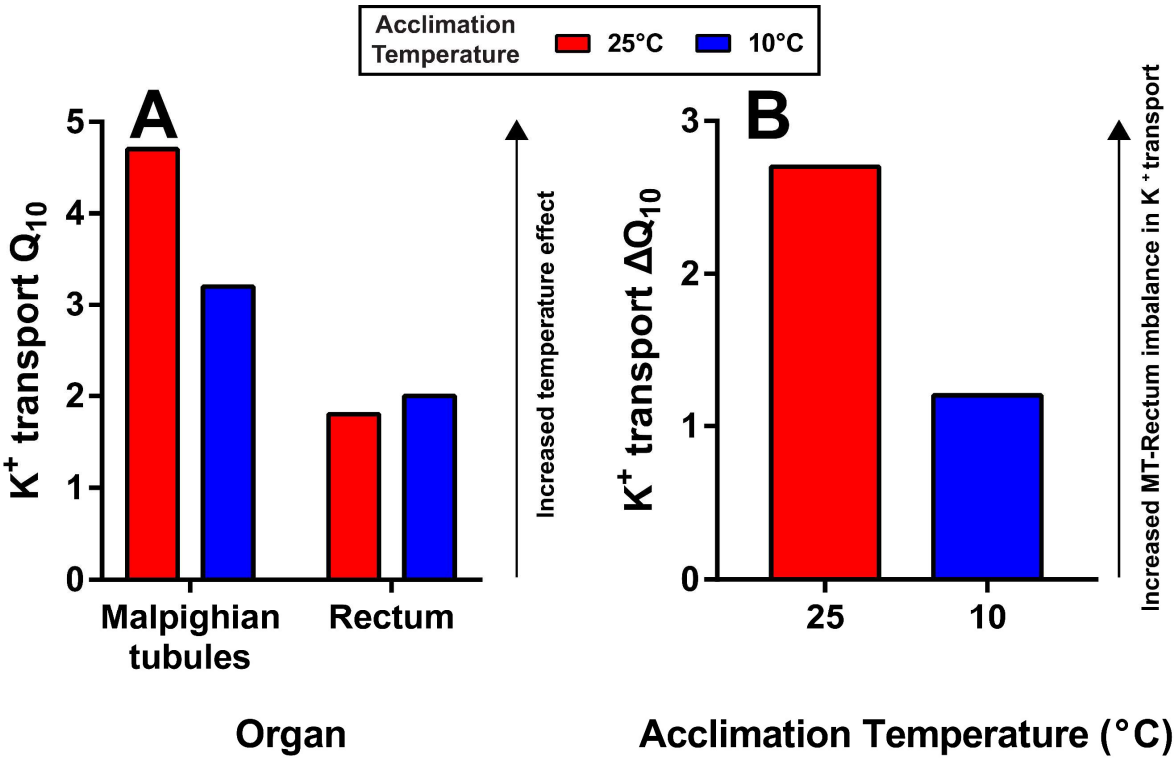

Figure 3.5. Warm acclimated *D. melanogaster* females experience a greater mismatch in K^+ transport between the Malpighian tubules and the rectum at low temperatures. (A) Q_{10} of K^+ transport of the Malpighian tubules and the rectum of warm and cold acclimated flies. Q_{10} represents the rate of change of K^+ transport following a 10°C increase in temperature. (B) ΔQ_{10} between Malpighian tubules and the rectum of warm and cold acclimated flies. ΔQ_{10} was calculated as the difference between the Q_{10} of Malpighian tubule and rectum K^+ transport and signifies the relative effect of temperature on each organ's K^+ transport. The degree of deviance of ΔQ_{10} from 0 signifies the degree of imbalance in the effect of temperature on the Malpighian tubules and the rectum. MT represents Malpighian tubules.

3.6 Cold acclimation decreased midgut and Malpighian tubule Na^+/K^+-ATPase and V-type H^+-ATPase activity

The enzymatic activity of Na^+/K^+-ATPase and V-type H^+-ATPase (relative to total protein) was assessed in the midgut, Malpighian tubules, and hindgut of warm- and cold-acclimated flies. Reductions in the maximal activity of both ATPases were noted in the Malpighian tubules and the midgut. Activity of Na^+/K^+-ATPase was 41% lower in the midgut (unpaired t-test; $P = 0.01$; n = 5), 53% lower in the Malpighian tubules (unpaired t-test; $P = 0.006$; n = 3) of cold-acclimated flies relative to those that were warm-acclimated. By contrast, no difference in Na^+/K^+-ATPase activity was noted between the acclimation groups in the hindgut (unpaired t-test; $P = 0.7$ n = 5; Figure 3.6A). In a similar pattern, V-type H^+-ATPase activity was 92% lower in the midgut (unpaired t-test; $P = 0.0004$; n = 3), 61% lower in the Malpighian tubules (unpaired t-test; $P = 0.01$; n = 5) and did not differ in the hindgut of cold-acclimated flies (unpaired t-test; $P = 0.9$; n = 5; Figure 3.6B).

Interestingly, differences in activity of both ATPases in the Malpighian tubules were largely driven by differences in protein content (unpaired t-test; $P = 0.01$; Figure 3.7A), despite a similar number of Malpighian tubules collected per group. When assessed independently of protein content, there was no significant difference in the enzymatic activity of either Na^+/K^+-ATPase (unpaired t-test; $P = 0.52$; Figure 3.7B) or V-type H^+-ATPase ($P = 0.32$) between flies from different acclimation temperatures. A similar comparison of total protein content revealed no significant differences in midguts (unpaired t-test; $P = 0.41$) or hindguts ($P = 0.30$) between flies from both acclimation groups.

To assess whether the difference in protein content may be driven by difference in Malpighian tubule size, the width and length of Malpighian tubules of flies from both

acclimation groups were assessed. Malpighian tubule length did not differ between the two acclimation groups (unpaired t-test; $P = 0.240$; Figure 3.8A), but the Malpighian tubules of cold-acclimated flies were significantly wider than those of warm-acclimated flies (unpaired t-test; $P = 0.007$; Figure 3.8B-C).

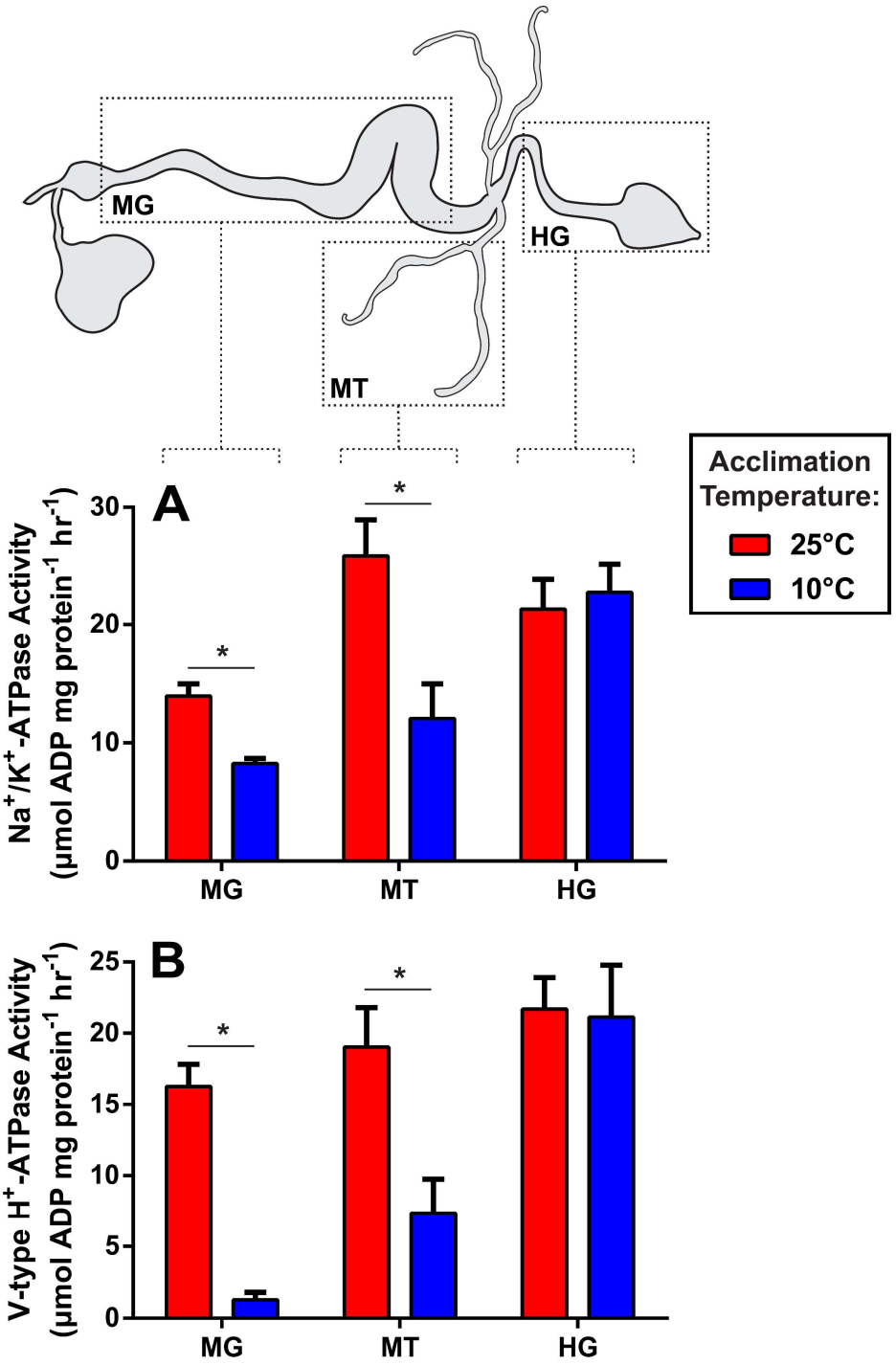

Figure 3.6. Cold-acclimation decreases the maximal activity of Na^+/K^+-ATPase and V-type H^+-ATPase in the midgut and Malpighian tubules of adult female *D. melanogaster*.
(A) Maximal enzymatic activity of Na^+/K^+-ATPase in the midgut (MG), Malpighian tubules (MT), and hindgut (HG) of warm- (red bars) and cold-acclimated (blue bars) flies. (B) Maximal enzymatic activity of V-type H^+-ATPase in the midgut, Malpighian tubules and hindgut in warm- and cold-acclimated flies. Bars represent mean ± SEM. Asterisks denote significant difference in enzymatic activity (unpaired t-tests; $P < 0.05$).

