Examining the Effects of Cold on Gut Epithelial Permeability in *Locusta migratoria*

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs
in partial fulfillment of the requirements of the degree of

Master of Science

in

Biology

Carleton University
Ottawa, Ontario

Abstract

When exposed to temperatures below their minimum tolerable temperature, the majority of insects succumb to a comatose state. If the exposure is relatively mild or brief, they are able to recover from this chill coma. However, in the event of harsh or prolonged cold stress, an accumulation of cold-induced tissue damage occurs and can ultimately result in mortality. These chill injuries have been consistently associated with a loss of organismal ion balance that occurs at low temperatures. In *Drosophila*, this imbalance is hypothesized to occur at least partly due to a cold-induced disruption of epithelial barriers along the paracellular pathway. However, the specific location of these barrier failures, and their role in solute leak, remain unknown. The primary goal of my research was therefore to use the migratory locust (*Locusta migratoria*) to investigate the relationships among chilling injury, ionoregulation, and gut barrier integrity in the cold. In Chapter 2, I used a fluorophore (FITC-dextran) to quantify paracellular leak across the gut epithelia, in both the serosal-mucosal and mucosal-serosal directions. Interestingly, leak appears to be unidirectional, favouring the mucosal to serosal route of movement in the cold. I then investigated the origin of barrier failure along the gut using FITC-dextran, although no differences were found between rates of FITC-dextran leak in the cold and under control conditions. As a whole, these data generate exciting new hypotheses regarding the mechanisms of cold-induced barrier failure. In Chapter 3, I explored the use of a second marker of paracellular permeability (mPEG-FITC) for quantifying epithelial permeability in locusts. Here, I provide evidence which suggests that mPEG-FITC is not a suitable marker of permeability, at least in locusts. It is my hope that together, these experiments will enhance not only our understanding of the underlying physiological mechanisms of chill susceptibility and tolerance, but will also expand our knowledge of insect paracellular barriers in groups other than Diptera.

Acknowledgments

I am extremely thankful for my wonderful partner, Matt. You have supported me throughout this entire journey that is a Master's and I cannot thank you enough for being 'by my side', even from across the country. You have been, and continue to be, my rock when I need you the most – to give me that extra push to keep going through the ups and downs, to be my guinea pig as I prep for presentations, and much more. I would also like to thank my amazing family for all of the unconditional love and support that they have given me, both in life and throughout these last two years.

To Heath MacMillan: I could not have asked for a better supervisor. You have pushed me to achieve more than I thought I could, and have been instrumental in helping me to develop not only as a scientist, but also as a communicator. You have opened up many opportunities for me during my time here and I am truly grateful that you went above and beyond to do so. I cannot thank you enough for your endless patience, dedication, and guidance, or all that you have done for me. I also appreciate all of the time and energy that you put into both your students and into making this lab an amazing place to work. I am proud to have been a part of the MacMillan Lab.

I would also like to thank my wonderful committee members, Jessica Forrest and Jeff Dawson. Thank you to Jessica for all of her support, input, and cheerfulness throughout our meetings. And to Jeff for not only his support and input on my thesis, but also for his unofficial role as mentor in the lab and classroom (despite his questionable worm dissection skills!).

My experience here at Carleton would not have been complete without the incredible community within the Biology Department. Thank you to Jenny Bruin for chairing my thesis defense, and for being such a positive role model. Thank you to Jayne Yack for taking the time to serve on my defense committee. Thank you to Joan Mallet for all of her advice and wisdom.

To Sarah Anne Szabototh, Roisin Holahan, Courtney Lindsay, and Andras Dobai for answering my many questions. And to all of the other amazing people that I have come to know over my Masters: Fatima Haider, Myriam Hoyeck, Erin Vanzyl, Chris Bergin, Laura Corrigan, Kevin Xiong, Jackie Lebenzon, Lauren Rowsey, Cynthia Cheney, Alex Cheslock, Mahmoud El-Saadi, Serita Fudlosid, Hannah Davis, Jessica Carrington, Marshal Ritchie, Dawson Livingston, Irfan Dhanidina, Ravneet Hansi, Bassam Helou, Hirva Patel, Mirvat Noubani, Mat Roloson, and the many other friends and colleagues who have supported me over the past two years – thank you.

I am truly lucky to have all of you enriching my life.

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CT_{min}	Critical thermal minimum
CCO	Chill coma onset
V_m	Membrane potential
SD	Spreading depolarization
CNS	Central nervous system
CCRT	Chill coma recovery time
MTs	Malpighian tubules
NKA	Na^+/K^+-ATPase
VA	V-ATPase
SJs	Septate junctions
pSJs	Pleated septate junctions
sSJs	Smooth septate junctions
FITC-dextran	Fluorescein isothiocyanate-dextran
ISME	Ion-selective microelectrodes
mPEG	Fluorescein PEG

1 General Introduction

1.1 The Importance of Understanding Insect Cold Physiology

At an estimated 4-5 million (known and unknown) different species and accounting for 80% of the known animal species, insects dominate Earth's fauna (Costello et al., 2012; Hill, 1997). As a result, these ectotherms play important roles in nearly all ecosystems, ranging from barren deserts to lush tropical forests and the freezing poles (Chown and Terblanche, 2006; Hill, 1997). Insects also heavily influence our world economies; Pollination alone, which is mostly done by insects, enhances production in 75% of globally important crops, contributing to an over $500 billion industry (Breeze et al., 2016; Gallai et al., 2008; Klein et al., 2007). Meanwhile, as the global appetite for animal protein continues to rise and the availability of arable land wanes, insects are being increasingly promoted as a sustainable alternative to conventional protein production systems (Berggren et al., 2019; Premalatha et al., 2011). Insects are also well suited for use in environmental impact assessments due to their ubiquitous presence and general ease of sampling. Environmental disturbances within ecosystems often impact insect roles as predators, herbivores, and parasites (among others), and their responses to these perturbations can be monitored (Rosenberg et al., 1986).

While beneficial insect species exits, there are also those which cause widespread damage to human and animal health, as well as global agricultural interests (Hill, 1997). For instance, vector-born diseases such as malaria and zika are becoming increasingly common and are of major concern in many countries (Epstein et al., 2002; Velázquez-Castro et al., 2018). Together, these insect-spread diseases account for over 1.5 million deaths per year and 17% of the estimated global burden of all infectious diseases (Rua and Okech, 2019). Similarly, pests like aphids, locusts, and beetles (e.g. the Colorado potato beetle) pose great risk to the health of

crops and forests, as well as their overall productivity (Oerke, 2006). The emerald ash borer (*Agrilus planipennis*) for example, is responsible for the widespread destruction of millions of North American ash trees, totaling billions in reparations and management (Poland and McCullough, 2006). Because of the many and varied ways insects impact our lives, it is critically important that we understand the factors that influence their ranges and abundance.

As they are ectotherms, insect distribution patterns are highly dependent on the temperature of their ambient environment. Such restrictions are present as temperature affects not only insect survival, but also growth, population dynamics, and reproduction (Chown and Terblanche, 2006; Colinet et al., 2015). It is therefore not surprising that insect geographic distribution is tightly coupled to their thermal tolerance, as further supported by the strong positive relationship between the lowest temperature a species can tolerate and the lowest temperature within its environment (Hazell et al., 2010; Kellermann et al., 2012; Kimura, 2004; Overgaard and MacMillan, 2017). Consequently, insect distributions are changing as global surface temperatures rise, resulting in the movement of invasive species, disease vectors, and pests beyond their normal ranges (Addo-Bediako et al., 2000; Chown and Terblanche, 2006). For example, *Aedes aegypti* (the yellow-fever mosquito) is the main carrier of yellow fever, dengue, and chikungunya (among others) and has classically been observed in tropical and subtropical areas worldwide. However, their distribution range has recently expanded to higher latitudes, increasing the spread of *Aedes*-borne diseases (Rua and Okech, 2019). Thus, to better manage these harmful populations, there is a pressing need to predict and/or limit insect movement (Chown et al., 2012; Hunter, 2019). However, to take these steps we must first understand the physiology that determines thermal tolerance (Chown et al., 2012). While work is being conducted to understand how upper thermal limits dictate insect physiology and behaviour, the

main objective of this thesis is to contribute to the current understanding of how low temperatures impact insects.

1.2 Cold Tolerance in Insects

Insect cold tolerance has been classically divided into two categories: freeze tolerant and freeze avoidant insects. The former is characterized by the insect's ability to withstand ice formation in the extracellular fluid, while the latter is based on the ability to avoid freezing by lowering the freezing point of their extracellular fluids (Overgaard and MacMillan, 2017; Sinclair, 1999). The presence and extent of these two strategies is often dependent on life stage and season, although exceptions exist where a given strategy is maintained year-round (Toxopeus and Sinclair, 2018). Recently however, attention has turned towards a third group, known as chill susceptible insects. Chill susceptible insects suffer from negative effects of chilling at temperatures above the freezing point of their extracellular fluids (Andersen et al., 2018; Findsen et al., 2013; Overgaard and MacMillan, 2017). I briefly examine components of freeze tolerant and avoidant insects before more comprehensively discussing chill susceptible species (which include the migratory locust, the focus of this thesis).

1.2.1 *Freeze Tolerance*

To survive in a frozen state, freeze tolerant insects must be able to overcome the potential lethal effects of freezing. For instance, extracellular freezing can often result in a redistribution of water and solutes across cell membranes, reduced cell volumes caused by water outflow, and concentrated solute levels in remaining liquid compartments (Toxopeus and Sinclair, 2018). Together, these effects of freezing can lead not only to compromised membrane structure and cellular organization, but also to damaged protein structure and function (Lovelock, 1953;

Mazur, 1984; Storey and Storey, 1988; Toxopeus and Sinclair, 2018). Regardless of the other effects, ice crystal growth alone can pierce and/or tear tissues (Toxopeus and Sinclair, 2018). Furthermore, rapid rewarming can cause cell swelling and rupture from rehydration (Gao and Critser, 2000). With such high stakes, it is important to have the ability to control the location and rate of ice formation (Raymond and Wharton, 2016).

While there is no single known prerequisite for tolerating ice formation, many freeze tolerant insects are known to accumulate various cryoprotectants such as glycerol and ice-nucleating agents (among others) (Toxopeus and Sinclair, 2018). These protectants are thought to help slow crystal formation and preserve cell function and integrity in the face of low temperatures (Izumi et al., 2006; Tsumuki and Konno, 1991). In the woolly bear caterpillar (*Pyrrharctia isabella*), for instance, hemolymph glycerol levels can reach over 800 mM (Marshall and Sinclair, 2011). Such high glycerol levels are hypothesized to reduce overall ice content and the probability of intracellular ice formation, in turn allowing these insects to recover from up to seven days in a frozen state (Layne et al., 1999; Marshall and Sinclair, 2011; Toxopeus and Sinclair, 2018). Similarly, ice-nucleating agents, which initiate ice formation, can localize crystals to compartments that are able to tolerate the resulting mechanical damage (Zachariassen, 1985). In the natural setting, these agents can help insects like the New Zealand alpine weta (*Hemideina maori*), and sub-Antarctic beetles (*Hydromedion sparsutum* and *Perimylops antarcticus*) survive for extended periods of time at -10 and -6.5°C, respectively (Wilson and Ramlov, 1995; Worland and Block, 1999). Surprisingly, extreme examples of freeze tolerant insects exist, where no apparent cryoprotectants are used. *Heleomyza borealis* (Diptera) for instance, overwinter as larvae in Arctic environments, and can survive temperatures below -60°C (Worland et al., 2000). This survival in the absence of cryoprotectants is possibly due to

mechanisms related to a preconditioned hypometabolic state that occurs prior to the start of winter (Worland et al., 2000). Despite the myriad of adaptations that freeze tolerant insects possess to survive in the cold, it is important to note that freeze avoidant insects often have similar lower lethal temperatures (the temperature at which 50% of individuals survive an acute cold stress) as those that are freeze tolerant (Andersen et al., 2015a; Sinclair, 1999). This suggests more than one way for insects to successfully endure low temperatures.

1.2.2 *Freeze Avoidance*

While freeze tolerant insects are able to survive freezing, those that are freeze avoidant depress the freezing point of their extracellular fluids (the supercooling point) in hopes of avoiding the injuries and stress that come with freezing (Overgaard and MacMillan, 2017; Zachariassen, 1985). These animals can therefore survive sometimes extreme low temperatures due to various physiological adaptions, including the synthesis of antifreeze proteins, dehydration, and or an increase in body osmolality via the accumulation of polyols (e.g. glycerol), sugars (e.g. trehalose), salts (Duman, 2002; Overgaard and MacMillan, 2017; Salt, 1961; Sinclair et al., 2003; Zachariassen, 1985). For instance, willow cone gall larvae (*Rhabdophaga strobiloides*) produce large quantities of glycerol which decreases its supercooling point from -26.5°C during the summer to -56.1°C in the winter (Miller, 1982; Miller and Werner, 1980). Similarly, desiccation in insects like the Arctic springtail (*Onychiurus arcticus*) triggers the production of trehalose (Worland et al., 1998). These two means of cryoprotection combined result in an organism that can withstand -30°C without freezing as all osmotically active water has been lost (Holmstrup and Sømme, 1998; Sinclair et al., 2003; Worland et al., 1998). Finally, antifreeze proteins also help to mitigate freezing by inhibiting ice nucleators in both the hemolymph and the gut (Duman, 2002; Sinclair et al., 2003). Antifreeze

proteins can substantially improve an insect's ability to avoid freezing. In a beetle (*Dendroides canadensis*), for example, freezing does not occur until body temperatures reach -26°C in the winter (Horwath and Duman, 1984; Sinclair, 1999).

1.2.3 *Chill Susceptibility*

In stark contrast to insects that are freeze avoidant or tolerant, chill susceptible insects are negatively affected by low temperatures well above the freezing point of their extracellular fluids (Sinclair, 1999). This cold tolerance strategy can be found in the majority of hexapods, including crickets (*Gryllus pennsylvanicus*), fruit flies (*Drosophila melanogaster*), locusts (*Locusta migratoria*), moths (*Thaumatotibia leucotreta* and *Cydia pomonella*), beetles (*Alphitobius diaperinus*), and mosquitos (*A. aegypti*) – many of which experience similar adverse effects in the cold (Overgaard and MacMillan, 2017). As these ectotherms are chilled beyond their minimum tolerable temperature (critical thermal minimum; CT_{min}), they experience a loss of coordinated neuromuscular movements. Shortly after, they succumb to a state of complete but reversible paralysis (chill coma), where they remain for the duration of the cold exposure (Armstrong et al., 2012; Boardman et al., 2012; Carrington et al., 2013; Chidawanyika and Terblanche, 2011; Colinet et al., 2011; MacMillan and Sinclair, 2011; Robertson et al., 2017). The onset of chill coma (chill coma onset; CCO) presents an unavoidable problem for these insects as they are "ecologically dead" and therefore vulnerable to predation in this comatose state.

Classically, there has been much debate surrounding the physiological mechanisms that drive the onset of chill coma, principally whether it is caused by muscle or nervous system impairment, or both. For instance, previous correlative evidence has demonstrated how cold-induced depolarization of muscle membrane potential (V_m) results in a loss of muscle

excitability, and that this coincides with CCO (Coello Alvarado et al., 2015; Koštál et al., 2006; Overgaard and MacMillan, 2017). Along with locusts, this "muscle before nervous" hypothesis has been further supported in *D. melanogaster* and bees (*Apis mellifera*), where a decreased excitability of the extensor tibialis (locusts and *Drosophila*) and flight muscles (*A. mellifera*) were observed alongside V_m depolarization (Findsen et al., 2014; Findsen et al., 2016; Hosler et al., 2000). However, some species of *Drosophila* are able to maintain normal muscle V_m at temperatures below CCO, which contradicts it as a mechanism of coma onset (Hosler et al., 2000; MacMillan et al., 2014). By contrast, evidence supporting nervous system before muscle impairment has also been documented. This depolarization in the central nervous system (CNS), commonly referred to as spreading depolarization (SD), has been linked to surges in extracellular K^+ concentrations, as well as neuronal and glial depolarization in *L. migratoria* and *D. melanogaster* (Andersen et al., 2015b). The onset of SD has also been correlated with the initial loss of coordinated muscle movement that occurs at CT_{min}, with a complete loss of muscle function occurring shortly after (Armstrong et al., 2012; Robertson et al., 2017). SD is therefore thought to be strongly associated with the silencing of neural activity, and may play a role in CCO (Andersen and Overgaard, 2019; Andersen et al., 2018; Robertson et al., 2017). In fact, recent evidence in several *Drosophila* species has provided further convincing support for SD as the cause of CCO over muscle impairment. Here, the temperatures at which SD events occurred corresponded to the temperatures of CCO and CT_{min} for each drosophilid (Robertson et al., 2017). Additionally, Andersen and Overgaard (2018) found that a loss of CNS function in more cold tolerant drosophilids was followed closely by a loss of muscle V_m and that CNS function recovered before muscle polarization occurred. Together, these findings have important implications, especially in the context of measuring chill susceptibility.

Along with CCO and CT_{min}, the time it takes an animal to recover the ability to stand post-cold stress (chill coma recovery time; CCRT), and their condition 24 h later (survival) are common means through which chill susceptibility can be quantified (see Fig. 1.1 for a summary of measurement timing relative to cold exposure and ion movement; Overgaard and MacMillan, 2017). While each of these measurements occur through distinct mechanisms, they are typically associated with a local and/or systemic loss of ion balance (Coello Alvarado et al., 2015; MacMillan et al., 2012; Robertson et al., 2017).

Under optimal conditions, the hemolymph of most insects contains high and low concentrations of Na^+ and K^+, respectively (Coello Alvarado et al., 2015; MacMillan et al., 2012; Robertson et al., 2017). During chill coma however, these insects typically experience a loss of ion (most notably Na^+, K^+, and Cl^-) and water homeostasis. During prolonged chilling, a net leak of hemolymph Na^+ and water (which follows Na^+ osmotically) into the gut lumen occurs, resulting in the concentration of K^+ remaining in the hemolymph (Andersen et al., 2017; Gerber and Overgaard, 2018; MacMillan et al., 2015a; Ravn et al., 2019). The simultaneous leak of some intracellular K^+ down its concentration gradient into the hemocoel worsens this problem, altogether resulting in the systemic cell depolarization (including muscle cells) seen at low temperatures. For an insect to recover from chill coma, a reestablishment of hemolymph ion content and volume must therefore occur, driven by the movement of Na^+ (and water) from the gut lumen back to the hemocoel (Coello Alvarado et al., 2015; MacMillan et al., 2015a). As ion and solute balance once again becomes tightly regulated, the restoration of muscle V_m occurs, eventually enabling the quantification of CCRT and survival.

