Investigating a link between cold stress and bacterial leak from the gut of *Locusta migratoria*

by

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Abstract

Following prolonged low temperature exposure, chill-susceptible insects can incur chilling injuries that manifest as motor deficits and tissue damage. This tissue damage is thought to be driven by a loss of ion and water homeostasis, characterized by elevated potassium concentration in the hemolymph (hyperkalemia). The insect gut plays a major role in the maintenance of ion and water balance, and gut epithelial barrier function is compromised during chilling, which can contribute to the leak of water and solutes across the gut wall. The insect gut also houses an abundant bacterial population which can contribute to the host's cold tolerance. In recent years, however, a growing number of studies have reported increased activation of the insect immune system following a cold stress, suggesting that cold stress may cause bacterial infection. For my research, I hypothesized that prolonged cold stresses result in the leak of bacteria from the gut into the surrounding hemolymph as a possible explanation for the immune activation. With locusts as my model insect, I used an *E. coli* strain that expresses a fluorescent marker (*GFPmut3*) to track bacterial leak from the gut. After a period of feeding on wheat that was soaked in a concentrated solution of the bacteria, locusts were exposed to -2°C for varying lengths of time before hemolymph was extracted and plated on agar media to facilitate bacterial colony growth. Surprisingly, I found no evidence of bacterial leak as no colonies were observed regardless of cold exposure length. My research strongly suggests that gut barrier integrity is well maintained even after severe cold stresses and is sufficient enough to prevent the leak of whole bacteria across the gut wall, which opens up other possible explanations as to why immune activation occurs following cold exposures in insects.

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Section 1: Introduction

1.1 *Insects in the environment*

Insects make up a large portion of Earth's known and unknown animal species (Stork, 2018). Found in many ecosystems of varied climates, they can play an important role in providing ecosystem or agricultural services such as pollination, seed dispersal, and biocontrol of pests, to name a few (Schowalter et al., 2018; Symondson et al., 2003). On the other hand, some insects act as pests and may pose threats to human or plant health (Brewer et al., 2019; Cohuet et al., 2010; Kamgang et al., 2020; Poland and McCullough, 2006; Safranyik et al., 2010).

All insects are ectotherms, which means that their internal body heat is primarily derived from their external environment. An insect's thermal environment plays a key role in regulating its survival, reproduction, and other biochemical processes (Tattersall et al., 2012). Because insect life history is so intimately tied to temperature, the thermal environment can also determine the geographical distribution of these ectothermic animals (Sunday et al., 2011). Studies have demonstrated a correlation between the highest or lowest temperatures an insect species can tolerate, and the temperature of the environment (Addo-Bediako et al., 2000; Andersen et al., 2015; Hoffmann et al., 2002; Kellermann et al., 2012), strongly suggesting that both high and low temperature limits are key factors in determining a species' latitudinal limits. These findings have become increasingly crucial to understanding ectotherm biogeography in an era of global climate change, as insects are frequently being observed in areas beyond their normal ranges (Bale and Hayward, 2010). One major driving force behind these extensions in ranges is an increase in minimum and mean winter temperatures over the past few decades, which has allowed some species to successfully move into, and overwinter in, new areas past their normal latitudinal range (Bale and Hayward, 2010; Sparks et al., 2005).

Climate change not only affects the average temperature of regions, but it may also lead to greater and more frequent extremes in high and low temperatures throughout seasons (Easterling et al., 2000). These stochastic anomalies in temperature can be an important factor in accelerating the spatial movement of species (Meehl et al., 2000; Parmesan et al., 2000). However, their establishment and persistence in these new areas hinge on the availability of important resources, a suitable climate, and the ability to successfully overwinter. Most species typically do not successfully establish themselves in new areas due to a change in local environmental conditions and resource availability, and thus die (Battisti et al., 2006; Loxdale and Lushai, 1999). As low temperatures are a deciding factor in limiting the distribution of most insects, phenotypic plasticity in the face of low temperatures strongly contributes to the persistence of insects in cold environments (Hoffmann et al., 2005; Marshall et al., 2020; Overgaard and MacMillan, 2017; Sgrò et al., 2016).

The extended geographic range of insect pests and disease vectors is of particular concern in many countries, but of equal concern is how climate change can affect pollinators and other useful insects in ecosystems (Memmott et al., 2007; Porter et al., 1991). To better predict the response of insects to a changing climate, it is important to understand how the thermal tolerance of these organisms is tied to their physiology (Chown et al., 2010). Generally, cold tolerance is a stronger predictor of insect distribution than heat tolerance (Addo-Bediako et al., 2000; Andersen et al., 2015; Kimura, 2004). Although both heat and cold stresses are harmful to insects, my thesis focuses on low temperature stress and aims to contribute to our overall understanding of the lower thermal limits of insect physiological systems.

1.2 Insect cold tolerance

Although all insects are ectotherms, not all species tolerate low temperatures in the same manner. The current consensus classifies insects into three different strategies based on how they survive low temperatures or freezing: freeze-avoidant, freeze-tolerant, and chill-susceptible (Lee, 1989; Sinclair et al., 2015). Freeze avoidant insects use biochemical mechanisms that suppress the formation of ice in their extracellular fluid, or hemolymph, which leads to their body fluids having a relatively lower (more negative) freezing point compared to the other two groups. Freeze-tolerant insects, however, do not suppress ice formation; instead, they can survive ice formation in their bodies, and even promote ice formation at higher temperatures and initiate freezing (Sinclair, 1999). Freeze-tolerant insects survive the cellular *processes* involved in cooling, freezing, and thawing, as well as the challenges that come with them (Lee, 1989; Sinclair et al., 2015; Toxopeus and Sinclair, 2018). Insect from both these groups exhibit a range of physiological adaptations that allow them to avoid or tolerate freezing, including the production of cryoprotectants and antifreeze proteins, self dehydration, or the removal of ice-nucleating agents (organisms or substances that encourage ice formation) (Bale, 1987; Clark et al., 2009; Duman, 2003; Overgaard and MacMillan, 2017; Zachariassen, 1985). It is unclear if the mechanisms of freeze-avoidance and freeze-tolerance are consistent among insect taxa, as these strategies of cold tolerance have evolved multiple times (Sinclair et al., 2003; Toxopeus and Sinclair, 2018).

Chill-susceptible insects make up the majority of the Class Insecta and include the model fruit fly (*D. melanogaster*) and migratory locust (*Locusta migratoria*). Unlike freeze-avoidant and freeze-tolerant insects which incur injuries related to freezing, chill-susceptible species suffer chilling injuries at low temperatures above the freezing point of their body fluids

(Overgaard and MacMillan, 2017). These injuries are thought to be driven by cell death as a result of membrane phase transitions and the loss of ion homeostasis (Andersen et al., 2017; Bayley et al., 2018; Carrington et al., 2020; Košťál et al., 2004; MacMillan and Sinclair, 2011b). The severity of chilling injuries tend to increase with cold duration and intensity, hence both short cold stresses and prolonged, but more mild cold stresses may harm the organism (Nedvĕd et al., 1998).