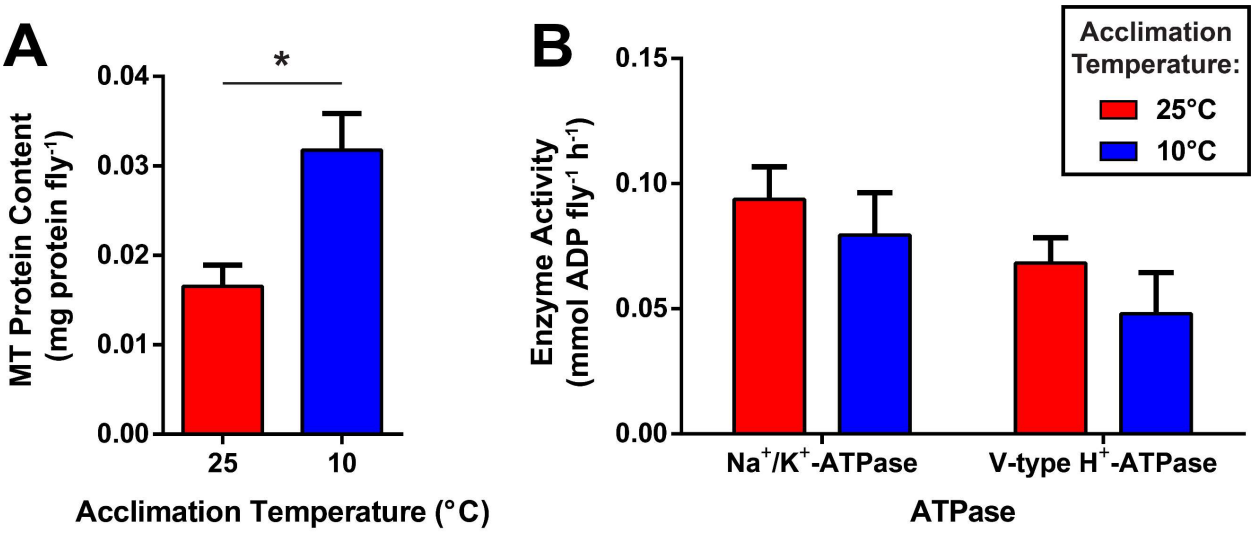

Figure 3.7. Differences in Malpighian tubule protein content underlie apparent changes in ion-motive ATPase activity in female *D. melanogaster*. (A) Malpighian tubule protein content per fly in warm- and cold-acclimated flies (n = 5 sets of Malpighian tubules from 30 flies per acclimation group). Cold-acclimated files had higher tubule protein content (unpaired t-test; P = 0.01). (B) Maximal enzymatic activity of Na^+/K^+-ATPase and V-type H^+-ATPase per fly in warm- and cold-acclimated flies. Maximal enzymatic activity did not differ between acclimation treatment (unpaired t-tests; P >0.3). Bars represent mean ± SEM. Asterisk denotes a significant difference.

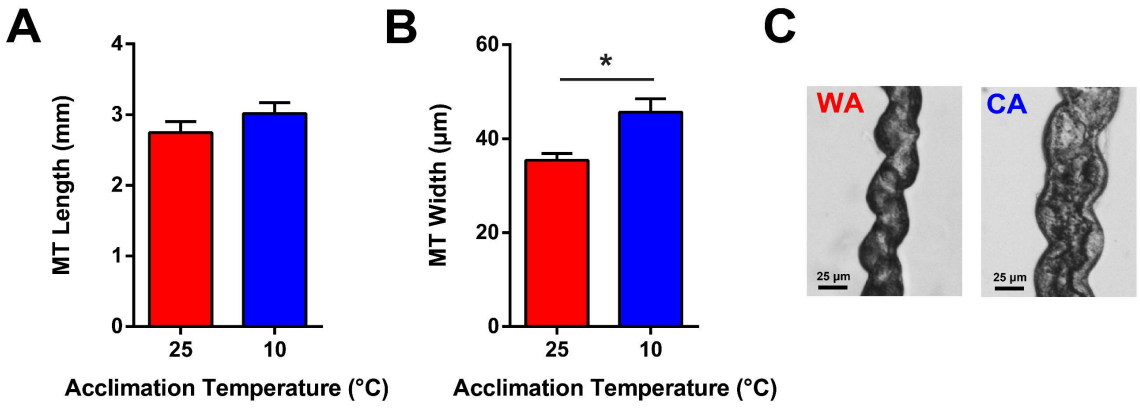

Figure 3.8. Cold-acclimated adult *D. melanogaster* females have wider Malpighian tubules of the same length. Malpighian tubule (A) length and (B) width assessed in warm- (WA) and cold-acclimated (CA) flies (n = 7 tubules per group). The Malpighian tubules of cold-acclimated flies are wider but not longer than those of warm-acclimated flies. (C) Example image of warm-acclimated (left) and cold-acclimated (right) Malpighian tubule illustrating alterations in MT size. Bars in panels A and B represent mean ± SEM. Asterisk denotes a significant difference (unpaired t-test; P < 0.01).

Table 3.1. Statistical results for all assessments described in sections 3.1, 3.2, 3.4, and 3.6. DF = degrees of freedom.

Trait	Variable	Statistical Test	Statistic (DF)	*P*-value	Related Figure
Survival	Cold exposure (0°C) time	Logistic regression	Z () = -13.59	**< 0.0001**	3.1A
	Acclimation temperature		Z () = -9.35	**< 0.0001**	
Critical thermal minimum (CT_{min})		Unpaired student's t-test	t (38) = 6.21	**< 0.0001**	3.1B
Chill-coma recovery time (CCRT)			t (34) = 7.90	**< 0.0001**	3.1C
Haemolymph [K^+], warm-acclimated flies	Cold exposure (0°C) time	Linear regression	R^2 (1, 6) = 0.77	**= 0.0045**	3.2A
Haemolymph [K^+], cold-acclimated flies			R^2 (1, 4) = 0.83	= 0.117	
Haemolymph [K^+]		ANCOVA	F (1, 10) = 27.24	**= 0.0004**	
Survival, warm-acclimated flies	Haemolymph [K^+]	Linear regression	R^2 (1, 6) = 0.83	**= 0.0180**	3.2B
Survival, cold-acclimated flies			R^2 (1, 4) = 0.63	**= 0.0117**	
Survival		ANCOVA	F (1, 10) = 20.44	**= 0.0011**	
Mean Midgut K^+ flux	Exposure temperature	2-way ANOVA	F (1, 20) = 0.19	= 0.67	3.4A
	Acclimation temperature		F (1, 20) = 0.33	= 0.57	
	Exposure x Acclimation temperature		F (1, 20) = 0.20	0.61	
Mean Ileum K^+ flux	Exposure temperature		F (1, 36) = 1.30	= 0.26	3.4B
	Acclimation temperature		F (1, 36) = 4.31	**= 0.045**	
	Exposure x Acclimation temperature		F (1, 36) = 6.37	**= 0.016**	
Mean Rectum K^+ flux	Exposure temperature		F (1, 32) = 4.29	**= 0.047**	3.4C
	Acclimation temperature		F (1, 32) = 4.33	**= 0.045**	

Trait	Variable	Statistical Test	Statistic (DF)	*P*-value	Related Figure
Mean Rectum K^+ flux	Exposure x Acclimation temperature	2-way ANOVA	F (1, 32) = 0.80	= 0.38	3.4C
Midgut K^+ flux, MG1, 6°C	Acclimation temperature	Holm-Sidak multiple comparison test	t (64) = 1.48	= 0.60	$3.4A_1$
Midgut K^+ flux, MG2, 6°C			t (64) = 0.94	= 0.88	
Midgut K^+ flux, MG3, 6°C			t (64) = 0.16	= 0.99	
Midgut K^+ flux, MG4, 6°C			t (64) = 0.41	= 0.99	
Midgut K^+ flux, MG5, 6°C			t (64) = 0.42	= 0.99	
Midgut K^+ flux, MG6, 6°C			t (64) = 0.38	= 0.99	
Midgut K^+ flux, MG1, 23°C			t (126) = 1.92	= 0.30	$3.4A_2$
Midgut K^+ flux, MG2, 23°C			t (126) = 0.87	= 0.81	
Midgut K^+ flux, MG3, 23°C	Acclimation temperature	Holm-Sidak multiple comparison test	t (126) = 0.96	= 0.81	$3.4A_2$
Midgut K^+ flux, MG4, 23°C			t (126) = 0.78	= 0.81	
Midgut K^+ flux, MG5, 23°C			t (126) = 0.42	= 0.81	
Midgut K^+ flux, MG6, 23°C			t (126) = 1.75	= 0.35	
Na^+/K^+-ATPase activity, midgut	Acclimation temperature	Unpaired student's t-test	t (6) = 4.09	**= 0.0064**	3.6A
Na^+/K^+-ATPase activity, Malpighian tubules			t (8) = 3.28	**= 0.0113**	
Na^+/K^+-ATPase activity, hindgut			t (8) = 0.41	= 0.6933	
V-type H^+-ATPase activity, midgut			t (6) = 7.10	**= 0.0004**	3.6B
V-type H^+-ATPase activity, Malpighian tubules			t (8) = 3.17	**= 0.0132**	
V-type H^+-ATPase activity, hindgut			t (8) = 0.14	= 0.8953	
Malpighian tubule protein content per fly			t (8) = 3.21	**= 0.0124**	3.7A
Na^+/K^+-ATPase activity per fly, Malpighian tubules			t (8) = 0.67	= 0.5206	3.7B

Trait	Variable	Statistical Test	Statistic (DF)	*P*-value	Related Figure
V-type H^+-ATPase activity per fly, Malpighian tubules	Acclimation temperature	Unpaired student's t-test	t (8) = 1.05	= 0.3239	3.7B
Malpighian tubule length			t (12) = 1.24	= 0.2401	3.8A
Malpighian tubule width			t (12) = 3.23	**= 0.0072**	3.8B

Table 3.2. Results of two-way ANOVAs assessing the effects of acclimation and exposure temperatures on fluid secretion rate, $[Na^+]$ in secreted fluid, $[K^+]$ in secreted fluid, Na^+ secretion rate, K^+ secretion rate, and the ratio of Na^+: K^+ in the secreted fluid. DF = degrees of freedom.