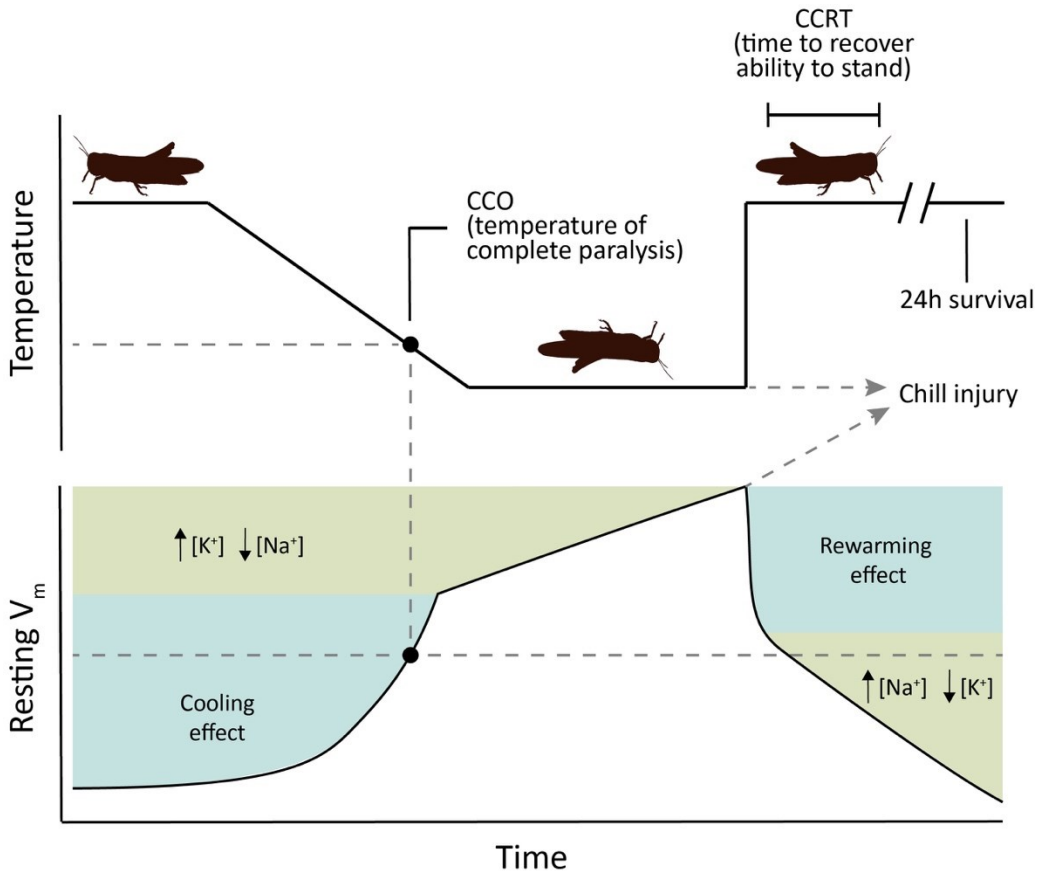

Figure 1.1. A representation of the chilling process in chill susceptible insects, like the migratory locust. During these processes, the onset of chill coma and the development of tissue damage (chilling injuries; *top*) are tightly correlated with a depolarization of resting membrane potential (V_m; *bottom*). As an insect is cooled, K^+ and Na^+ regulation is lost. Over time, the gradient of K^+ across the cell membrane fails, and a marked depolarization in membrane potential occurs, worsened over time by the continuous increase of hemolymph [K^+]. Once past a threshold potential, the silencing of neuromuscular activity occurs, often signaling the onset of chill coma (CCO). Ion balance is further disrupted the longer an insect spends in the cold, eventually triggering chill injury formation (*grey arrows*). However, once the insect is returned to optimal conditions, they can often recover from chill coma (and the resulting injuries) upon the restoration of hemolymph solute and water regulation (i.e. restored high and low hemolymph Na^+ and K^+ concentrations, respectively). Measurements of chill susceptibility taken post-cold stress have therefore been associated with the restoration of V_m. Here, the time taken for an insect to recover the ability to stand is termed chill coma recovery time (CCRT) and the post-stress assessment of the insect's condition is termed survival (often 24 h later). Figure adapted from (Overgaard and MacMillan, 2017).

In the event of a prolonged or harsh cold exposure however, chill susceptible insects may not recover once returned to near-optimal conditions. During such bouts of cold, insects often accumulate cold-induced tissue damage known as chilling injuries (MacMillan et al., 2012). These injuries are typically identified at the organismal level through a loss of coordination, permanent limb paralysis, or mortality, and occur in the presence of both high extracellular [K^+] (hyperkalemia) and low temperature (Coello Alvarado et al., 2015; Koštál et al., 2006). Recent evidence in *L. migratoria* supports that these chilling injuries develop proportionally to the degree of muscle membrane depolarization, resulting in greater rates of muscle injury over time (Andersen et al., 2017; Bayley et al., 2018; MacMillan et al., 2015b). Furthermore, this depolarization triggers the opening of voltage-gated Ca^{2+} channels which increases intracellular [Ca^{2+}] in muscle fibres (Bayley et al., 2018). The influx of Ca^{2+} that ensues has been linked to the development of cold-induced injury, and is proposed to occur through the downstream processes activated by the Ca^{2+} surge, possibly via apoptotic or necrotic mechanisms (Andersen et al., 2017; Bayley et al., 2018; MacMillan et al., 2015b). Failure to maintain ion and water balance in the cold can therefore result in organismal injury or mortality and as a result, understanding the mechanisms through which this ionoregulatory collapse occurs is important to understanding chill susceptibility. By taking advantage of the natural or inducible variation in insect cold tolerance, we can surmise the cause of these events, which furthers understanding of cold tolerance and chill susceptibility.

In a natural setting, insects are constantly adjusting physiologically (acclimatizing) to the daily fluctuation of temperature below and above their activity thresholds that occur (Nicotera and Orrenius, 1998; Yi and Lee, 2011; Yi et al., 2007). This preexposure to non-lethal temperatures in turn offers some degree of protection in the event of exposures that might

otherwise be lethal. In controlled settings, insects held over extended periods of non-lethal cold (acclimation) can therefore be used to understand the underlying physiology of cold tolerance phenotypes, like differences in gene expression and metabolic pathways between warm and cold acclimated insects (Salt, 1961). Cold acclimation in *D. melanogaster*, for example, results in the differential regulation of nearly one third of the transcriptome and almost half of the metabolome, thus revealing aspects of cold tolerance physiology that may not have been easily observed otherwise (MacMillan et al., 2016). Furthermore, differences between acclimation treatments (warm vs. cold) can also provide insight to how ionoregulation occurs within chill susceptible insects at low temperatures (MacMillan et al., 2016). For instance, cold acclimated *Gryllus* crickets are better able to defend against the loss of Na^+ and water to the gut lumen during cold exposure compared to warm acclimated crickets (Andersen et al., 2017; Atwal, 1960; Coello Alvarado et al., 2015; Koštál, 2004; Koštál et al., 2006; MacMillan et al., 2017; Smith, 1970). Similarly, cold acclimated *D. melanogaster*, *L. migratoria*, firebugs (*Pyrrhocoris apterus*), and cockroaches (*Nauphoeta cinereal*) are able to maintain low K^+ concentration in their hemolymph during cold exposures, with some barely experiencing CCO or gut barrier failure, unlike their warm acclimated counterparts (Coello Alvarado et al., 2015; MacMillan et al., 2017). Cold acclimation can therefore mitigate the damage caused by chilling to ion and water balance within these chill susceptible insects, which confers an increased cold tolerance. Together, this body of evidence suggests that the inability to maintain homeostasis in the cold can result in chilling injury and mortality, and that cold tolerance can be improved through an improved ability to maintain ion balance in the cold.

1.3 Maintenance of Ion and Water Balance

Across insects, various gut structures exist which reflect the diverse range in diet and feeding modes (Andersen et al., 2017; Koštál, 2004; Koštál et al., 2006; MacMillan et al., 2015a; MacMillan et al., 2017). For instance, kissing bugs (*Rhodnius prolixus*) possess expanded anterior midguts, which allows them to quickly absorb excess fluid from their blood meals into the hemolymph that is later excreted (Chapman, 2013). By contrast, *Drosophila*, which typically feed on decaying matter, often have long narrow guts characteristic of improving nutrient absorption and limiting water uptake (Engel and Moran, 2013; Farmer et al., 1981). The gut tract of *L. migratoria*, which is specialized in digesting plants, possesses larger foregut and hindgut than midgut segments (Chapman, 2013; Engel and Moran, 2013). This structure allows the bolus to be sufficiently broken down in the foregut prior to further digestion and enables large quantities of KCl, NaCl, and water to be absorbed into the hemolymph across the hindgut to minimize water loss (Audsley et al., 2013; Chapman, 2013; Loveridge, 1968; Phillips, 1964). Despite this wealth of variation in gut morphology, the basic means through which insects regulate ion and water balance remain similar.

Homeostasis within these arthropods is principally regulated through the transport and diffusion of ions and water across ionoregulatory epithelia, most notably Malpighian tubules (MTs), midgut, and hindgut epithelia (Engel and Moran, 2013; Lemaitre and Miguel-Aliaga, 2013). Under near-optimal conditions, transport epithelia rely on strict electrochemical gradients established by electrogenic transporters (Phillips et al., 1987). In the cold however, the reaction rate of these active transport enzymes decreases, while the passive movement of solutes and water remains relatively unaffected (Ellory and Willis, 1982; MacMillan et al., 2015c). If left unchecked, the resulting disruption in net transport/leak rates across ionoregulatory epithelia can

be lethal. As gut epithelia are crucial for maintaining homeostasis within insects, where and how these ionoregulatory processes fail is of great interest. Additionally, due to the specialized regulatory role each section of the gut possesses, it is possible that only a portion of it is responsible for the deterioration of homeostasis observed under sub-optimal conditions. Thus, to better understand the role each gut section plays in solute and water maintenance, I examine the key ionoregulatory properties of their epithelia in this section, including aspects of both transcellular and paracellular movement across the gut epithelia (see Fig. 1.2 for an overview of solute and water regulation along the gut). It is important to note however, that while digestion and breakdown of carbohydrates can occur in the foregut, this segment of the digestive tract acts primarily as a passage through which the bolus travels before reaching the remainder of the gut for digestion and nutrient absorption (Dadd, 1970; Holtof et al., 2019). As such, it will not be discussed further in this chapter.

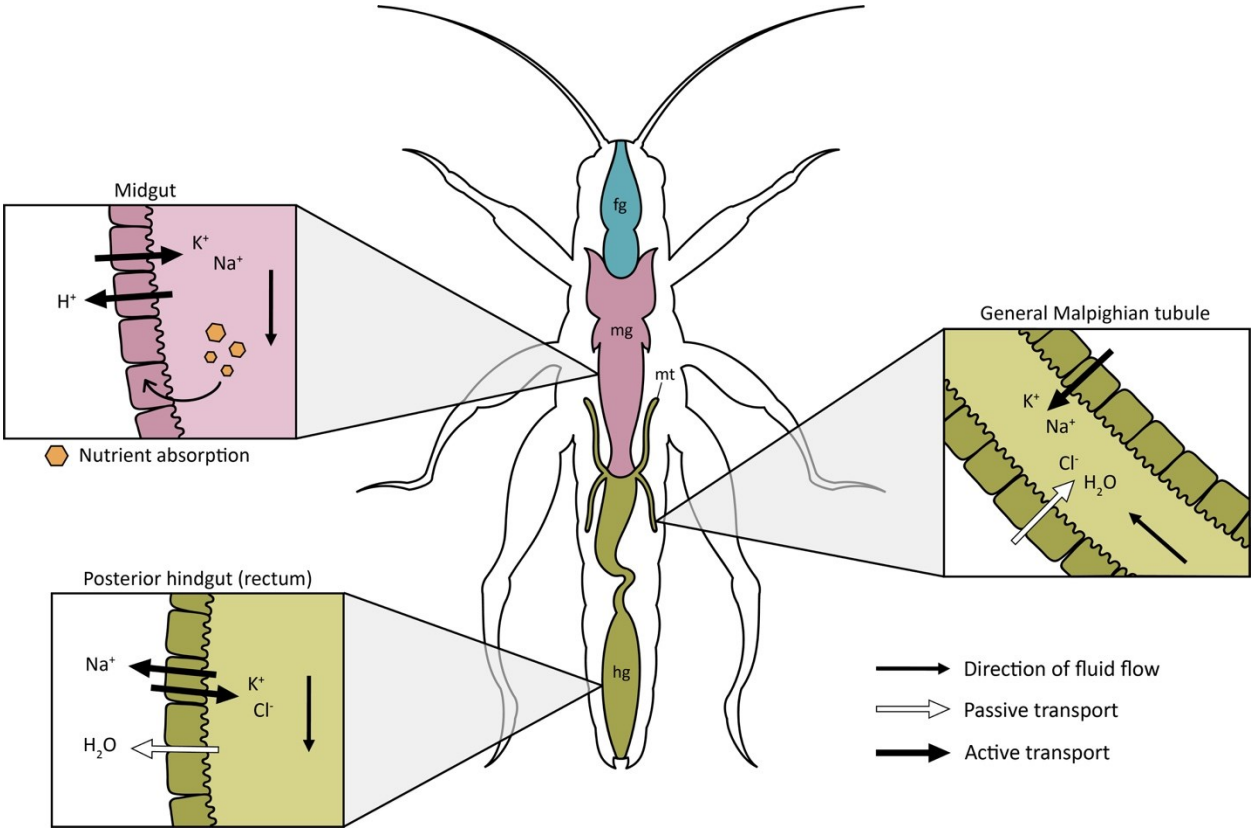

Figure 1.2. An overview of solute and water homeostasis across the hemolymph (white) and ionomotive epithelia (darkened cells) in a general phytophagous insect. For simplicity, the gut is sectioned into its general components, where the foregut (*fg*) is in blue, the midgut (*mg*) is in pink, and the Malpighian tubules (*mt*) and hindgut (*hg*) are in green. Aside from preliminary digestion and breakdown of food, the foregut typically plays a minimal role in solute regulation. Across the midgut, electrochemical gradients established by ion movement energize the digestion and absorption of macronutrients (e.g. carbohydrates, proteins, etc.). Together, the Malpighian tubules and hindgut make up the insect renal system, and use a combination of active and passive transport to regulate ion and water movement (and therefore maintain homeostasis) across epithelia. Figure synthesized using information from a variety of sources, including Holtof et al., 2019, Denecke et al., 2018, O'Donnell, 2017, Phillips et al., 1987, and Oschman and Wall, 1969.

1.3.1 *Structure and Function of Midgut Epithelia*

Unlike the epithelium of the fore- and hindguts, which are ectodermally derived, the midgut epithelium is endodermal in origin (Engel and Moran, 2013; Lemaitre and Miguel-Aliaga, 2013). The insect midgut epithelium also lacks the cuticular layer found along the other two segments, and is instead protected by an acellular structure known as the peritrophic matrix (PM) (Chapman, 2013; Denecke et al., 2018). In well-fed orthopterans like *L. migratoria*, a new PM is secreted approximately every 15 minutes, resulting in the formation of overlapping layers resembling a telescope as old PMs are gradually passed posteriorly through the gut (Baines, 1978; Lehane, 1997). This chitinous structure creates a barrier between the food bolus and the midgut itself, and helps protect midgut cells from mechanical damage (Denecke et al., 2018). The PM therefore divides the midgut into two spaces: the endoperitrophic space, where the initial digestion of macronutrients such as carbohydrates, proteins, and lipids occurs, and the ectoperitrophic space, where the assimilation of digestive end products takes place along the apical side of the midgut epithelium (Holtof et al., 2019). Cells along the ectoperitrophic space are often tall, columnar, and form microvilli on their luminal side which maximize nutrient, ion, and water absorption (Chapman, 2013; Denecke et al., 2018). Such absorption across the midgut epithelia (along with secretion along the Malpighian tubules; discussed below) is often powered by electrogenic transporters (Klein et al., 1991).

Unlike most vertebrate epithelia, which rely on Na^+/K^+-ATPase (NKA) activity to transport ions, epithelia along the apical side of the insect midgut typically rely on energy supplied by a combination of other electrogenic transporters (Harvey et al., 1983). Together, V-ATPases (VA; or proton pumps) and cation/H^+-antiporters (which do not require ATP for energy) cycle protons and K^+ (or Na^+) from cell to lumen (Klein et al., 1991; Moffett and Koch,

1992; O'Donnell, 2017; Wieczorek, 1992; Wieczorek et al., 1989). In action, VAs and cation/H^+-antiporters along the midgut of the tobacco hornworm (*Manduca sexta*) retract H^+ from the gut lumen while driving K^+ in, effectively creating a net electrochemical gradient across which molecules like amino acids can be absorbed into cells (Castagna et al., 1998; Holtof et al., 2019). There are however NKAs present along the midgut of insects such as *D. melanogaster*. These ATPases play a supporting role in adjusting Na and K levels within the cells and lumen, while VAs do the 'heavy lifting' to energize ion transport across the gut (D'Silva et al., 2017; O'Donnell, 2017). Regardless, once these molecules have been absorbed, they can either be further processed inside the epithelial cells, or absorbed into the hemolymph through the basal membrane for transport to their designated site of action or storage (Holtof et al., 2019).

1.3.2 *Urine Production in the Malpighian Tubules*

Following the midgut are the Malpighian tubules (MTs), which are blind-ended and tube-shaped diverticula of the gut that act as a primary site of ion and water regulation in insects (Dow et al., 1998). Despite their small scale, insect MTs can transport water and ions at rates that greatly exceed any other known animal tissues (gram for gram; Maddrell and O'Donnell, 1992). The blood-sucking kissing bug (*R. prolixus*) for instance, can take in a blood-meal in volumes over 12 times its own body mass, and must be able to quickly deal with the osmotic and ionic challenges such large meals bring. The MTs of these insects are therefore capable of replacing total cell ion (such as Cl^-) and water volumes every 5 and 15 seconds, respectively (Adams, 1999; Beyenbach, 2003). In addition to the variation in osmotic and ionic demands imposed by lifestyle, MT structures differ in morphology between insect genera, ranging from simple tubules in *Calliphora* (blowflies) to those differentiated into secreting and absorbing regions in *Tenebrio* (mealworm beetles), *Rhodnius*, and *Pieris* (common garden butterflies; Berridge and Oschman,

1969; Grimstone et al., 1968; Henson, 1939; Irvine, 1969; Wigglesworth and Salpeter, 1962). Further variation can be found in MT cell types. Many insects, including dipterans, hymenopterans, and lepidopterans possess tubules composed of two cell types: principal cells, which mediate active cation and water transport, and secondary (stellate) cells, which promote transcellular water transport and Cl^- movement (Beyenbach, 2003; Dow et al., 1998; Halberg et al., 2015; O'Donnell et al., 1998). Insects belonging to the ancestral Exopterygota such as orthopterans and hemipterans however, appear to lack stellate cells; ionoregulation across this lineage may therefore occur across tubules composed of solely principal cells (Halberg et al., 2015). Despite their differences, these tubules are often considered to be somewhat analogous to vertebrate kidneys due to their role of generating primary urine (metabolic waste) via ion and water secretion.

Insect hemolymph typically contains high and low concentrations of Na^+ and K^+, respectively (Overgaard and MacMillan, 2017). By contrast, one characteristic feature of their primary urine is that it often contains extremely concentrated levels of K^+ and low Na^+, relative to the hemolymph (Berridge and Oschman, 1969). It is important to note that diet and overall hemolymph composition can modify this Na^+/K^+ ratio, however, urine formation in herbivorous insects like the migratory locusts, which excrete predominantly K^+, will be discussed here. To maintain these ratios of cations, basolateral NKAs, apical VAs, cation/H^+-antiporters, Na^+-K^+-Cl^- cotransporters (NKCC), and paracellular channels (Cl^- and water) work in concert to regulate transepithelial ion and water secretion along the MTs (Harvey et al., 1983; Ianowski and O'Donnell, 2006; O'Donnell, 2017). Through basolateral NKCCs and NKAs (among other transporters), Na^+, K^+, and Cl^- enter principal cells in the distal end of the MTs (Ianowski and O'Donnell, 2004). From here, intracellular carbonic anhydrases are stimulated to produce

protons from the hydration of CO_2, which are then pumped into the lumen by apical VAs (Edwards and Patton, 1967; Maddrell and O'Donnell, 1992; O'Donnell, 2017). With excess protons available within the lumen, cation/H^+ antiporters trade H^+ for now intracellular Na^+ and K^+ (Harvey, 2009; Wessing et al., 1997). Such a positive voltage potential within the lumen drives Cl^- paracellularly from the hemolymph, after which water follows osmotically (Harvey, 2009; Ianowski and O'Donnell, 2006; O'Donnell and Maddrell, 1983; Phillips, 1970). The resulting urine exits the MTs, mixing with the gut contents (typically near the anterior hindgut and posterior midgut) before passing posteriorly to the hindgut for further solute and water modification (Phillips et al., 1987; Wigglesworth, 1932).