As temperatures drop below their critical thermal minimum (CT_{min}), which differs among species, chill-susceptible insects exhibit a loss of voluntary motor control. Continued cold exposure eventually leads to the onset of chill-coma, a state of complete but reversible paralysis (Andersen et al., 2015; Hazell and Bale, 2011; MacMillan and Sinclair, 2011; Rodgers et al., 2010). An insect in chill-coma will remain completely immobile and accumulate chilling injuries so long as temperatures remain below its CT_{min} , eventually dying if the injuries are too severe. If the insect does not die and is returned to a benign environment, chill-coma can be reversed and the insect may regain neuromuscular function. After a period of time, the insect regains the ability to stand and move again. This time taken, known as chill-coma recovery time (CCRT), is directly correlated with how long the insect remains in chill-coma (MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017). When profiling an insect's cold tolerance, CCRT can be used as a quantitative measure. An injury or mortality assay can also be used a period of time after measuring CCRT, usually a day or more post-cold exposure, in order to quantify the extent of chilling injuries. This assay typically employs a point-based scale to assess an insect's movement or behaviour, with more injured insects exhibiting greater degrees of chilling injuries (Brzezinski and MacMillan, 2020; Carrington et al., 2020; El-Saadi et al., 2020; MacMillan et al., 2014). Both CCRT and survival/injury assays are useful tools when used together, as they are

commonly used by ecologists to find patterns or differences in thermal tolerance at the population or species level of an insect (Davis et al., 2021; MacMillan et al., 2015a; Ransberry et al., 2015; Slatyer and Schoville, 2016)

1.3 *The insect gut and Malpighian tubules*

The progression of neuromuscular failure at low temperatures is thought to occur as a result of a disruption to ion and water balance. In insects, the gut plays a major role in regulating the active and passive flow of solutes such as sodium (Na^+) and potassium (K^+) ions, as well as controlling the passive movement of water, at benign conditions (MacMillan and Sinclair, 2011b; MacMillan et al., 2017; Yerushalmi et al., 2018). Structurally, the insect gut is divided into three regions: foregut, midgut, and hindgut (Figure 1.3.1; Brzezinski and MacMillan, 2020). The foregut and hindgut are derived from ectodermal cells and lined with cuticle. The midgut, however, is derived from endodermal cells, lacks the cuticular lining, and contains a peritrophic matrix, an acellular layer secreted at the junction of the foregut and midgut. The peritrophic matrix protects the epithelial cells of the midgut from injury caused by ingested food (Linser and Dinglasan, 2014).

All three sections of the gut contribute to the process of digestion in insects. The foregut serves to mechanically break up food, and can even act as a storage area. The bulk of digestion and nutrient absorption happens in the midgut; any water remaining is reabsorbed in the hindgut before the digested bolus passes into the rectum and is excreted (Linser and Dinglasan, 2014; Livingston et al., 2020; Phillips et al., 1987). In the hindgut, water moves between the gut lumen (interior) and the hemocoel (body cavity of the insect containing hemolymph, a fluid analogous to blood of chordates) down the concentration gradient transcellularly through channels or paracellularly past structures called septate junctions (Izumi and Furuse, 2014; Jonusaite et al.,

2016). These junctions are ladder-like protein complexes located between gut epithelial cells that regulate the passive movement of solutes and water (MacMillan et al., 2017; O'Donnell, 2008; Phillips et al., 1987). Ion movement occurs between the midgut/hindgut and hemolymph in two ways: transcellularly via active transport by membrane-bound ion transporters, or paracellularly past the septate junctions. Under optimal environmental conditions, hemolymph volume and sodium (Na^+) concentration remain high, while potassium (K^+) concentration remains low to maintain ion and water homeostasis (D'Silva et al., 2017; Harvey et al., 1983; MacMillan and Sinclair, 2011b; MacMillan et al., 2015b; Overgaard and MacMillan, 2017). Posterior to the midgut lie strand-like appendages known as Malpighian tubules. These structures serve as analogues to human kidneys and play a major role, alongside the hindgut, in the maintenance of ion and water homeostasis via urine production in the insect body (Beyenbach, 2003; Dow et al., 1994). Structurally, Malpighian tubules can differ between species in relation to environmental adaptations, but mainly consist of principal and stellate cells. Principal cells regulate the active transport of Na^+ and K^+ into the tubule lumen via membrane-bound transporters, while stellate cells transcellularly transport water and chloride (Cl^-) ions (Beyenbach, 2003).

Low temperatures are detrimental to a chill-susceptible insect, in part, because they slow the rate of active ion transport in the gut and Malpighian tubules. As a cold exposure continues, active transport rates pass a critical threshold where they cannot counterbalance the passive leak of solutes and water (Overgaard et al., 2021). Ion and water homeostasis then become disrupted when a net leak of Na^+ and water into the gut lumen occurs, reducing hemolymph volume (MacMillan and Sinclair, 2011b). Epithelial barriers in the gut also become disrupted which exacerbates the cold-induced leakage (Brzezinski and MacMillan, 2020). This concentrates the K^+ in the hemolymph and is further made worse by the leak of K^+ from the interior of cells to the

extracellular space (Andersen et al., 2013). Cell membrane potential is heavily dependent on low extracellular $[K^+]$, so these events lead to a depolarization of cell membranes in the insect tissues (e.g. muscle and nerve cells) (MacMillan et al., 2014; Armstrong et al., 2012). Reduced neuronal excitability in the central nervous system leads to impaired nerve signalling and the progressive loss of neuromuscular function and motor control observed in chill-coma (Andersen et al., 2017; MacMillan et al., 2015c; Overgaard and MacMillan, 2017; Robertson et al., 2017; Yerushalmi et al., 2018). Continued exposure to the cold leads to further membrane depolarization and an increased concentration of K^+ in the hemolymph, eventually activating voltage-gated calcium (Ca^{2+}) channels. This leads to an influx of Ca^{2+} into cells, which can trigger apoptotic or necrotic muscle tissue death, leading to cold-induced chilling injuries (Bayley et al., 2018). If the injuries are not severe to the point that the insect dies, when returned to a warm environment, it will start to recover from chill-coma by restoring hemolymph volume and ion concentrations, re-establishing water and ion homeostasis. When active transport rates in the Malpighian tubule and gut start to increase again during rewarming, water and Na^+ from the gut lumen start to move back into the hemolymph, reducing its concentration of K^+ and restoring muscle membrane potential. The insect then fully or partially regains motor function, thus recovering from chill-coma (MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). Because of the effects of gut barrier disruption on the loss of ion and water homeostasis at low temperatures, the ionoregulatory and barrier functions of the insect gut are now thought to be central to insect chill tolerance (MacMillan, 2019).