Trait	Variable	Statistic (DF)	*P*-value
Secretion Rate	Acclimation temperature x Exposure temperature	F (3, 70) = 6.6	**= 0.0006**
	Exposure temperature	F (3, 70) = 91.6	**< 0.0001**
	Acclimation temperature	F (1, 70) = 21.5	**< 0.0001**
$[Na^+]$ in Secreted Fluid	Acclimation temperature x Exposure temperature	F (3, 66) = 5.7	**= 0.0016**
	Exposure temperature	F (3, 66) = 22.5	**< 0.0001**
	Acclimation temperature	F (1, 66) = 54.2	**< 0.0001**
$[K^+]$ in Secreted Fluid	Acclimation temperature x Exposure temperature	F (3, 67) = 3.7	**= 0.0157**
	Exposure temperature	F (3, 67) = 7.6	**= 0.0002**
	Acclimation temperature	F (1, 67) = 17.3	**< 0.0001**
Na^+ Secretion Rate	Acclimation temperature x Exposure temperature	F (3, 68) = 4.5	**= 0.0065**
	Exposure temperature	F (3, 68) = 117.1	**< 0.0001**
	Acclimation temperature	F (1, 68) = 5.1	**= 0.0265**
K^+ Secretion Rate	Acclimation temperature x Exposure temperature	F (3, 69) = 3.3	**= 0.0239**
	Exposure temperature	F (3, 69) = 57.0	**< 0.0001**
	Acclimation temperature	F (1, 69) = 12.1	**= 0.0009**
Na^+:K^+ Ratio in Secreted Fluid	Acclimation temperature x Exposure temperature	F (3, 66) = 6.8	**= 0.0005**
	Exposure temperature	F (3, 66) = 7.7	**= 0.0002**
	Acclimation temperature	F (1, 66) = 28.3	**< 0.0001**

4. Discussion

4.1 Cold acclimation mitigates hyperkalemia and improves the cold tolerance of female *D. melanogaster*

Cold acclimation improves the cold tolerance of *D. melanogaster* females and mitigates the degree of cold-induced hyperkalemia. Specifically, cold acclimated flies entered chill coma at a lower temperature (lower CT_{min}), recovered faster from a chill coma (lower CCRT), and survived longer at 0°C (Figure 3.1A-C). These results are consistent with improvements in chill tolerance (CCRT, CT_{min}, and survival) in a variety of insects following cold acclimation, including *D. melanogaster* (Andersen et al., 2017a; Koštál et al., 2011; Overgaard et al., 2008; Ransberry et al., 2011). As previously found in firebugs, crickets, cockroaches, and fruit flies, cold-induced haemolymph $[K^+]$ elevations were greatly mitigated following cold acclimation (Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2015c). Mitigated cold-induced hyperkalemia is consistent with the observed improvements in CCRT and survival, that have consistently been related to the prevention of cold-induced hyperkalemia (Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2015a; Yerushalmi et al., 2016). Thus, this population of *D. melanogaster* responds to a cold acclimation treatment similarly to previous studies on this species (with improved chill tolerance and mitigated cold-induced hyperkalemia). I therefore assessed whether the ionoregulatory organs of *D. melanogaster* are involved in facilitating this preservation of K^+ balance.

While CT_{min} is unrelated to the degree to which haemolymph $[K^+]$ is disturbed in the cold, its reduction illustrates that multiple interacting mechanisms work together in flies beyond simply the prevention of cold-induced hyperkalemia, to result in ecologically beneficial whole-body phenotypes. Since CT_{min} is thought to be determined by cold-induced depolarizations, this

change suggests that the V_m of cold-acclimated flies is less sensitive to cold-induced depolarizations. In fact, this was found to be the case in cold acclimated locusts and cold tolerant *Drosophila* species, since both experience reduced cold-induced muscle depolarizations (Andersen et al., 2015b; Andersen et al., 2017a). In chill-susceptible insects it is thought that muscle depolarization results from the reduction of Na^+/K^+-ATPase activity in the cold, reducing the electrogenic contribution (V_e) to V_m (see section 1.2.1). Cold-acclimated flies may overcome this issue, for example, by reducing their reliance of V_e for the determination of their resting membrane potential or by increasing Na^+/K^+-ATPase activity to retain the functionality of this pump in the cold.

As previously found between *Drosophila* species of varying cold tolerance, haemolymph $[K^+]$ elevations are dramatically reduced (Figure 3.2). In the current study, when compared at a similar survival rate of ~0.54, warm-acclimated flies experienced a ~340% elevation in haemolymph $[K^+]$ while cold-acclimated flies experienced a reduction of ~3% in haemolymph $[K^+]$. This finding is also consistent with a previous study on *D. melanogaster* acclimated at 15°C that similarly demonstrated reduced cold-induced hyperkalemia (MacMillan et al., 2015a). This raises an important question: does cold-induced hyperkalemia kill cold-acclimated flies or is the eventual rise in haemolymph $[K^+]$ merely a consequence of K^+ leak from cells that have died of other causes? The evidence from the current study supports the latter, as no haemolymph $[K^+]$ change was seen at 54% survival, suggesting that an alternative mechanism leads to cold-related death prior to the onset of hyperkalemia. However, further studies are required to delineate this process.

4.2 Physiological plasticity of the Malpighian tubules improves K^+ clearance

In insects, the Malpighian tubules are responsible for the formation of the primary urine and act as the main excretory organ, and thus play a central role in organismal iono- and osmoregulation (Larsen et al., 2014). We used Ramsay assays and measured secreted fluid ion concentrations with ion-selective microelectrodes to assess temperature effects on fluid, Na^+, and K^+ secretion following thermal acclimation. As predicted, the Malpighian tubules of cold-acclimated flies maintained K^+ secretion rates at low temperatures better than those from warm-acclimated flies, which would facilitate K^+ clearance in the cold (Figure 3.3E). This resulted in preserving the $[Na^+]$:$[K^+]$ ratio of the secreted fluid from tubules of cold-acclimated flies, while warm-acclimated flies experienced a 4-fold increase in the $[Na^+]$:$[K^+]$ ratio (Figure 3.3F). Reduced K^+ secretion in the tubules of warm-acclimated flies would reduce their capacity for K^+ clearance and thus likely contribute to the accumulation of haemolymph $[K^+]$.

The Malpighian tubules are energized by temperature-sensitive ATPases, and the basal rate of fluid secretion across the Malpighian tubules dramatically decreases in the cold (MacMillan et al., 2015d; Ramsay, 1954). In an apparent compensatory response, following one week at 10°C, the fluid secretion rate of tubules from cold-acclimated flies was elevated at 5°C, 10°C, and 25°C, but not at 0°C, relative to warm-acclimated flies (Figure 3.3A). For instance, the secretion rate of tubules from cold-acclimated flies increased by a factor of ~9 from 0.05 nL min^{-1} to 0.46 nL min^{-1} at their acclimation temperature of 10°C. These rates are still below those of tubules from warm-acclimated flies at room temperature (~50%) demonstrating that exposure to 10°C for 1-week results in partial compensation of fluid secretion rates. However, at 0°C, the temperature used for the analysis of CCRT and chill survival, there was no difference in fluid secretion rates but ion secretion rates differed.

To my knowledge, this is the first assessment of the role of Malpighian tubules in *D. melanogaster* cold acclimation, but both MacMillan *et al.* (2015d) and Andersen *et al.* (Andersen et al., 2017b) assessed the role of the tubules in cold tolerance among five *Drosophila* species raised under common garden conditions (21-22°C). Together, these results suggest that thermal acclimation and adaptation may work through shared or similar physiological mechanisms in the Malpighian tubules. Both cold adaptation and acclimation reduce (or entirely prevent) haemolymph $[K^+]$ disturbance in the cold (MacMillan et al., 2015d; MacMillan et al., 2017). In both cases more cold tolerant flies better maintained tubule K^+ secretion and consequently the ratio of $[Na^+]$:$[K^+]$ while warm adapted or acclimated flies experienced preferential secretion of Na^+ over K^+ (Andersen et al., 2017b; MacMillan et al., 2015d). In addition to the direct removal of haemolymph K^+, maintenance of the $[Na^+]$:$[K^+]$ ratio likely also mitigates hyperkalemia by better preserving haemolymph volume via the retention of Na^+ ions which act as key haemolymph osmolytes (MacMillan et al., 2015a). Thus, chill tolerance in cold adapted and acclimated flies appears to be at least partially improved by maintained tubule K^+ secretion at low temperature. Lastly, in contrast to the present study, recent work by Des Marteaux et al. (2018) found that cold acclimation reduces Malpighian tubule fluid secretion rates in crickets, suggesting that mechanisms of thermal acclimation may vary between different chill-susceptible insects.