1.3.3 *Absorption and Excretion Along the Hindgut*

The hindgut is composed of the pylorus, ileum and rectum, and is the main site of selective reabsorption of water and ions within insects (Chapman, 2013; Engel and Moran, 2013; Holtof et al., 2019; Phillips et al., 1987; Ramsay, 1971). The pylorus lies at the anterior margin of the hindgut and acts as the point of attachment for the MTs (Chapman, 2013; Holtof et al., 2019). Following this junction is the ileum, which connects the pylorus with the rectum and can contain important symbiotic microorganisms. In some insects such as *Drosophila*, mosquitoes, and aphids, small communities housed within the ileal cuticle along the long narrow guts may act to improve nutrient absorption and water retention (Chapman, 2013; Engel and Moran, 2013). Within others, like termites and scarab beetles, these symbionts contribute largely to the breakdown of plant polysaccharides for use in aerobic metabolism (Douglas, 2015). The final section, the rectum, is the main site for osmotic regulation in the hindgut. This structure resembles an enlarged sac with regions of columnar epithelial cells known either as rectal pads (six) arranged radially around the rectum (e.g. cockroaches and orthopterans), or as rectal

papillae (e.g. dipterans; Chapman, 2013; Oschman and Wall, 1969; Ramsay, 1971). It is at the specialized epithelia of these rectal pads that the majority of water and solute reabsorption occurs (Phillips et al., 1987; Ramsay, 1971).

Across rectal pad cells, water, ions (Na^+, K^+, and Cl^-) and other small molecules are absorbed readily from the KCl-rich primary urine into the hemocoel (Oschman and Wall, 1969; Phillips and Dockrill, 1968). A thin, porous cuticle however, lines the rectum and excludes larger molecules, retaining them in the rectal lumen for excretion (Phillips and Dockrill, 1968). The apical plasma membrane lies below this cuticle and is lined with Cl^- pumps which transport Cl^- from the lumen into rectal epithelial cells (O'Donnell and Simpson, 2008; Phillips et al., 1988; Spring et al., 1978). Such a negative voltage within cells combined with the disparity between lumen and hemolymph [K^+] drives Na^+ and K^+ passively across the rectal pads via apical channels (Phillips et al., 1987). Within the convoluted intercellular channels, NKAs pump intracellular Na^+ into the paracellular space in exchange for K^+ to generate high Na^+ concentrations (Oschman and Wall, 1969). Na^+ therefore moves into the hemocoel and is followed closely by water across the hindgut epithelium via osmosis (water reabsorption) (Phillips, 1981; Phillips et al., 1987; Wall and Oschman, 1970). Waste remaining within the rectal lumen is excreted shortly thereafter.

1.3.4 *Septate Junctions - Barriers of Paracellular Permeability*

As solutes and water move (or leak) passively along the paracellular pathway, they must overcome obstacles known as septate junctions (SJs) to reach their destination. Similar to vertebrate tight junctions, these invertebrate structures are specialized cell-cell junctions that largely determine the permeability of the paracellular pathway (Izumi and Furuse, 2014; Jonusaite et al., 2016). Additional evidence suggests that these SJs are also involved in

maintaining tissue integrity and adhesiveness (Lane and Flores, 1988). However, unlike vertebrate tight junctions, which have 'kissing points' of contact between adjacent epithelia, SJs are ladder-like in structure (see Fig. 1.3; Farquhar and Palade, 1963; Gilula et al., 1970). Arthropod epithelia typically have two known types of SJs: pleated and smooth. The most common SJ described are the pleated SJs (pSJs), which are generally observed in ectodermally derived epithelia such as the foregut, hindgut, trachea, and glia (Carlson et al., 2000; Jonusaite et al., 2016; Kukulies and Komnick, 1983). These SJs are 2-3 nm wide, while the second type of SJs, smooth SJs (sSJs) are 5-20 nm wide (Jonusaite et al., 2016; le Skaer et al., 1987; Noirot-Timothée and Noirot, 1980). Smooth SJs, on the other hand, are typically found in epithelia of endodermal origins like the midgut and Malpighian tubules (Jonusaite et al., 2016; Noirot-Timothée et al., 1978).

The ladder-like SJs prevent leak of a myriad of compounds across epithelia, typically those of larger size. For instance, studies in *Drosophila* observed that fluorescently-labelled dextrans over 10 kDa are unable to pass between epithelial cells (Lamb et al., 1998). Conversely, sSJs in the Malpighian tubules of *R. prolixus* are readily permeable to a range of smaller molecules, including sucrose (342 Da), inulin (~7000 Da), mannitol (~183 Da), and polyethylene glycol (4000; Jonusaite et al., 2016; le Skaer et al., 1987; O'Donnell and Maddrell, 1983). The ability to selectively and dynamically control solute diffusion across epithelia is therefore critical for maintaining separation between the organism and the outside environment (i.e. the gut lumen; Izumi and Furuse, 2014; Jonusaite et al., 2016; MacMillan et al., 2017). This is even more evident in instances where barrier function has been compromised. In *Drosophila*, for example, age-dependent loss of gut barrier integrity or traumatic brain injury have been associated with lethal disruptions of barrier properties (Izumi and Furuse, 2014; Jonusaite et al.,

2016; Katzenberger et al., 2015; Rera et al., 2012). Similar lethal alterations in barrier integrity have been observed under suboptimal environmental conditions such as hypoxia and cold exposure (Huber et al., 2001; MacMillan et al., 2017; Sun et al., 2015). In the latter exposure however, cold-acclimated (pre-treated) *Drosophila* appear better equipped to survive the cold and demonstrated reduced paracellular leak of a fluorescent probe from their gut lumen to their hemolymph when compared to warm-acclimated flies (MacMillan et al., 2017). Such improvements in cold tolerance associated with barrier regulation suggests that SJ function and gut barrier integrity may be important to insect chill tolerance.

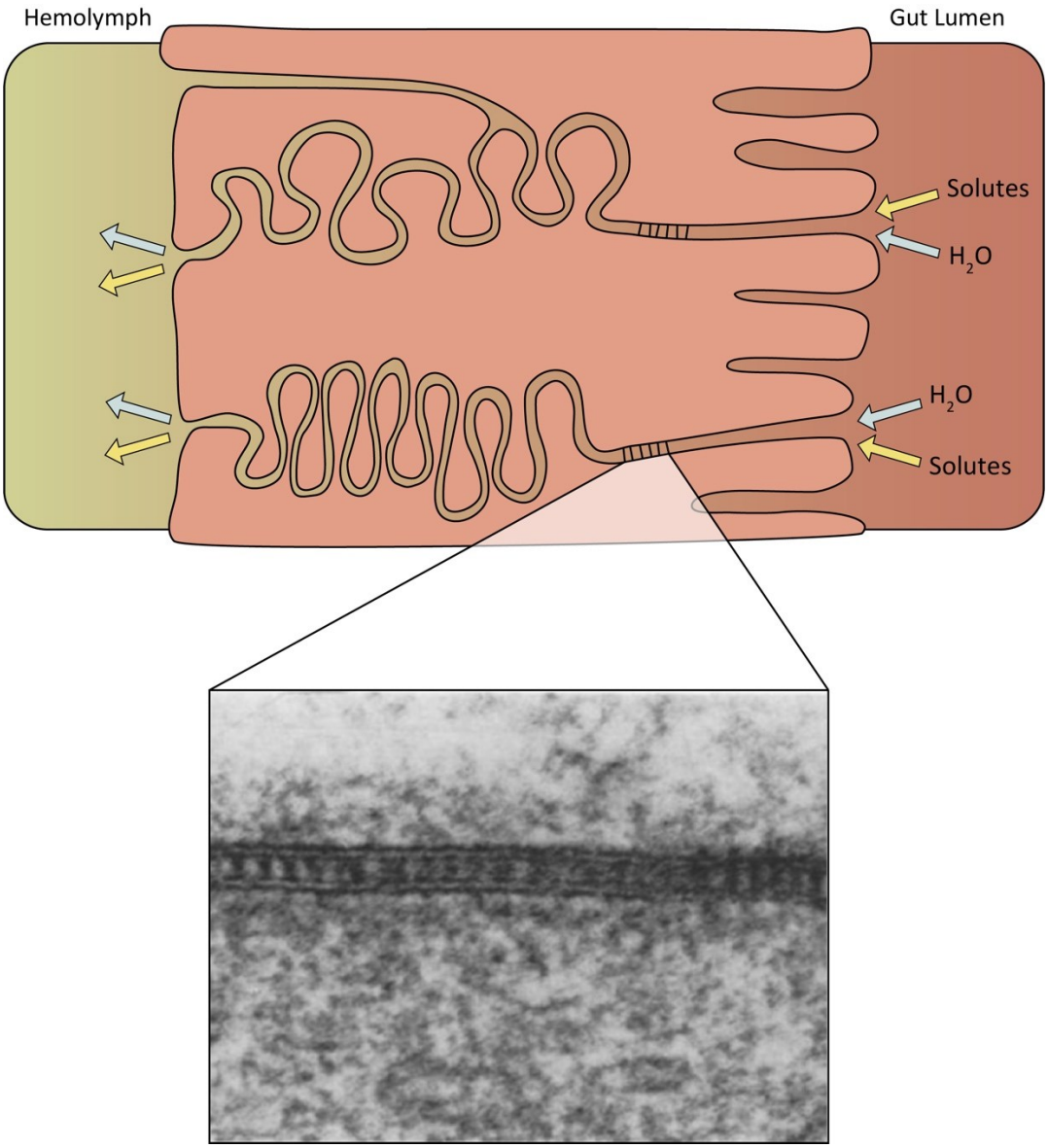

Figure 1.3. A general schematic of insect septate junctions (SJs) along the gut. In cross-section, these structures are often ladder-like in appearance and span 2-20 nm between adjacent cells. SJs are located along the apical borders of epithelial cells and largely determine the permeability of the paracellular pathway by dynamically and selectively controlling solute diffusion across epithelia (typically by solute size). Electron microscopic image from Flower and Filshie, 1975. Illustration adapted from Des Marteaux et al., 2018.

1.4 Thesis Overview

In this thesis, I investigated the role of paracellular barriers of the gut in insect chill susceptibility using the migratory locust (*L. migratoria*) as my experimental system of choice. As an experimental system, the migratory locust is advantageous as they are chill susceptible insects with a broad geographic distribution, ranging from tropical to temperate zones (Hunter, 2019; Jing and Kang, 2003). Such a widespread distribution allows for the potential application of knowledge gleaned to insect populations across various climates and environmental conditions. It is important to note that the locusts used in this thesis have been both bred in captivity and supplemented with lab-bred locusts for nearly 50 years (Jeff Dawson, personal communication). Nevertheless, the chill susceptibility data presented in Chapter 2.3.1 indicate that chill tolerance within the Carleton locust colony remains variable, and that it has not been greatly impacted by their breeding conditions. While further work should be conducted to verify the applicability of this work to wild populations, these lab-bred locusts have been deemed suitable models for my thesis. Using these locusts, my research will not only further our understanding of mechanisms underlying insect chill susceptibility and tolerance, but may also enhance our knowledge of the basic biology of paracellular barriers in orthopterans. To my knowledge, no work (beyond general characterization) has been conducted on locust paracellular junctions.

Recent evidence in *D. melanogaster* suggests that the loss of ion homeostasis that typically occurs with chilling is, at least partly, caused by disruption of epithelial paracellular barrier function along the gut (MacMillan et al., 2017). While we now know that gut barriers fail in the cold, we do not know where along the gut this is occurring. Due to the specialized functions of each segment of the gut tract (foregut, midgut, hindgut), it is possible that only a

portion of the gut is responsible for this cold-induced leak. The primary objectives of my research were therefore to determine 1) whether chilling damages locust gut epithelial integrity, and 2) where along the gut (if present) this loss of barrier integrity is occurring, focusing on the paracellular aspects of this damage. Similar to experiments conducted on *D. melanogaster*, the majority of my experiments used a common marker of paracellular permeability, FITC-dextran, to visualize loss of barrier function along the paracellular pathway. This marker has been used in both vertebrate and invertebrate models such as fruit flies (*D. melanogaster*), rats (*Rattus norvegicus*), and zebrafish (*Danio rerio*), demonstrating the ability to permeate several tissues, including the gut (Bagnat et al., 2007; Condette et al., 2014; MacMillan et al., 2017). Below, I summarize the main contents of each chapter.

In Chapter 2, I investigate the presence of cold-induced barrier failure at low temperatures in the migratory locust and seek to isolate the resulting leak across the gut. Initially, when FITC-dextran was injected into the hemolymph, negligible concentrations of the marker were found within the gut, while the majority of the injected FITC-dextran remained in the hemocoel. This suggested that gut barrier failure was not occurring in my cold-exposed locusts. However, when tested for barrier failure in the opposite direction (i.e. the locusts were fed FITC-dextran), marker concentrations increased in the hemolymph over time in the cold. Thus, locusts, like *D. melanogaster*, do experience a loss of barrier function, although it is likely unidirectional. With this in mind, follow up experiments to isolate this barrier failure prepared sections of the gut as FITC-dextran-filled 'gut sacs,' simulating a fed state *ex vivo*. Interestingly, no differences were found between the rates of marker leak over time in the cold and room temperature, or between the gut sections. While contrary to my initial predictions, these results allow for new

hypotheses regarding the mechanism of cold-induced barrier failure within insects to be addressed.

In Chapter 3, I further explore why FITC-dextran (3-5 kDa) was unable to diffuse from the hemocoel into the gut lumen. I therefore repeated experiments using a smaller, but also common marker of paracellular permeability known as mPEG-FITC (350 Da) to see if the former probe was simply too large to pass across the gut paracellularly. Once control locusts (held at room temperature) were injected however, it was quickly apparent that locusts are able to remove the marker, possibly via energy-dependent transport or solute drag. Experiments conducted in the cold corroborated these results. Here, mPEG-FITC was detected both within the gut and the hemolymph, however, concentrations remained constant over time in the cold. As no fluctuations in marker concentrations were observed in the cold, this further supports that the marker is being actively cleared from the hemocoel to be excreted (and this process is temperature sensitive) rather than moving passively into the gut lumen. Although the means through which mPEG-FITC is cleared from the locust system remain unknown, it is clear that this marker is not a suitable for quantifying epithelial permeability (at least in locusts).

Collectively, the studies I present in this thesis provide further evidence for the importance of ionomotive epithelia in chill susceptible insects beyond Diptera, and open up new directions through which we can continue to investigate the physiology of cold tolerance (discussed in Chapter 4). With my work and the future directions that stem from it, I hope to further our understanding of the physiological mechanisms that underlie chill susceptibility and physiology.

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2. The Effects of Chilling on Gut Epithelial Barrier Integrity in *L. migratoria*

2.1 Introduction

Chill susceptible insects, like locusts, are those that succumb to the negative effects of cooling at temperatures well above the freezing point of their extracellular fluids (Overgaard and MacMillan, 2017). When these ectothermic animals are chilled, they reach a temperature at which a state of neuromuscular paralysis (chill coma) occurs, known as chill coma onset temperature (CCO) (Andersen et al., 2018). The insects remain in this reversible comatose state for the duration of a cold exposure, and can often recover once the stressor (brief or mild cold) has been removed. The time taken to regain the ability to stand following chill coma is termed chill coma recovery time (CCRT) (David et al., 1998; Gibert et al., 2001; Robertson et al., 2017). Both CCO and CCRT are regularly used as non-lethal means of quantifying chill susceptibility in various groups of insects, such as crickets (*Gryllus pennsylvanicus*), caterpillars (*Pringleophaga marioni*), fruit flies (*Drosophila melanogaster*), locusts (*Locusta migratoria*), and firebugs (*Pyrrhocoris apterus*) (Andersen et al., 2013; Andersen et al., 2017a; Chown and Jaco Klok, 1997; Coello Alvarado et al., 2015). In the event of a particularly harsh or prolonged exposure, an accumulation of cold-induced tissue damage (chilling injuries) can occur (Coello Alvarado et al., 2015; Koštál et al., 2006; MacMillan and Sinclair, 2011). Chilling injuries typically manifest as a loss of coordination, permanent limb paralysis, or mortality. Quantifying an insect's condition or survival following cold stress is therefore another common measure of tolerance. It can be accomplished by using a scoring system (typically as dead/alive, or a range of conditions from dead to alive) to indirectly measure the degree of injury sustained, typically 24 h after removal from the cold (Findsen et al., 2013; Overgaard and MacMillan, 2017; Ravn et al., 2019). Similar to CCO and CCRT, post-chilling survival has been used as a measure of chill tolerance

in a variety of insect systems, including *D. melanogaster*, springtails (*Orchesella cincta*), and the rusty grain beetle (*Cryptolestes ferrugineus*), among others (Koštál et al., 2011; Nedved et al., 1998; Smith, 1970). While each of these means of measuring chill susceptibility work via distinct mechanisms, they are all typically associated with a local or systemic loss of ion balance, suggesting that ionoregulatory failure may be a principal cause of chill susceptibility (Armstrong et al., 2012; Bayley et al., 2018; Findsen et al., 2013; Koštál et al., 2006; MacMillan et al., 2015a).

In insects, organismal ion homeostasis is under tight regulation by both the Malpighian tubules (MTs) and gut epithelia (MacMillan and Sinclair, 2011). Briefly, the gut has three main regions (foregut, midgut, and hindgut; see Fig. 2.1 for general locust anatomy), each with its own specialized functions. The foregut lies at the anterior margin of the gut and consists of flattened and undifferentiated cells consistent with the lack of absorption or secretion that takes place in the region (Chapman, 2013). Instead, the foregut generally acts as a passage through which food travels, although muscular breakdown and salivary digestion of the bolus can occur (Dadd, 1970). From the foregut, food travels to reach the remainder of the gut to be digested (Engel and Moran, 2013; Naikkhwah and O'Donnell, 2012). Cells along the midgut (region following the foregut) are actively involved in digestive enzyme production and secretion, acting as the primary site of digestion and nutrient, ion, and water absorption (Chapman, 2013; Yerushalmi et al., 2018). Following the midgut are the MTs, blind-ended tube-shaped diverticula of the gut that are somewhat analogous to the vertebrate kidneys (Chapman, 2013). These tubules are one cell layer thick and contain a multitude of cation exchangers and pumps (Maddrell and O'Donnell, 1992; O'Donnell and Ruiz-Sanchez, 2015). Through these ionoregulatory pumps (e.g. V-ATPase, the proton pump that primarily energizes transport at the MTs) and channels, ions such

as K^+, Na^+ and Cl^- are driven from the hemolymph to the tubule lumen. For instance, K^+ is secreted into the tubule lumen which helps to maintain a low K^+ (generally 10-15 mM) environment where nerves and muscles can function (Andersen et al., 2017a; Andersen et al., 2018; Gerber and Overgaard, 2018; Harvey and Zerahn, 1972; Yerushalmi et al., 2018). These means of ionoregulation across the MTs promote an osmotic gradient that favours the movement of water and unwanted waste products or toxins into the tubule lumen (secretion), producing primary urine (Maddrell and O'Donnell, 1992). Finally, the hindgut (composed of the ileum and rectum) is the most posterior region of the gut, and is the main site for the absorption of water and solutes (Phillips et al., 1987). Because of this, cell membranes along the intercellular spaces of the hindgut are rich in Na^+/K^+-ATPases, which generate high [Na^+] in the paracellular space that drives water across the hindgut via osmosis, permitting water reabsorption and production of a dry feces (Des Marteaux et al., 2018; Phillips, 1981; Wall and Oschman, 1970).