1.4 Gut Microbiome and Immune System

Gut epithelial cells and septate junctions act as barriers that separate the internal contents of the gut from the external hemocoel. In addition to regulating the flow of water and ions, the

gut also houses an abundant microbial community in its lumen which is primarily composed of bacteria and yeasts (Dillon and Dillon, 2004; Padilla, 2016; Wong et al., 2011). The gut can be considered an open system in the sense that it can be invaded by other microbes through ingestion, thus affecting its microbial composition (Douglas, 2009; Douglas, 2015). Diet plays a role in shaping the composition of the gut microbiome, and different compositions can be observed among insect species and across seasons (Douglas, 2015; Ferguson et al., 2018).

Recent studies have illustrated how gut bacteria and yeasts may affect an insect's survival positively or negatively at low temperatures. For example, *D. melanogaster* with a healthy gut flora exhibit significantly increased cold tolerance (Henry and Colinet, 2018; Moghadam et al., 2018; Padilla, 2016). On the other hand, ingested bacteria in the gut of beetles and moths may act as ice-nucleating agents and negatively impact their cold tolerance by initiating extracellular freezing (Lee et al., 1992; Lee et al., 1994; Tang et al., 2004). Because bacteria may act as ice-nucleating agents, some insects have evolved an adaptation whereby they evacuate their gut contents before overwintering (Cannon and Block, 1988; Krunić and Radović, 1974). These studies illustrate an important role that the gut microbiome plays in the thermal tolerance of insects.

Not all bacteria are benign or beneficial. Like that of humans, the insect immune system detects, responds to, and eliminates pathogenic bacteria from the body. Peptidoglycan, found in the cell walls of gram-positive and gram-negative bacteria, as well as lipopolysaccharide, found in the outer membrane of gram-negative strains, elicit an immune response from the host (Charles and Killian, 2015). In the case of a pathogenic attack, the specific immune response depends on which of the three immune pathways are activated (Toll, Imd, or JNK, though some crossover can occur). The Toll pathway responds to gram-positive bacteria, the Imd pathway

responds to gram-negative bacteria, and the JNK pathway contributes to the production of antimicrobial peptides. A fourth pathway, the JAK/STAT pathway, produces immune signalling molecules (cytokines) after septic injury to tissues and promotes hemocyte differentiation, but does not seem to be directly involved in fighting pathogens (Bang, 2019; Lemaitre and Hoffmann, 2007).

The insect immune system can produce cellular or humoral components depending on the type of foreign substance that needs to be eliminated. For example, cellular defenses, in the form of circulating hemocytes, can phagocytose and encapsulate invaders (Strand, 2008). Humoral immunity includes the production of antimicrobial peptides from the fat body (and, in some insects, from hemocytes), and coagulation and melanisation of the hemolymph (Dunn, 1990). Peptidoglycan recognition proteins in the hemolymph, upon detecting bacteria, induce the expression of antimicrobial peptides and trigger the melanisation process via the prophenoloxidase cascade (Choe et al., 2002; González-Santoyo and Córdoba-Aguilar, 2012). The melanin formed then helps to entrap and surround bacteria by producing nodules, or tight stacks of hemocytes (Dubovskiy et al., 2016).

It is logical that an artificially-induced bacterial challenge would trigger an immune response in insects, and this has been clearly demonstrated previously (Adamo, 2004; Charles and Killian, 2015; Rowley et al., 1990; Salehipour-shirazi et al., 2017). However, immune activation in *D. melanogaster* has also been reported after subjecting flies to a cold stress, though the underlying mechanisms behind this phenomenon are still unclear (Sinclair et al., 2013). This response is mainly characterized by an increased expression of genes in the Toll and/or Imd pathways, such as *attacin*, *diptericin*, *drosomycin*, and *metchnikowin* (Salehipour-shirazi et al., 2017; Štětina et al., 2019). In overwintering crickets (*G. veletis*), increased hemocyte

concentration in the hemolymph, along with counterintuitively reduced (rather than increased) melanisation, has also been observed (Ferguson et al., 2018). However, it is difficult to generalize the effects of low temperatures on cellular or humoral immunity in insects due to inconsistencies in the immune response among more cold tolerant insect species. In an ecological context, seasonal changes may result in higher or lower hemolymph concentrations of hemocyte and melanin in freeze-avoidant and freeze-tolerant species (Ferguson and Sinclair, 2017). Thus, from the current literature, the relationship between low temperature environments and immune activity remains blurred.

It is known that gut epithelial barriers fail in the cold, yet most research has focused on the leak of non-microbial matter, such as solutes, across the compromised barriers. In a study looking at septate junction dysfunction following mechanical stress, Katzenberger et al. (2015) showed that traumatic brain injury led to intestinal barrier failure in *D. melanogaster*, as measured by the leak of a blue, organic dye into the hemocoel. Furthermore, injury resulted in increased expression of the antimicrobial peptide genes *Attacin*, *Diptericin*, and *Metchnikowin*, which are also upregulated after a cold stress. This establishes a link between mechanical injury, gut barrier failure, and immune activation caused by bacteria in insects, but this has not been directly tested in the context of low temperature stress.

Since 1) the insect gut contains a community of bacteria, 2) paracellular barriers get leaky in the cold in chill susceptible insects, and 3) cold stress activates the insect immune system, I hypothesized that cold-induced damage to the gut epithelial barriers may lead to immune activation as a result of bacterial leakage into the hemocoel. My thesis aims to address an unexplored avenue in cold-induced immune activation in insects by investigating the potential for leak of bacteria from the gut into the hemolymph following a cold stress in locusts (*L.*

migratoria), a model chill-susceptible insect. In order to track the movement of bacteria across the gut wall, I developed a novel protocol that involved feeding locusts a fluorescent strain of *Escherichia coli* before exposing them to low temperatures, after which I tested for the presence of the bacteria in their hemolymph.

Figure 1.3.1. Diagram of the gut anatomy of locusts. The foregut, midgut, and hindgut all contribute uniquely to the process of digestion. Ion and water homeostasis are maintained by the renal system, comprised of the hindgut and Malpighian tubules. Figure adapted from Brzezinski and MacMillan (2020).