4.3 Rectal K^+ reabsorption is lower in cold-acclimated flies

Whereas the Malpighian tubules serve as the primary site for ion and fluid excretion into the gut, the midgut and hindgut absorb ions and water from the gut into the haemolymph (D'Silva et al., 2017; Larsen et al., 2014). As such, I hypothesized that reduced K^+ absorption in the cold across these epithelia would alleviate cold-induced hyperkalemia. To assess K^+

absorption across the gut I utilized the scanning ion selective electrode technique to measure K^+ flux at 6°C and 23°C across the midgut and hindgut (ileum and rectum). No difference in midgut K^+ flux was found between acclimation treatments in all six assessed sites at 6°C (Figure 3.4A_1) or 23°C (Figure 3.4A_2). Additionally, neither acclimation nor exposure temperature impacted mean midgut K^+ flux (Figure 3.4A). In contrast to the midgut, differences in K^+ flux were observed in the hindgut. Temperature had no effect on K^+ flux at the ileum of cold-acclimated flies but ileal K^+ flux of warm-acclimated flies was higher at 23°C compared to 6°C (Figure 3.4B). The most pronounced effect of both acclimation and exposure temperatures was observed in the rectum, where K^+ flux in the recta of cold-acclimated flies was significantly lower than that of warm-acclimated flies at both temperatures (Figure 3.4C). Additionally, K^+ flux at the rectum was reduced at 6°C regardless of acclimation group and this is consistent with reduced metabolic demand in the rectum of grasshoppers in the cold (Palazzo and August, 1997). The K^+ flux across rectal pads of five *Drosophila* species was also reduced in low temperatures regardless of the degree of chill tolerance of the species (Andersen et al., 2017b). The reduction of rectal K^+ reabsorption at low temperatures likely assists in preventing hyperkalemia and is thus consistent with the hypothesis of the current study that cold acclimation mitigates hyperkalemia by reducing net K^+ uptake in the main ionoregulatory epithelia of *D. melanogaster*.

4.4 Haemolymph K^+ balance depends on the integrated functions of the Malpighian tubule and the rectum

Under homeostatic conditions, the Malpighian tubules and rectum, as the primary sites of K^+ transport, must work in a synchronous and complementary manner to maintain organismal K^+ balance. Thus, to estimate the degree of cold-induced disruption to whole body K^+ balance, a

comparison between their relative inhibition at low temperature is informative; a mismatch in the effects of temperature on these two organs would lead to an imbalance in haemolymph [K^+] in the cold. In warm acclimated flies, the Q_{10} of Malpighian tubule K^+ secretion was 4.7 and the Q_{10} of rectal K^+ flux was 1.8, resulting in a ΔQ_{10} of 2.7 (Figure 3.5). Hence the cold had a higher effect on Malpighian tubule function than on the rectum. In contrast, in cold acclimated flies, the Q_{10} of Malpighian tubule K^+ secretion was 3.2 and the Q_{10} of rectal K^+ flux was 2.0, resulting in a ΔQ_{10} of 1.2 (Figure 3.5). Therefore, even with cold acclimation, the cold has a greater effect on Malpighian tubule function compared to the rectum; however, cold acclimation reduces the imbalance in K^+ transport between these two organs such that K^+-clearance is more severely affected in the cold, in comparison to rectal K^+ absorption, in warm acclimated flies compared to cold acclimated flies. As a result, I would expect that K^+ would accumulate in the haemolymph of warm acclimated flies more rapidly, as observed in the current study. These results are also informative in that they show that the Malpighian tubules are more temperature sensitive than the rectum and exhibit a greater degree of adjustment to K^+ transport following cold acclimation both in terms of thermal sensitivity and in absolute K^+ transport rates. This supports the idea that reduced Malpighian tubule K^+ clearance is a central problem for *D. melanogaster* at low temperatures, as is the case for crickets (Des Marteaux et al., 2018), and that its preservation is thus beneficial to the development of chill tolerance.

4.5 The effects of cold acclimation on ion transport are independent of V-type H^+- and Na^+/K^+-ATPase activity.

I hypothesized that cold acclimation would mitigate the loss of ion balance through the alteration of active ion transport in ionoregulatory organs. To test this, I measured the activities

of V-type H^+-ATPase and Na^+/K^+-ATPase in isolated midguts, Malpighian tubules, and hindguts of warm- and cold-acclimated flies.

In *D. melanogaster*, an apically-located V-type H^+-ATPase is the primary driver of Malpighian tubule fluid secretion, while a basolateral Na^+/K^+-ATPase contributes 10-19% of fluid secretion and is responsible for increasing the $[K^+]$:$[Na^+]$ ratio in the secreted fluid (Linton and O'Donnell, 1999). Thus, to account for increased Malpighian tubule fluid secretion and the preservation of K^+ secretion over a variety of temperatures, I expected that the activities of both ion-motive ATPases would increase in cold-acclimated flies. Contrary to this hypothesis, however, the activity of both ATPases significantly decreased in cold-acclimated flies when compared to their warm-acclimated counterparts when these activities were standardized by total protein content of tubules (Figure 3.7A-B). However, when ATPase activities were expressed per individual organ, no significant differences in activities were found (Figure 3.7B). Each sample contained the same number of tubules, so I measured Malpighian tubule size and found that cold-acclimated tubules are significantly wider than warm-acclimated tubules (Figure 3.8A-C). Similar measurements of the Malpighian tubules revealed no such size differences in chill tolerant *Drosophila* species when corrected for total body mass (Andersen et al., 2017b). Currently, it is unclear whether this increased tubule width in cold-acclimated flies stems from hypertrophy, hyperplasia, or simply the enlargement of the Malpighian tubule lumen, and whether this size difference is indeed the cause of increased protein content. Further studies are also required to elucidate whether these morphological changes have any functional relevance. Regardless, I found no evidence to support the hypothesis that functional changes in ion transport would be mediated by changes in ion-motive ATPase activity, suggesting that an alternative mechanism is responsible for the functional differences (e.g. increased fluid secretion

and K^+ transport rates) that were observed in the Malpighian tubules of cold-acclimated flies (discussed below). This is of particular interest, because in crickets, Na^+/K^+-ATPase activity increased in the Malpighian tubules despite a decrease in fluid secretion in the cold (Des Marteaux et al., 2018).

At the ureter-gut junction, Malpighian tubule and midgut contents mix prior to their entry into the hindgut, where ions are actively reabsorbed into the haemolymph, resulting in concentrated excreta (Larsen et al., 2014). Unlike the Malpighian tubules, however, our basic mechanistic understanding of insect rectal function is weak. In cockroaches, locusts, and flies, the main site of ion reabsorption occurs at areas of thickened epithelia known as rectal pads (Larsen et al., 2014). Studies of ion transport across locust rectum suggest that an apical V-type H^+-ATPase at least partially energizes epithelial ion transport (Gerencser and Zhang, 2003; Phillips et al., 1996). Na^+/K^+-ATPase is also highly expressed in *D. melanogaster* hindgut, second in abundance only to the Malpighian tubules, and has been localized to the basolateral membrane in *A. aegypti*, yet its function remains unknown (Chintapalli et al., 2007; Patrick et al., 2006). As is the case in crickets (Des Marteaux et al., 2018), the activities of Na^+/K^+- and V-type H^+-ATPase in the hindgut did not differ between the acclimation treatments in the current study, suggesting an alternative mechanism is responsible for altered K^+ flux across the ileum and rectum. As with the tubules and hindgut, a mismatch also exists between ion-motive ATPase activity and midgut K^+ flux. While mean midgut K^+ flux did not change with acclimation treatment, both Na^+/K^+- and V-type H^+-ATPases decreased in activity.

Cumulatively, these misalignments between ion-motive ATPase activity and ion transport suggest that other mechanisms affect epithelial transport following cold acclimation. Such alternative mechanisms may include: (1) changes to the plasma membrane environment known

to affect key transport proteins such as Na^+/K^+-ATPase (reviewed by Hazel (1995)), (2) changes in paracellular permeability which may mediate cold-induced ion leak in the cold (Andersen et al., 2017b; MacMillan et al., 2017), (3) changes in endocrine control of ion- and osmoregulation (Terhzaz et al., 2015), (4) changes in mitochondrial ATP production in the cold (Colinet et al., 2017) or (5) changes in the thermal sensitivity of Na^+/K^+- or V-type H^+-ATPase. Since all enzyme activity assays in this study were conducted at 25°C, the thermal sensitivity of these enzymes was not determined here. However, MacMillan (2015b) previously showed that no difference in the thermal sensitivity of Na^+/K^+-ATPase exists following cold-acclimation in *D. melanogaster*. The thermal sensitivity of V-type H^+-ATPase following cold acclimation remains a possibility that should be assessed in future studies.

5. Conclusions

As previously demonstrated in a variety of chill-susceptible insects, cold acclimation led to reduced CT_{min}, faster recovery from a chill coma, and reduced degree of cold-induced increases in haemolymph $[K^+]$ in *D. melanogaster*. This improvement in haemolymph K^+ balance in the cold coincided with increased Malpighian tubule K^+ and fluid secretion rates at low temperatures. In parallel, reabsorption of K^+ was reduced in the rectum but unchanged in the midgut of cold-acclimated flies in comparison to warm-acclimated flies. Together, these changes illustrate that cold-acclimated flies have a greater capacity for K^+ clearance than warm-acclimated flies in the cold and support an important role for these ionoregulatory organs in the prevention of cold-induced hyperkalemia following cold acclimation. Furthermore, measurement of the activities of Na^+/K^+-ATPase and V-type H^+-ATPase revealed no clear link to K^+ transport across the midgut, Malpighian tubules, or hindgut, suggesting that modulation of these organs following cold acclimation is mediated through an alternative mechanism. These results lend

support to the role of plasticity of the Malpighian tubule and the rectum in the cold acclimation of chill-susceptible insects, and the independence of this functional plasticity to the modulation of Na^+/K^+-ATPase and V-type H^+-ATPase.

