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## Thermal Biology of Insect Immunity and Host-Microbe Interactions

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

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## Abstract

The influence of temperature on interactions with pathogenic or symbiotic microbes is a driving force behind the survival of insects under climate change. However, we know little of how insects physiologically respond to these pressures. In temperate climates, winter dominates the thermal landscape; thus, I am particularly interested in how cold interacts with insect responses to microbes. Here I explore the thermal biology of the insect immune system and the impacts of cold on host-microbe interactions. First, I demonstrate that acute exposure to cold activates selective components of immunity in *Drosophila melanogaster*, as a compensatory response to trade-offs or injury. Next, I show that cold acclimation decreases immune function at low temperatures in *Gryllus veletis* at the same time that cold tolerance increases. I conclude that this is a trade-off between immunity and the response to cold. Third, I demonstrate that immune activity varies seasonally in insects, but that each species responds differently. These shifts were likely driven by species-specific responses to multiple overwintering pressures. Fourth, I demonstrate that thermal plasticity in both *Gryllus veletis* and the fungal pathogen *Metarhizium brunneum* contribute to the outcome of infection. Further, fluctuating temperatures produce different outcomes of infection than constant temperatures, but we can predict these outcomes based on additive thermal performance under constant conditions. Lastly, I observe that the composition of the hindgut microbiome in *Gryllus veletis*, containing both beneficial and pathogenic microbes, shifts irreversibly across seasons. Further, microbial shifts coincide with changes in both cold tolerance and immune activity, which indicate that there is a functional relationship between the microbiome and host survival of low temperatures. Overall, changes in temperature are inextricably linked to changes in insect responses to both pathogenic and symbiotic microbes, which has likely selected for an adaptive physiological connection between insect immunity and the response to cold. I demonstrate that the connection between physiological responses to abiotic and biotic pressures modify our interpretation of phenotype. Therefore, we cannot rely on a univariate and species-isolated understanding of how insects respond to temperature if we are to predict the impact of climate change on their fitness.

**Keywords** Insect, pathogen, winter, thermal variation, season, phenotypic plasticity, trade-offs, microbiome, infection

## Co-authorship statement

Chapter 2 (modified for format) is published in the *Journal of Insect Physiology* with Golnaz Salehipour-shirazi and Dr. Brent Sinclair as co-authors. Golnaz contributed significantly to the experimental design and conducted the haemocyte counts, qPCR, and melanisation assays. I share first authorship with Golnaz on this manuscript as her contributions to the experiments were the basis of her Master's thesis. I contributed to experimental design, conducted the experiments on phenoloxidase activity, bacterial clearance, and survival of fungal infection. I contributed to the analysis and synthesis of the data and discussion, and wrote the bulk of the manuscript. Dr. Brent Sinclair contributed significantly to the experimental design, ideas, and discussion of this chapter, and provided extensive editorial input on the manuscript.

Chapter 3 (modified for format) is published in the journal *Oecologia* with Dr. David Heinrichs and Dr. Brent Sinclair as co-authors. Dr. Heinrichs contributed to the experimental design and provided methodological expertise. Dr. Sinclair contributed significantly to the experimental design, ideas, and discussion of this chapter, and provided extensive editorial input on the manuscript. I contributed to experimental design, performed the experiments, analysed the data, and wrote the bulk of the manuscript.

Chapter 4 (modified for format) is in press in the *Journal of Experimental Zoology-A* with Dr. Brent Sinclair as co-author. Dr. Sinclair contributed significantly to the experimental design, ideas, and discussion of this chapter, and provided extensive editorial input on the manuscript. I contributed to experimental design, performed the experiments, analysed the data, and wrote the bulk of the the manuscript.

Chapter 5 is in preparation for submission to *American Naturalist* with Dr. Brent Sinclair as a co-author. Dr. Sinclair contributed significantly to the experimental design, ideas, and discussion of this chapter. I contributed to experimental design, performed the experiments, analysed the data, and performed the bulk of the writing of the manuscript.

Chapter 6 is in preparation for submission to *Proceedings of the Royal Society B* with Pranav Dhakal, Dr. Carol Bucking, and Dr. Brent Sinclair as co-authors. Mr. Dhakal conducted the analysis of the microbial sequencing data. Dr. Bucking contributed to the experimental design and provided both expertise and equipment. Dr. Sinclair contributed significantly to the experimental design, ideas, and discussion of this chapter. I contributed to experimental design, performed the experiments, and wrote the bulk of the manuscript.

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*For Madeline – whose smiles have a curious way of curing sleep deprivation*

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## List of Abbreviations

- AMP: Antimicrobial peptide
- ANOVA: Analysis of variance
- BCA: Bicinchoninic acid
- CA: Cold acclimated
- cDNA: Complementary deoxyribonucleic acid
- CCR: Chill coma recovery
- CFU: Colony-forming units
- CHC: Circulating haemocyte concentration
- CT: Constant temperature
- CT<sub>max</sub>: Critical thermal maximum
- CT<sub>min</sub>: Critical thermal minimum
- DF: Degrees of freedom
- DNA: Deoxyribonucleic acid
- EDTA: Ethylenediaminetetracetic acid
- FM: Field microcosm
- FT: Fluctuating temperature
- FTR: Fluctuating thermal regime
- FTRA: Fluctuating-thermal-regime acclimated
- FTR1A: Fluctuating-thermal-regime one acclimated
- gDNA: genomic deoxyribonucleic acid
- GV: Grey value
- HSD: Honestly significant difference

LB: Lysogeny broth

L-DOPA: L-3,4-dihydroxyphenylalanine

LM: Lab microcosm

LPS: lipopolysaccharide

mRNA: Messenger ribonucleic acid

MID: Multiple identifier

OTU: Operational taxonomic unit

PAMPs: pathogen-associated molecular patterns

PBS: phosphate-buffered saline

PC: Post cold exposure

PI: Post-infection

PCoA: Principal Coordinates Analysis

PCR: Polymerase chain reaction

PO: Phenoloxidase

ProPO: Prophenoloxidase

PRR: pathogen-recognition receptors

RNA: Ribonucleic acid

rRNA: Ribosomal ribonucleic acid

TPC: Thermal performance curve

T<sub>opt</sub>: Thermal optimum

SCP: Supercooling point

UPGMA: Unweighted pair group method with arithmetic mean

SEM: Standard error of the mean

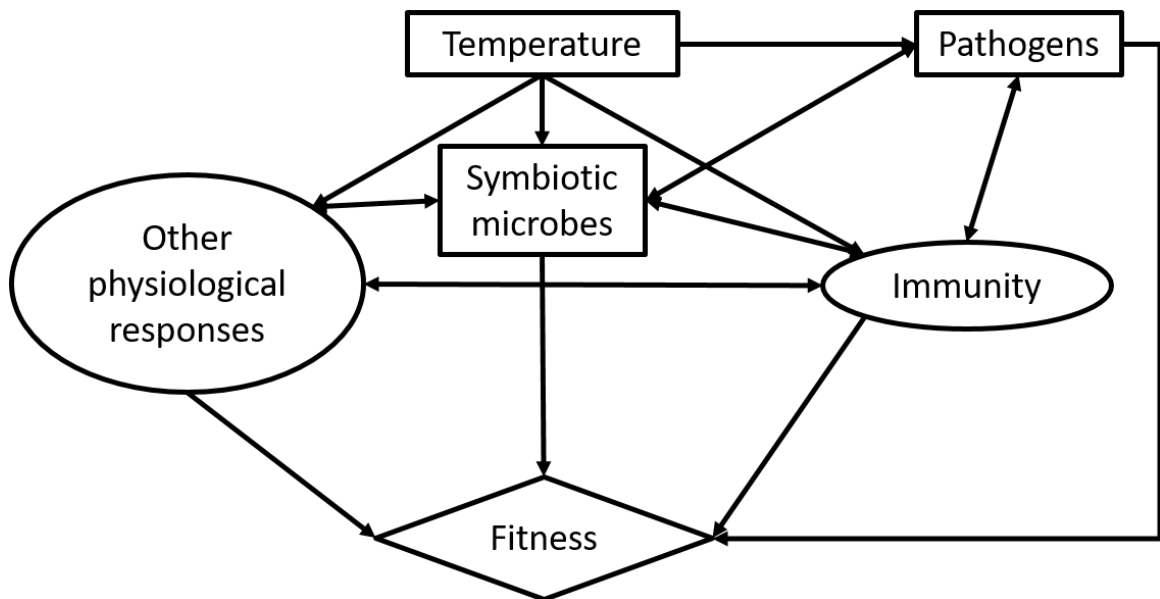
WA: Warm acclimated

# Chapter 1

## 1 Introduction

As a result of a changing climate, animal survival is currently challenged by simultaneous shifts in multiple abiotic and biotic environmental pressures (Kaunisto et al., 2016). Ectotherms, who cannot defend their internal body temperatures, are particularly vulnerable to changes in temperature associated with climate change (Deutsch et al., 2008). Temperature will directly affect the physiology of ectotherms, and also modify their experience of other environmental pressures (Deutsch et al., 2008; Harvell et al., 2002; Pachauri and Meyer, 2014). For example, the global decline of amphibian species is linked to the combination of temperature-driven increases in pathogen growth with temperature-driven declines in amphibian immunity (Raffel et al., 2012; Rohr and Palmer, 2013; Rohr and Raffel, 2010). Further, temperature-driven disruptions in communities of symbiotic microbes can modify ectotherm physiology and their ability to survive under climate change (Weis, 2008). Thus, ectotherm success depends on their ability to cope with integrated changes in temperature and microbial pressures (Fig. 1.1). However, we currently understand very little of how insects respond to multiple environmental pressures, and have particularly ignored the influence of biotic pressures such as pathogens (Kaunisto et al., 2016).