Through the continuous ionoregulatory actions of the alimentary canal, the hemolymph of most insects contains high and low concentrations of Na^+ and K^+, respectively, at optimal or near-optimal thermal conditions (Engel and Moran, 2013; MacMillan et al., 2015b; Maddrell and O'Donnell, 1992). In cold conditions, however, the activity of ionoregulatory enzymes like V-ATPases and Na^+/K^+-ATPases is suppressed (Bayley et al., 2018; Hosler et al., 2000; Mandel et al., 1980; McMullen and Storey, 2008; Moriyama and Nelson, 1989; Yerushalmi et al., 2018). Over time, this temperature-induced suppression of transcellular ion transport often results in a net leak of hemolymph Na^+ and water (which follows Na^+ osmotically) to the gut lumen, effectively concentrating the K^+ that remains in the hemolymph (MacMillan et al., 2014). Some intracellular K^+ simultaneously leaking down its concentration gradient into the extracellular space worsens this problem (Andersen et al., 2017; MacMillan et al., 2014). As K^+

concentrations rise in the hemocoel (hyperkalemia), the gradient of K^+ across the cell membrane is lost, and a marked depolarization in membrane potential occurs, eventually resulting in the activation of voltage-gated Ca^{2+} channels (Andersen et al., 2017a; Bayley et al., 2018; MacMillan et al., 2015a). The influx of Ca^{2+} that ensues is proposed to initiate a crippling cascade which causes a deterioration of cellular integrity, likely through apoptosis (Mattson and Chan, 2003; Nicotera and Orrenius, 1998; Yi and Lee, 2011; Yi et al., 2007). Failure to maintain ion and water homeostasis in the cold (termed "ionoregulatory collapse") can therefore ultimately result in organismal chilling injury or death. In turn, understanding the biochemical mechanisms underlying this failure is critical to understanding chill susceptibility. While a cold-induced failure of transcellular transport is one likely mechanism of chilling injury and is under active investigation, ions do not cross epithelia solely via transcellular pathways (Donohoe et al., 2000; MacMillan et al., 2016a; O'Donnell and Maddrell, 1983).

In addition to the active movement of ions and passive transport of solutes and water through cells (transcellular pathways; e.g. via channels), the gut also relies on the passive movement, or leak, of these molecules through the paracellular pathways between adjacent cells (Jonusaite et al., 2016; le Skaer et al., 1987). Septate junctions (SJs) are specialized cell-cell junctions analogous to vertebrate tight junctions that largely determine the permeability of these paracellular pathways (Jonusaite et al., 2016). Arthropod epithelia generally have two types of SJs: pleated and smooth. The former are typically observed in ectodermally-derived epithelia such as the foregut and hindgut, and are 2-3 nm wide, while smooth SJs are found in endodermally-derived tissues like the midgut, and are 5-20 nm wide (Jonusaite et al., 2016). To date, the majority of SJ studies has been conducted on *Drosophila*, including the identification of SJ types, associated proteins, and SJ influence in cold tolerance plasticity (Izumi and Furuse,

2014; MacMillan et al., 2016b; MacMillan et al., 2017). Notably, cold acclimated (pre-treated) flies, which are generally more cold tolerant than their warm acclimated counterparts, have an upregulation of 60% of genes encoding known or putative fly SJ proteins (MacMillan et al., 2016b). Furthermore, MacMillan et al. (2017) demonstrated that cold-acclimated (10°C) flies had reduced paracellular leak of a fluorescent probe from their gut lumen to their hemolymph, when compared to warm-acclimated (25°C) flies. This reduction in paracellular leak during cold stress was associated with increased cold tolerance at the organismal level. Together, these studies suggest that cold exposure can cause increased rates of leak through the paracellular barriers. However, because flies were fed the probe for these experiments (and the gut was completely loaded with the probe upon cold exposure) the precise site of this leak and the mechanisms that drive it remain unclear, as does whether this a problem experienced by all insects, or just *D. melanogaster*.

Here, we investigated the effects of chilling on ionomotive epithelial integrity in a chill susceptible insect, the migratory locust (*Locusta migratoria*). As previously observed in *Drosophila*, we hypothesized that chilling disrupts septate junctions (SJs) in locust gut epithelia and that this effect leads to paracellular leak across the gut epithelia. Due to the often temperature-sensitive nature of ionoregulatory enzymes, and their dense concentration along ionomotive epithelia, we also hypothesized that this disruption in barrier integrity is limited to transport-rich segments along the locust gut such as the midgut and hindgut. To address these hypotheses, we first tested the degree of chill susceptibility in our locusts by measuring their performance post-cold exposure. We then used the fluorescently-labelled marker, FITC-dextran to characterize directionality in cold-induced paracellular leak, and to locate areas of leak along the locust gut.

2.2 Methods

2.2.1 *Experimental System*

All experiments were conducted using male and female adult locusts (*Locusta migratoria*) aged 3-4 weeks post-final ecdysis. Locusts were obtained from a continuously breeding colony maintained at Carleton University in Ottawa, ON. This colony is reared under crowded conditions on a 16 h:8 h light:dark cycle at 30°C with 60% relative humidity (see Dawson et al., 2004). All animals had *ad libitum* access to a dry food mixture (oats, wheat bran, wheat germ, and powdered milk), and fresh wheat clippings supplied three times per week.

2.2.2 *Chill Coma Recovery Time and Survival*

The degree of chill susceptibility of the locust colony was quantified using both their chill coma recovery time (CCRT) and the degree of injury/mortality 24 h following exposure to -2°C. On the day of the experiment, locusts were collected from the colony, sexed by eye, and individually placed into 50 mL polypropylene tubes. These tubes were sealed using lids with small holes to allow access to air for the duration of the experiment. Excluding controls, all locusts were suspended using a Styrofoam rig in a cooling bath (Model AP28R-30, VWR International, Radnor, USA) containing a circulating ethylene glycol:water mix (3:2) preset to 20°C and cooled to -2°C at a rate of -0.20°C min^{-1}. Both bath temperature and locust internal body temperature were monitored; the former via internal probes, and the later via inserted type-K thermocouples (TC-08 interface; *PicoLog* software version 5.25.3) located at the junction of the head and thorax of representative locusts (that were not used further in the experiments). Locusts were then left undisturbed for 2, 6, 24, or 48 h upon which arbitrarily selected groups of locusts were removed from the cold and returned to room temperature (23°C). In order to monitor CCRT, insects were removed from their tubes and gently placed on the surface of a table

and observed for the time taken to recover from chill coma. Animals were stimulated by gentle puffs of air from a transfer pipette every minute and were marked as having fully recovered when standing on all six limbs. Observation time was limited to 60 min; any locusts not meeting this criterion were marked as having not recovered.

After 60 min, the locusts were returned to their respective tubes along with a dry food mixture (oats, wheat bran, wheat germ, and powdered milk) and water (supplied in microcentrifuge tubes with cotton) and left for 24 h at room temperature (23°C). An assessment of 24 h survival post-cold exposure was performed using a scoring system of 0 to 5, similar to that described by MacMillan et al. (2014). Briefly, scores were defined as follows: 0: no movement observed (i.e. dead); 1: limb movement (slight leg and or head twitching); 2: greater limb movement, but unable to stand; 3: able to stand, but unable or unwilling to walk or jump; 4: able to stand, walk, and or jump, but lacks coordination; and 5: movement restored pre-exposure levels of coordination.

2.2.3 *Quantification of Serosal to Mucosal Paracellular Leak and Ion Imbalance*

To measure paracellular permeability in the gut epithelia of locusts, we monitored the movement of a fluorescently-labeled molecule in both the serosal (hemolymph) to mucosal (lumen) direction, and the mucosal to serosal direction. All experiments used FITC-dextran (3-5kDa, Sigma Aldrich, St. Louis, USA) a commonly used probe for determining paracellular permeability in both invertebrate and vertebrate models such as fruit flies (*D. melanogaster*), rats (*Rattus norvegicus domesticus*), and zebra fish (*Danio rerio*) (see MacMillan et al., 2017; Condette et al., 2014; Bagnat et al., 2007).

Experiments conducted in the serosal to mucosal direction (from the hemolymph to the gut lumen) were done both to identify the presence of leak across the gut epithelia and isolate the

area across which leak occurred. Protocols for this novel leak assay were developed and honed over the course of preliminary trials. In the final assay, FITC-dextran was dissolved in locust saline (in mmol l^{-1}: 140 NaCl, 8 KCl, 2.3 $CaCl_2$ Dihydrate, 0.93 $MgCl_2$ Hexahydrate, 1 NaH_2PO_4, 90 sucrose, 5 glucose, 5 trehalose, 1 proline, 10 HEPES, pH 7.2) resulting in a final FITC-dextran concentration of 3.84 x 10^{-3} M (selected based on standard curves from the preliminary trials). Using a 25 µL Hamilton syringe, 20 µL of this solution was injected into the hemocoel ventrally at the junction of the thorax and first abdominal segment of locusts. Pilot experiments revealed that neither the 20 µL injection nor the FITC-dextran itself impacted locust performance or survival (survival for control locusts with and without FITC-dextran injection was scored as 5). Following the protocol for CCRT (outlined above), animals were suspended in a cooling bath preset to 20°C and cooled to -2°C at a rate of -0.2°C min^{-1}. Insects were then held at -2°C (or room temperature for controls) and left undisturbed for 2, 6, 24, or 48 h.

Locusts were individually removed from the cooling bath and dissected within 15 min of their target cold exposure duration for tissue collection. Animals were sacrificed by decapitation before removing all limbs and wings. The thorax and abdomen (containing the internal organs) were placed in a petri dish lined with silicone elastomer (Sylgaard 184 Silicone Elastomer Kit, Dow Chemical, Midland, USA) and containing locust saline. A longitudinal incision was made in the anterior to posterior direction along the ventral side to expose the gut. With the body wall pinned back, the tracheae and Malpighian tubules were then cleared away to access the gut tissue. The gut was then cut into three segments: anterior, central, and posterior (see Fig. 2.1). Briefly, the anterior segment was defined as the foregut to the anterior midgut caeca, the central segment as the posterior midgut caeca to the midgut-hindgut junction, and the posterior segment as the midgut-hindgut junction to the rectum. To avoid excessive leak of gut contents during

collection, segments were gently pinched with dissecting forceps at both ends before excision. Upon removal, segments were washed briefly in saline (while retaining their contents) to remove any excess dextran-saturated hemolymph, and placed in microcentrifuge tubes containing 500 µL of locust saline. Samples were subsequently homogenized (OMNI International Tissue Master 125 120 V, Kennesaw, USA; approximately 3 min or until solution is uniform), sonicated (Qsonica Sonicators Model CL-188, Newton, USA; 3 x 5 s bursts with 10 s rests), and centrifuged for 5 min at 10,000 × *g*. A 100 µL aliquot of the resulting supernatant was collected and transferred to a 96-well plate (Corning Falcon Imaging Microplate; black/clear bottom) for fluorescence spectrophotometry (Ex: 485 nm, Em: 528 nm; BioTek Cytation 5 Imaging Reader, Winooski, USA). Concentrations of FITC-dextran in the samples were determined by reference to a standard curve of FITC-dextran in locust saline, and control samples confirmed that tissues from locusts that were not injected with the probe had negligible fluorescence.

Hemolymph extraction experiments were performed on separate locusts over identical exposures to determine how much FITC-dextran was being lost from the hemolymph over time. Similar to the above protocols, a new set of locusts were injected with the FITC-dextran solution and suspended at -2°C for 2, 6, 24, or 48 h in a circulating cooling bath, while controls were held at room temperature. After their designated exposures, hemolymph samples were collected using methods adapted from Findsen et al. (2013). Briefly, locusts were pricked dorsally using a dissecting probe at the junction of the head and thorax before using a 50 µL capillary tube to collect the hemolymph (as described for hemolymph ion measurements). A 2 µL aliquot of hemolymph was pipetted into 96-well plates (Corning Falcon Imaging Microplate; black/clear bottom), diluted 50-fold with saline, and analyzed for FITC-dextran content via fluorescence spectrophotometry. Pilot experiments showed no interference from the saline when measuring

fluorescence. To maximize use of the large volumes of available hemolymph, these locusts were also used to collect data on cold-induced ion movement over time.

An additional 10 µL of hemolymph from each animal was collected using a 50 µL capillary tube. Samples were promptly vortexed and flash frozen in liquid nitrogen to avoid coagulation of the hemolymph and stored at -80°C until experiments. All samples were vortexed once again prior to testing. Hemolymph Na^+ and K^+ concentrations were measured using ion-selective borosilicate microelectrodes (TWI150-4, World Precision Instruments, Sarasota, USA). No interference from the FITC-dextran was found when measuring ions. The ion content of hemolymph both with and without FITC-dextran was measured in control (room temperature) locusts across 48h, and statistical analysis revealed no significant differences in ion concentrations between the two groups (Linear model, $F_{1, 10} = 0.302$, $P = 0.594$). Our Na^+-selective microelectrodes were constructed using 100 mM NaCl backfill solution and Na^+ ionophore II cocktail A (Sigma Aldrich), while K^+ selective microelectrodes contained 100 mM KCl backfill solution and K^+ ionophore I cocktail B (Sigma Aldrich). Microelectrodes were calibrated using standards of 10 mM and 100 mM of NaCl or KCl (osmolality adjusted with LiCl) for their respective measurement. These standards were also used to calculate the ion concentration in samples of hemolymph from the obtained voltage measurements using the following formula:

$$C_f = C_L \cdot 10^{\Delta V/S},$$

where C_f is the final concentration in mM, C_L is the concentration (in mM) of the lowest standard used for the data point of interest, ΔV is the difference (mV) between the sample of interest and the lowest standard, and S is the slope (the difference in mV between the two standards). Only microelectrodes with a slope between 50 and 60 mV were used for all experiments (Na^+: 51.2 ±

0.1; K^+ 55.5 ± 0.4).

2.2.4 *Measuring Paracellular Leak in Fed Locusts*

Hemolymph extractions were also used (on a separate set of locusts) to measure leak across the gut epithelia in the mucosal to serosal direction. Looking back on the literature, we noticed that experiments using models such as mosquitos (*Aedes aegypti* and *Anopheles gambiae*) and rats (*Rattus norvegicus domestica*) administered FITC-dextran orally to test for paracellular leak (Condette et al., 2014; Edwards and Jacobs-Lorena, 2000; Pantzar et al., 1993). To test whether the lack of FITC-dextran movement in the serosal to mucosal direction was due to this key difference in methodology, we took a different approach. Instead of FITC-dextran injections, we fed locusts a mixture of dry food (oats, wheat bran, wheat germ, and powdered milk) saturated with a solution of FITC-dextran in water (9.6×10^{-4} M) for 24 h prior to experiments. Similar to experiments in the opposite direction, all animals were exposed to -2°C for 2, 6, 24, or 48 h. Hemolymph was sampled and analyzed as above following removal from the cold.

2.2.5 *Isolation of Barrier Failure Across the Gut*

As we could only detect leak of the probe out of (and not into) the gut, an important question remained: where along the gut is this barrier disruption occurring? To determine the location of this leak, a gut sac approach adapted from Hanrahan et al. (1984) was applied to each of the three gut segments (Fig. 2.1; anterior, central, and posterior). On the day of the experiment, locusts were sacrificed by decapitation, and prepared for dissection by removing all appendages. The thorax and abdomen were placed in a Sylgaard-lined petri dish and locust saline was used to keep tissues moist during dissections. An incision made in the anterior to posterior direction was made along the ventral side to pin open the body cavity. All structures aside from

the gut tract were cleared away before the segments were isolated.

To suspend each isolated gut segment within our system, a heat flared polyethylene tube (PE tube; VWR ID x OD: 0.023 x 0.038", Radnor, USA) was inserted and tied into the anterior margin of the section. Once secure, standard locust saline was injected through the PE tube to thoroughly rinse out the gut contents. A second heat flared PE tube was then inserted and tied into the posterior margin of the segment. Preparations were kept in a petri dish containing continuously oxygenated (95% O_2, 5% CO_2, Praxair, Danbury, USA) room temperature saline until all three segments had been prepared for suspension. Once complete, a 9.6 x 10^{-4} M FITC-dextran solution was injected via PE tube into each preparation until it had filled both PE tubes, ensuring the lumen was filled with the saline containing the probe. Each preparation was suspended in a beaker containing 25 mL of continuously oxygenated locust saline, which acted as our serosal environment (see Fig. 2.2 for detailed schematic). After a 30 min rest period at room temperature to allow for tissue stabilization, the beaker was moved into the cooling bath (preset at 0°C) and monitored for 5 h (Gerber and Overgaard, 2018). Preparations were then removed from the cooling bath and monitored for an additional 2 h at room temperature. This was done to determine whether leak was exacerbated or alleviated by rewarming. Aliquots of 90 µL were collected directly from the beakers once an hour for the duration of the experiment and analyzed immediately post-collection for fluorescence as before. The results obtained from fluorescent analyses were then plotted to obtain leak rates per cm^2 of gut tissue for all preparations. Briefly, the slope (([FITC-dextran] (µmol) against Time (h)) of each gut sac sample and measurements of tissue length and width (treated as cylindrical surface area) were used to calculate leak rates per cm^2 of gut tissue. Log_{10} of the data were used for all statistical analyses.

2.2.6 *Data Analysis*

All data, excluding rates of FITC-dextran gut leak (*ex vivo* gut sac preparations) and concentrations of FITC-dextran found in the gut (*in vivo* FITC-dextran injection experiments; outlined below), were analyzed using linear models in R Studio version 1.2.1335 (https://www.rstudio.com). The effects of time in the cold on gut leakiness (quantified by FITC-dextran movement) were analyzed using a linear mixed effects model via the lmer() function in R (lme4 and lmerTest packages for R). Time and segment were treated as fixed effects, while each individual locust (*ex vivo* gut sac preparations) or locust sex (*in vivo* FITC-dextran injection experiments) was treated as a random effect to account for variability in locust gut leakiness per individual or sex. All data were analyzed with time as both a continuous and categorical factor, however, the outcomes of these two approaches were identical. As such, all results presented in this section treat time as a continuous factor. Finally, paired t-tests were done to compare the rates of FITC-dextran leak/cm^2 of gut tissue in the cold and post-cold in *ex vivo* gut sac preparations. The level of statistical significance was 0.05 for all analyses, while all additional values presented are mean ± standard deviation.

2.3 Results

2.3.1 *Chill Coma Recovery Time and Survivability*

The chill susceptibility of our locust colony was confirmed by measuring chill coma recovery time and 24 h survival post-cold exposure. After 2 h of cold exposure, all animals recovered from chill coma to standing position (CCRT) within 10-18 min. However, recovery time significantly increased as exposure times grew longer (Fig. 2.3a; Linear model, $F_{1, 22} = 31.3$, $P < 0.001$). This trend persisted until the last time point (48 h at -2°C), at which point no locusts

recovered the ability to stand within 60 min. Similarly, survival rates decreased with longer cold exposures, leading to nearly 100% mortality after 48 h at -2°C (Fig. 2.3b; Linear model, $F_{1, 38}$ = 199, $P < 0.001$).

2.3.2 *Hemolymph Ion Content*

Ion selective microelectrodes were used to determine the effects of cold exposure on extracellular ion balance over the course of our experiments. While the concentration of Na^+ in the hemolymph decreased significantly as time at -2°C increased up until approximately 24 h (Fig. 2.4b; Linear model, $F_{1, 23} = 10.1$, $P = 0.004$), hemolymph K^+ concentrations significantly increased, doubling from 11.8 mM to 23.3 mM over 48 h at -2°C (Fig. 2.4a; Linear model, $F_{1, 23} = 27.8$, $P < 0.001$).