6. Appendix A: Assessment of cold tolerance of the adult arboviral disease vector mosquito, *Aedes aegypti*

6.1 Introduction

The mosquito *Aedes aegypti* resides in tropical and subtropical regions where it serves as an arboviral disease vector for Zika, Chikungunya, yellow fever, and dengue (Aitken et al., 1979; Bhatt et al., 2013; Gonzales et al., 2015; Kraemer et al., 2015). The global distribution of *A. aegypti* is closely related to environmental temperatures (Brady et al., 2013; Brady et al., 2014; Kraemer et al., 2015), and thus particularly with current climate change models predicting increases in average global temperatures and a greater frequency of extreme thermal events (Easterling, 2000; Williams et al., 2015), there is growing interest in understanding the thermal limitations of *A. aegypti*.

Low temperatures have been shown to affect all stages of the *A. aegypti* life cycle, adversely affecting a variety of life history traits including development rate, reproductive success, and survival (Carrington et al., 2013; Davis, 1931; Rueda et al., 1990; Thomas et al., 2012; Tun-Lin et al., 2000; Yang et al., 2009). The effect of temperature on *A. aegypti* success is quite pronounced, as survival from egg to adult drops from 92% at 20°C to only 3% at 15°C (Rueda et al., 1990). Of the different life stages of *A. aegypti*, the eggs are the most tolerant to cold, surviving and hatching following cold exposures up to 24 h at -2°C or 1 h at -17°C (Davis, 1931; Thomas et al., 2012). Accordingly, *A. aegypti* have been documented to successfully overwinter as far north as Washington D.C. and Indiana (Hawley et al., 1989; Lima et al., 2016). Like other poikilotherms, larval *A. aegypti* experience slowed development, delayed and decreased pupation, and increased mortality with decreasing temperatures (Brady et al., 2014; Carrington et al., 2013; Tun-Lin et al., 2000; Yang et al., 2009). Overall development time from

egg to adult is reduced 5.5 times with a 20°C change in temperature ($Q_{10} \sim 2.3$) from 7.2 days at 35°C to 39.7 days at 15°C. Lastly, adult *A. aegypti* experience increased mortality, decreased oviposition rate, and overall reduced fecundity at 15°C (Tun-Lin et al., 2000). To date, studies of temperature effects on *A. aegypti* have largely focused on consequences to reproductive success, with little work assessing the only life stage capable of disease transmission – the adult. Here, I assess the cold tolerance of adult *A. aegypti* and investigate whether and how it is affected by (1) cold acclimation, (2) sex, (3) and blood-feeding.

Since mosquitoes succumb to low temperature exposures prior to the onset of freezing of their bodily fluids, they are considered chill-susceptible insects. Notably, however, great variation in thermal tolerance exists within chill-susceptible insects and can stem from evolutionary adaptation, seasonal variation, long-term acclimation, or even acute exposure to low temperatures (rapid cold-hardening) (Chown and Terblanche, 2006; Hoffmann et al., 2003; Kelty and Lee Jr, 2001; Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2015a). To date, the capacity for thermal plasticity at low temperature (cold acclimation) has been demonstrated in many chill-susceptible insects including fruit flies, cockroaches, and crickets (Koštál et al., 2006; MacMillan et al., 2012; Yerushalmi et al., 2018). Cold acclimation affects a variety of phenotypes in chill susceptible insects including decreased temperature of coma onset (CT_{min}), decreased time to recover following removal from a cold exposure (CCRT), and increased survival following a cold exposure (Coello Alvarado et al., 2015; Gibert and Huey, 2001; Koštál et al., 2004). While little research has been conducted on the effect of cold acclimation in *A. aegypti*, studies of the Asian tiger mosquito, *Aedes albopictus* (Shepard et al., 2009) have demonstrated increased cold tolerance in eggs following cold acclimation (Hanson and Craig, 1994). Furthermore, this response to cold acclimation was only found in temperate populations

of *A. albopictus* and not tropical populations, and is thought to contribute to the northward expansion of the species' range (Hanson and Craig, 1994; Hanson and Craig, 1995; Rochlin et al., 2013; Romi et al., 2006).

In addition to the effect of cold acclimation, the sex-specificity of cold tolerance in *A. aegypti* is important to understand, particularly with respect to disease transmission which is solely done by the blood feeding females. To date, studies of the effect of sex on insect cold tolerance have yielded mixed results with variability between different species. For example, while studies of the wheat weevil (*S. granaries*) and the red flour beetle (*T. castaneum*) showed better cold tolerance in males (Edwards, 1958; Fields et al., 1998), studies of fruit flies (*D. melanogaster* and *D. suzukii*) and beetles (*C. pusillus* and *C. ferrugineus*) demonstrated greater cold tolerance in females (Jakobs et al., 2015; Jean David et al., 1998; Kawamoto, 1989; Williams, 1954).

In this study, I also assess the effect of blood-feeding on the cold tolerance of *A. aegypti*. In my previous work, I showed that both acute and chronic dietary salt stress can impact the cold tolerance of *D. melanogaster* (Yerushalmi et al., 2016). Blood feeding is similar to dietary salt supplementation in that it is also an ionic load, yet unlike salt supplementation, blood is regularly consumed by female *A. aegypti*, and thus its impact as an ecologically relevant challenge on the cold tolerance of *A. aegypti* is of interest. In contrast to the immediate effects of low temperatures, prolonged cold exposure (that may lead to injury or death) is often accompanied by large disruptions to ion and water homeostasis (Andersen et al., 2013; Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b; MacMillan et al., 2015c). When chill susceptible insects are exposed to low temperatures, Na^+ leaks down its concentration gradient, away from the haemolymph and into the gut (Koštál et al., 2004; MacMillan and Sinclair,

2011b). Since Na^+ is a major haemolymph osmolyte in many insects, water passively follows into the gut, leading to an overall reduction in haemolymph water content (Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2012). This reduction in haemolymph volume in addition to proposed K^+ leak from tissues (Andersen et al., 2013; Des Marteaux and Sinclair, 2016; Findsen et al., 2013; MacMillan et al., 2015a) leads to elevated concentrations of K^+ in the haemolymph (Koštál et al., 2004; Koštál et al., 2006; MacMillan et al., 2015c; Yerushalmi et al., 2016). The intake of a blood meal affects mosquito ionoregulation in a variety of ways which may alter their cold tolerance. First, the ingestion of a blood meal, greater in weight to their own, presents an ionoregulatory challenge for *A. aegypti* which occurs largely in two stages: an initial Na^+ load from the blood plasma followed by a surge of K^+ from lysed erythrocytes (Clements, 1992; Pacey and O'Donnell, 2014; Williams et al., 1983). Second, blood meals also increase the permeability of the midgut, where for example, mice antibody molecules were found in the haemolymph following a blood meal (Hatfield, 1988). Both acute dietary salt intake (both NaCl and KCl) and increased gut paracellular permeability have been previously shown to increase CCRT in *D. melanogaster* by Yerushalmi et al. (2016) and MacMillan et al. (2017), respectively. In addition to these effects, however, diuresis begins immediately following the ingestion of a blood meal, peaking within minutes of blood meal intake and remaining elevated for about 30-60 mins (Stobbart, 1977; Williams et al., 1983). Excretion is primarily driven by the activation of the Malpighian tubules via various diuretic hormones (Coast, 2009). The current thesis demonstrates that increased Malpighian tubule activity is associated with better CCRT as shown in cold-acclimated *D. melanogaster* (Yerushalmi et al., 2018). While the combination of these proposed effects may act in interacting and opposing ways to lead to the observed phenotype, I hypothesize that the effect of blood-feeding will depend on the stage of the digestion of a blood

meal. During the initial phase, I hypothesize that blood feeding will speed up chill coma recovery by (1) reducing Na^+ leak into the gut (via the alteration of the gut Na^+ gradient) and (2) increasing Malpighian tubule K^+ secretion. In contrast, following the lysis of erythrocytes, I hypothesize that increased K^+ leak into the gut will result in gradual increases in chill coma recovery time with increased time following a blood meal.

Thus, in the present study I aim to build onto current knowledge of *A. aegypti* cold tolerance by measuring the effects of temperature acclimation, sex, and blood feeding on the cold tolerance of adult *A. aegypti*. I quantify cold tolerance via three main cold tolerance parameters: critical thermal minimum (CT_{min}), chill coma recovery time (CCRT), and survival following cold exposure.

6.2 Methods

The *Aedes aegypti* population used in the current study was previously described by Durant and Donini (2018) and was reared at York University as previously described by Misyura et al. (2017) with slight modifications. Eggs were hatched in 2 L of dechlorinated tap water and fed 6 mL of a premade food solution composed of 1.8 g liver powder and 1.8 g of inactive yeast in 500 mL of reverse-osmosis water daily. The population was kept at room temperature (22±1°C) with a 12:12 h light:dark cycle. Water was changed every four days. Pupa were isolated daily and given 1-2 days to mature prior to the placement of 40 ± 10 pupa in small open-top containers with ~60 mL of dechlorinated tap water. The open-top containers were then placed within custom made (18 cm long x 15 cm wide x 10 cm tall) enclosed containers (with a netted section to allow for air flow) allowing the pupa to emerge over a period of two-days (48 h). A premade sugar water solution (40 g of sucrose in 250 mL of tap water) was placed in each container to allow for adults to feed. Following 48 h given for emergence, any remaining

pupa were removed, and the containers were separated into two different acclimation treatments: cold-acclimation (15°C) and warm-acclimation (25°C). This ensured all adults were 1-2 days old upon the initiation of the acclimation treatments. Both acclimation groups were maintained on a 12:12 h light:dark cycle. All experiments were then conducted following a five-day period of exposure to each acclimation treatment, 6-7 days post-emergence (Figure 6.1).