In some cases, insects have evolved mechanisms that link the responses to integrated environmental pressures (Sinclair et al., 2013). In cross-talk responses, a shared signaling pathway leads to distinct physiological responses. In cross-tolerance responses, one physiological response may protect the organism against distinct pressures (Sinclair et al., 2013). In both cases, the animal need only experience one pressure (e.g. temperature) to initiate protection against one or more pressures (e.g. pathogens). For example, cold exposure can increase insect immune activity, independent of infection. This physiological response may represent cross-talk between the response to cold and the response to pathogens associated with winter (Sinclair et al., 2013). Thus, changes in immunity following cold exposure provide an opportunity to explore the physiology underlying the response to integrated abiotic and biotic pressures in insects.



**Figure 1.1 A simplified schematic of the interconnected nature of environmental pressures: temperature and microbes.** Temperature may directly affect pathogen growth or virulence, while simultaneously affecting host physiology that is both directly (e.g. immunity) and indirectly (e.g. influence of other physiological activity on immunity) related to the defense against pathogens. As temperature changes, physiological activities will either be directly affected by temperature (e.g. thermal sensitivity, damage) or plastic, thereby changing their response to the environment, their relationship with connected physiological systems, and, ultimately, their fitness.

I approach this thesis with the overarching hypothesis that insects adjust their physiology in response to environmental temperature to cope with both the change in temperature as well as concomitant changes in both pathogenic and symbiotic microbial pressures. I explore this hypothesis by examining temperature-driven changes in the insect immune system. I investigate why cold might activate the immune system, how thermal and seasonal acclimation affect immune activity, and how both the host and pathogen respond to the thermal environment to determine the outcome of their interaction. In particular, I focus on thermal plasticity in host immunity. Finally, I explore changes in the insect gut microbiome in relation to season and temperature to begin to understand how the microbiome might influence overwintering immune activity.

## 1.2 The importance of insects and their thermal environments

Insects are the most diverse and successful group of terrestrial animals, with myriad positive and negative impacts on ecosystem and economy. For example, Losey and Vaughan (2006) estimate that insect ecological services – even before including their impacts on agriculture and health – are worth \$57 billion annually in the United States. Insects provide numerous ecosystem services – in particular: decomposition, food sources, biocontrol, herbivory, dispersal, and pollination. Further, many act as medically-important vectors of disease of both humans and wildlife (Beatty and Marquardt, 1996). Thus, due to their ubiquity and importance in maintaining ecosystem function, it is essential to understand how insects interact with the multiple pressures associated with their thermal environments. We can then use our understanding of their physiology to predict how changes in the thermal environment will influence their distribution, abundance, and ecosystem function.

### 1.2.1 Multiple challenges associated with overwintering

Insects in temperate, polar, and alpine regions can spend more than half of their lives experiencing and responding to multiple environmental stressors associated with overwintering (Williams et al., 2015). Temperature is the main driver of overwintering

stress, as it determines the rate of biological processes, whether or not an animal will cross physiological thresholds (e.g. injury), and influences the occurrence and severity of other environmental stressors (Williams et al., 2015). In particular, temperature will drive the availability of water and resources in the winter, the rate of energy use, and the incidence of, or susceptibility to, pathogen and predation stress (Williams et al., 2015). Thus, temperature creates interactions between multiple, overwintering environmental pressures – such as resource availability, pathogens, and energy use. Insects must thus respond to these interacting pressures simultaneously to survive.

As ectotherms, insects are unable to defend their internal body temperature from changes in the external environment. Therefore, low temperatures slow the rate of biochemical reactions, and physiological activities, such as growth and reproduction, slow or cease in the cold (Tattersall et al., 2012). Further, at both above- and sub-zero temperatures, ectotherms may experience injury associated with exposure to cold (Tattersall et al., 2012). Chilling injury, which is injury unrelated to freezing, can occur above the freezing point and is categorized into both acute and chronic injury. Acute injury occurs on the scale of minutes to hours and includes membrane phase transitions (Drobnis et al., 1993) and apoptosis (Yi and Lee, 2004). Chronic injury occurs over days to weeks and is largely characterised by a disruption of ion and water balance (Košťál, 2004; MacMillan, 2013). Low temperatures can also initiate protein misfolding and denaturation, leading to loss of function (Tattersall et al., 2012).

When temperatures are low enough to promote the formation of ice, insects risk tissue damage and death from both intracellular and extracellular ice formation. In large part, injury from freezing is likely related to the osmotic dehydration of cells and concentration of solutes during freezing (Lee, 1989). As such, freezing stress is closely related to desiccation stress that accompanies a lack of biologically available water during the winter. Indeed, there appears to be cross-tolerance in the mechanisms that underlie cold- and desiccation-tolerance (Sinclair et al., 2013). Further, freezing injury may also arise from mechanical damage of membranes and macromolecules by recrystallisation (Knight and Duman, 1986). Both cold- and freezing-induced damage to tissues such as the gut may also leave the insect vulnerable to infection by microbes that live within the gut and

are normally blocked from entering the body cavity of the host (MacMillan and Sinclair, 2011; Marshall and Sinclair, 2011; Sinclair et al., 2013).

During overwintering, most insects are unable to feed. To fuel their basal metabolism and physiological responses to stress through the winter and into spring, insects must rely on energy stores accumulated during the previous growing season (Hahn and Denlinger, 2007). Because temperature will directly affect metabolic rate in insects – even when metabolism is suppressed, as is common during winter dormancy (Hahn and Denlinger, 2007) – warmer or colder winters will either increase or decrease energy use throughout the winter, respectively. These changes in winter temperature will thereby affect available energy stores and overwintering success (Irwin and Lee, 2003; Williams et al., 2015; Williams et al., 2012).

Although winter is often considered a time of “suppression” (of metabolism, reproduction, feeding, and activity), some organisms remain active at low temperatures – including pathogens, which can threaten the survival of overwintering insects. For example, various strains of the fungal entomopathogens *Beauveria bassiana* and *Metarhizium* sp. are cold active, found in the overwintering sites of insects, and lead to overwintering mortality (Bidochka et al., 1998; personal observation; Mills, 1981; Steenberg et al., 1995). Further, entomopathogenic fungi such as *Beauveria bassiana* may require hosts to survive the winter, as it does not survive well for prolonged periods of time in the soil (Bidochka et al., 1998); thus, insects may be likely to be infected throughout the overwintering periods. Some pathogens are low-temperature specialists, such as the bacteria *Yersinia enterocolitica* which only becomes insecticidal at low temperatures (Bresolin et al., 2006).

The symbiotic microbes associated with insects and other ectotherms will experience the same shifts in temperature as their host. Seasonal changes in temperature may then influence their community composition and functionality (Carr et al., 1976). Because the physiology of ectotherms is a product of their interaction with their symbiotic microbes (Douglas, 2015), changes in their community composition and function will then affect host survival. For example, hibernation in frogs disrupts the microbial community of the



gut and selects for opportunistic pathogens, leading to increased overwintering mortality (Carr et al., 1976). Further, physiological changes in the host that accompany the response to changes in season may also influence the microbial community. For example, new physiochemical conditions in the gut as a result of fasting during hibernation selects for a shift in the bacterial community of heterothermic ground squirrels (Carey et al., 2013). In insects, temperature and season are likely to elicit shifts in the community composition of the gut microbiome. However, we currently know little to nothing about how the thermal environment shapes the microbial communities associated with insects, or how shifts in these communities influence physiology and fitness.

### 1.2.2 The response to low temperatures and related overwintering challenges

The ability to respond to overwintering challenges is often plastic, such that insects acquire tolerance to cold and other stressors through seasonal acclimatisation (Sinclair et al., 2015). Seasonal acclimatisation is triggered by a – sometimes complex – array of cues that elicit phenotypic changes in insects; the most common cues are changes in temperature and photoperiod (Košťál, 2006; Sinclair et al., 2015). These “real world” conditions can be difficult to reproduce in the laboratory; however, short-term acclimation (days to weeks) and hardening (hours) can be used to elicit plasticity and study the physiology underlying responses to low temperature and other overwintering pressures (Sinclair et al., 2015).