2.3.3 *Serosal to Mucosal Leak*

To quantify the presence of paracellular leak across the gut epithelia in the cold (from the hemocoel into the gut), samples were taken from each gut segment (Fig. 2.1, anterior, central, and posterior) and analyzed for fluorescent content following marker injection. Interestingly, while FITC-dextran concentrations significantly increased in the gut over time (Fig. 2.5a; Linear model, $F_{1, 77} = 7.85$, $P = 0.006$), less than 1% of the total injected marker appeared within the gut after 48 h in the cold. Using summary data from both the gut leak assay (0.006 ± 0.0003 mg/mL) and hemolymph extraction experiments (following section; 1.85 ± 0.117 mg/mL), we estimate that approximately 0.34% of total FITC-dextran injected into the hemolymph leaked across the gut epithelia into the lumen during cold exposure. This method also made it possible for us to isolate potential sites of barrier loss along the gut. In addition to the minute movement of FITC-dextran over time, there were no significant differences in marker concentration among the three gut segments (Linear model, $F_{2, 77} = 1.76$, $P = 0.179$). Similarly, no significant interaction was

found between the time spent at -2°C and the segment type (Linear model, $F_{2, 77} = 1.57$, $P = 0.214$). Finally, total concentrations of FITC-dextran found in the gut did not significantly differ between treatment and control locusts at 24 h (Fig. 2.5b; Linear model, $F_{4, 1} = 0.531$, $P = 0.758$). There was, however, a significant difference between the total concentration of FITC-dextran sampled at 0 h and 24 h in both treatments (Linear model, $F_{1, 11} = 25.4$, $P < 0.001$).

Loss of marker from the hemolymph was investigated to corroborate levels of cold-induced leak into the gut and to determine whether our locusts were capable of metabolizing the probe. There was a significant loss of FITC-dextran from the hemolymph over time in the cold and at room temperature (Fig. 2.5c; Linear model, $F_{1, 39} = 5.26$, $P = 0.027$). However, there was no significant difference in marker movement between the two treatment groups (Linear model, $F_{1, 39} = 0.142$, $P = 0.708$). We also observed no significant interaction between marker concentration over time in the cold and room temperature conditions (Linear model, $F_{1, 39} = 0.138$, $P = 0.712$).

2.3.4 *Mucosal to Serosal Leak*

Traditionally, studies examining paracellular leak of FITC-dextran and other large markers like inulin involve the oral administration of probes to the animals – a stark contrast to our serosal to mucosal (probe injection) approach. These differences in methods may account for our initial finding that paracellular barriers are maintained in the cold (at least in locusts). To address this possibility, we again examined cold-induced leak, however, this time in the mucosal to serosal direction. Locusts were fed a dry food mixture saturated with water containing a set concentration of FITC-dextran and sampled for marker content. Unlike the minimal FITC-dextran leak that occurred in the serosal to mucosal direction, these experiments yielded a significant and near linear increase in hemolymph FITC-dextran concentration over time in the

cold (Fig. 2.6; Linear model, $F_{1, 28} = 15.4$, $P < 0.001$). There was an approximately 32-fold difference in hemolymph FITC-dextran levels between control locusts and those that spent 48 h at -2°C.

2.3.5 *Isolation of Barrier Failure*

With the tendency of cold-induced leak to seemingly occur unidirectionally, a modified gut sac technique was used isolate the location(s) of gut barrier failure. Here, each segment (Fig. 2.1; anterior, central, and posterior) was suspended and injected with FITC-dextran to quantify leak in the mucosal to serosal direction. Samples were periodically collected from the surrounding "serosal" environment and analyzed for marker content. At 0°C, no significant differences between leak rate/cm^2 tissue were found between the segments (Fig. 2.7; Linear model, $F_{2, 15} = 1.03$, $P = 0.379$). The same was true for segments held at room temperature over the course of experiments (Linear model, $F_{2, 10} = 0.989$, $P = 0.406$). Furthermore, contrary to our predictions, treatment (-2°C vs 23°C) did not have a significant effect on the leak rate/cm^2 of tissue in any gut segment (Linear model, $F_{2, 30} = 1.10$, $P = 0.346$). There were also no main effects of either the leak rate/cm^2 tissue between segments (Linear model, $F_{2, 30} = 0.882$, $P = 0.424$) or of the treatment (Linear model, $F_{1, 30} = 1.04$, $P = 0.315$). Finally, no significant differences were found between the leak rate/cm^2 of tissue at 5 h of cold and the leak rate/cm^2 of tissue at 2 h of 23°C in either of the three segments (anterior: two-tailed $t_5 = 0.668$, $P = 0.534$; central: two-tailed $t_5 = 1.19$, $P = 0.288$; posterior: two-tailed $t_5 = 1.27$, $P = 0.261$).

2.4 Discussion

Chill susceptible insects experience adverse effects of chilling at low temperatures that occur in the absence of ice formation. Consequences of cold exposure for these insects, like chill

coma and tissue damage, are consistently associated with a disruption of ion and water balance (Overgaard and MacMillan, 2017). Although temperature effects on active ion transport processes are likely critical drivers of organismal failure, another potential contributor is cold-induced deterioration of paracellular barrier components known as septate junctions (SJs). In this study, we provide evidence for the presence of unidirectional cold-induced paracellular leak. While the gut leaks *in vivo*, we demonstrate that each section of the gut leaks at similar rates in the cold as it does at room temperatures within an *ex vivo* setup. To our knowledge, this is the first evidence of paracellular leak in cold-exposed insects other than *Drosophila*, and the first to report details comparing events of cold-induced barrier failure along the gut as a whole in the cold. Our findings support epithelial barrier function as a contributing factor in insect chill tolerance.

2.4.1 *Gut Epithelial Integrity Appears to be Maintained in the Serosal to Mucosal Direction*

Similar to trends observed in *Drosophila*, we predicted that a large and rapid increase in gut FITC-dextran concentration would occur over time in the cold (MacMillan et al., 2017). However, when each gut segment was analysed for marker content, we found that cold stress induced minimal leak from the hemolymph into the gut (Fig. 2.5a). Samples of hemolymph assayed for FITC-dextran levels also revealed little loss of the marker over time in the cold (Fig. 2.5c). Such a retention of FITC-dextran in the hemocoel both in the cold and at room temperature across 48 h provides evidence that dextran is not readily metabolized in locusts. Studies conducted in the past reveal that compounds like juvenile hormone (JH) and JH analogues are metabolized between 2 to 24 h after administration in desert locusts (*Schistocerca gregaria),* kissing bugs (*Rhodnius prolixus*), and mice (*Mus musculus*) (Ajami and Riddiford, 1973; Slade and Zibitt, 1972; White, 1972). While we do see a loss of FITC-dextran from the

hemolymph, the majority of the injected marker was retained in the hemocoel after a prolonged period of time. Based on this reasoning, we excluded FITC-dextran metabolism as a plausible explanation for our nominal marker movement.

This lack of marker movement is smaller still when compared to results in *Drosophila*, where even cold-acclimated (and more cold tolerant) flies exhibited 10.5-fold increases in hemolymph FITC-dextran levels in the cold (Andersen et al., 2017b; MacMillan et al., 2017). While in lesser concentrations, other macromolecules such as inulin (approx. 5000 kDa) have also been shown to leak across the gut in fifth instar desert locusts (*S. gregaria*; desert locust) (Zhu et al., 2001). Furthermore, areas along the midgut of larval *Aedes aegypti* (yellow fever mosquito) are permeable to FITC-dextran as large as 148 kDa (Edwards and Jacobs-Lorena, 2000). This permeability to large molecules is not a phenomenon limited to invertebrates. On the contrary, numerous intestinal permeability experiments have been done in vertebrate models such as mice and rats using FITC-dextran – the vast majority of which support the ability of FITC-dextran to diffuse across areas of the gut (Pantzar et al., 1993; Woting and Blaut, 2018). While our findings clearly differ from those previously reported, it is important to note that they are consistent with the inability for FITC-dextran to permeate the locust rectal wall (Gerber and Overgaard, 2018). In this case, however, gut preparations were exposed to a short-term cold stress. Exposure to more prolonged bouts of cold may yield different results. A plausible explanation for this lack of marker movement in our locusts may lie in the structure of FITC-dextran itself. Permeability across the paracellular pathway is largely determined by septate junctions (SJs), which span an intercellular space of 50-200 Å (5-20 nm; Jonusaite et al., 2016). By comparison, the 4 kDa FITC-dextran used in this study is approximately 14 Å and should therefore be able to cross through this pathway unhindered. However, as FITC-dextran is bulky,

polar, and uncharged, it may be physically incapable of permeating the paracellular pathway, even under cold stress (Matter and Balda, 2003).

2.4.2 *Directionality of Cold-Induced Loss of Barrier Function*

While our experiments originally examined movement in the serosal to mucosal direction to isolate gut leak, it is common practice to assess leak via the opposite route. In addition to *Drosophila*, studies spanning an array of models from kissing bugs (*Rhodnius prolixus*) to mice (*Mus musculus*) and killifish (*Fundulus heteroclitus*) have documented the movement of various markers through the paracellular pathways in the mucosal to serosal direction across gut epithelia (Andersen et al., 2017b; le Skaer et al., 1987; MacMillan et al., 2017; O'Donnell and Maddrell, 1983; O'Donnell et al., 1984; Wood and Grosell, 2012; Woting and Blaut, 2018). The lack of FITC-dextran movement in the serosal to mucosal direction may therefore be attributed to the route of administration.

To distinguish between the presence of strictly unidirectional leak and a lack of leak, we fed locusts a dry food-dextran mixture 24 h prior to cold exposure. In stark contrast to our previous results, we observed a significant increase in hemolymph dextran concentrations over time at -2°C, resulting in a near 18- and 32-fold rise in total FITC-dextran levels after 24 h and 48 h in the cold, respectively (Fig. 2.6). Interestingly, the former increase of FITC-dextran under cold stress was similar to that seen across 24 h in FITC-dextran-fed *Drosophila*, where a 10.5-fold increase in marker concentration was observed (MacMillan et al., 2017). Meanwhile, in the opposite direction (serosal to mucosal), leak across the gut of our locusts resulted only in a 2-fold increase in the cold relative to concentrations in animals prior to cold exposure (Fig. 2.5). These data therefore suggest that cold-induced leak does occur in locusts, but occurs unidirectionally across the gut epithelia during cold stress.

It is well-documented that cold exposure causes not only a loss of ion and water balance, but also a depolymerization of cytoskeletal components such as actin (Belous, 1992; Callaini et al., 1991; Des Marteaux et al., 2018; Kayukawa and Ishikawa, 2009; Kim et al., 2006). This protein is critical to ion transport regulation, often acting as an anchor point to which transport proteins attach on both the basal and apical borders of epithelial cells (Cantiello, 1995a; Cantiello et al., 1991; Janmey, 1998; O'Donnell, 2017; Sasaki et al., 2014). Damage to the actin cytoskeleton can therefore create a cascade of detrimental effects within an organism. For instance, disruptions in the actin cytoskeleton have been shown to inactivate K^+ channels in human melanoma cells (Cantiello et al., 1993). Similarly, in rat kidneys, failure of the cytoskeletal system stimulates Na^+/K^+-ATPase activity such that it has an increased affinity for Na^+ (Cantiello, 1995b). Such a disruption of ionoregulation could in turn directly compromise water and Na^+ reabsorption within insects - especially in the cold.

In addition to its role in regulating ion transport, actin has also been linked to the maintenance of tissue integrity as a key component of SJ structure (Lane and Flores, 1988; Woods and Bryant, 1991). As SJs are typically located on the apical borders of epithelial cells, cold-induced disruption in SJs, as seen with *Drosophila* (MacMillan et al., 2017), may be caused by cold-induced disassembly of the cytoskeletal network (Belous, 1992; Harvey and Zerahn, 1972). Coupled with a failure of ion transporters and channels on the apical borders, such a loss of tissue integrity could lead to a functionally "funnel-like" cavity along the mucosal side of gut epithelia. This further deterioration of barrier integrity in the cold may exacerbate the damage and leak of gut contents into the hemocoel and trigger the cascade of events which result in the damage and death typically seen in cold-exposed insects. It is important to note that the surface of gut epithelial cells may differ in transport and SJ properties, potentially resulting in only a

section or sections along the gut vulnerable to cold-induced structural damage (Cioffi, 1984; Harvey and Zerahn, 1972). Nevertheless, such structural deterioration along the mucosal side of the gut epithelia may therefore account for why FITC-dextran, despite its large and bulky composition, is able to move from the gut into the hemocoel, but not in the opposite direction.

2.4.3 *Similarity Between Leak Rates in the Cold and at Room Temperature*

Contrary to our initial predictions, cold exposure did not significantly impact leak rates per cm^2 of gut tissue when compared to our controls. Additionally, all three segments had similar rates of FITC-dextran leak at their respective treatment temperatures (Fig. 2.7). These results are also contrary to our previous data, which provide evidence for unidirectional FITC-dextran leak in the cold. A plausible explanation for the discrepancies observed between experiments may lie in differences in experimental setups; whereas the former experiments measured marker leak *in vivo*, this gut sac technique was conducted *ex vivo*. Removing the segments from their natural environment may have therefore isolated the tissue from the processes which trigger cold-induced barrier disruption along the gut. Such differences between experimental systems has also been demonstrated in other fields. When examining the vessel wall thickness of human intracranial arteries for instance, measurements varied between *in vivo* and *ex vivo* (Harteveld et al., 2018). One speculation of the cause of this variance is the lack of vessel wall displacements, which typically occur during the cardiac cycle, in *ex vivo* samples (Harteveld et al., 2018). Similarly, discrepancies have been found between locust *ex vivo* and mammalian *in vivo* models for *E. coli* K1 meningitis invasion of the CNS, although such results may also be the cause of distinct organisms (Mokri-Moayyed et al., 2008). In the case of our gut sacs, conditions in which cold-induced ion imbalance occurs may not be present *ex vivo*.

It is well-known that water and ion balance is lost within chill susceptible insects in the cold (Andersen et al., 2017a; Coello Alvarado et al., 2015; MacMillan et al., 2015c; Overgaard and MacMillan, 2017). As time in the cold increases, K^+ accumulates in the insect hemocoel. The accompanying depolarization of membrane potential and activation of voltage-gated Ca^{2+} channels that occur are hypothesized to ultimately result in cell (and organismal) death (Andersen et al., 2018; Bayley et al., 2018; Mattson and Chan, 2003; Yi and Lee, 2011; Yi et al., 2007). Within our system, gut segments were suspended in a Na^+-rich locust saline to most closely mimic the hemocoel, while FITC-dextran was dissolved in distilled water. It is quite possible that the cold-induced damage that typically occurs in the cold may not have had the chance to present due to the maintenance of low extracellular K^+ in our preparation. To verify this, experiments could be repeated using a high K^+-FITC-dextran saline within the gut lumen. A second possibility for measuring no differences between treatments could pertain to the cold exposure itself. According to our chill susceptibility data (Fig. 2.3a and b), locust condition post-cold stress begins to have significant negative effects around 24 h at -2°C. At this point, 60% of locusts have recovered from chill coma within the allotted time period. The following day, individual condition was also impacted, ranging from seemingly normal to near-death states. Similar results have previously been reported in *L. migratoria* by Andersen et al. (2017) and *Gryllus* crickets by MacMillan et al. (2012; CCRT only). In these orthopterans at least, it would seem that 24 h in the cold, and the coinciding loss of ion balance that occurs, is a critical time point for distinguishing between individual chill tolerance. Consequently, 5 h at 0°C may not be harsh enough to elicit any negative effects on the organism.

2.4.4 *Conclusions*

In the present study, we show that locusts, like *D. melanogaster*, experience cold-induced leak when exposed to low temperatures. In doing so, we provide not only clear evidence that this leak is unidirectional across the gut, but also evidence suggesting that this process of barrier disruption in the cold is more integrated than we initially predicted (i.e. damage is occurring *in vivo* that is absent *ex vivo*). Together these data reinforce the importance of interpreting results in the context of the organism as a whole system, and provide a new means through which leak across the gut can be studied. Furthermore, we propose that future experiments be carried out by first exposing locusts (whole organism) to 24 h of cold stress prior to dissection for suspension as separate gut sac preparations. This way, damage accrued over the 24 h of cold exposure may present, allowing us to determine which segment(s) has become leaky.

2.5 Figures

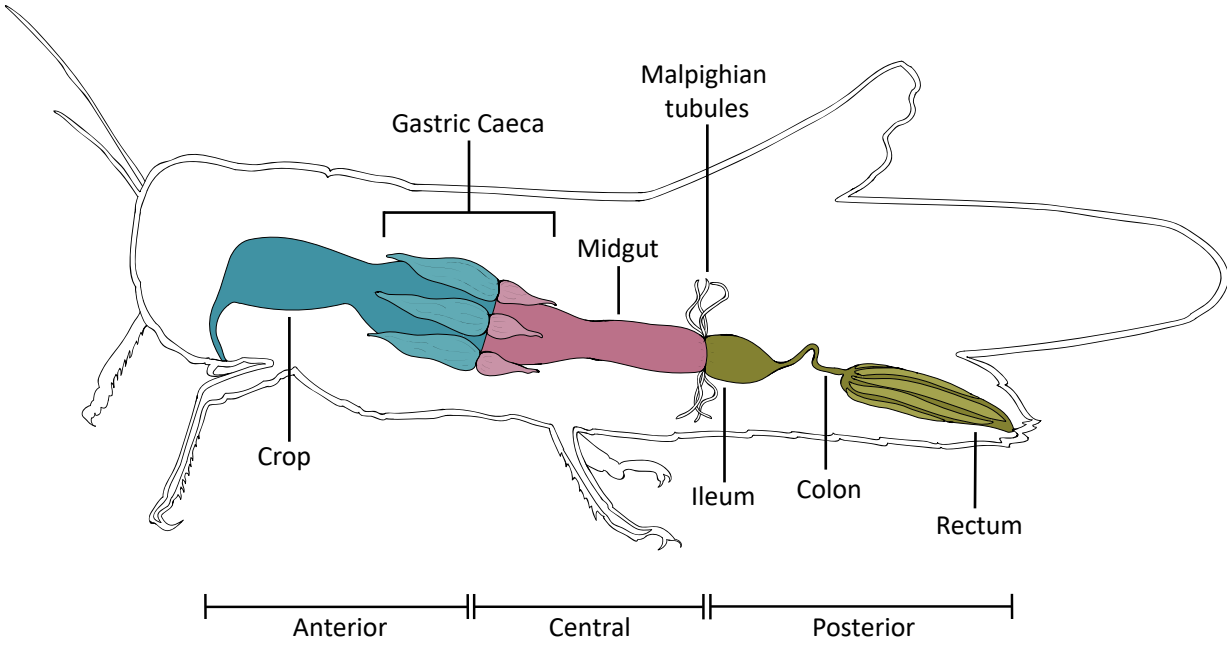

Figure 2.1. A schematic of the locust (*L. migratoria*) gut tract sectioned into three segments (anterior, central, and posterior). Relative to the locust gut anatomy, the segments were determined as follows: anterior – foregut to the anterior midgut caeca; central – posterior midgut caeca to the Malpighian tubules (removed; the midgut-hindgut junction); posterior – Malpighian tubules to the rectum. Figure illustrated from observation.