Section 2: Methods

2.1 Locust rearing

Locusts (*L. migratoria*) used in the experiments were obtained from a colony maintained at Carleton University, Ottawa, ON. Rearing conditions closely resembled those described in Brzezinski and MacMillan (2020). The colony was reared on a 16 h:8 h light:dark cycle at 28°C at 60% relative humidity, under crowded conditions. Locusts were held in custom-built wooden cages with a wire mesh bottom. Eggs were laid in sand-filled plastic cups that females oviposited in through an opening in the wire mesh. The egg cups were collected three times a week, covered in cling wrap, and placed in an incubator (VWR 1545; Marshall Scientific, Hampton, NH, USA) at 33°C for ten days before placing the cups in clean cages to allow the eggs to hatch. All experiments were done with locusts at 3-4 weeks old after final ecdysis. All locusts were fed a dry mixture of oats, wheat germ, wheat bran, and dry milk powder, as well as fresh wheat clippings, three days a week.

2.2 Quantifying cold tolerance using chill-coma recovery time (CCRT) and injury score

To quantify cold tolerance in the locust colony, both CCRT and degree of chilling injuries (injury score) were used as measures. The methodology used here was slightly modified from Brzezinski and MacMillan (2020). Locusts from the colony were collected during the morning (or afternoon for 12 and 36 h cold exposures, see below) of the experiment and placed individually in 50 mL polypropylene falcon tubes. Holes were made in the lids of the tubes, which provided locusts access to air for the duration of the experiment. Control locusts were placed in an incubator (Isotemp BOD Refrigerated Incubator 3720A; Thermo Fisher Scientific, ON, Canada) with dry oat mixture and fresh wheat clippings for 48 h at 25°C. Locusts

undergoing cold stresses were randomly assigned to four cold exposure groups that were left in a refrigerated circulating bath (Model AP28R-30; VWR International, Radnor, PA, USA) for 12, 24, 36, or 48 h. Cold exposures were done by suspending the falcon tubes containing the locusts in a Styrofoam rig and placing the rig in the bath filled with 100% ethylene glycol. The bath was pre-set to 25°C and cooled to -2°C at a rate of -0.25°C min⁻¹. Locusts in the Control, 24 h, and 48 h exposure groups were placed in the bath together. Two days post-treatment, locusts in the 12 and 36 h groups were then placed in the bath together. Bath temperature was monitored throughout the duration of the cold exposure using type-K thermocouples (TC-08 Data Logger; Picotech, Texas, USA). Upon the completion of a group's cold exposure, locusts, in their comatose state, were removed from their tubes and placed on their side on a clean bench at room temperature (25°C). To measure CCRT, each locust was closely monitored for the time taken to regain neuromuscular function and stand on all six legs, which was recorded as that locust's CCRT. A 90 min cut-off point was used, after which any locust that had failed to stand on all legs was marked as "unrecovered". The control and treatment groups each contained eight locusts (four males and four females).

After 90 min, locusts were placed in clean 50 mL polypropylene tubes with dry oat mixture and fresh wheat clippings and left to recover in the incubator at 25°C. After one day (24 h) of recovery post-cold exposure, an injury assessment was done using a 5-point scale adapted from MacMillan et al. (2014). Scores were defined as follows: 0: no movement observed (dead); 1: limb movement (leg and/or head twitching); 2: greater limb movement (leg, head, and whole body twitching), 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 similar to pre-exposure levels of coordination. After scoring injury, locusts were returned to their

respective tubes with replenished oats and wheat clippings and placed in the incubator. The injury assessment was repeated three more times for each locust two, three, and four days (48, 72, 96 h) post-cold exposure to assess latent chilling injuries likely caused by infection from bacteria in the hemolymph.

2.3 *Fluorescent bacteria feeding protocol*

I developed a novel feeding assay to investigate bacterial leak from the gut of locusts following a cold stress. Protocols for this procedure were developed using a mutant fluorescent strain of *E. coli*, *GFPmut3* (λ_{max} excitation: 500 nm, λ_{max} emission: 513 nm) which contains a green fluorescent gene on a plasmid alongside an ampicillin-resistant gene (Chalova et al., 2008; Zhao et al., 2008). Initial trials involved growing bacteria in liquid medium overnight, using the resultant bacterial culture to soak dry oats or wheat, and immediately feeding the soaked food to locusts. I tested different combinations of wheat, oats, and bacterial solution concentrations (via centrifugation) to ensure an abundance of the fluorescent bacteria throughout the locust gut. However, all of these trials resulted in 1) the locusts not eating the food when oats were used, 2) the locusts eating very little food when oats were used, or 3) the locusts eating the food overnight when wheat was used. In the event that the wheat was eaten, an absence of the fluorescent bacteria in the gut was confirmed via plating the gut contents (see below). This suggested that the concentration of bacteria in the wheat was insufficient to guarantee ample fluorescent *E. coli* in the lumen of the locust gut overnight. Furthermore, trials where the locusts did not eat the food, or ate very little, suggested that they were either already satiated, or strongly preferred the wheat over the oats.

In the final assay, an overnight culture of *GFPmut3 E. coli* was grown in LB Broth containing ampicillin (1 μ L ampicillin/1 mL media) in an incubator at 37°C (Model MIR-154;

PHC Corporation, Wood Dale, IL, USA). Then, 125 mL of medium was centrifuged at 8000 rpm (9730 x g) for 15 min (Sorvall RC 6 Plus; Thermo Scientific, Waltham, MA, USA). The supernatant was discarded, and the bacterial pellet was then resuspended in 1.25 mL of distilled water in a 2.0 mL centrifuge tube, effectively concentrating the bacterial solution 100-fold. Ten 3.0 cm strands of freshly cut wheat were added to the tube and allowed to soak for 24 h in an incubator at 37°C. Simultaneously, locusts that were to be used in the experiments were moved to a separate cage for 24 h with no wheat or oats to stimulate hunger. Each locust was then placed in separate small deli plastic containers, with holes in the lids, along with their own bacteria-soaked wheat where they fed for 24 h in an incubator at 25°C.

Before carrying out cold exposures, and to ensure the validity of the feeding protocol, I confirmed presence of the fluorescent bacteria in the gut following the feeding period. A qualitative analysis was done by homogenizing and plating the gut contents on Ampicillin-containing LB plates, after feeding on the bacteria-soaked wheat overnight. Locusts (3 male, 3 female) were removed from the incubator after the 24 h feeding period and very briefly (1 s) submerged in 70% ethanol. Locusts were decapitated with sterile scissors, and their wings and limbs were removed. The remaining thorax and abdomen were placed in a 100 mL petri dish with an elastomer base (Sylgard 184 Silicone Elastomer Kit). A longitudinal incision was made in the anterior to posterior direction on the animals' ventral side to expose the gut. In a 100 mm petri dish with an elastomer filled base (Sylgard 184), the Malpighian tubules, fat body, and trachea were cleared first before excising the foregut, midgut, and hindgut (Figure 1). Both ends of each segment were gently pinched with sterile forceps to mitigate leak of gut contents before removal. Gut segments were then very briefly washed (1-2 s) in 70% ethanol to remove any bacteria on the outer gut surface as a result of possible contamination during dissection.