In terms of their ability to withstand low temperatures and freezing, insects can be broadly categorized as chill-susceptible, freeze-avoidant, or freeze-tolerant. Chill-susceptible insects will die from chilling injury unrelated to freezing and thus must avoid or protect themselves from low-temperature exposure; freeze-avoidant insects will die upon freezing and thus must maintain body temperatures above their freezing point; finally, freeze-tolerant insects are able to tolerate internal ice formation and must instead control the formation of ice within the body and the freeze-thaw transition (Lee, 1989). Insects may also respond behaviorally to low temperatures, and choose microhabitats that allow them to maintain their body temperatures above critical, lethal, points.

In response to, or in preparation for, exposure to low temperatures, insects may perform physiological shifts to protect themselves from injury and death associated directly or indirectly with exposure to low temperatures (Lee, 1989). One of the hallmarks of cold-tolerance is the ability to maintain ion and water homeostasis in the cold, through mechanisms that are currently under investigation (MacMillan et al., 2015; MacMillan and Sinclair, 2011; MacMillan et al., 2012). To stabilise membranes and maintain fluidity at low temperatures, insects may modify membrane fatty acid composition (Košťál et al., 2003), and accumulate cryoprotectants, such as polyols. Cryoprotectants (among other molecules, such as heat shock proteins) also act to protect proteins and macromolecules from misfolding and denaturation associated with both low temperatures and desiccation (Lee, 1989; Sinclair et al., 2013). Moreover, cryoprotectants may act colligatively to depress the supercooling point (freezing point), thereby protecting freeze-avoidant insects from reaching the freezing point of their body fluids (Lee, 1989; Tattersall et al., 2012).

Freeze-avoidant insects may also directly alter the community composition of their microbiomes to increase cold tolerance. Particular species of bacteria in the gut can act as ice-nucleating agents (e.g. *Pseudomonas* spp.) and, if left unchecked, will catalyse the lethal formation of ice within the insect (Lee and Costanzo, 1998). In response, some insects actively void or mask ice-nucleating bacteria from the gut (Olsen and Duman, 1997). Because the immune system is responsible, in part, for controlling the composition of the gut microbiome (Douglas, 2015), it could play a role in the control of ice-nucleating bacteria. However, the mechanism underlying the ability to control the presence or activity of ice-nucleating bacteria is unclear (Olsen and Duman, 1997; Worland et al., 2000).

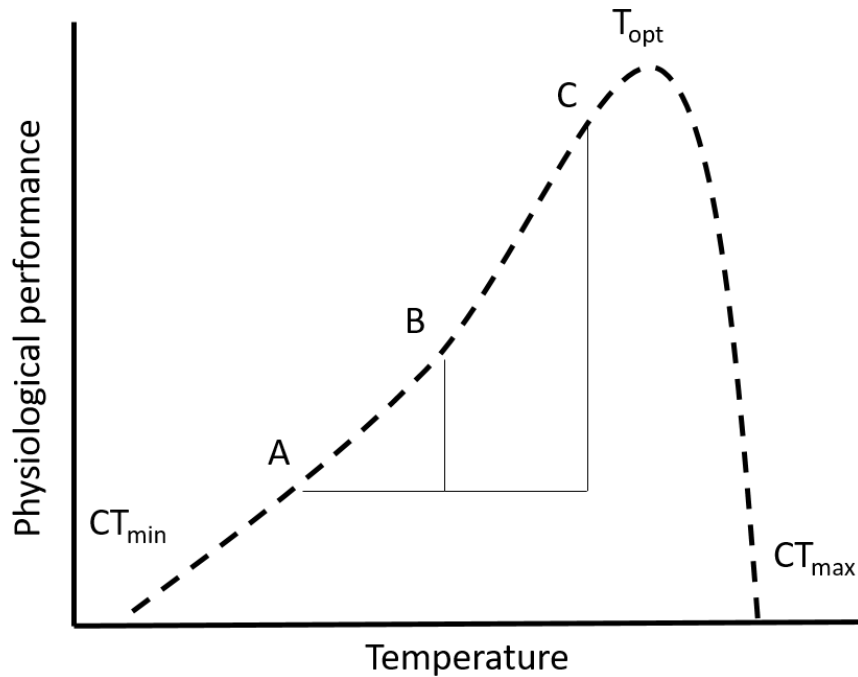
To conserve energy over the winter, many insects enter diapause or states of dormancy and quiescence, during which they suppress their activity and basal metabolism. This allows them to mitigate the starvation pressures associated with a lack of food availability in the winter (Hahn and Denlinger, 2007). Diapause is also characterised by simultaneous increases in resistance to cold and related stressors, such as desiccation (Rinehart et al., 2006), and possibly pathogens (Ragland et al., 2010).

Although we know that parasites and pathogens remain active at low temperatures and can be a source of overwintering mortality (Mills, 1981; Steinmann et al., 2015), we know little of how insects respond to pathogen stresses during winter. The immune system remains functional at low temperatures during diapause (Nakamura et al., 2011), and activity may be up-regulated to combat pathogen stress during dormancy (Ragland et al., 2010). For example, *Drosophila* sp. and *Allonemobius socius* are more likely to survive infection when they are acclimated to winter conditions (Fedorka et al., 2013; Kutch et al., 2014; Toxopeus et al., 2016), diapausing flesh flies, *Sarcophaga crassipalpis*, up-regulate the expression of genes coding for immune peptides (Ragland et al., 2010), and insects with stronger immune responses are more likely to survive the winter (Caron and Myers, 2008; Krams et al., 2011a).

Conversely, honeybees, *Apis mellifera* down-regulate immune activity and are more susceptible to viral infection (Steinmann et al., 2015) and damselflies (*Hetaerina americana*) have reduced resistance to bacterial pathogens (Córdoba-Aguilar et al., 2009) during the winter. Immune activity is costly (Ardia et al., 2012; Freitak et al., 2003), and decreased immune activity may manifest from an energetic conflict between immunity and overwintering energy conservation (Vesterlund et al., 2014). Similarly, decreased activity may result from a trade-off between immunity and other physiological responses, such as the response to cold (Linderman et al., 2012). Overall, modifications to immune activity during the winter are likely to be part of the response to multiple pressures associated with overwintering in insects. These changes in immune activity will then affect overwintering success both as a direct response to pathogens, and indirectly through the relationship of immunity to the rest of the physiology of the insect (Fig. 1.2).

### 1.2.3 The importance of temperature variability

Ectotherm performance responds non-linearly to temperature, such that performance accelerates with increasing temperature toward an inflection point, then switches to a decelerating relationship under which performance rapidly declines beyond an optimum (Fig. 1.2; Sinclair et al., 2016). Most terrestrial ectotherms will experience thermally variable environments and their physiological performance will fluctuate asymmetrically



**Figure 1.2 Typical thermal performance curve.** An example of the non-linear relationship of ectotherm performance and temperature. The curve is bounded by the critical thermal maxima ( $CT_{max}$ ) and minimum ( $CT_{min}$ ). The curve accelerates in the concave section towards an optimum ( $T_{opt}$ ), after which it quickly decelerates in the convex section. Due to this non-linear relationship, a change in temperature of the same magnitude (e.g. A to B, or B to C) results in changes in performance of differing magnitudes (a property known as Jensen's inequality).

with changes in temperature (Colinet et al., 2015). Due to the non-linearity and accelerating/decelerating nature of thermal performance, a change in temperature of the same magnitude will disproportionately affect performance depending on where along the thermal performance curve this change in temperature falls (i.e. Jensen's inequality, Fig. 1.2). Thus, only considering instantaneous performance at constant temperatures will provide a radically different estimate of the effects of temperature on performance than including fluctuating temperatures (Colinet et al., 2015).

The differential responses by insects to fluctuating temperatures (FTs) versus constant temperatures (CTs), will also depend on whether or not fluctuations occur between permissive temperatures, or cross physiological thresholds leading to stress (Colinet et al., 2015; Marshall and Sinclair, 2012). For example, fluctuations within permissive temperatures can accelerate development (Fischer et al., 2011), whereas fluctuations to temperatures that cause injury are deleterious to development (Garcia-Ruiz et al., 2011). Further, fluctuating temperatures may also act as a prophylactic signal for changes in the environment. For example, FTs trigger increased expression of genes coding for immune peptides, independent of infection, which suggests that FTs act as signal for changes in pathogen pressure associated with the thermal environment (Torson et al., 2015). However, the evolutionary reasons why FTs might alter immunity or the outcome of infection are unclear.