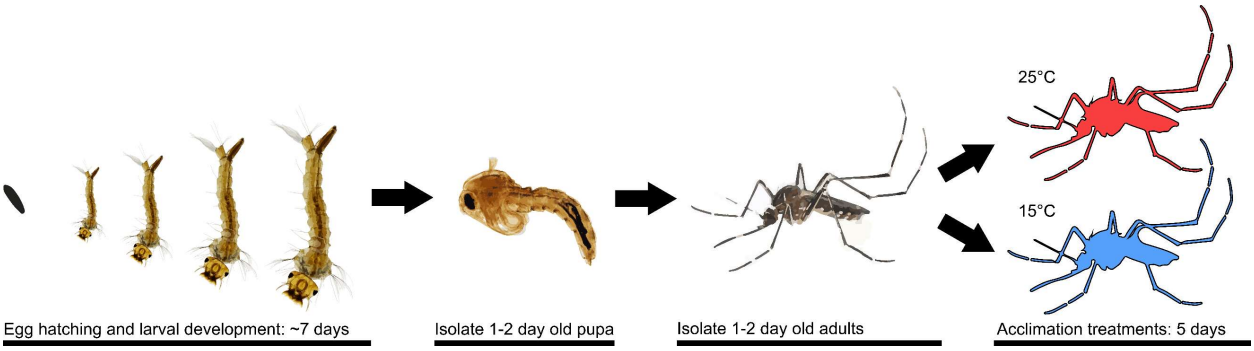

Figure 6.1. Mosquito rearing and acclimation schedule. Eggs were hatched in dechlorinated tap water and fed daily until larval pupation. Pupa were isolated daily and placed in custom built containers to facilitate their emergence into adults. Following a two-day period, any remaining pupa were removed, and the containers were separated into warm (25°C) or cold (15°C) acclimation treatments. All experiments were then conducted following five-days of thermal acclimation, 6-7 days post-emergence.

To assess critical thermal minimum (CT_{min}), individual mosquitoes were anesthetized under CO_2 and placed in 4 mL glass vials in a temperature-controlled bath (model MX7LL, VWR International, Mississauga, Canada) containing a 1:1 ethylene glycol:water mixture. The temperature of the bath was initially set to 25°C and then ramped down at a rate of 0.13°C min^{-1} while mosquito movement was carefully monitored. The temperature of the bath was independently monitored with a pair of type-K thermocouples connected to a computer running Picolog (version 5.25.3) via a Pico TC-07 interface (Pico Technology, St. Neots, UK). The temperature at which movement stopped following perturbation with a plastic probe was recorded as the mosquito's CT_{min}.

Chill coma recovery time (CCRT) was determined following 6 h at 2°C. Upon removal from the cold exposure, mosquitoes were sexed and placed in 4 mL enclosed glass containers at room temperature (22±1°C) and observed for 120 minutes. The duration of time required for a mosquito to stand on all legs following its removal from the cold was recorded as its CCRT. To assess the effect of blood feeding on CCRT, sugar-water mixture was removed from the cages of warm-acclimated mosquitoes 24 h before blood feeding. Mosquitoes were exposed to warm sheep blood for 20 min through a thinly stretched parafilm membrane. The mosquitoes were then either given a 0, 40, or 160 min (or alternatively 20, 60, or 180 min from the onset of blood-feeding) period prior to the initiation of the cold treatment of 6 h at 2°C. Mosquitoes that did not feed during the 20 min blood exposure period were used as an internal control.

Chilling-survival was assessed following 6 h cold exposures of varying temperatures. Exposure temperatures varied between acclimation groups to include temperatures that result in survival proportions ranging from 0% to 100%. To this end, cold-acclimated mosquitoes were exposed to -4°C, -3°C, -2.5°C, -2°C, -1°C, 0°C, 1°C, and 2°C and warm-acclimated mosquitoes were exposed to -2°C, -1°C, -0.5°C, 0°C, 1°C, and 2°C. Immediately upon removal from the cold exposure mosquitoes were isolated in 4 mL enclosed glass containers and left at room temperature (22±1°C) for 18 h to recover. Following this, the mosquitoes were assessed such that those that were able to stand were considered alive while those that were unable to stand were considered dead.

CCRT and CT_{min} were analyzed using two-way ANOVAs followed by Sidak's multiple comparison test for comparison within each sex and each acclimation group. Survival following cold stress was analyzed using a generalized linear model with a binomial error distribution. CCRT following blood-feeding was assessed using a two-way ANOVA. CCRT and CT_{min}

analyses were conducted on GraphPad Prism version 7.00 (GraphPad Software, La Jolla, USA). The assessment of chilling survival was conducted in R version 3.4.3 (R Core Team, 2015).

6.3 Results

The CT_{min} of mosquitoes was significantly affected both by acclimation temperature (2-way ANOVA; $F(1, 27) = 37.1$, $P < 0.0001$; Figure 6.2), and sex ($F(1, 27) = 8.33$, $P = 0.0076$), as well as the interaction of the two factors ($F(1, 27) = 4.80$, $P = 0.037$; Figure 6.2). More specifically, cold acclimation significantly lowered mean CT_{min} of female mosquitoes by 3.0°C (Sidak's multiple comparison test; $P = 0.035$), and males by 6.4°C ($P < 0.0001$). Sex-related effects on CT_{min} were only found within warm-acclimated mosquitoes where the mean CT_{min} of females was lower by 3.9°C in comparison to males ($P = 0.0046$). No difference in CT_{min} was found between cold-acclimated males and females ($P = 0.84$).

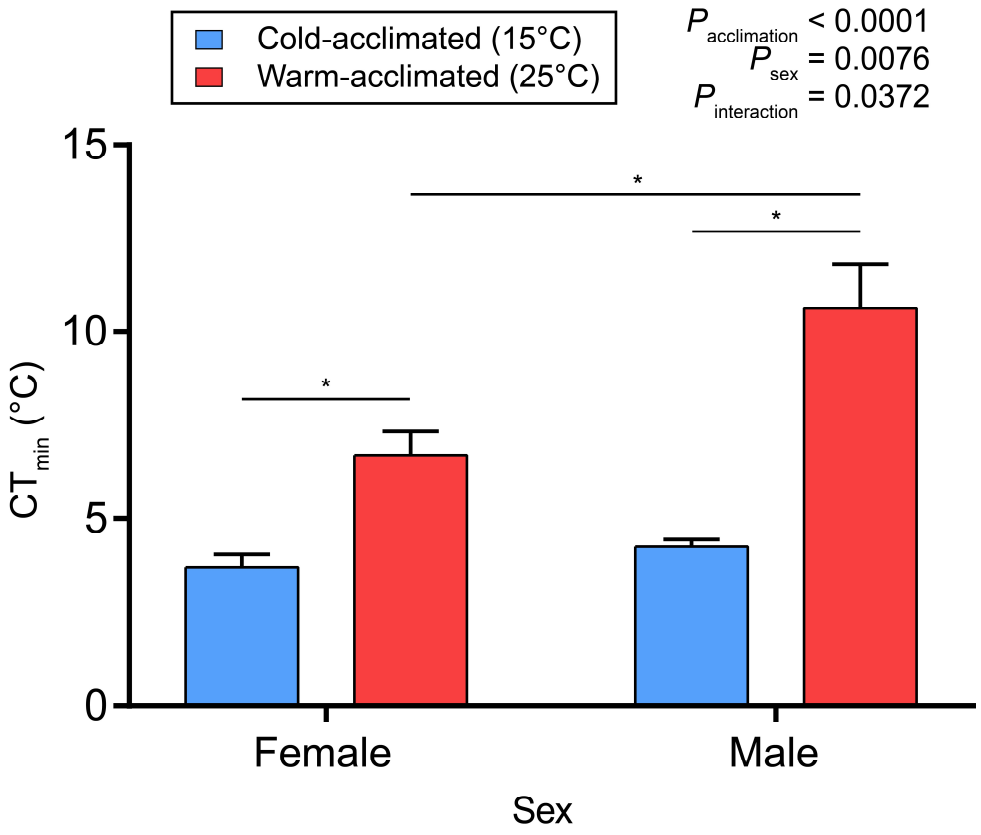

Figure 6.2. Cold acclimation decreases the critical thermal minima (CT_{min}) of female and male *A. aegypti* adults. Following a 5-day acclimation period at 15°C (cold acclimation) or 25°C (warm acclimation), flies were sexed and isolated under CO_2 into 5 mL glass vials. CT_{min} was measured via visual assessment of isolated mosquitoes cooled at a rate of 0.13°C/min in a cooling bath and recorded as the temperature of complete cessation of movement. Bars represent mean ± SEM. Asterisks denote significant difference (Sidak's multiple comparison test; $P < 0.05$).

Mosquito CCRT was affected by both acclimation temperature (2-way ANOVA; $F(1, 112) = 39.7$, $P < 0.0001$; Figure 6.3) and sex ($F(1, 112) = 4.45$, $P = 0.037$) with no significant interaction effect ($P = 0.79$). More specifically, cold acclimation significantly decreased mean CCRT by 34.5 min in females (Sidak's multiple comparison test; $P < 0.0001$) and by 31.7 min in males ($P = 0.0001$).