Segments were placed in 2.0 mL centrifuge tubes containing 1.5 mL of sterile locust saline (in mmol L⁻¹: 140 NaCl, 8 KCl, 2.3 CaCl₂ Dihydrate, 0.93 MgCl₂ Hexahydrate, 1 NaH₂PO₄, 90 sucrose, 5 glucose, 5 trehalose, 1 proline, 10 HEPES, pH 7.2). Segments were then homogenized (Tissue Master 125; Omni International, Kennesaw, GA, USA) for three minutes or until the solution became uniform. After briefly vortex mixing, a 150 µL aliquot of the homogenized solution was spread on an LB agar plate containing Ampicillin. Plates were then incubated overnight at 37°C. Multiple fluorescent colonies were observed on the plates after plating all three gut segments (Figure 2.3.1), which qualitatively confirms the presence of the fluorescent *E. coli* in the gut after feeding on the wheat.

After finalizing the protocol, I needed to ensure that the 24 h fasting protocol and bacterial load in the gut did not increase or decrease the degree of chilling injuries after a severe cold stress. To do this, I carried out another survival assay similar to the one described previously. Ten strands of freshly-cut wheat were soaked in either 1.25 mL of the 100X bacterial solution, or 1.25 mL of distilled water (control) in a 2.0 mL centrifuge tube for 24 h at 37°C. Simultaneously, locusts were isolated for 24 h to induce starvation. Locusts were then allowed to feed on either type of wheat for 24 h at 25°C, after which they were exposed to -2°C for 48 h. An injury assessment was then carried out for four days post-cold exposure.

2.4 *Investigating cold-induced bacterial leak from the gut*

To test whether bacteria in the gut leak into the hemolymph during cold stress a separate group of locusts were suspended in a cooling bath at -2°C for 12, 24, 36, or 48 h following 24 h of feeding on the wheat soaked in the fluorescent bacteria solution. Control locusts were kept in the incubator at 25°C for 48 h with wheat and oats provided *ad libitum*. Immediately after the exposures, hemolymph was collected using techniques adapted from Findsen et al. (2013). Using

a sharp probe, locusts were pricked dorsally at the head-thorax junction. A 50 μ L capillary tube was then used to collect hemolymph via capillary action at the site of injury. By inserting a pipette tip at the end of the capillary tube, 10 μ L of hemolymph was drawn and pipetted into 190 μ L of sterile locust saline in a 1.5 mL centrifuge tube. This was repeated with an additional 10 μ L of hemolymph from the same locust that was pipetted into another tube containing 190 μ L of sterile locust saline. After briefly vortex mixing, one of the 200 μ L solutions was spread on an LB agar plate with Ampicillin, and the other solution was spread on an LB agar plate without Ampicillin. Plates containing Ampicillin would select for the fluorescent *E. coli* strain, while plates without Ampicillin would support the growth of any microbes able to grow on the medium. Both plates were then incubated at 37°C. The plates were checked for colony growth every day for four days. Each experimental group contained six locusts (3 male, 3 female), except for the 48 h cold treatment group, which contained ten locusts (5 male, 5 female).

Bacterial leak could occur following, rather than during, a cold stress, so I completed a follow up experiment to test for bacterial leak following a 6 h rewarming period after the cold stress. Cold exposures were done in an identical manner as described above. Immediately following the cold exposures, however, locusts were placed in small plastic deli containers (9.9 cm x 4.6 cm) with freshly cut wheat and dry oat mixture in excess. The containers with the locusts were then placed in the incubator and left to recover at 25°C for 6 h. Hemolymph extractions and plating were then performed as described previously, checking for bacterial growth every day for four days. Each experimental group contained six locusts (3 male, 3 female).

2.5 *Effect of cold stress on gut tissue viability*

In order to relate the results of the gut leak assay to the health and integrity of the gut epithelial tissue, I refined a protocol for a LIVE/DEAD cell viability assay adapted from previous studies that assessed insect tissue viability (Andersen et al., 2017; Bayley et al., 2018; MacMillan et al., 2015b). A section of the posterior foregut and anterior hindgut were used as representative sections of the gut to quantify cell damage. Locusts were exposed to -2°C for 12, 24, 36, or 48 h. Control locusts were kept in an incubator at 25°C for 48 h, with fresh wheat and dry oat mixture provided ad libitum. Immediately following treatment, locusts were removed from the cold and had their head, wings, and limbs removed. A longitudinal incision was then made on the ventral side in the anterior to posterior direction. Locusts were then submerged under 50 mL of locust saline in a 100 mm petri dish with an elastomer base. Insect pins were used to fully expose the locust body cavity. After removing the anterior foregut and discarding it, micro dissecting scissors were then used to cut the cylindrically-shaped posterior foregut on the ventral side, exposing the gut interior. A section of the foregut tissue was then excised, briefly washed (1-2 s) in a separate saline solution and placed on a clean glass slide with the inner gut epithelia facing upwards. The same procedure was done with the anterior hindgut after removing the midgut.

To quantify cellular viability, a LIVE/DEAD sperm viability kit (Thermo Fisher Scientific, ON, Canada) containing two fluorescent stains was used. SYBR 14 (excitation λ_{max} : 475 nm, emission λ_{max} : 516 nm) is membrane permeable and binds to nucleotides in the nuclei of healthy cells. Propidium iodide (excitation λ_{max} : 535 nm, emission λ_{max} : 617 nm) is membrane impermeable and only binds to the nuclei of cells whose membranes have been compromised, outcompeting SYBR 14 in the process. A 50 μL solution of SYBR 14 (40 μM) was added to the tissue on the slide for 10 min, after which a 50 μL solution of propidium iodide (84 μM) was

added for 10 minutes. The solutions were removed from the slide and a cover slip was placed on top of the tissue. Using a fluorescent microscope (Invitrogen EVOS Digital Colour Fluorescence Microscope; Thermo Fisher Scientific, ON, Canada), live cells fluoresce green and dead cells fluoresce red. Cell viability can then be calculated using software such as ImageJ (Schindelin et al., 2012) to output the number of live and dead cells in the gut tissue samples, as has been done in previous studies which assessed cell viability (MacMillan et al., 2015b; Andersen et al., 2017; Bayley et al., 2018).

Testing of this protocol was done on hindgut tissue from a male locust that was not exposed to the cold (Figure 2.5.1). However, due to insufficient locusts in the colony to carry out the experiment, the final data could not yet be collected. Additionally, more effort is needed to clearly distinguish individual cells and produce clearer images through the procedure.