### 1.3 The insect immune system

Infection of an insect by viruses, bacteria, fungi, and protozoan/metazoan parasites can occur through the exoskeleton or via ingestion and respiration. Therefore, the cuticle (covering the exterior of the insect and extending into the trachea, foregut, and hindgut) and epidermis offer the first line of defense against pathogens and parasites (Gillespie and Kanost, 1997). The thickness of the pro-cuticle layer, the degree of tensile strength and hardening by sclerotisation, and the composition of the waxy layer all contribute to resistance against pathogens that are able to penetrate the exoskeleton to gain access to the haemocoel (e.g. fungal pathogens; Hajek and Leger, 1994). Infection can also occur through the spiracles (Hajek and Leger, 1994), or through penetration/invasion of the gut

following ingestion (e.g. bacteria, viruses, and malaria-causing protozoan parasites in the genus *Plasmodium*). The gut is lined with cuticle in all areas except for the midgut, where (in most, but not all, insects) a chitin-protein peritrophic membrane is formed. This membrane allows the passage of small particles (e.g. digestive enzymes, water, salts, organic molecules) while protecting the gut epidermis from harsh food particles and (in some cases) pathogens that are unable to breach the peritrophic membrane (Richards and Richards, 1977). However, many pathogens and parasites are able to breach these barriers, and so insects must also employ their cellular and humoral immune systems as the footmen of their defense.

To mount an effective immune response, an animal must be able to recognise ‘self’ from ‘non-self’. In most animals, including insects, pathogens are recognised by cell-surface receptors and circulating immune proteins and carbohydrates (e.g. lectins; Gillespie and Kanost, 1997) that bind to conserved pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) – an endotoxin from the outer membrane of Gram-negative bacteria. Binding of PAMPs to a host cell triggers a signal transduction cascade that leads to changes in gene expression (e.g. the production of antimicrobial peptides) or changes in activity by the cell (e.g. initiation of phagocytosis, release of cytokines). Epithelial cells and fat body cells are able to recognise and react to invaders (Gillespie and Kanost, 1997; Welchman et al., 2009); however, immune cells called hemocytes are employed as the dominant watchdogs and defenders in the open circulatory system (Lavine and Strand, 2002; Marmaras and Lampropoulou, 2009).

Haemocytes can be separated into different classes, each of which may be specialised to a particular function (Lavine and Strand, 2002; Strand, 2008): for example, granulocytes are largely responsible for phagocytosis, whereas oenocytoids are non-adhesive and more likely to participate in the release of phenoloxidase and the production of melanin (Strand, 2008). Haemocytes exist in circulation, but may also adhere to tissues (sessile hemocytes) or rest in storage organs (e.g. haematopoietic organ) as either matured or progenitor cells (Lavine and Strand, 2002; Strand, 2008). Haemocytes are responsible for recognition of invaders via pattern-recognition receptors (PRRs) either on the surface of

the cell itself that bind to PAMPs, or by recognition of PAMP-PRRs by cell-surface protein receptors, leading to signal transduction and the release of cytokines that trigger increased/modified haemocyte activity and, sometimes, expression of antimicrobial peptides (Lavine and Strand, 2002). Upon infection, hemocytes are released into circulation from adherence to tissues or from the hematopoietic organ, and may also replicate in circulation (Lavine and Strand, 2002). Circulating hemocytes are recruited to the site of injury or infection to perform phagocytosis, encapsulation, and nodule formation.

In most insects, the enzyme phenoloxidase (PO) is synthesized and stored within hemocytes in its zymogen form, prophenoloxidase (proPO), although PO or proPO may also circulate constitutively and extracellularly in the haemolymph (González-Santoyo and Córdoba-Aguilar, 2012). Upon immune insult, serine proteases induce a conformational change of proPO to active PO, which then converts phenols to quinones that subsequently polymerize to form melanin. Through this cascade, free radicals and other cytotoxic molecules are produced, which can contribute to killing pathogens, but also cause self-damage. Thus, melanisation responses are tightly regulated and highly localised (González-Santoyo and Córdoba-Aguilar, 2012). The melanisation response is a general and fast-acting response and can be used in defense against insults as small as bacteria to as large as a parasitoid egg or other large metazoan parasite. Melanin itself acts to surround and “suffocate” the pathogen – cutting it off from available nutrients and preventing further distribution through the body, effectively reducing the proliferation and infectivity of the pathogen until it dies (Gillespie and Kanost, 1997).

Antimicrobial peptides and proteins (AMPs) are responsible for cell-free destruction of invading microbes (e.g. bacteria and fungi) and are mainly produced in the fat body upon infection (Dunn, 1986), although they may also be produced by hemocytes and epithelial cells in both the epidermis and intestinal tract (Davies et al., 2014; Davies et al., 2012; McGettigan et al., 2005), and circulate constitutively in low levels in the haemolymph (e.g. lysozyme; Dunn, 1986). In general, inducible AMPs are used as a second line of defense, as activity lags because it requires RNA synthesis (Haine et al., 2008). In insects,

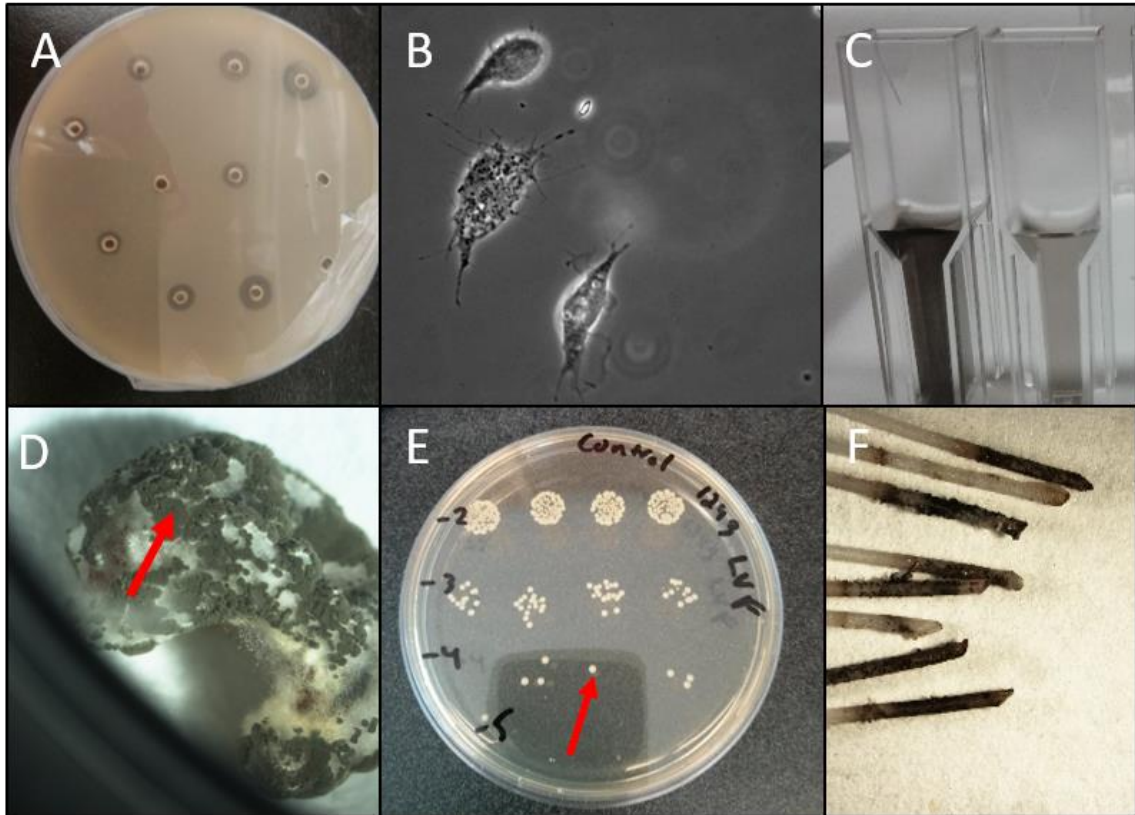
AMPs are produced mainly through one of three pathways: Toll, Imd, and JAK/STAT – each of which correspond to the response of different classes of pathogens. Generally, Toll is responsible for the response to Gram-positive bacteria and fungi, Imd for the response to Gram-negative bacteria, and JAK/STAT for the response to tissue damage and viruses (Lemaitre and Hoffmann, 2007). However, particular antimicrobial peptides, such as Drosomycin, can be produced through both the Toll and Imd pathways (Lemaitre and Hoffmann, 2007).

Because immune activity is costly (Ahmed et al., 2002; Ardia et al., 2012; Freitak et al., 2003) and may also cause self-damage (Moreno-Garcia et al., 2014), insects may also employ tolerance as a mechanism to cope with pathogen infection. In this case, the immune system is not fully activated in the face of pathogen infection. Instead, when the damage of immune activation (either directly or *via* costly defense) is greater than the threat of damage by pathogens, the insect will instead tolerate non-lethal levels of pathogen activity (Moreno-Garcia et al., 2014).