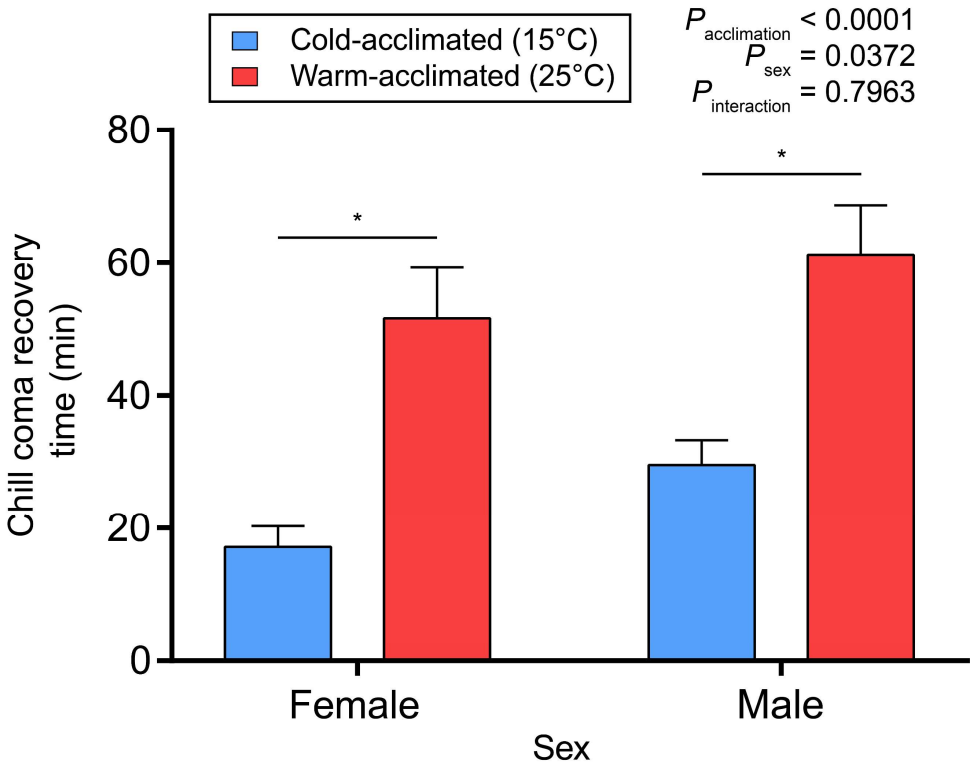

Figure 6.3. Chill coma recovery time decreases following cold acclimation and is sex-dependent. Following a 5-day acclimation period at 15°C (cold acclimation) or 25°C (warm acclimation), CCRT was measured following 6 h at 2°C. CCRT was considered as the time required for mosquitoes to regain balance and stand on all legs without external disturbance following removal from the cold. Bars represent mean ± SEM. Asterisks denote significant difference (Sidak's multiple comparison test; $P < 0.05$).

Chilling survival was significantly affected by both acclimation temperature (Logistic regression; $Z = -7.1$, $P < 0.0001$; Figure 6.4) and sex ($Z = -2.5$, $P = 0.014$). The temperature resulting in 50% survival (LT_{50}) was lowest in cold-acclimated flies at -1.93±0.15°C and -1.32±0.19°C in females and males, respectively. In contrast, the LT_{50} of warm-acclimated flies was ~1.5°C higher at 0.03±0.20°C and 0.29±0.18°C for females and males, respectively.

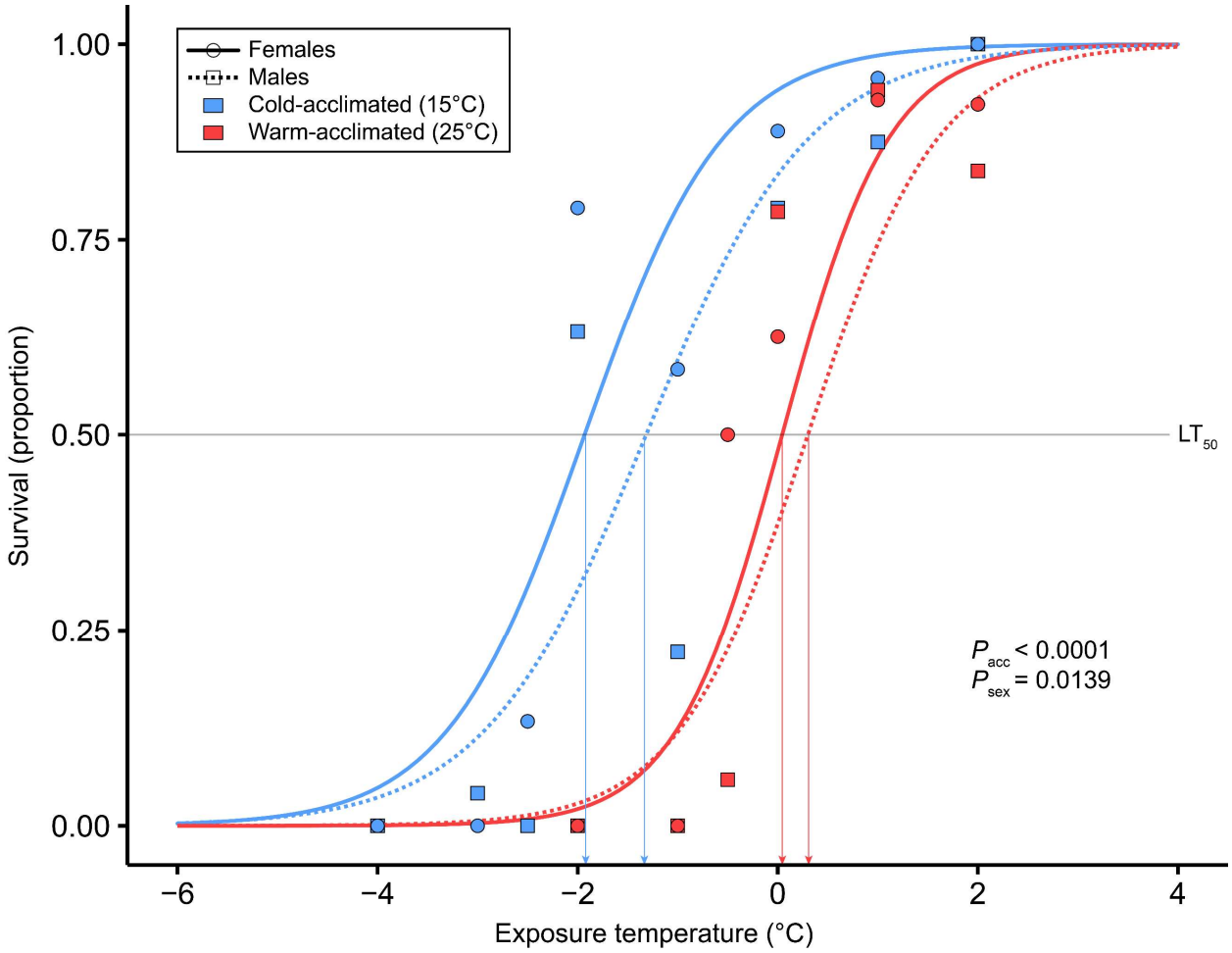

Figure 6.4. Chilling survival of *A. aegypti* increases following cold-acclimation and is sex-dependent. The chilling survival of warm (25°C; red) and cold (15°C; blue) acclimated mosquitoes was assessed following 6 h exposures to varying temperatures. Survival was assessed in both females (solid lines, circles) and males (dashed lines, squares). Symbols represent the proportions of surviving mosquitoes from the denoted group (n = 6 – 28 mosquitoes per symbol). Lines represent logistical models of the survival curves of each group. McFadden pseudo R^2 were 0.47, 0.47, 0.58, and 0.42 for CA female, CA males, WA female, and WA males, respectively. Note: unlike R^2, a McFadden pseudo $R^2 > 0.4$ represents a good fit (McFadden, 1978).

To assess the effect of blood feeding on mosquito cold tolerance, individuals were blood-fed prior to an acute cold exposure and assessment of CCRT. It was noticed, however, that despite the removal of sugar-water one day prior to blood-feeding (as was done for warm-acclimated mosquitoes), <5% of cold-acclimated mosquitoes fed (Figure 6.5A) in comparison to

~50% feeding amongst warm-acclimated females. Thus, the effect of blood feeding was assessed in warm-acclimated flies only at 20, 60 or 180 min following the introduction to the blood meal. No differences were found in the CCRT of unfed and blood-fed mosquitoes exposed to 6 h at 2°C following 20, 60, or 180 min following the onset of blood feeding (two-way ANOVA; $F(1, 59) = 0.027$; $P_{feeding} = 0.87$).

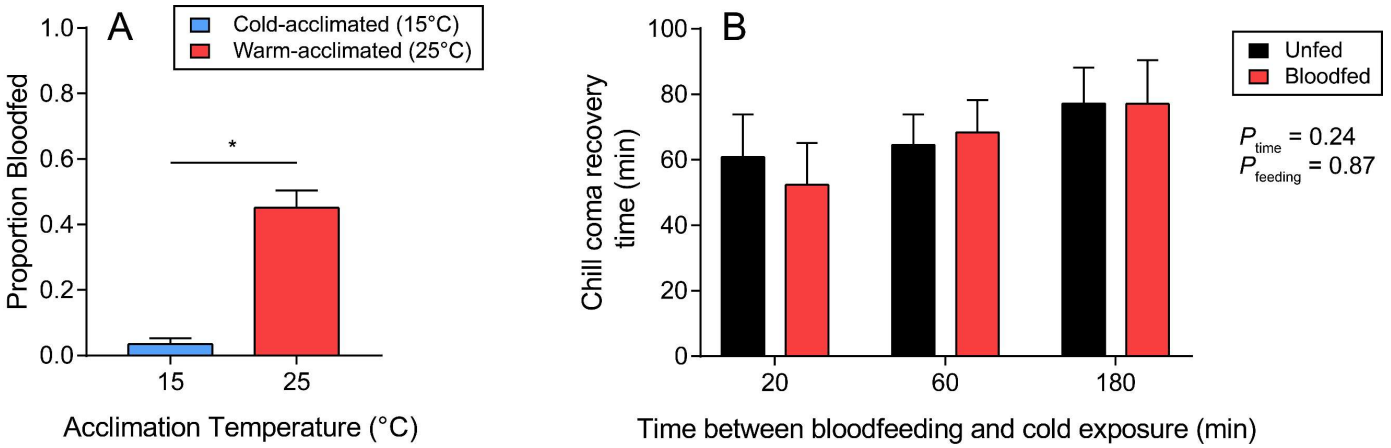

Figure 6.5. Chill coma recovery time (CCRT) is unaffected by blood feeding in warm-acclimated (25°C) *A. aegypti* females. (A) Proportion of blood-feeding warm and cold acclimated mosquitoes. (B) CCRT of warm acclimated female *A. aegypti* 20, 60, and 180 min following the onset of blood feeding. CCRT was measured as the time to regain balance and stand on all legs following removal from the cold exposures. The CCRT of blood-fed mosquitoes was compared with mosquitoes of the same cohort that did not feed during the 20 min blood exposure. Bars represent mean ± SEM.