2.6 Data analysis

CCRT and injury scores were analyzed in R Studio version 3.5.3 (R Core Team, 2019). The distribution and variance of residuals were assessed using Shapiro-Wilk tests and Q-Q plots, which supported the use of non-parametric tests. The effect of cold duration on CCRT was analyzed with a Kruskal-Wallis (KW) test followed by pairwise Wilcoxon tests with the Benjamini-Hochberg (BH) correction. Since the assumption of normality was not met, a generalized linear model (glm) with a Poisson distribution was used to test for the effect of cold duration and assessment day on injury scores. A KW test followed by pairwise Wilcoxon tests (with BH correction) were used to test for significant differences in injury scores on the first and fourth assessment days following the cold exposures. Again, because of the non-normal distribution of the data, a glm with a Poisson distribution was used to examine the effect of bacterial feeding on chilling injuries. Cold duration was held as a fixed categorical variable,

while assessment day was held as a continuous variable (in their respective analyses). Values presented on graphs are shown as mean $\pm$ standard deviation, with the α -level being 0.05 for all statistical tests. For the gut leak assay, no statistics were used to analyze bacterial growth on plates due to the lack of colonies.

Figure 2.3.1. Example growth of *GFPmut3 E. coli* on Ampicillin agar plates after homogenizing three gut regions following 24 h of feeding. Locusts were dissected after feeding on the *GFPmut3* bacteria-soaked wheat for 24 h. The **A)** foregut, **B)** midgut, and **C)** hindgut were excised, homogenized, and plated on Ampicillin plates to look for the growth of fluorescent bacterial colonies under a UV lamp (n = 6 locusts). Images were taken from one individual to represent presence of bacteria from all three gut regions.

Figure 2.5.1. Example LIVE/DEAD staining of anterior hindgut tissue from a male locust that underwent no cold stress (a trial experiment). After dissecting out the gut tissue of interest, SYBR 14 and propidium iodide solutions are added to stain live and dead cells, which fluoresce green and red, respectively. The tissue is then examined under a fluorescence microscope. Due to the “blending” of cells together and lack of clear discrimination between individual cells, the protocol is currently under refinement.

Section 3: Results

3.1 *Chill-coma recovery time and injury score*

Cold tolerance in locusts was quantified by measuring CCRT immediately following removal from the cold, as well as measuring injury 1, 2, 3, and 4 days post-cold exposure. As the length of cold exposures increased, CCRT significantly increased from approximately 19.3 mins after 12 h at -2°C to 80.4 mins after 48 h at -2°C (for those locusts that recovered within the 90 min cut off period; KW, $\chi^2 = 17.67$, $P < 0.001$). Longer cold exposures also resulted in fewer locusts recovering before the 90 min cut-off. Exposures at -2°C for 12 or 24 h yielded a 100% recovery rate, which decreased to 75% at 36 h and 37.5% at 48 h (Figure 3.1.1).

Chilling injuries were quantified using injury scores (as described in the Materials and Methods; Figure 3.1.2). Similar to CCRT, longer cold exposures led to higher degrees of injury (GLM, $F_{4,140} = 44.62$, $P < 0.001$). Over the four days following the cold stresses, an increase in the degree of injury was seen as well (GLM, $F_{3,140} = 4.30$, $P = 0.006$). However, this was only seen when including the 48 h cold exposure group in the statistical model. No significant interaction between the two variables of ‘injury score’ and ‘day of assessment’ was seen, meaning that differences in cold exposure durations did not influence rates of survival decline over the four day period (GLM, $F_{12,140} = 0.89$, $P = 0.561$). From these results, I expected that, if cold-induced bacterial leak across the gut was observed, it would be most pronounced in the 48 h cold-exposed group.

3.2 *Effect of bacterial feeding on chilling injuries*

After developing the bacterial feeding protocol, an injury assay was done to assess whether the ingested *E. coli* amplified or mitigated cold-induced chilling injuries after a 48 h

cold exposure (Figure 3.1.3). No significant difference in the degree of injury was found in bacteria-fed locusts (2.63 ± 1.69 , day 1; 0.75 ± 1.75 , day 4) compared to locusts that ate wheat without additional *E. coli* present (2.50 ± 1.77 , day 1; 0.50 ± 1.41 , day 4) (GLM, $F_{2,84} = 0.36$, $P = 0.702$).

3.3 Bacterial leak across the gut following cold exposures

Immediately following a cold exposure, hemolymph samples were taken from locusts and spread on LB agar plates with or without ampicillin to look for *GFPmut3* colony growth exclusively or total colony growth. After checking the plates for growth every day for four days, to my surprise, no bacterial colonies were seen on any plates (LB or ampicillin) regardless of cold exposure duration (Table 3.3.1).

In a follow-up experiment to examine bacterial leak following a period of rewarming, locusts were left to recover with food at benign temperature for 6 h following the cold exposure, after which hemolymph was collected and plated as before. Similarly, no bacterial colonies were observed on any plates regardless of cold exposure duration (Table 3.3.2).

Figure 3.1.1. The effect of cold stress duration on chill-coma recovery time (CCRT) in *L. migratoria*. After locusts were exposed to -2°C for 12, 24, 36, or 48 h (n = 8 in each group), they were observed for 90 mins and were marked as having recovered when they were able to stand on all six legs. Locusts that were not able to stand after 90 mins were marked as “dead”. Values above the dashed black line represent the number of locusts in each cold exposure group that recovered before 90 min. Groups sharing the same letter are not significantly different. Solid black points represent mean values $\pm$ s.d for each treatment group, while open black points represent the CCRT of each individual locust tested.

Figure 3.1.2. The effect of cold stress duration on chilling injuries in *L. migratoria*. After locusts were chilled for 12, 24, 36, or 48 h (n = 8 in each group), 24 h post-treatment, a 5-point survival scale was used to assess the extent of chilling injuries. Scores were defined as follows: 0: no movement observed (dead); 1: limb movement (leg and or head twitching); 2: greater limb movement (leg, head, and whole body twitching), 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 similar to pre-exposure levels of coordination. The survival assessment was then repeated three additional times; 48, 72, and 96 h post-treatment. Bar plots show mean injury scores **A**) one day and **B**) four days after cold stress ($P < 0.001$ for both plots). Groups sharing the same letter are not significantly different. Open data points represent individual locusts. **C**) Plot showing the decline in survival over the four day period for each group. Data points shown represent mean values. Error bars in all plots represent s.d.

Figure 3.1.3. Bacterial feeding does not amplify or mitigate chilling injuries in *L. migratoria*. Over a 24 h period, locusts (n = 8 in both groups) were fed wheat that was soaked in either a concentrated solution of *GFPmut3 E. coli* or distilled water (control; CTL). Locusts were then exposed to -2°C for 48 h. An injury assay was then performed over a period of four days following the cold exposure to assess chilling injuries. Data points represent mean values. Error bars represent s.d.