### 1.3.2. Measuring immune activity

Because insects possess both constitutive and induced immune activity, we can quantify both the potential for an immune response (i.e. without introducing a pathogen) as well as realised responses against introduced pathogens (Fedorka et al., 2007). Constitutive levels of circulating hemocytes (Fig. 1.3B), phenoloxidase activity (Fig. 1.3C), humoral antimicrobial activity (Fig. 1.3A) and AMP gene expression give us an indication of the baseline immunocompetence of an insect, as well as an indication of what kinds of activity might be elevated following either exposure to stress unrelated to pathogens, or a pathogen challenge. We can also measure whole-animal responses to pathogens that are likely to encompass several branches of the immune system, such as survival of fungal infection (Fig. 1.3D) or clearance of bacteria (Fig. 1.3E). Further, insects are also able to tolerate immune insult, as opposed to activating immunity to resist infection (Moreno-Garcia et al., 2014). Tolerance is difficult to characterise and quantify, but can explain





**Figure 1.3 Examples of common measures of immune activity in insects**

**A. Lysozyme activity.** Agar plates contain small holes from which extracted haemolymph diffuses into a homogenous distribution of Gram-positive bacteria. If lysozyme is present, will destroy the bacteria and create a clear ring (Vilcinskas and Matha, 1997). **B. Phase-contrast of hemocytes.** Circulating hemocytes can be quantified from haemolymph samples to provide a measure of immune activity (Rolff and Siva-Jothy, 2003). **C. Phenoloxidase activity.** Phenoloxidase activity can be measured spectrophotometrically using dopamine as a substrate. As dopamine is converted into melanin, the solution changes from clear to brown or black, with darker colours indicating a higher level of enzyme activity (Adamo, 2004). **D. Survival of fungal infection.** Fungi are one of the main pathogens that insects encounter, thus their survival of fungal infection is a suitable measure of disease resistance or tolerance (Rolff and Siva-Jothy, 2003). Further, infection can be verified post-mortem as the fungus will grow from the haemolymph to the cuticle with distinct sporulation (e.g. green spores indicate *Metarhizium* sp.; indicated by red arrow). **E. Bacterial clearance.** We can quantify levels of antimicrobial activity in the haemolymph in *in vitro* or *in vivo* by challenging the insect with an antibiotic-resistant bacteria that can be selected for on an agar plate containing antibiotics (Haine et al., 2008) (red arrow indicates a colony-forming unit of bacteria). **F. Melanisation response.** We can quantify the strength of the melanisation response against a dummy parasite, such as a small piece of nylon filament (Krams et al., 2011b).

increased survival of infection without significant increases in measures of immune activity (Moreno-Garcia et al., 2014). Lastly, it is important to measure several aspects of immunity when considering broad changes to immunocompetence. Changes in immunity are often disparate among different components of the immune response; phenoloxidase activity may increase while the expression of AMPs decrease (Adamo et al., 2016). Similarly, increases in constitutive immunity may not translate into increases in disease resistance (Fedorka et al., 2007). Thus, measuring only one component of activity does not provide an accurate picture of how overall immunity changes.

### 1.3.3 Eco-immunology: the interaction between host and environment

Ecological immunology, or eco-immunology, is a relatively new discipline, defined by Sheldon and Verhulst (1996), that seeks to understand how the external environment influences immunity and disease resistance. The immune system is not only responsive to pathogens, but also to various other changes in the abiotic and biotic environment (Sadd and Schmid-Hempel, 2008; Sheldon and Verhulst, 1996), and including via physiological events (e.g. moulting) (Table 1.1). In particular, the immune system appears to be tightly – albeit complexly – linked to the stress response in insects (among other animals), with both immunosuppressive and immunoenhancing effects (Adamo, 2014, 2016). For example, because the stress response and the immune response share resources such as lipid transport proteins, activation of the flight-or-fight stress response can suppress immunity via resource-based trade-offs (Adamo et al., 2008). Conversely, disease resistance can be enhanced as a by-product of the stress response, whereby intracellular stress responses that lead to cellular protective mechanisms (e.g. expression of heat shock proteins and detoxification enzymes) indirectly improve disease resistance (Davies et al., 2014; Davies et al., 2012). The immune system is also responsive to tissue damage that may accompany a stressful environment (both in the presence and absence of infections), whereby damage-associated molecular patterns (DAMPs) from damaged, dead, or apoptotic cells trigger immune activity (Babcock et al., 2008).

**Table 1.1** Examples of environmentally-driven changes in immune activity independent of infection.

<b>Environmental/ physiological change</b>	<b>Hypotheses for links</b>	<b>Example</b>
<b>Stress</b>		
<i>Crowding</i>	Prophylactic response to increased pathogen stress in crowding	<i>Tenebrio molitor</i> increase cuticular melanisation <sup>1</sup> and resistance to fungal pathogen <sup>2</sup> at high densities
<i>Temperature</i>	Trade-offs, resource allocation, damage	Repeated freezing and thawing increases survival of fungal infection in <i>Pyrrharctia isabella</i> <sup>3</sup>
<i>Flight or fight</i>	Liberate resources for stress response	Flight stress reconfigures immune investment in <i>Gryllus texensis</i> <sup>4,5,6,7,8</sup>
<i>Physical stress</i>	Priming for infection	Mild shaking in <i>Galleria mellonella</i> increases haemocyte density, expression of genes coding for immune peptides and enzymes, and increases resistance to <i>Candida albicans</i> <sup>9</sup>
<i>Tissue damage</i>	Repair	Aseptic tissue damage induces systemic immune response in <i>Drosophila melanogaster</i> <sup>10</sup>
<i>Starvation</i>	Resource availability	Starved bumble bees <i>Bombus terrestris</i> are less likely to survive infection <sup>11</sup>
<b>Feeding/digestion</b>	<i>Increase:</i> response to potential incoming pathogens <i>Decrease:</i> trade-offs with metabolic demands of digestion and/or egg production	Blood-feeding induces haemocyte proliferation and increases survival of low levels of bacterial infection, but reduces survival at high concentrations <sup>12</sup>
<b>Temperature (non-stressful)</b>	Prophylactic signal for pathogen pressure	Fluctuating temperatures induce expression of genes coding for immune peptides <sup>13</sup>
<b>Season</b>	<i>Increase:</i> Prophylactic response to increased pathogen pressure; by-product of enhanced melanisation for desiccation resistance <i>Decrease:</i> Trade-offs and resource limitation	Acclimation to autumn-like conditions increases phenoloxidase activity and survival of bacterial infection in <i>Allonemobius socius</i> <sup>14</sup> ; Survival of infection is lower in winter in <i>Haeterina americana</i> <sup>15</sup>
<b>Diapause</b>	Prophylactic response to increased pathogen stress	Diapause induction increases expression of genes coding for immune peptides in <i>Sarcophaga</i>

		<i>crassipalpis</i> <sup>16</sup> and <i>Lymantria dispar</i> <sup>17</sup>
<b>Ageing/moulting</b>	Trade-offs, resource allocation	Immunity senesces with age <sup>18</sup> , fluctuates with hormonal control of development <sup>19</sup> , and varies with age <sup>20</sup>
<b>Mating/Reproduction</b>	Trade-offs, resource allocation	Sexual activity in male <i>Drosophila melanogaster</i> decrease immunocompetence <sup>21</sup>
<b>Diet</b>	Resource/substrate availability	Consumption of acorns increases immune activity and survival of fungal infection compared to diet of cow dung in <i>Thorectes lusitanicus</i>