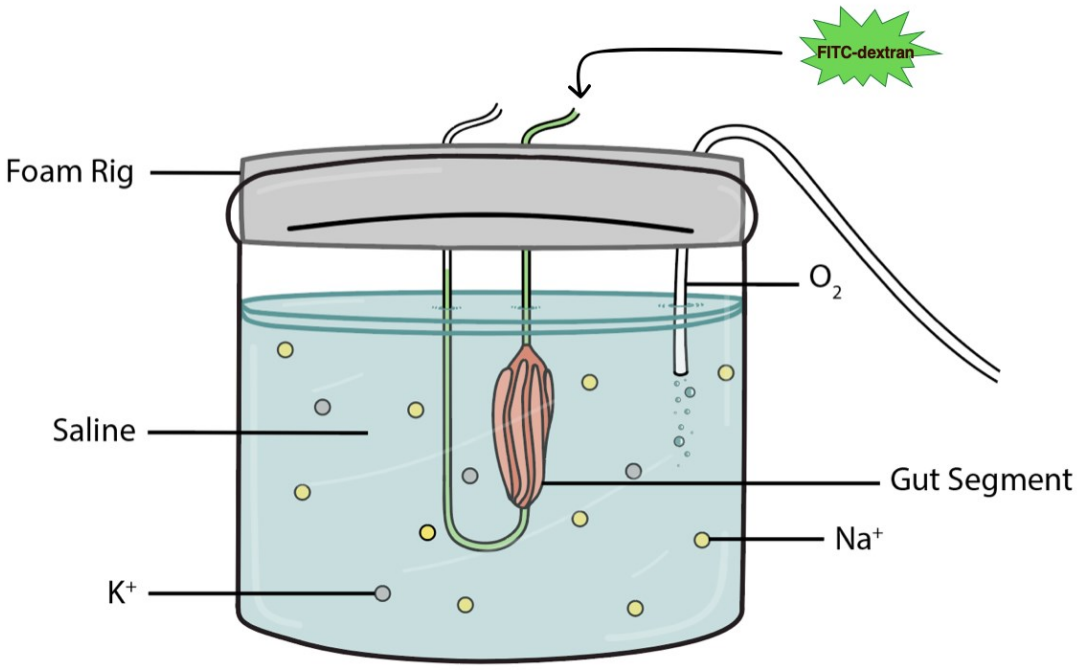

Figure 2.2. A schematic of the gut sac setup for *L. migratoria*. Briefly, segments (anterior, central, or posterior) were fixed to a polyethylene tube and suspended in an oxygenated saline. FITC-dextran was added to the mucosal side of the sac via tubing, and the system exposed to 0℃ for up to 5 h, followed by 2 h at room temperature (23°C). Figure illustrated from observation.

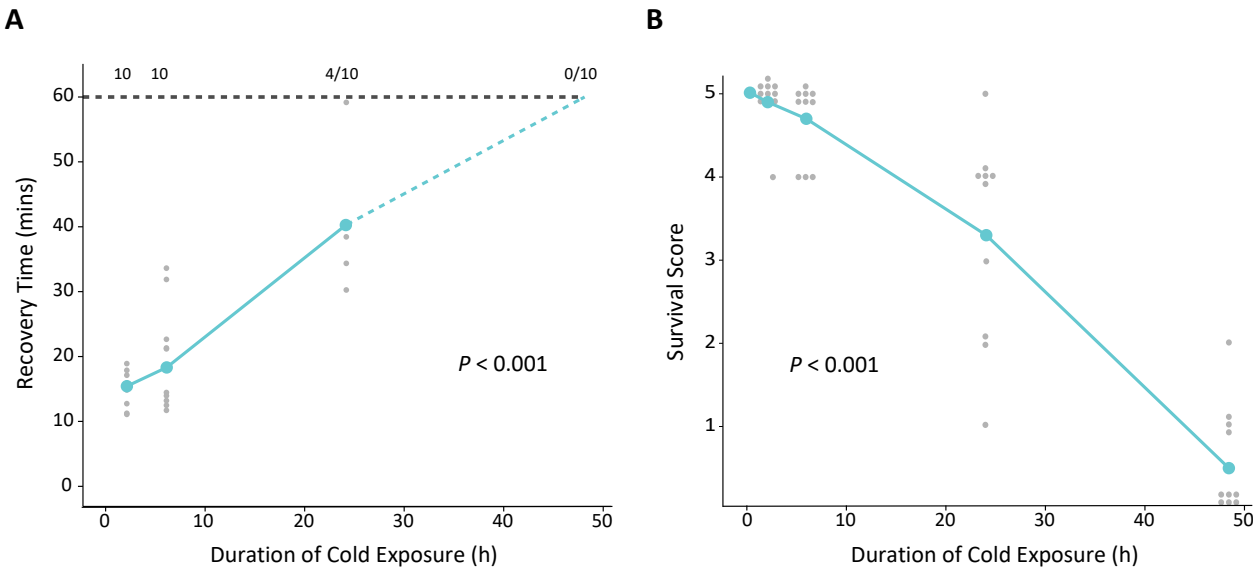

Figure 2.3. Locusts (*L. migratoria*) are chill susceptible insects. **A)** Chill coma recovery time (CCRT) of locusts (mixed sexes; n = 10 per time point) held at -2 ℃ for 2, 6, 24, or 48 h. Locusts were observed for 60 mins following cold exposure and were marked as having recovered when standing on all six limbs. Values above the dotted black line represent the number of recovered locusts at each time point. The solid blue line represents mean values per time point, while the grey dots represent each data point per time point. **B)** Locust condition (survival) following exposure to -2 ℃ for 0, 2, 6, 24, or 48 h (n = 10 per time point). Survival score was based on the following: 0: no movement observed (i.e. dead); 1: limb movement (leg and or head twitching); 2: moving, but unable to stand; 3: able to stand, but unable or unwilling to walk or jump; 4: able to stand, walk, and or jump, but lacks coordination; and 5: movement restored pre-exposure levels of coordination. Values are mean ± standard deviation. The solid blue line represents mean values per time point, while the grey points represent each sample per time point. To show all data points, dots are clustered around their respective score (where applicable).

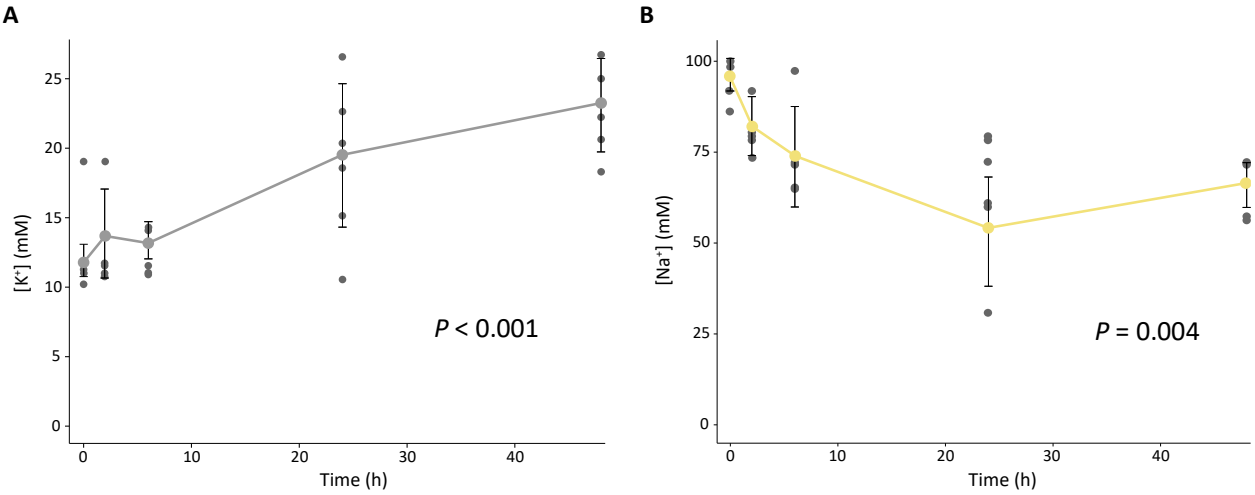

Figure 2.4. Cold-induced ion imbalance occurs in locusts (*L. migratoria*). **A)** Changes in locust hemolymph K^+ concentrations over time spent at -2°C (n = 5-6 locusts per time point). **B)** Samples of locust hemolymph Na^+ concentrations over time spent at -2℃ (n = 4-6 locusts per time point). Light dotted lines represent control (23°C) data points. Values are mean ± standard deviation. Dark grey points represent each sample taken per time point. Error bars not shown are obscured by the symbols.

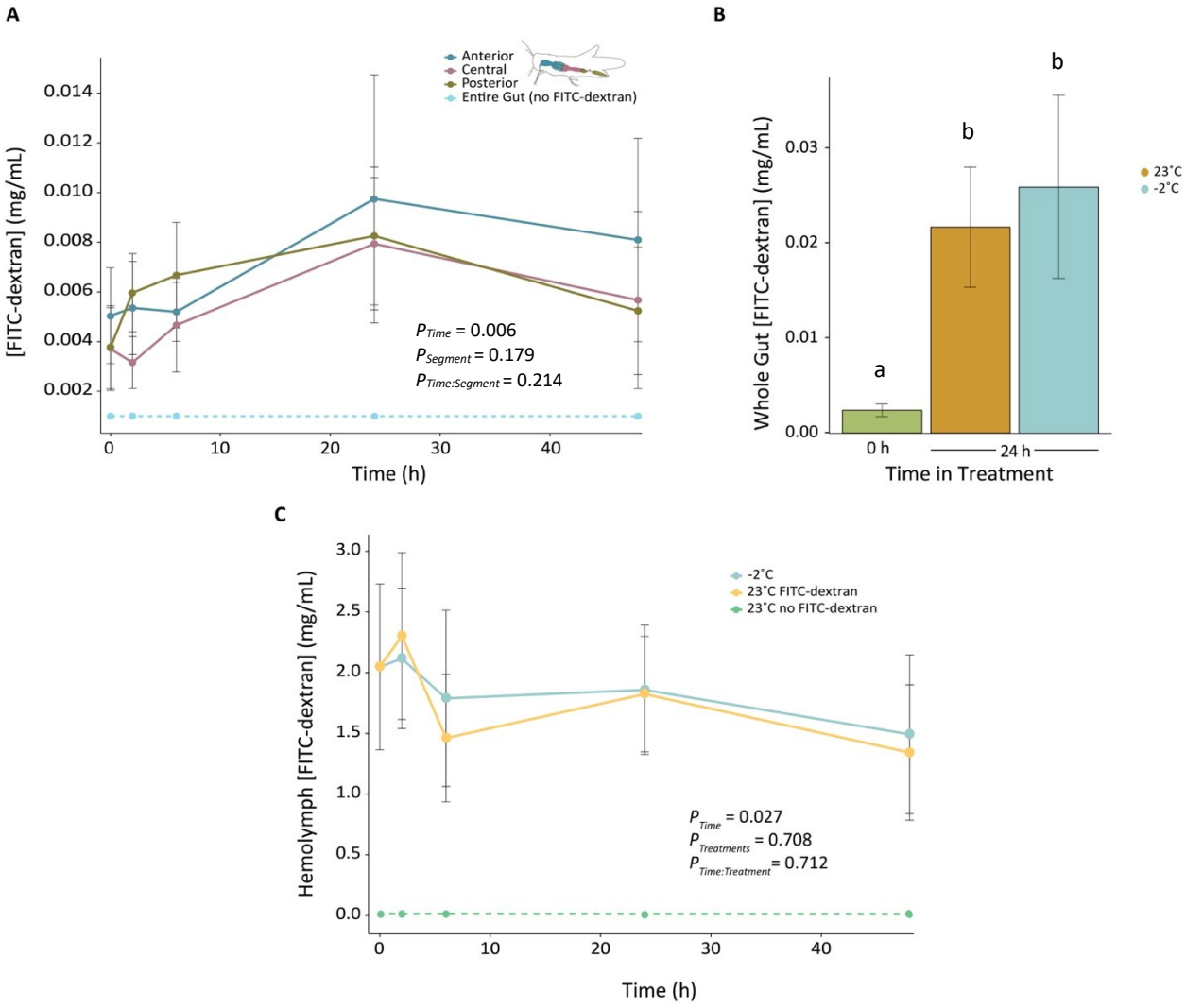

Figure 2.5. Minimal cold-induced leak of FITC-dextran occurs across the gut epithelia of *L. migratoria* in the serosal to mucosal direction. **A)** Concentration of injected FITC-dextran (mg/mL) present in to each gut segment (anterior, central, and posterior; n = 6 per group) after exposure to -2°C for 2, 6, 24, or 48 h. **B)** Mean values representing the total FITC-dextran content within the gut (sum of anterior, central, and posterior segments) at 24 h of either 23℃ (control) or -2℃ (n = 4-6 per group). Letters denote a significant difference (P_{Time} < 0.001; $P_{Treatment}$ = 0.758). **C)** Concentration of injected FITC-dextran remaining in the hemolymph over 48h in the cold (-2℃) and at room temperature (n = 4-6 per group). Values are mean ± standard deviation. Error bars not shown are obscured by the symbols.

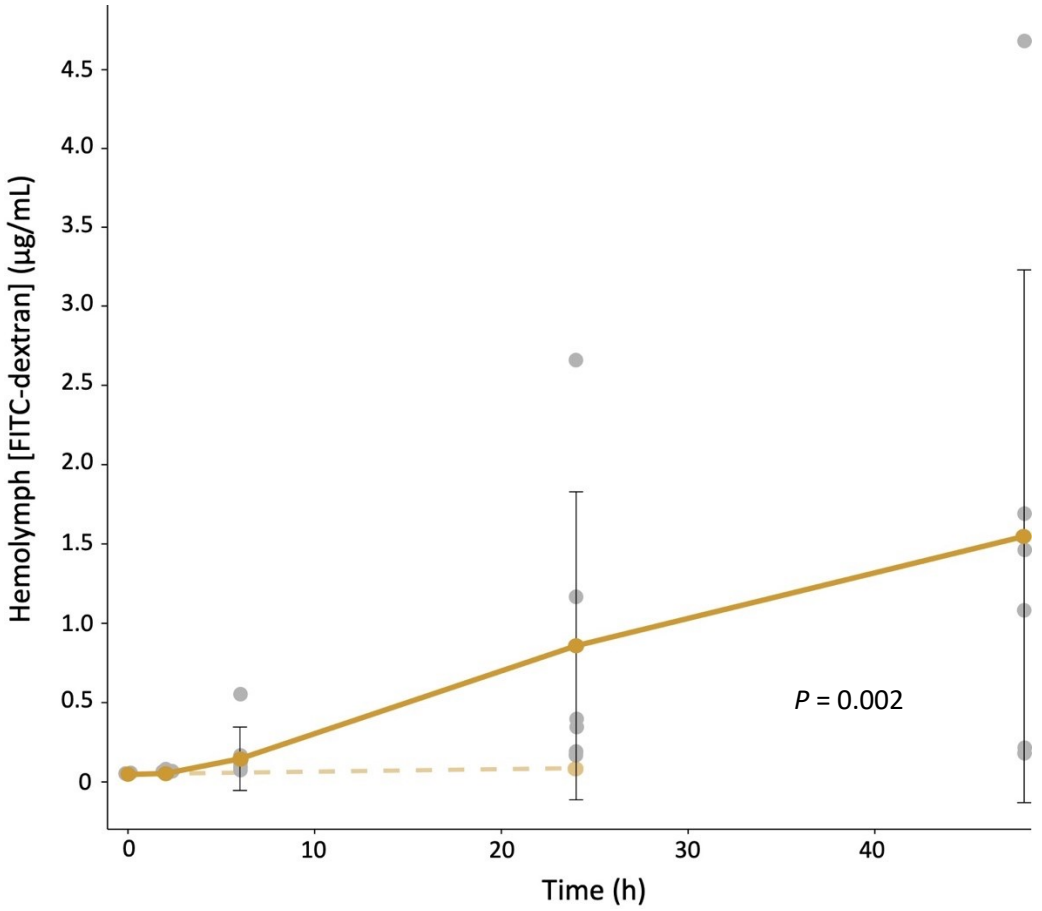

Figure 2.6. FITC-dextran is leaking in the mucosal to serosal direction across the gut of *L. migratoria*. Levels of hemolymph FITC-dextran (µg/mL) following oral administration of the marker and either 2, 6, 24, or 48 h at -2°C (n = 6 per time point). Values are mean ± standard deviation. Grey points represent each sample taken per time point. The dotted line represents mean values of sampled control locusts up to 24h (n = 5 per time point). Error bars not shown are obscured by the symbols.

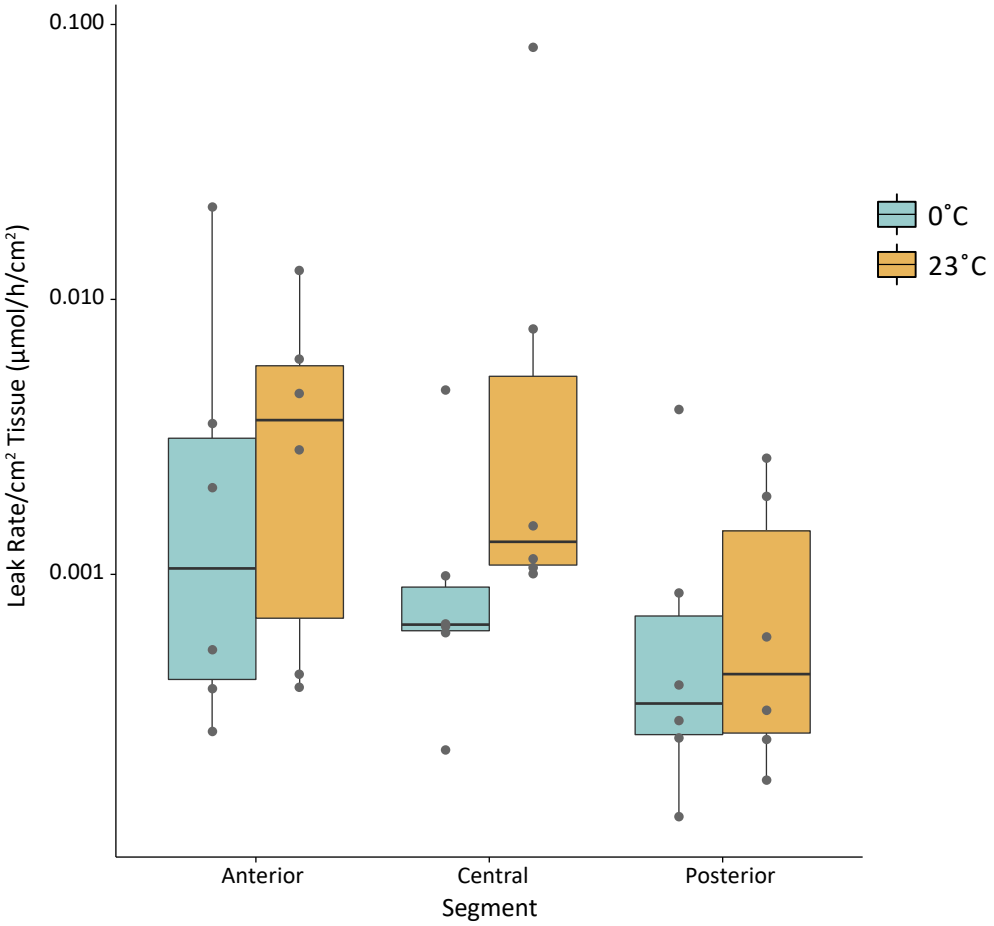

Figure 2.7. Leak rates of FITC-dextran per cm^2 of tissue appear similar both in the cold and at room temperature. Box plots of FITC-dextran (log_{10} scale) leak rates per cm^2 of tissue appearing in the surrounding saline shown at treatment (0°C) and room temperatures for each of the three segments (anterior, central, and posterior) prepared as gut sac filled with FITC-dextran (n=6 samples per treatment, with n=12 overall per segment). Box plot midlines represent median values. Grey points represent each sample taken per treatment. No significant interaction was found between the treatment and the leak rate/cm^2 of each gut segment (Linear model, $F_{2, 30} = 1.10$, $P = 0.346$). Additionally, no main effects of treatment or gut segment were observed (Linear model, $F_{1, 30} = 2.23$, $P = 0.315$; Linear model, $F_{2, 30} = 0.882$, $P = 0.424$, respectively). Finally, no differences were found between the leak rate/cm^2 of tissues held at 5 h of cold and the leak rate/cm^2 of tissue held afterwards at 2 h of 23°C in any of the three segments (anterior: two-tailed $t_5 = 0.668$, $P = 0.534$; central: two-tailed $t_5 = 1.19$, $P = 0.288$; posterior: two-tailed $t_5 = 1.27$, $P = 0.261$).