6.4 Discussion

In the present study, I show that the cold tolerance of adult *A. aegypti* is plastic and sex-dependent and that CCRT is unaffected by blood feeing within 20 -180 min of feeding.

Five-days of cold-acclimation at 15°C improved the cold tolerance of adult *A. aegypti* in all assessed traits, decreasing CT_{min} and CCRT, and increasing chilling survival. This finding is consistent with other chill-susceptible insects, including tropical species such as *Nauphoeta cinerea* and tropical *Drosophila* species (Koštál et al., 2006; Overgaard et al., 2011),

demonstrating that our population of *A. aegypti* is similarly thermally plastic. The ability of a tropical species like *A. aegypti* to exhibit thermal plasticity at low temperatures seems counterintuitive. However, this finding is consistent with previous work in *Drosophila*, which showed that both tropical and widespread species are similarly capable of thermal plasticity and that it is rather innate differences that underlie their varying cold tolerance (Overgaard et al., 2011). Furthermore, the ability of this population to acclimate to mild cold exposures is consistent with the climate of their origin state of Florida, which can reach a minimum of ~10°C during the winter months (Davis et al., 1994; Eisen and Moore, 2013; Kraemer et al., 2015). Although *A. aegypti* have been shown to overwinter as far north as Washington D.C. based on genomic data, it is uncertain which life stage they were in and what microclimates they experienced (Lima et al., 2016).

In the current study, female *A. aegypti* were found to be more cold tolerant than males as they exhibited lower CT_{min} and CCRT and increased chilling survival. The most pronounced difference was found in warm acclimated mosquitoes, where females entered a chill coma at 6.7°C while males entered a chill coma at 10.6°C. Interestingly, this difference of 3.6°C in CT_{min} was reduced following cold acclimation to only 0.6°C, suggesting that cold acclimation reduces sex-specific differences in CT_{min} (Figure 6.2). CCRT was found to be ~10 min shorter in females of both acclimation groups (Figure 6.3). Lastly, sex was found to significantly affect chilling survival, such that the LT_{50} was 0.61°C and 0.23°C lower in females than in males in cold- and warm-acclimated mosquitoes, respectively (Figure 6.4). Overall, cold acclimation reduced the gap in CT_{min} but not in CCRT and survival among males and females. This supports the hypothesis that different mechanism underlie chill coma entry, chill coma recovery, and chilling survival (reviewed by Overgaard and MacMillan, 2017). Furthermore, these findings are

consistent with previous studies of *Drosophila* that have similarly shown that females exhibit faster recovery and better chilling survival than males (Jakobs et al., 2015; Jean David et al., 1998). While the current data provides little insight of the mechanism that underlies this sexual dimorphism in cold tolerance, some previously suggested possibilities include differences in weight (Edwards, 1958) and haemolymph solutes such as trehalose and free amino acids (Fields et al., 1998). Increased weight of female *A. aegypti* may be indicative of a higher haemolymph volume which may act as a buffer against cold-induced ion imbalances. Alternatively, differences in haemolymph solutes such as trehalose and free amino acids may impact cold tolerance by acting as haemolymph osmolytes. For example, previous work on *C. ferrugineus* revealed that cold acclimated males are more cold tolerant and have increased free amino acids and trehalose levels than their female counterparts (Fields et al., 1998). The elevation of osmolytes has previously been associated with increased chill tolerance in chill-susceptible insects and is thought to act by reducing the reliance on Na^+ as a major haemolymph osmolyte and consequently mitigating water loss in response to Na^+ leak at low temperatures (Lalouette et al., 2007; Michaud and Denlinger, 2007; Overgaard et al., 2014).

To test the effect of blood-feeding on the cold tolerance of *A. aegypti*, I exposed females to 6 h at 2°C at 20, 60, or 180 min following the onset of a blood meal. It was noticed that while ~50% warm acclimated females fed, cold acclimated females did not feed (Figure 6.5A). In contrast to my current findings, Carrington et al. (2013) demonstrated that *A. aegypti* raised at 16°C blood-feed (albeit on human blood as opposed to sheep blood) but that this occurred only four days post emergence in contrast to one day post emergence in mosquitoes raised at 26°C. In the current study, however, all females were 6-7 days post-emergence and thus this alone does not explain the discrepancy in feeding proportions. Consumption of a blood-meal initiates the

gonotrophic cycle in *A. aegypti* and thus reduced feeding at low temperatures may explain the complete absence of egg laying by females at 16°C (Briegel, 1985; Carrington et al., 2013). This result also suggests that the risk of disease transmission in *A. aegypti* is reduced at lower temperatures due to their reduced probability of feeding, which is the mode of infection of vertebrates. Within warm-acclimated mosquitoes, neither the time duration post-feeding nor the blood-meal was found to impact CCRT (Figure 6.5B). MacMillan et al. (2012) have previously shown that in crickets, CCRT is associated with the degree of cold-induced hyperkalemia and the ability of an insect to actively re-establish normal haemolymph [K^+]. Assuming mosquito cold tolerance is governed by similar mechanisms, a lack of difference in CCRT following blood-feeding suggests that the net balance between the elevation of haemolymph K^+ and its restoration is unaltered. This would encompass three possibilities: (1) the degree of haemolymph hyperkalemia and capacity for ion reestablishment are unaffected by blood-feeding, (2) severe hyperkalemia in blood-fed insects is offset by a greater capacity for ion reestablishment, or that (3) mild hyperkalemia is offset by a poorer capacity for ion reestablishment. Vertebrate blood is hypoosmotic to insect haemolymph and high in Na^+. As a result, with the intake of a blood meal, mosquitoes are challenged with the dilution of their haemolymph and the influx of sodium (Williams et al., 1983). To overcome this challenge, diuresis immediately follows a blood-meal, initially excreting high amounts of Na^+ and water (Efron, 1988; Williams et al., 1983). It remains to be known when K^+ is released from lysed cells in *A. aegypti* but this can be estimated by the ionic composition of the urine. As diuresis progresses, the ion composition of urine changes, increasing in the ratio of [K^+]:[Na^+] 30 min and 60 min following a blood-meal, suggesting that at the tested times, at least some K^+ has been released (Williams et al., 1983). Based on this, both at the 60 and 180 min treatments (and potentially the 20 min), mosquitoes likely face increased

K^+ in the gut and haemolymph, which likely increases the degree of cold-induced hyperkalemia. However, preceding this K^+ load is the rapid post-prandial diuresis, which peaks within 6 min of a blood meal (Williams et al., 1983). As mentioned earlier, while initially high amounts of Na^+ are secreted, within 30-60 minutes, the ratio of $[K^+]$:$[Na^+]$ in the urine increases, with $[K^+]$ peaking at 14 times initial urine $[K^+]$ (Williams et al., 1983). This result is similar to the overall effect of the rectum and Malpighian tubules of cold-acclimated flies where increased K^+ secretion and reduced K^+ reabsorption led to net removal of K^+ and improved CCRT (Yerushalmi et al., 2018). Therefore, it is possible that this increase in K^+ excretion may oppose increased severity of cold-induced hyperkalemia to result in unchanged CCRT. At this point, however, additional studies are required to (1) examine whether *A. aegypti* experience cold-induced hyperkalemia and (2) further delineate the physiological impact of a blood-meal on passive ion leak across the gut and determine how the ionoregulatory response to the blood meal may impact cold-induced hyperkalemia.

6.5 Conclusions and future directions

The results presented herein demonstrate that the cold tolerance of adult *A. aegypti* is affected by exposure temperature and more moderately by sex but not by blood feeding. Despite primarily inhabiting tropical and subtropical regions where temperatures are relatively high and more stable than temperate regions, adult *A. aegypti* display thermal plasticity, as evidenced by marked improvements in CT_{min}, CCRT, and survival following 5-days at 15°C. This finding is consistent with many other chill-susceptible insects – such as *D. melanogaster* as evidenced by the current thesis – that improve in all the mentioned traits (Yerushalmi et al., 2018). It was also found that, in comparison to males, female mosquitoes are generally more cold tolerant, albeit it is a smaller effect size than thermal acclimation. Lastly, blood feeding was thought to affect

CCRT by introducing an ionoregulatory challenge that would amplify the ion disturbances experienced by insects at low temperatures, or alternatively better prepare mosquitoes by stimulating their excretory organs in advance of the cold treatment. Interestingly, no difference was found in CCRT at 20, 60, or 180 min following the onset of blood feeding.

Further assessment of the physiological mechanisms that underlie the cold tolerance of *A. aegypti* is still required. First, measurements of haemolymph [K^+] following cold exposure are needed to determine whether *A. aegypti*, like other insects, experience hyperkalemia at low temperatures. If this is the case, assessments of the Malpighian tubules and the rectum would be required to determine the nature of the ionoregulatory responses of cold acclimated mosquitoes that may similarly assist in the prevention of hyperkalemia in this species.

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