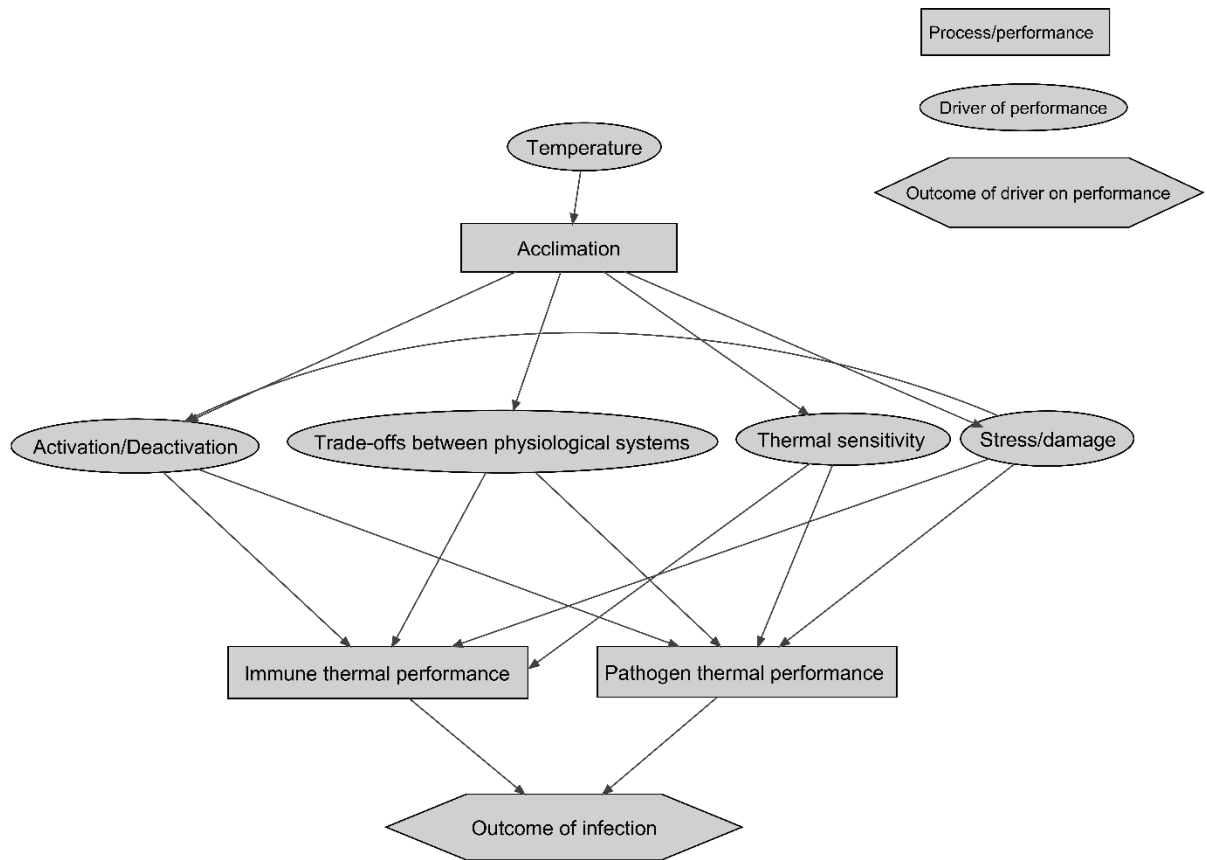
<sup>1</sup>. (Barnes and Siva-Jothy, 2000); <sup>2</sup>. (Wilson and Cotter, 2008); <sup>3</sup>. (Marshall and Sinclair, 2011); <sup>4,5,6,7,8</sup>. (Adamo, 2010, 2014, 2016; Adamo et al., 2008); <sup>9</sup>. (Mowlds et al., 2008); <sup>10</sup>. (Pastor-Pareja et al., 2008); <sup>11</sup>. (Moret and Schmid-Hempel, 2000) <sup>12</sup>. (Castillo et al., 2011) <sup>13</sup>. (Torson et al., 2015); <sup>14</sup>. (Fedorka et al., 2013); <sup>15</sup>. (Córdoba-Aguilar et al., 2009); <sup>16</sup>. (Ragland et al., 2010) ; <sup>17</sup>. (Lee et al., 2002); <sup>18</sup>. (Daukste et al., 2012); <sup>19</sup>. (Flatt et al., 2008); <sup>20</sup>. (Prasai and Karlsson, 2012); <sup>21</sup>. (McKean and Nunnery, 2001); <sup>22</sup>. (Verdu et al., 2013)

Overall, the immune system is responsive to changes in the environment via trade-offs between physiological systems responding to multiple pressures and the direct effects of the environment on immune function (Table 1.1). In some cases, the environment may signal for a change in pathogen pressure that initiates a prophylactic response by the insect (Table 1.1). Further, immune activity is costly (Ahmed et al., 2002; Ardia et al., 2012; Freitak et al., 2003; Moret and Schmid-Hempel, 2000) and is thus highly dependent on resource availability, leading to the potential for energetic trade-offs between the response to multiple environmental pressures (Table 1.1; Catalan et al., 2012). Finally, the individual effects of environmental variables on immunity may produce different phenotypes when combined; for example, high temperatures can increase PO activity when insects are reared on high quality food, but decrease PO activity if food quality is poor (Lee et al., 2008).

#### 1.3.4 Thermal ecology of immunity and infection

Because insects are ectotherms, and the immune system relies on temperature-dependent cell and enzyme activities, increases or decreases in temperature will directly increase or decrease immune activity simply based on thermodynamics (Murdock et al., 2012). Insects may take advantage of this relationship with temperature behaviourally – for example, by employing behavioural fever, during which insects bask to increase their body temperature and fight infection by increasing the rate of immune activity (and/or increase temperature beyond the thermal optimum of the pathogen) (Elliot et al., 2002). Conversely, low temperatures slow the activity of immune enzymes and cells, although they remain active (Nakamura et al., 2011). The outcome of infection across temperatures will then depend on the thermal sensitivity of the immune system (Fig. 1.4), and how its performance matches or mismatches the thermal performance of its pathogens (Fig. 1.4; Thomas and Blanford, 2003).

Independent of thermodynamics, changes in temperature lead to state changes in immune activity in the host, as well as pathogen infectivity. In particular, exposure to low temperatures can activate immune activity (see Section 1.3.5; Sinclair et al., 2013) and increase virulence of some pathogens (Bresolin et al., 2006). Conversely, immunity can trade-off with other physiological systems, resulting in temperature-mediated changes in



**Figure 1.4 The effect of temperature on infection outcome .** Each of the effects of temperature on immunity and pathogen performance are mediated through thermal plasticity achieved via acclimation. The outcome of infection will ultimately depend on the match or mismatch in thermal performance of the host and pathogen under different thermal environments. Thermal performance of each will depend on thermal sensitivity, activation or deactivation signals that temperature may provide, as well as any trade-offs between the response to multiple pressures or damage that temperature may signal for or produce.

other physiological responses. For example, infection increases the time it takes for *Drosophila melanogaster* to recover from cold exposure (Linderman et al., 2012). Further, because thermal acclimation can modify thermal sensitivity (Williams et al., 2012), or lead to state changes in physiological activity and phenotype (MacMillan et al., 2015), the outcome of infection should also depend on the thermal history and plasticity of both hosts and pathogens (Fig. 1.4).

### 1.3.5 Cold-activation of the immune system

Cold exposure is one environmental pressure that appears to activate immunity in insects (summarised in Table 1.2). For example, cold-exposed *Drosophila melanogaster* increase survival in the face of fungal infection (Le Bourg et al., 2009), and upregulate expression of immune-related genes, including those coding for antimicrobial peptides (Zhang et al., 2011). This cold-associated upregulation of immune activity may have ecological relevance. For example, an enhanced encapsulation response is associated with higher winter survival in water striders (Krams et al., 2011a). Conversely, bacterial infection increases chill coma recovery time (i.e. reduces cold tolerance) in *Drosophila melanogaster* (Linderman et al., 2012). Thus, there appear to be links between the responses to cold and infection, although the nature of those responses – and their adaptive significance – have not been thoroughly explored. There are at least four non-exclusive hypotheses that could account for the evolution of cold-immune links in insects. Two are non-adaptive (a non-specific general stress response and a by-product of selection for behavioral fever) and two are adaptive (protection against opportunistically-pathogenic gut flora and pathogen-host mismatches in low temperature performance).

#### *1) Immune activation is non-adaptive, but a consequence of a general stress response*

Although there is considerable variation in the molecular and physiological responses by insects to different abiotic stresses (Harrison et al., 2012), there are clearly some general stress responses, and upregulation of the immune system in response to cold and overwintering may simply be associated with those shared response pathways. However, unnecessary immune activation should be selectively disadvantageous due to the

**Table 1.2 Evidence of the relationship between low temperature and the immune response in insects**

<b>Species</b>	<b>Evidence</b>	<b>Reference</b>
<b>Diptera</b>		
<i>Drosophila melanogaster</i>	1. Repeated cold exposure increases resistance to fungal infection	1
	2. Acute, chronic, and repeated cold exposure elicit differential but increased expression of genes coding for immune peptide	2
<b>Hymenoptera</b>		
<i>Megachile rotundata</i>	Cold exposure increases expression of immune genes and reduces incidence of chalk brood disease	3
<b>Hemiptera</b>		
<i>Aquarius naja</i>	Stronger melanisation is associated with better overwintering survival	4
<b>Coleoptera</b>		
<i>Tribolium castaneum</i>	Cold shock of parental generation increases bacterial resistance in offspring	5
<b>Lepidoptera</b>		
<i>Galleria mellonella</i>	Mild cold shock induces expression of immune genes and increases circulating hemocytes	6
<i>Pyrrharctia isabella</i>	Repeated freezing and thawing increases resistance to fungal infection	7
<i>Trichoplusia ni</i>	Increased resistance to <i>Bacillus thuriensis</i> is correlated with increased overwintering survival	8

<sup>1</sup>(Le Bourg, 2011; Le Bourg et al., 2009) <sup>2</sup>(Zhang et al., 2011) <sup>3</sup>(Xu and James, 2012) <sup>4</sup>(Krams et al., 2011) <sup>5</sup>(Eggert et al., 2015) <sup>6</sup>(Mowlds and Kavanagh, 2008) <sup>7</sup>(Marshall and Sinclair, 2011) <sup>8</sup>(Caron and Myers, 2008)



energetic costs associated with mounting an immune response (Moret and Schmid-Hempel, 2000). In addition, increased overwinter survival of water striders with strong immune responses (Krams et al., 2011) suggests that, in at least some species, there may be a fitness advantage to winter immune activation.