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3. Re-examining mPEG-FITC (350 Da) as a Marker of Epithelial Permeability in the Migratory Locust

3.1 Introduction

In addition to the movement of solutes and water through cells (transcellular pathways), ionomotive epithelia also rely on the passive movement, or leak, of these molecules between adjacent cells (paracellular pathway; Jonusaite et al., 2016; O'Donnell and Maddrell, 1983). To effectively maintain a separation between the internal and external environments (e.g. the gut lumen), paracellular pathways must selectively and dynamically control solute diffusion across epithelia (Izumi and Furuse, 2014; Jonusaite et al., 2016; MacMillan et al., 2017). Classically, a range of molecules have been used to characterize permeability along these pathways and to determine just how selective they are. For instance, the permeability of the Malpighian tubules (MTs) of *R. prolixus* has been thoroughly tested using a range of small molecules such as sucrose (342 Da), inulin (~7000 Da), and polyethylene glycol (PEG; 4000 Da; Jonusaite et al., 2016; le Skaer et al., 1987; O'Donnell and Maddrell, 1983). Molecules of a higher molecular weight, such as dextrans over 10 kDa, have also been used to measure permeability between epithelial cells in *D. melanogaster*, although these were unable to cross the epithelia (Lamb et al., 1998). Furthermore, while larger, the short chain structure of PEG has been shown to cross epithelia more readily than the bulky yet small sucrose (le Skaer et al., 1987). Together, permeability across the paracellular pathway appears to be dictated largely by molecule size and structure (O'Donnell and Maddrell, 1983; Bjarnason et al., 1995; O'Donnell et al., 1984). As a result, relatively low-molecular weight molecules such as mPEG-FITC (mPEG; 350 Da) are popular markers for epithelial permeability in a range of animal models.

mPEG, like FITC-dextran (used in Chapter 2), has been long considered an ideal marker for epithelial permeability in various organisms due to its wide range of administration routes and stability (Chadwick et al., 1977). Consequently, it has been widely used in vertebrate models such as humans (*Homo sapiens*), pigs (*Sus scrofa domesticus*), rats (*Rattus norvegicus*), killifish (*Fundulus heteroclitus*), and trout (*Oncorhynchus mykiss*) to measure permeability across the gastrointestinal tract as well as the gills (Chadwick et al., 1977; Kansagra et al., 2003; Lane and Corrigan, 2006; Maxton et al., 1986; Robertson and Wood, 2014; Wood and Grosell, 2012). In the latter non-aquatic animals, PEG (400 Da) is typically used to examine disorders in which intestinal barrier function has been compromised, such as Crohn's disease or celiac sprue (Krugliak et al., 1989). Similarly, studies involving trout and killifish often use PEG to visualize gill permeability, and solute and water movement across the intestine (Robertson and Wood, 2014; Wood and Grosell, 2012). This marker has also been used in insect models like the kissing bug (*R. prolixus*), silkworms (*B. mori*), and mosquitos (*A. aegypti*) to investigate paracellular permeability along tissues such as the midgut and the MTs (Fiandra et al., 2009; Jonusaite et al., 2017; le Skaer et al., 1987). While insects are known to transport small molecules like dyes (e.g. amaranth, indigo carmine) and organic acids (e.g. fluorescein) across their MTs (Bresler et al., 1990; Maddrell et al., 1974; O'Donnell et al., 2003), the transport of mPEG and PEGs of other molecular weights has yet to be demonstrated (to my knowledge; Self et al., 1995). Thus, mPEG (like FITC-dextran) appears to be a useful marker to measure gut epithelial permeability in *L. migratoria*.

In Chapter 2, I used a fluorescently-labelled dextran (FITC-dextran; 3-5 kDa) to explore cold-induced barrier failure in the migratory locust. Interestingly however, I found that FITC-dextran did not freely leak into the gut lumen, but did leak from the gut lumen into the

hemocoel. To explore why this marker was unable to diffuse from the hemolymph into the gut lumen, I used mPEG (350 Da) to re-measure epithelial permeability along the gut. As this marker is both smaller and less sterically hindering than FITC-dextran, I predicted that cold-induced paracellular leak would be observed, regardless of the route of administration (e.g. lumen to hemocoel and *vice versa*). My experiments involving marker injection in Chapter 2 were therefore repeated, this time using mPEG instead of FITC-dextran.

3.2 Methods

3.2.1 *Experimental System*

All experiments used male and female adult locusts (*Locusta migratoria*) aged 3-4 weeks post-final moult. Locusts were obtained from a continuously breeding colony maintained at Carleton University in Ottawa, ON. The colony is reared under crowded conditions on a 16 h:8 h light:dark cycle at 30°C with 60% relative humidity (see Dawson et al., 2004). All animals had *ad libitum* access to a dry food mixture (oats, wheat bran, wheat germ, and powdered milk), while fresh wheat clippings were supplied three times per week.

3.2.2 *Measuring Cold-Induced Leak into the Gut*

To examine cold-induced paracellular permeability along locust gut epithelia in the serosal to mucosal direction (hemocoel to lumen), mPEG-FITC was used (mPEG; MW 350 Da, NANOCS, Boston, USA). Protocols for the injection and measurement of the marker were identical to FITC-dextran from Chapter 2. Briefly, a 25 µL Hamilton syringe was used to inject locusts ventrally at the junction of the thorax and first abdominal segment with 20 µL of a 3.84 x 10^{-3} M mPEG-saline solution (in mmol l^{-1}: 140 NaCl, 8 KCl, 2.3 $CaCl_2$ Dihydrate, 0.93 $MgCl_2$ Hexahydrate, 1 NaH_2PO_4, 90 sucrose, 5 glucose, 5 trehalose, 1 proline, 10 HEPES, pH 7.2). As with the FITC-dextran, neither the 20 µL injection nor the mPEG itself impacted locust

performance or survival (survival for control locusts with and without mPEG after 48 h was scored as 5). Animals were then suspended in a cooling bath (Model AP28R-30, VWR International, Radnor, USA) preset to 20°C and cooled to -2°C at a rate of -0.2 min^{-1}, where they were left undisturbed for 2, 6, 24, or 48 h (n = 6 per time point). Locusts were individually removed from the cooling bath and dissected within 15 mins of their target cold treatment time for collection of the anterior, central, and posterior gut segments (refer to Fig. 2.1 for segment breakdown). Samples were subsequently homogenized (OMNI International Tissue Master 125 120 V, Kennesaw, USA; approximately 3 min or until solution is uniform), sonicated (Qsonica Sonicators Model CL-188, Newton, USA; 3 x 5 s bursts with 10 s rests), and centrifuged for 5 min at 10,000 × *g*. A 100 µL aliquot of the resulting supernatant was collected via micropipette and transferred to a 96-well plate (Corning Falcon Imaging Microplate; black/clear bottom) for fluorescence spectrophotometry (Ex: 485 nm, Em: 528 nm; BioTek Cytation 5 Imaging Reader, Winooski, USA). Concentrations of mPEG in the samples were determined by reference to a standard curve. Control samples confirmed that tissues from locusts that were not injected with the marker had negligible fluorescence.

3.2.3 *mPEG Hemolymph Extractions*

Similar to experiments done with FITC-dextran, hemolymph extractions were performed on locusts in the cold to determine how much mPEG was being lost from the hemolymph over time. Protocols for injection are identical to those described above. Briefly, new locusts were injected with the mPEG solution and suspended for 2, 6, 24, or 48 h at -2°C in a circulating cooling bath, while controls were held at room temperature (23°C; n = 6 per exposure time in each treatment). Hemolymph samples were collected after the designated exposure time using methods adapted from Findsen et al. (2013). Briefly, locusts were pricked dorsally at the junction

of the head and thorax to access the hemolymph. A 50 µL capillary tube was then used to collect the hemolymph, 2 µL of which was transferred into 96-well plates (Corning Falcon Imaging Microplate; black/clear bottom) and diluted 50-fold with saline. Samples were then analyzed for mPEG content via fluorescence spectrophotometry as previously mentioned. As with the FITC-dextran, pilot experiments showed no interference from the saline when measuring fluorescence.

3.2.4. *Data Analysis*

All data (excluding mPEG movement in the serosal to mucosal direction) were analyzed using linear models in R Studio version 1.2.1335 (https://www.rstudio.com). The effects of time in the cold on gut leakiness (measured via mPEG movement into the gut) were analyzed using a linear mixed effects model via the lmer() function in R (lme4 and lmerTest packages for R). Time and gut segment were treated as a fixed effect and each individual locust was treated as a random effect. Data were analyzed with time as both a continuous and categorical factor, however, no differences were seen between the two. As such, all results presented in this section treat time as a continuous factor. The level of statistical significance was assumed to be 0.05 for all analyses, while all additional values presented are mean ± standard deviation.

3.3 Results

3.3.1 *Gut Leak Assays with mPEG*

Analysis of mPEG was conducted in a manner identical to that of FITC-dextran in Chapter 2. However, unlike the results seen with FITC-dextran, which showed little marker movement, mPEG concentrations significantly decreased in the gut over time at room temperature, resulting in over a 200-fold difference in posterior segment mPEG levels between 0 h and 48 h (Fig. 3.1a; main effect of time: Linear model, $F_{1, 28} = 10.9$, $P = 0.003$). Significant

differences between mPEG levels in each gut segment were also observed (main effect of segment: Linear model, $F_{2, 56} = 24.1$, $P < 0.001$). Additionally, a significant interaction was seen as the effect of time depends on the segment in question (Linear model, $F_{2, 56} = 9.52$, $P < 0.001$). In the cold however, marker concentrations did not significantly increase in the gut over time, remaining instead rather constant across the 48 h at -2°C **(**Fig. 3.1b; main effect of time: Linear model, $F_{1, 84} = 0.206$, $P = 0.651$). Interestingly, significant differences in mPEG levels were observed between each of the gut segments (Linear model, $F_{2, 84} = 54.4$, $P < 0.001$). For instance, there was approximately a 30- and 5-fold difference in mPEG levels between the posterior segment and the anterior and central segments, respectively, over 48 h in the cold. However, as these differences remained constant across time in the cold, it is not surprising that the effects of time didn't depend on the segment (Linear model, $F_{2, 84} = 0.495$, $P = 0.611$).

3.3.2 *Hemolymph Extractions*

Loss of mPEG from the hemolymph was investigated to see if similar concentrations of mPEG were presenting in the gut over time. Unlike the FITC-dextran experiments where minimal amounts of the probe were lost from the hemolymph, mPEG concentrations significantly decreased over time both at -2°C and room temperature (Fig. 3.2; Linear model, $F_{1, 58} = 9.82$, $P = 0.003$; Linear model $F_{1, 28} = 6.53$, $P = 0.016$, respectively). While differences over time between mPEG concentrations at room temperature and in the cold were also found (Linear model, $F_{1, 56} = 9.76$, $P = 0.003$), no significant interaction was found as the effect of time didn't depend on the treatment (Linear model, $F_{1, 56} = 0.297$, $P = 0.588$).

3.4 Discussion

3.4.1 *mPEG Movement Across the Epithelia is Temperature-Dependent*

In Chapter 2, the degree of leak across gut epithelia in cold-exposed *L. migratoria* was explored using FITC-dextran. However, this marker was shown to leak unidirectionally in the cold, favoring movement out of the gut lumen and into the hemolymph (mucosal to serosal) rather than in the opposite direction. Given little FITC-dextran leak occurred in the serosal to mucosal direction, I speculated that the large molecular size of FITC-dextran (3-5 kDa) may limit its effectiveness as a marker of paracellular permeability in locusts. It is well known that macromolecule permeability along epithelia is determined by molecular weight and structure (Bjarnason et al., 1995; O'Donnell et al., 1984). As a result, I conducted identical gut leak and hemolymph extraction experiments, this time using mPEG (350 Da) as a smaller and less sterically-hindering molecule. An additional characteristic of mPEG that makes it an appealing marker of paracellular permeability is its apparent lack of transporter, resulting in the inability to be transported into intracellular spaces (Self et al., 1995). Diffusion through the paracellular pathway therefore seems to be the only plausible avenue for this marker. In this study, however, I demonstrate the ability for the locust to successfully and rapidly clear this molecule from its hemolymph, presumably via some direct or indirect mechanism of energy-dependent transport.

In stark contrast to the results observed with FITC-dextran, control locusts rapidly cleared mPEG from their gut tract. As early as 15 mins at room temperature (i.e. sampled after marker injection into the hemocoel; reported as time 0 h), there already exist clear differences in mPEG concentrations between the posterior, and the central and anterior segments (Fig. 3.1a). At this time point, the posterior section contains nearly four times more mPEG than the central, and 13 times more than the anterior. At 2 h, the discrepancy between these three segments is larger

still as these probe levels increase by approximately 11 and 22-fold when compared to the central and anterior segments, respectively. Furthermore, the marker is nearly cleared from the gut between 2 and 6 h, leaving minimal traces of mPEG in each of sections after 6 h. Such an influx of mPEG into the gut at 0 h suggests that following injection, the marker is in the process of being cleared from the hemolymph for excretion in the hindgut. Indeed, such removal from the hemolymph was confirmed via hemolymph extractions, which revealed a near total clearance of the marker over time (Fig. 3.2). Similarly rapid clearance of mPEG from the animal system has been reported in kissing bugs (*R. prolixus*), trout (*O. mykiss*), humans (*Homo sapiens*), and rats (*R. norvegicus*) (Bjarnason et al., 1995; Krugliak et al., 1989; le Skaer et al., 1987; Maxton et al., 1986; Robertson and Wood, 2014). Experiments were next repeated, this time at low temperatures to observe mPEG leak under suboptimal conditions.

At -2°C, mPEG concentrations in the posterior segment were highest shortly following marker injection and remained fairly stable over 48 h (Fig. 3.1b). Compared to the anterior and central sections, levels of mPEG in the posterior section were nearly 30 and 5 times greater, respectively. This presence suggests that, although the molecule may have been transported into the gut lumen prior to chilling, the cold-induced suppression of active transport may be responsible for this steady state of mPEG measured (Ellory and Willis, 1982; MacMillan and Sinclair, 2011; MacMillan et al., 2015; Yerushalmi et al., 2018). Additionally, due to the constant levels of this small marker observed in the gut, it is reasonable to infer that no passive movement is occurring in the cold and that there is an absence of paracellular barrier disruption along the tract (at least in a way that permits mPEG movement). Conversely, if any transport and leak is occurring in the cold, marker leak is not exceeding transport rates as levels are constant

within the gut. With such results, it is therefore possible that mPEG movement at room temperature is related to or mediated by some form of energy-dependent transport.

An important component of the insect renal system is the Malpighian tubules (MTs). These blind-ended tubules function as a primary site of ionoregulation in insects, and are thus capable of transporting ion and water at incredible rates (Maddrell and O'Donnell, 1992). At the MTs, electrochemical gradients maintained by electrogenic transporters and channels create a positive voltage potential within the tubule lumen. As a result, Cl^- is driven into the tubule lumen paracellularly, followed closely by water which follows osmotically (Harvey, 2009; Ianowski and O'Donnell, 2006; O'Donnell and Maddrell, 1983; Phillips, 1970). The high rates of paracellular water flux that occur may explain how mPEG is cleared from the hemolymph under control conditions; mPEG movement in control locusts may be due to solvent drag, or the movement of solutes entrained by the flow of water (Maddrell and O'Donnell, 1992). Under cold conditions, the cessation of mPEG movement may therefore result from the decrease in paracellular water movement that occurs (decreased water flux as an effect of the cold-induced suppression of ionoregulatory activity; Ellory and Willis, 1982; Yerushalmi et al., 2018). It is important to note that solvent drag of PEG has been previously reported across the rat intestine however, this same PEG movement has proven to be absent in other animal systems, like the common killifish (*F. heteroclitus*; Krugliak et al., 1989; Wood and Grosell, 2012). While solvent drag represents a plausible explanation for mPEG movement into the gut lumen, alternative possibilities exist which involve the direct influence of ATP-dependent transport.

Due to their choice of diet, phytophagous insects, like the migratory locust, are constantly exposed to unwanted and sometimes toxic compounds, including insecticidal proteins and peptides (Denecke et al., 2018). As a result, they must employ a range of mechanisms through

which these foreign compounds are safely removed from their systems. One such mechanism of xenobiotic removal is by transporter proteins that are able to actively pump these molecules out of or into cells (Denecke et al., 2018). To date, members of two families of transporters have been associated with xenobiotic transport in animals: the ATP-binding cassette (ABC) family, which uses energy from ATP breakdown to pump molecules out of the cell; and the solute carrier (SLC) family, which are often associated with the pumping of compounds into cells (Hediger et al., 2013; Wilkens, 2015). Proteins from these two transporter families have been well characterized, typically in the context of small molecule pharmaceutical (< 1000 Da) transport and absorption across the human intestine (Denecke et al., 2018). Here, members of each family have been observed both on the apical and basolateral sides of the membrane (Estudante et al., 2013; Giacomini et al., 2010). However, evidence in *D. melanogaster*, lepidopterans, and mosquitos (*Anopheles stephensi*) suggests that orthologs of these transporters exist along the insect midgut, and possibly function to transport compounds like insecticides (Bretschneider et al., 2016; Harrop et al., 2014; Pearce et al., 2017; Qi et al., 2016; Xiao et al., 2016). Additionally, ABCs have been reported in *L. migratoria*, although these proteins were associated with maintaining cuticular barrier integrity and desiccation resistance (Yu et al., 2017). Nevertheless, it is not beyond the realm of possibility that a foreign marker like mPEG could be removed from the hemocoel by similar transporters.

A third means through which insects are able to clear xenobiotics from their systems is endocytosis (Casartelli et al., 2008). Here, compound-membrane interactions trigger the inward budding of the apical membrane, creating a vesicle containing the target compound. This vesicle is eventually transported to the basolateral membrane for removal via exocytosis (Bechara and Sagan, 2013). While endocytosis can often occur in response to the binding of specific vesicle-

triggering receptors, how exactly specificity for xenobiotic uptake in insects is determined is not always clear (Denecke et al., 2018). In cells from the midgut of *Bombyx mori*, for instance, a putative ortholog of a mammalian receptor was linked to the endocytotic uptake of fluorescently-labelled albumin (FITC-albumin; Casartelli et al., 2005; Casartelli et al., 2008). By contrast, little is known regarding what triggers the endocytotic uptake of the bioinsecticide FITC-*Aea*-TMOF (*Aedes aegypti* Trypsin Modulating Oostatic Factor), also in *B. mori* (Fiandra et al., 2009). Additional work should therefore be conducted to determine how exactly mPEG is being transported across the gut epithelium. To do so, cross sections of the gut could be examined under a fluorescence microscope for the presence of mPEG-filled vesicles, which would suggest mPEG clearance at room temperature occurs through endocytosis over transporter-mediated removal. Alternatively, Ramsay fluid secretion assays could be used to monitor mPEG secretion in isolated MTs, allowing one to determine whether mPEG is saturating transporters along these tubules (Ramsay, 1953).