Table 3.3.1. Bacterial colony counts after plating locust hemolymph immediately following cold exposures. After feeding fluorescent bacteria to locusts and exposing them to -2°C for 12, 24, 36, or 48 h, a sample of hemolymph was immediately extracted and plated on both LB and Ampicillin plates to test for *GFPmut3 E. coli* leak from the gut. Male and female locusts in each group were used in a 1:1 ratio.

Cold duration (h)	Sample size (locusts)	LB total colony count	Ampicillin total colony count
Control	6	0	0
12	6	0	0
24	6	0	0
36	6	0	0
48	10	0	0

Table 3.3.2. Bacterial colony counts after plating locust hemolymph 6 h following cold exposures. After feeding fluorescent bacteria to locusts and exposing them to -2°C for 12, 24, 36, or 48 h, locusts were allowed to recover at benign temperature for 6 h before hemolymph was extracted and plated on both LB and Ampicillin plates. Male and female locusts in each group were used in a 1:1 ratio.

Cold duration (h)	Sample size (locusts)	LB total colony count	Ampicillin total colony count
Control	6	0	0
12	6	0	0
24	6	0	0
36	6	0	0
48	6	0	0

Section 4: Discussion

4.1 General Discussion

A number of recent studies have reported immune activation, characterized by cellular or humoral immune responses or upregulation of immune genes, following cold stresses. With the current knowledge regarding gut leak and tissue injury in the cold, I hypothesized that prolonged cold exposures would lead to bacterial leak from the gut lumen of *L. migratoria* into the hemocoel.

First, I exposed locusts to increasing durations of low temperature (-2°C) and quantified their injury scores over four days following the cold stress. Longer cold stresses resulted in higher degrees of injury, which agrees with previous work done in locusts (Andersen et al., 2017; Brzezinski and MacMillan, 2020). Latent chilling injuries manifested in locusts exposed to -2°C for 48 h, quantified by increasing injury scores over a three day period after the first survival assessment. A similar pattern was seen in *D. melanogaster* exposed to a relatively long cold stress (25 h) at 0°C ; higher injury scores (and more severe chilling injuries) one day after the cold stress led to significantly more deaths the following three days after removal from the cold (El-Saadi et al., 2020). Chilling injuries in *D. melanogaster* can manifest as damage to the midgut epithelia (MacMillan et al., 2017); chilling injuries in locusts can manifest as damage to muscle fibers (specifically the mesothoracic posterior tergocoxal muscle, muscle M90; Snodgrass, 1929) and the hindgut (Bayley et al., 2018; MacMillan et al., 2015b). Cellular viability in the muscle tissue of locusts remained significantly low following a 24 h rewarming period after a cold stress (MacMillan et al., 2015b). These results from *D. melanogaster* and *L. migratoria* suggest that chilling injuries do not fully heal, and may even continue to worsen in the days following removing the insect from a severe cold stress.

Chilling, in insects, leads to the leak of solutes across the gut epithelia (MacMillan and Sinclair, 2011b; MacMillan et al., 2017; Yerushalmi et al., 2018). If damage is occurring to the gut tissue following severe chilling, it is possible larger gut contents (i.e: bacteria or their cell walls) may also leak into the hemolymph and into other tissues in the body, leading to inflammation (Brandt et al., 2004). One way in which the insect immune system eliminates pathogenic matter is via the production of cytotoxic compounds (Nappi and Vass, 1993). However, this response also has the capacity to harm organs that are near the site of infection. This was demonstrated in beetle larvae (*Tenebrio molitor*), where melanization in response to a foreign entity (nylon) in the insect body resulted in collateral damage to the Malpighian tubules near the site of the immune attack (Sadd and Siva-Jothy, 2006). Thus, if bacterial leak across the gut epithelia occurs after a cold stress, the subsequent immune response to clear the hemolymph of infection may injure nearby tissues (like the Malpighian tubules) in the process. As the tubules play an important part in regulating ion and water homeostasis, especially following a cold stress, this would provide a possible explanation for the worsening injuries observed in *D. melanogaster* and *L. migratoria* after chilling. From the results of my experiments, bacterial leak following cold exposures is unlikely to occur, but the leakage of fragments of bacterial cell walls remains a possible explanation for cold-induced immune activation in insects.

To test for movement of bacteria from the gut to the hemolymph, I fed locusts a fluorescent strain of *E. coli* before exposing them to the cold. Contrary to my expectation, there was a complete absence of bacteria in the hemolymph, both immediately following a cold stress and following a warm recovery period. As the ability of the immune system to clear bacteria from the hemolymph is significantly reduced at low temperatures (Ferguson and Sinclair, 2017), and hemolymph was plated immediately after the cold stress in one of my experiments, it is

unlikely that the absence of bacteria shown from my results were a result of immune-related bacterial clearance. In this case, there are two scenarios that are likely: 1) gut barriers maintain their integrity despite damage to the gut epithelia, or 2) gut barriers remain undamaged by the cold. It has previously been shown that gut tissue becomes damaged following low temperature exposure, but septate junctions (paracellular barriers) also exhibit increased leakiness during and after a cold spell (Brzezinski and MacMillan, 2020; MacMillan et al., 2017). However, the size of *E. coli* ($\sim 1 \times 3 \mu\text{M}$; Reshes et al., 2008) may prevent it from crossing the septate junctions, even when they are compromised, since intercellular spaces are only approximately 20 nm wide when healthy (Jonusaite et al., 2016). Thus, it is much more likely that although gut tissue damage occurs following a cold stress, sufficient paracellular barrier integrity is maintained, and this serves to prevent bacterial leakage in the cold.

Data from Katzenberger et al. (2015) showed that traumatic brain injury in *D. melanogaster* induces septate junction failure, which in turn led to the leak of gut bacteria into the hemolymph and triggered an immune response. In the same study, genome-wide association analysis uncovered single-nucleotide polymorphisms in genes associated with post-traumatic injury (*grh*, *bbg*, and *scrib*). These genes play an important role in the formation and integrity of gut epithelial barriers (Bilder and Perrimon, 2000; Bonnay et al., 2013; Narasimha et al., 2008), and variations in the nucleotide sequences of these genes were linked to differences in mortality rates following injury in separate isogenic fly lines. This provides evidence for bacterial leak across the gut as a result of septate junction failure after a traumatic brain injury. It is currently unclear if any changes in the expression of these genes occur following a cold or mechanical stress. Nonetheless, these results suggest that the underlying physiology of gut barrier failure during a cold stress differs from that following mechanical trauma.