### *2) Selection for behavioral fever links immune responses and thermal biology*

Behavioural fever is a thermoregulatory response to infection by insects that improves their survival of infection (Thomas and Blanford, 2003). There is some evidence that this thermoregulatory behavior is mediated by eicosanoids (Bundey et al., 2003) which may also play a role in general insect thermal biology and responses to infection (Stanley, 2006). It is possible that there has been selection for cross-talk in eicosanoid signaling pathways associated with behavioral fever, and that this cross-talk persists in a non-adaptive fashion at low temperatures as well. A better understanding of the signaling pathways associated with low temperature responses in insects will allow exploration of this hypothesis.

### *3) Tissue damage during cold exposure leads to immune challenge*

Both chilling and freezing injury in insects are accompanied by physical damage, particularly to the gut and Malpighian tubules (MacMillan and Sinclair, 2011; Marshall and Sinclair, 2011; Yi and Lee, 2003). Wounding itself initiates immune activity (Gillespie et al., 1997), and damage to the gut could allow the gut flora to enter the haemocoel (MacMillan and Sinclair, 2011), directly activating antimicrobial responses. Thus, there may have been selection for (adaptive) pre-emptive immune activation, because cold is frequently associated with wounding and/or invasion of microbiota from the gut.

#### 4) *Protection against cold-active pathogens*

Many insects overwinter in diapause, with consequent suppression of metabolic rate, disruption of water and ion homeostasis, and an inability to behaviorally avoid parasites and pathogens (MacMillan and Sinclair, 2011; Rider et al., 2011). If the natural flora, pathogens, or parasites are less inhibited by low temperatures than the host, then there exists an opportunity for these organisms to outpace the host immune system, much as is hypothesized for immune suppression during mammalian hibernation (Bouma et al., 2010). Thus, there may have been selection for a baseline level of immune activation throughout the winter to provide protection against cold-active pathogens, or for activation of immune responses immediately upon re-warming.

Regardless of whether cold-related immune activation has an adaptive evolutionary origin, with the exception of melanization responses to gut damage, there are few mechanisms of immune protection that overlap with the postulated cellular mechanisms of damage from cold. Therefore, the links between cold exposure (and overwintering in general) and immune upregulation are likely the result of cross-talk among the pathways, as has been postulated for immune interactions with many other stress signals in *Drosophila* (e.g. Davies et al., 2012) and *Tribolium castaneum* (Altincicek et al., 2008). Further, the connection between cold and immunity will likely be mediated by, or affect, overwintering energy use, the response to low temperatures, and the ability to respond to pathogens, thereby determining overwintering survival and success.

### 1.5 Summary and objectives

Insects are faced with multiple, co-occurring stressors, pressures, and changes in their environments that influence their physiology. For example, overwintering insects face cold, desiccation, starvation, and pathogen stresses simultaneously (Williams et al., 2015). In particular, we have largely neglected the interaction between biotic pressures on insect success – such as temperature and pathogen pressure in overwintering insects. Cold stress can activate the immune system in insects, which suggests that there may be cross-talk between the physiological response to low temperatures and pathogens (Sinclair et al., 2013). The role of the immune system in contributing to overwintering success will

be directly and indirectly dependent on temperature (Fig. 1.4); however, we know little of the thermal biology of the insect immune system and how low temperatures affect the interaction between insect hosts and their pathogens.

## 1.6 Thesis overview

In this thesis I address two major questions: 1) Why does exposure to low temperatures change immune activity? and 2) How does the thermal environment change the interaction between insect hosts and their microbial environment (including pathogens)? I hypothesise that insects adjust their immune activity in response to low temperatures to cope with simultaneous changes in both temperature and microbial pressures. Further, I hypothesise that these temperature-driven changes in both microbes and their insect hosts shift the outcome of their interactions. To address these hypotheses, I have structured my thesis into five objectives, each of which is addressed in a single chapter. Chapters 2-6 in this thesis were prepared as separate manuscripts for publication; Chapters 2 and 3 are published, Chapter 4 has been peer-reviewed and is undergoing minor revisions, and Chapters 5 and 6 are in preparation for publication.

My first objective was to determine the extent of cold-activation of immunity in *Drosophila melanogaster* as a step in understanding why cold might activate immunity. In chapter 2 (“Does cold activate the *Drosophila melanogaster* immune system”), I investigate which components of the immune system are activated by cold and whether or not this differs depending on the type of cold exposure that the insect experiences (in this case, acute or sustained cold exposure). I find that acute cold exposure increases potential immunity, but either transiently decreases or has no effect on realised immunity. Sustained cold exposure has little to no effect on immunity at all. I conclude that cold activates parts of the immune system to compensate for cold-induced damage to or trade-offs between immunity and recovery from cold exposure.

My second objective was to understand if cold acclimation allows the immune system to adjust its performance in the cold. In chapter 3 (“Paradoxical acclimation responses in the thermal performance of insect immunity”), I investigate whether the immune system is

thermally plastic, and if cold-acclimation beneficially increase immune activity at low temperatures. I find that cold acclimation benefits locomotor activity at low temperatures, but paradoxically narrows the thermal performance curve of realised immune activity such that activity is decreased in the cold but maintained at higher temperatures. I conclude that immunity trades-off with preparation for cold exposure.

My third objective was to determine if there are generalizable or idiosyncratic modifications to immune activity in overwintering insects, which I addressed in three species of field-collected insects native to Ontario: the woolly bear caterpillar (*Pyrrharctia isabella*), the goldenrod gall fly (*Eurosta solidaginis*) and acorn weevils (*Curculio* sp.). In chapter 4 (“Insect immunity varies idiosyncratically during overwintering”) I explore seasonal patterns of several measures of immune activity among these species of insects in their juvenile, overwintering stages. I find that immune activity varies seasonally in all three, but that these patterns are largely species-dependent. Further, I find that that the temperature at which immune activity is measured may reflect different seasonal patterns of activity, and that microhabitat during overwintering affects the strength of immune activity in the spring. I conclude that seasonal immune activity varies by the types of overwintering pressures that are encountered, and will thus be susceptible to climate-change-induced modifications to winter environments.

My fourth objective was two-fold: 1) to determine how thermal history of both hosts and pathogens modified the outcome of infection under different thermal environments, and 2) to determine how and why fluctuating temperatures modify the outcome of infection compared to constant temperatures. I addressed this objective with *G. veletis* and the entomopathogenic fungus *Metarhizium brunneum* in chapter 5 (“Thermal variability and plasticity determine outcome of host-pathogen interactions”). I find that both host and pathogen thermal history shift the outcome of infection, and that acclimation to fluctuating temperatures strengthens host defense against pathogens. Further, the outcome of infection under fluctuating temperatures appears to be an additive outcome of host and pathogen thermal performance at each temperature that comprises the fluctuating regime. I conclude that experiments performed under constant temperatures can be used to

extrapolate the relative thermal performance of hosts and pathogens and thus predict the outcome of infection under fluctuating environments.

My final objective was to determine if the gut microbiome of insects fluctuates seasonally with exposure to low temperatures, and if this fluctuation corresponded with seasonal changes in immune activity. I explored this objective with *G. veletis* in chapter 6 “Overwintering modifies gut microbiome composition and immune activity in insects”. I find that the gut microbiome shifts and appears to select against aerobic bacteria and/or ice nucleating agents, while promoting the growth of the symbiont, *Wolbachia*. Further, immune activity is largely suppressed in the winter, but recovers in the spring. I conclude that host physiology shifts at the same time as the bacterial community; thus the connection between host physiology and microbiome function is likely to be altered across seasons.

In chapter 7 (general discussion), I synthesize the results from these five studies. I find that the relationship between cold and immunity is likely to be adaptive, and driven by trade-offs between these physiological responses and temperature-induced damage to immune activity. I suggest that predicting the outcome of biotic interactions under different thermal environments will depend on the thermal history of both players in the interaction, and the mechanisms underlying the responses to multiple environmental pressures.

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## Chapter 2

In this chapter I aim to understand why cold activates the immune system in *Drosophila melanogaster* by examining how multiple components of the immune system respond to two different types of cold exposure. This chapter is adapted, with permission, from the version published in *Journal of Insect Physiology*<sup>1</sup>.