3.4.2 *Conclusions*

mPEG has been widely used as a marker of paracellular permeability in both invertebrates and vertebrates due to its favourable properties, such as the absence of a known transporter (Chadwick et al., 1977; Kansagra et al., 2003; Lane and Corrigan, 2006; le Skaer et al., 1987). Here however, I provide clear evidence that would suggest otherwise; this common marker of permeability was quickly cleared from the hemocoel under room temperature conditions. While studies have previously observed high permeability of mPEG, it has yet to be characterized as being transportable (to my knowledge). This property was confirmed during cold exposure, which effectively halted movement across the gut and supported that a cold-induced suppression of mPEG transport had occurred. Regardless of the means of transport,

these cold stress results suggest that mPEG is unable to diffuse paracellularly unaided (in locusts). While the mechanisms through which mPEG removal are unknown, I propose that this marker is not ideal for experiments involving epithelial permeability, at least in locusts. This encompasses studies involving both the injection (where mPEG is cleared from the hemocoel) and the oral administration of mPEG (where any probe leaking into the hemocoel would be transported back into the gut lumen). Future studies should therefore focus not only on determining how mPEG is cleared from the locust system, but also on exploring whether this ability extends beyond Orthoptera.

3.5 Figures

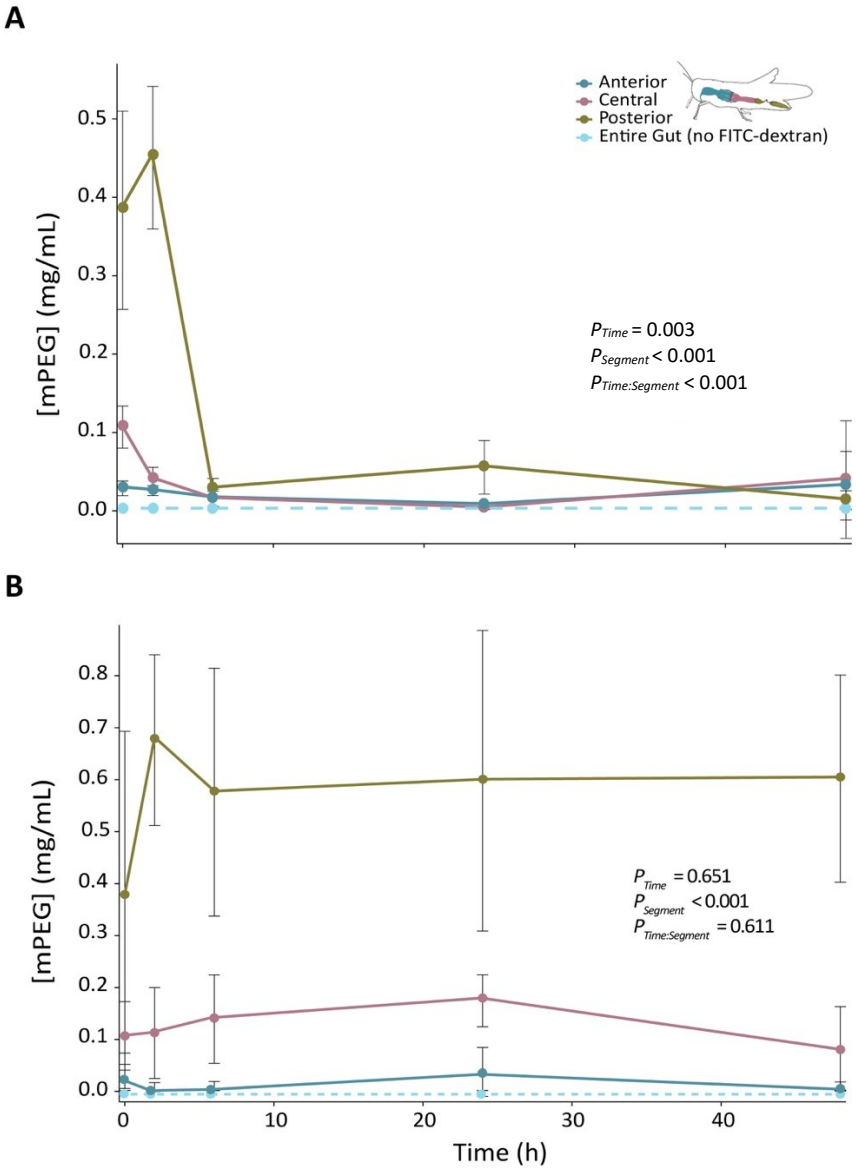

Figure 3. 1. Locusts (*L. migratoria*) are able to effectively clear mPEG from their hemolymph, and this movement appears to be temperature-sensitive. **A)** Concentration of mPEG appearing in each gut segment (anterior, central, and posterior; n = 6 per treatment group) post-injection over time at 23°C for 2, 6, 24, or 48 h. **B)** Concentrations of mPEG (mg/mL) present in each gut segment post-exposure to -2°C for 2, 6, 24, or 48 h (n=6 per treatment group). The dotted line represents samples of hemolymph without FITC-dextran. Values are mean ± standard deviation. Error bars not shown are obscured by the symbols.

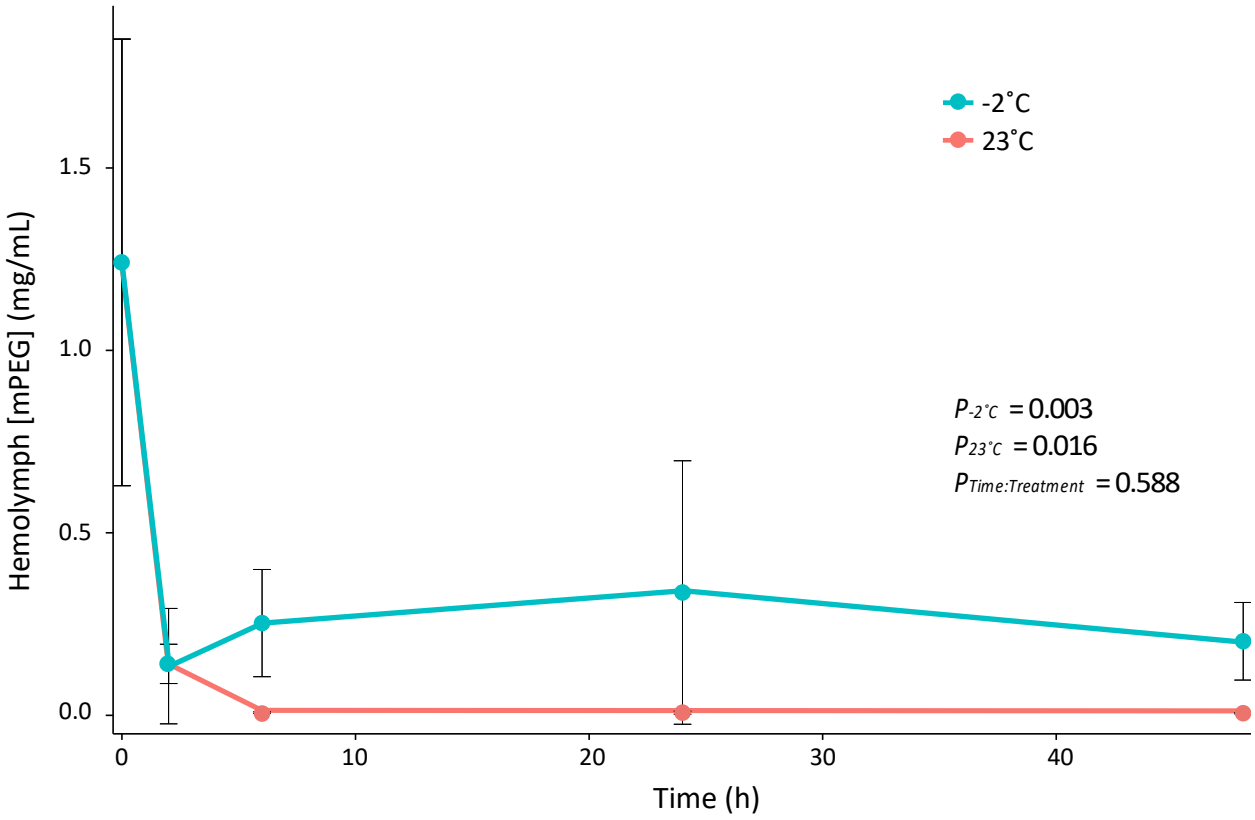

Figure 3.2. Differences between hemolymph mPEG concentrations in both cold-exposed and control locusts (*L. migratoria*). Animals were exposed to 2, 6, 24, or 48 h of either cold (-2°C) or room temperature (23°C; n = 6 per treatment group) conditions prior to hemolymph sampling. Values are mean ± standard deviation. Error bars not shown are obscured by the symbols.

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4. General Discussion

4.1 Thesis Summary

Chill susceptible insects, like the migratory locust, lose water and ion balance when exposed to low temperatures (Andersen et al., 2017a; Coello Alvarado et al., 2015; Findsen et al., 2013; Robertson et al., 2017). Recent evidence in *D. melanogaster* suggests that this loss of homeostasis is, at least partly, caused by the disruption of epithelial paracellular barrier function along the gut (MacMillan et al., 2017). While it is clear that gut barrier function is impaired in the cold, where along the gut this loss of integrity is occurring remains unknown. Due to the specialized roles each segment of the gut tract has (foregut, midgut, hindgut), it is possible that only a portion of the gut is responsible for cold-induced leak. I therefore sought to investigate 1) whether chilling impacts locust gut epithelial integrity, and 2) where along the gut this loss of barrier function is occurring, focusing on the paracellular pathway. In this thesis, I provided evidence that chilled locusts are unable to maintain gut barrier function and that cold-induced leak is unidirectional. That said, there is still much to be explored regarding paracellular barrier failure in the cold.

Using a common marker of paracellular permeability (FITC-dextran), I first investigated the effects of chilling on barrier function along the locust gut (discussed in Chapter 2). FITC-dextran was injected into the hemocoel prior to cold exposure to visualize not only leak from the hemolymph into the gut lumen over time at low temperature, but also where leak was most prominent along the gut. Surprisingly, negligible concentrations of FITC-dextran were found in the gut, while the majority of the injected probe was retained within the hemocoel. This suggested that gut barriers were intact during cold exposure. However, when tested in the opposite direction (i.e. locusts were fed FITC-dextran), marker levels within the hemolymph

increased over time in the cold. Thus, locusts, like *D. melanogaster*, also experience cold-induced barrier failure, although this leak is largely unidirectional. As FITC-dextran is a large (3-5 kDa), uncharged, and bulky marker, it may be physically incapable of leaking into the gut lumen from the hemocoel, even at low temperatures. In the opposite direction however, it is possible that chilling impacts tissue integrity along the apical borders of epithelial cells, creating a 'funnel-like' cavity that exacerbates leak into the hemolymph (see Chapter 2.4.1 and 2.4.2 for further discussion).

With the importance of this directionality in mind, follow up experiments to isolate barrier failure were designed to simulate a fed state *ex vivo*. This was done by preparing sectioned gut segments as FITC-dextran-filled 'gut sacs' from which I could measure the rate of marker leak into the 'hemolymph' over time. Here, no differences were found between the rates of probe leak over time in either the cold or at room temperature, or between the gut segments. In Chapter 2.4.3, I discussed the possibility that such results are due to the lack of K^+ movement across the epithelia, which may have ultimately prevented cold-induced tissue damage from occurring. Furthermore, the cold exposure used in these experiments alone may not have been harsh or prolonged enough to cause detectable leak.

In Chapter 3, I explored why FITC-dextran was unable to diffuse into the gut lumen from the hemocoel. To see if probe structure and size were preventing the movement of this large marker, I repeated experiments using mPEG-FITC (mPEG; 350 Da), a smaller, but also common marker of paracellular permeability. Once control locusts were injected with mPEG however, it was clear that locusts are able to transport it, possibly via some mechanisms of energy-dependent transport (e.g. active or endocytosis) or solvent drag. Experiments that I conducted at low temperatures corroborated these results. While mPEG was detected within both the gut and the

hemolymph, these concentrations remained constant over time when locusts were chilled. This finding supports the notion that mPEG is being actively cleared from the hemocoel for excretion rather than moving passively into the gut lumen, and that this process is temperature sensitive. While the mechanisms through which this mPEG transport occurs remain unknown, it is clear that it is not suited for quantifying epithelial permeability either through feeding or injections (at least in locusts).

Overall, my work contributes to the wealth of information surrounding the physiology of insect cold tolerance and has helped to expand our working knowledge of paracellular pathways in the gut. While the exact mechanisms behind cold-induced leak along these pathways are unknown, I present evidence which further supports the importance of paracellular barriers in maintaining homeostasis within insects. Hereafter, I propose some new directions for research based on my findings.

4.2 Revisiting Components of Paracellular Permeability

It is well known that barrier function along paracellular pathways is important for selectively and dynamically controlling solute diffusion across epithelia. While barriers along these pathways effectively maintain a separation between the organism and the outside environment (i.e. the gut lumen), injury or death can occur in the event of compromised barrier integrity (Izumi and Furuse, 2014; Jonusaite et al., 2016; MacMillan et al., 2017). In instances of ageing, hypoxia, or cold stress for example, the subsequent increase of gut epithelial permeability that occurs often leads to death in *D. melanogaster* (Huber et al., 2001; MacMillan et al., 2017; Rera et al., 2012; Sun et al., 2015). Due to importance of paracellular pathways, their composition along ionomotive epithelia such as the gut is under active investigation for their contribution to insect cold tolerance. One such component, known as septate junctions

(SJs), largely dictates the permeability of these pathways (Jonusaite et al., 2016). The SJs are specialized cell-cell junctions consistently linked to cold tolerance plasticity (Des Marteaux et al., 2017; MacMillan et al., 2016; MacMillan et al., 2017). For instance, cold-acclimated (pre-treated) *D. melanogaster* had reduced paracellular leak of FITC-dextran from their gut lumen to the hemocoel, and improved cold tolerance when compared to warm-acclimated flies (MacMillan et al., 2017). Similar to cold acclimated flies, reduced paracellular leak of FITC-dextran in the cold has also been observed in more cold tolerant species of *Drosophila* (e.g. *D. montana*) when compared to their warm-adapted allospecifics (e.g. *D. birchii* and *D. equinoxialis*; Andersen et al., 2017b). Additionally, differences in SJ appearance have been observed between cold- and warm- acclimation treatments, with cold acclimated flies possessing longer and more tortuous apical cell-cell contact regions than those that were warm-acclimated (MacMillan et al., 2017). While strong evidence for cold-induced paracellular leak in locusts is presented in Chapter 2, whether SJs themselves can be definitively linked to these events is not yet clear.

Classically, the majority of SJ work in insects has been conducted in dipteran genera like *Drosophila* and *Aedes*, and has revealed much information surrounding the identification and characterization of SJ types, as well as associated proteins (Izumi and Furuse, 2014; Jonusaite et al., 2016; Jonusaite et al., 2017; MacMillan et al., 2017; Yanagihashi et al., 2012; for examples in other Orders, see Des Marteaux et al., 2017; Kolosov et al., 2019; Lane and Flores, 1988; le Skaer et al., 1987). Little work (to my knowledge) has been done beyond the investigation of SJ morphology in locusts or other orthopterans (Eley and Shelton, 1976; Jarial, 1992; Kendall, 1969; Swales and Lane, 1985; but see Des Marteaux et al., 2017 for data on *G. pennsylvanicus*). Less information is available still on SJs in the locust gut specifically other than their basic

morphology (Jarial, 1992). I therefore suggest that further experiments in *L. migratoria* focus on exploring SJ plasticity and structure to investigate their influence (if any) in cold-induced paracellular leak. Such work could include the further characterization of SJs along the locust gut and the identification of how these SJs interact with the surrounding cytoskeleton (e.g. actin) under cold and control conditions (Belous, 1992; Harvey and Zerahn, 1972; Lane and Flores, 1988; MacMillan et al., 2017). A detailed exploration of differences in SJ abundance or composition among the gut segments (foregut, midgut, hindgut) may also help to identify areas prone to cold-induced barrier failure. Experiments also conducted post-acclimation (cold vs. warm) could reveal useful information regarding cold-induced responses in locust SJs, as well as the underlying mechanisms of thermal plasticity such as those involving ion and water movement along the paracellular pathway in the cold.

4.3 Small-Molecule Markers of Epithelial Permeability in Insects

While mPEG is a commonly used marker of paracellular permeability (Chadwick et al., 1977; Kansagra et al., 2003; Lane and Corrigan, 2006; le Skaer et al., 1987; Robertson and Wood, 2014), I provide evidence (discussed in Chapter 3) that this marker is not suitable for quantifying epithelial permeability, at least in locusts. Although these results were unexpected, I speculate that the mechanisms through which mPEG (350 Da) was rapidly cleared from the hemocoel (post-injection) are similar to those responsible for removing xenobiotics from the insect system. Phytophagous insects, like locusts, are constantly exposed to unwanted and sometimes toxic molecules, including insecticidal proteins, through their diet (Denecke et al., 2018). Their survival may therefore depend on the ability to clear these unwanted compounds from their body via mechanisms like carrier-mediated active transport, solvent drag, or endocytosis (Denecke et al., 2018). Through these means of xenobiotic clearance, insects are

able to remove compounds of various sizes, including insecticides such as amitraz (~293 Da), chlorpyrifos (~351 Da), abamectin (~873 Da), and *Amaryllis* lectin (12-50 kDa; a plant based lectin; Caccia et al., 2012; Lanning et al., 1996; Lara et al., 2015; Leslie et al., 2005; Xiao et al., 2016).

As measurements of insect epithelial permeability using mPEG have been typically been conducted under near-optimal conditions (to my knowledge; Jonusaite et al., 2017a; Jonusaite et al., 2017b; le Skaer et al., 1987; O'Donnell et al., 1984), it may have been difficult to distinguish between high mPEG permeability and the transport of mPEG across the gut. Thus, this marker has yet to be characterized as a transportable marker within these models (Self et al., 1995). The lack of mPEG movement across the gut epithelia at low temperatures, however, suggest that the membrane is not permeable to mPEG, and that it must somehow be transported into the gut lumen for excretion (discussed in Chapter 3.4). This brings into question the reliability of not only mPEG, but also other low molecular weight molecules as markers of epithelial permeability in insects, such as inulin or brilliant blue. In light of the incredible ability of insects to actively clear a variety of compounds from their hemolymph, I suggest that the permeability of any potential marker of paracellular leak be thoroughly explored under both normal (e.g. room temperature) and sub-optimal (e.g. cold) conditions prior to experimentation.

4.4 Applications within Insect Management

Since their popularization in the 1930s, insecticides have been widely used as a means to control unwanted insect populations (Le Goff and Giraudo, 2019). Today, as the distributions of insects worldwide continue to generally expand and/or shift poleward with rising global temperatures, there is a pressing need to predict and control the range of species that are harmful to human and animal well-being (Chown et al., 2012; Hunter, 2019; Rua and Okech, 2019).

Furthermore, to limit unintended impacts to ecosystems and the various organisms within them, it is important to design species-specific insecticidal compounds (Le Goff and Giraudo, 2019). As the paracellular pathways along the gut epithelia are needed to maintain compartmental separation within insects (Jonusaite et al., 2016), chemicals targeting proteins pertaining to components of these pathways (e.g. SJs), may be effective in reducing insect survival and fitness. While similarities between these SJ proteins may exist across insects, the exact genes coding for a specific protein may vary, allowing for the development of target-specific insecticides. Thus, understanding what dictates permeability along the gut epithelia would have promising applications in not only insect management, but also in adding to the current knowledge of how SJs contribute to cold tolerance.

4.5 Concluding Remarks

In addition to enhancing our understanding of the physiological mechanisms underlying chill susceptibility and tolerance, the research presented herein has expanded our current knowledge of paracellular barriers in groups beyond Diptera. As in *D. melanogaster*, locusts are unable to maintain gut barrier integrity when chilled, however, the resulting leak that occurs across the gut epithelia appears to be unidirectional. While the origin of this barrier failure remains unknown, I have generated exciting new hypotheses regarding the mechanisms of this cold-induced barrier failure that can now be explored. In the process of addressing my initial hypotheses, I was also able to develop novel means of measuring cold-induced leak within insects. My work has furthered our understanding of paracellular pathways in insects, which should help facilitate additional investigation into the role these pathways have in insect cold tolerance.

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