If cold stress does not result in bacterial leak from the gut, what mechanism is responsible for cold-induced immune activation? Since the relatively large size of bacteria ($\sim 1 \times 3 \mu\text{M}$ for *E. coli*, for example) may prevent them from bypassing the gut barriers, peptidoglycan from the cell wall may leak past the gut epithelia instead, as previously mentioned. The midgut of *D. melanogaster* contains lysozymes (Daffre et al., 1994), enzymes that cleave long peptidoglycan chains and break them up into smaller units (Callewaert and Michiels, 2010). The cell walls of gram-negative bacteria are thinner (1.5 – 15 nm; Vollmer et al., 2008) than those of gram-positive bacteria (20 – 80 nm; Reith and Mayer, 2011). *Acetobacter* and *Lactobacillus*, gram-negative and gram-positive bacteria, respectively, dominate the natural gut microbiome of adult *D. melanogaster* (Wong et al., 2011). It is possible that degradation of the relatively smaller cell walls of gram-negative bacteria in the gut via lysozymes can allow peptidoglycan to leak through the compromised septate junctions ($\sim 20 \text{ nm}$) and into the hemolymph. This may explain the increased expression of genes such as *attacin* and *diptericin* (Imd immune pathway), and *metchnikowin* (Toll and Imd pathway) (Salehipour-shirazi et al., 2017; Štětina et al., 2019) in insects following a cold stress, as the Imd immune pathway can respond to polymeric or monomeric peptidoglycan in gram-negative bacterial cell walls (Kaneko et al., 2004; Kleino and Silverman, 2014). If this is the case, then the latent chilling injuries following a cold stress may be a result of collateral damage to muscle or nerve tissues, or even organs, such as the Malpighian tubules, due to a melanization cascade response to peptidoglycan in the hemolymph (Kurata, 2010; Wang et al., 2019). Further research is needed to confirm whether cold stress leads to the leak of peptidoglycan across the gut barriers. This could be done by directly quantifying peptidoglycan in hemolymph samples after a cold exposure using an enzyme-linked immunosorbent assay (ELISA), or by measuring lysozyme activity from the hemolymph as an

indicator of the insect's humoral immune response to peptidoglycan (Ferguson et al., 2016; Flyg et al., 1987).

It is also entirely possible that immune activation after a cold stress may be entirely unrelated to bacterial leak, whole or partial. Inflammatory responses in the insect body can be triggered due to cellular injury, physical wounding, or starvation, all of which can be unrelated to bacteria (Becker et al., 2010; Márkus et al., 2005; Ming et al., 2014). In *D. melanogaster*, necrotic neuronal cells release reactive oxygen species that activate the Jun N-terminal kinase (JNK) pathway (Yang et al., 2013). This pathway triggers apoptosis in neighbouring cells and recruits hemocytes to the site of wounding where phagocytosis occurs to clear the area of debris (Marmaras and Lampropoulou, 2009; Shaukat et al., 2015). This may explain the increase in the number of circulating hemocytes following tissue damage or a low temperature exposure in *D. melanogaster* (Pastor-Pareja et al., 2008; Salehipour-shirazi et al., 2017), as cell death is a common consequence of cold stress in insects, including locusts, and has been measured repeatedly (Andersen et al., 2017; Bayley et al., 2018; MacMillan et al., 2015b; MacMillan et al., 2017; Carrington et al., 2020). Furthermore, immune activation occurs in *D. melanogaster* upon the detection of actin in the hemolymph. Actin is a protein constrained to cells where it acts to provide structural support, but is released into the hemolymph following cellular injury (Srinivasan et al., 2016). Thus, immune activation following a cold exposure may be a result of endogenous factors arising from cold-induced cell death that are unrelated to bacterial leak from the gut.

Although my experiments have shown that bacterial leak does not follow a severe cold stress, there is no indication as to whether gut tissue integrity is maintained. The mesothoracic posterior tergocoxal muscle in locusts is commonly used to assess muscle injury following cold

stresses (Andersen et al., 2017; Carrington et al., 2020; MacMillan et al., 2015b), yet only two studies to date have directly assessed the health of insect gut tissue following chilling (Bayley et al., 2018; MacMillan et al., 2017). These studies provide evidence of damage to the gut epithelia of *D. melanogaster* (particularly the midgut) and *L. migratoria* (hindgut) following a severe cold stress. In order to determine if gut epithelial injury occurred despite the lack of bacterial leak, I plan to quantify cell death in the foregut, midgut, and hindgut of *L. migratoria* following cold stress, using a LIVE/DEAD cell viability assay (Figure 3). This will help establish a clearer relationship between gut tissue viability and barrier integrity following a cold exposure.

With regards to the experimental design, one improvement would be to expose all locusts to their respective treatments at once, rather than in two separate periods, in order to further control for any effects of time or age on cold tolerance. Another improvement would be to randomly remove locusts from the bath at the 12, 24, 36, and 48 h marks rather than randomly assigning individuals to groups beforehand.

To provide further support to the study, one improvement would be to homogenize locust gut segments following cold exposures and plate their contents to better track the location of the fluorescent *E. coli*. A follow-up experiment can be done to investigate the effects of traumatic brain injury on locust survival and bacterial leak of the fluorescent marker. This would allow me to compare how different insect species respond to mechanical stress and whether gut leak of bacteria may arise as a result of a shared consequence of septate junction dysfunction after two forms of abiotic stress.

4.2 Conclusions

Due to increases in global mean winter temperatures, the northward expansion of some insect species has been observed (Breed et al., 2012; Parmesan and Yohe, 2003; Parmesan et al., 1999; Wilson et al., 2021). While thermal plasticity is important in the context of global climate change, overwintering success, which can be negatively affected by severely low temperatures, can also play a role in determining the northern range limits of insects (Breed et al., 2012; Lynch et al., 2014; MacQuarrie et al., 2019). As extreme low temperature events increase in frequency and winters become more variable (Easterling et al., 2000; Overland and Wang, 2016), knowing how insects are affected by low temperatures remains an important field of study in ecology.

Several research groups are actively examining how insects are physiologically affected by low temperatures, yet relatively little attention is paid to the role of host-microbe interactions in the causes and consequences of chilling injury. I hypothesized there was a direct role of gut bacterial leak in cold-induced immune activation and tested for the presence of bacteria in the hemolymph following cold stresses. Although no bacterial leak from the gut of *L. migratoria* was observed following a cold stress, my findings are useful in leading us to other testable hypotheses. One possible avenue worth exploring would be the leak of peptidoglycan across the gut to attempt to establish a relationship between gut bacteria and immune activation in insects. Future work may also investigate molecular signals involved in sterile inflammation as a result of immune signalling following necrotic or apoptotic cell death following cold exposures, an understudied and relatively poorly understood field as of yet.

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