### 2.1 Introduction

Understanding how the physiological responses by animals to multiple biotic and abiotic stressors are linked through cross-talk or cross-tolerance is key to understanding the multidimensional impacts of a changing climate (Kaunisto et al., 2016; Sinclair et al., 2013). For example, insects appear to increase immune activity after cold exposure (Marshall and Sinclair, 2011; Zhang et al., 2011), which could modify host-pathogen relationships with changing winters (Williams et al., 2015). However, it is unclear whether these putatively-linked responses to distinct stressors are non-adaptive by-products of a generalised stress response, or the result of a functional coadaptation reflecting a link between pathogens and cold (Sinclair et al., 2013).

#### 2.1.1 Why would cold activate the insect immune system?

There are several, non-mutually exclusive hypotheses to explain why cold exposure might increase immune activity in insects. Cold exposure may non-adaptively activate immunity through activation of pathways shared with the stress response (Sinclair et al., 2013); however, recovery from cold exposure is already energetically costly (MacMillan et al., 2012), and it should be selectively disadvantageous to increase the cost associated with cold exposure by unnecessarily increasing resistance to pathogens. Instead, cold-induced immune activity may be an adaptive response to immune stress associated with cold (Sinclair et al., 2013). Among adaptive responses, immune processes could be required for repair of damage from cold exposure, or the presence of cold-active

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<sup>1</sup> Salehipour-Shirazi, G.\* , Ferguson, L.V.\* , Sinclair, B.J., 2016. Does cold activate the *Drosophila melanogaster* immune system? *Journal of Insect Physiology* 96, 29-34.

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pathogens may have selected for increased immune activity following exposure to low temperatures (Sinclair et al., 2013).

To begin to understand the functional significance of cold-induced immunity, we can explore the effects of cold exposure on multiple components of the immune system. Measures of potential immunity (sensu Fedorka et al., 2007), such as gene expression, provide insight into affected pathways (e.g. Toll vs. IMD), and the potential for shared responses between the immune system and the stress response. Measures of realised immunity (sensu Fedorka et al., 2007) provide insight into whether or not the ability to fight or survive pathogen infection is affected by cold exposure, and thus whether immune activation may be a response to pathogen threat at low temperatures.

### 2.1.2 Different types of cold exposure could help explain cold-immunity relationship

The physiological responses to cold differ among insects and kinds of cold exposure (Zachariassen, 1985). Specifically, brief exposure to intense cold (e.g. 2 h at  $-5^{\circ}\text{C}$  in *Drosophila melanogaster*) is thought to cause cold shock injury, such as membrane phase transition (Drobnis et al., 1993) or initiation of apoptosis (Yi et al., 2007), while longer cold exposures appear to cause stress by disrupting ion and water balance (MacMillan et al., 2012). We can use these differences in the physiological response to different types of cold exposure to further explore the function of cold-induced up-regulation of immunity. For example, in *D. melanogaster*, short, prolonged, and repeated cold exposures elicit unique transcriptomic profiles including of the expression of genes associated with immunity (Zhang et al., 2011), which suggests that the relationship between cold and immunity depends on the physiological response to a particular type of cold exposure. Therefore, if a suite of immune responses is specific to a particular type of cold exposure, we can begin to infer how this activity is linked to the physiological response to cold, and thus why immune activity might be activated.

### 2.1.3 Objectives

To begin to understand why the immune system is linked to cold exposure, and what the consequences of this relationship may be, my objectives were to 1) determine if cold exposure activated immune activity, and 2) determine if acute cold exposure elicits a different immune response than sustained cold exposure. We quantified multiple components of both potential and realised immunity (Fedorka et al., 2007) in *Drosophila melanogaster* following two cold exposures that differed in both duration and temperature, and which are likely to provoke different physiological responses to cold, and which have previously been shown to elicit upregulation of immune-related genes (Zhang et al., 2011): acute (2h at -2°C) and sustained (10h at -0.5°C). Overall, we aim to use cold-activation of insect immunity as a system in which to tease apart the links between responses to abiotic and biotic stressors, and highlight the importance of considering the physiological connections between different stressors in insects.

## 2.2 Materials and methods

### 2.2.1 Rearing and cold exposures

An outbred mass-reared population of wild-type *Drosophila melanogaster* collected in London and Niagara-on-the-Lake, ON, Canada, in 2008 (described by (Marshall and Sinclair, 2010) was reared on a banana-cornmeal-agar medium at 21.5°C, 60% RH, under 14L:10D. Before beginning the experiment, we reared flies for several generations on a medium containing tetracycline and methylparaben (to eliminate *Wolbachia* sp., confirmed with PCR) (Carrington et al., 2010); flies used in experiments were then reared for at least four generations on banana-cornmeal-agar medium without antibiotics or antifungals. We collected newly-eclosed virgin females under CO<sub>2</sub>, and allowed them to recover for seven days to minimise the physiological effects of anaesthesia (Nilson et al., 2006). Flies were exposed to cold in groups of 10 in food vials (3-15 vials per experiment/exposure/treatment) in aluminium blocks cooled from refrigerated circulators (c.f. Nyamukondiwa et al., 2011); flies were in darkness during the cold exposure, thus cold exposures were performed during the hours in which flies would usually experience darkness. We exposed groups of flies to acute (-2°C, 2h) or sustained (-0.5°C, 10h) cold

in vials with food to maintain high humidity. Controls were handled identically to their corresponding cold-exposed group, but maintained at 21.5°C. After cold exposure, we returned flies to rearing conditions for 2-6h [depending on the immune response to be measured, and based on previous (Zhang et al., 2011) or preliminary experiments]. During this time all flies recovered (and were no longer in chill coma), and then we measured immunity.

## 2.2.2 Potential immunity

To determine the effect of cold exposure on potential immunity, we measured circulating haemocyte concentration (CHC) and phenoloxidase activity (PO) in haemolymph samples, and mRNA abundance of immune-related genes (IMD pathway: *attacin*, *cecropin*, *diptericin*; Toll pathway: *drosocin*, *drosomycin*; IMD and Toll: *defensin* and *metchnikowin*; Jak-STAT pathway: *Turandot-A* and *virus-induced RNA 1*). Statistical analyses were performed in SPSS. We used t-tests (CHC), t-tests with FDR correction (mRNA) or ANOVA (PO activity) to compare differences in potential immunity among treatments.

### 2.2.2.1 Circulating haemocyte counts

To estimate CHC, we collected haemolymph (n = 10 per treatment) following MacMillan and Hughson (2014), diluted haemolymph in anticoagulant buffer [0.55 % W/V cresyl violet, 0.5 % ethylene-diamine-tetraacetic acid in phosphate buffered saline (PBS)] and counted hemocytes in a Neubauer improved haemocytometer at 400× magnification.

### 2.2.2.2 PO activity

We collected haemolymph following MacMillan and Hughson (2014) and pooled haemolymph from 8-10 flies under oil, for a final volume of 0.1 µL (n = 3-5 pooled samples per treatment). we diluted the haemolymph in 10 µL of PBS, snap-froze the samples in liquid nitrogen, and stored them at – 80 °C until use. We measured PO activity spectrophotometrically using L-dihydroxyphenylalanine (L-DOPA; 4 mg/mL) as the substrate. We expressed phenoloxidase activity as change in absorbance at 492 nm /min / µL, obtained during the linear portion of the reaction (Wilson et al., 2001).

### 2.2.2.3 Measurement of relative mRNA abundance with qPCR

To investigate the expression of genes associated with Toll, IMD, and JAK/STAT pathways, we quantified the expression of genes related to products or intermediate components of pathways by quantitative real-time polymerase chain reaction (RT-qPCR) (Bing et al., 2012). For each cold exposure treatment, we snap-froze flies in liquid nitrogen 6 h following cold exposure (Zhang et al., 2011) and extracted and pooled RNA from 30 frozen flies per sample (n = 5 pooled samples per treatment) using Trizol Reagent. We dissolved the extracted RNA pellet in 60  $\mu$ l RNasefree water and determined the total RNA concentration and purity ratio at 260 and 280 nm absorbance using a NanoDrop 2000 spectrophotometer and associated software. Only samples with a 260/280 ratio of 1.98 or more were used. To remove DNA contaminants, we treated the RNA samples with DNase. We mixed one microgram RNA from each sample with 1  $\mu$ l of DNase I Amp Grade and 1  $\mu$ l of 10X DNase I Reaction Buffer. After incubation at room temperature for 15 min, we added 1  $\mu$ l of 25 mM EDTA incubated the samples at 65 °C for 5 min to inactivate the DNase. We cooled the samples on ice before using them for cDNA synthesis. To synthesize cDNA, we added 2  $\mu$ l of oligodT, 4  $\mu$ l qScript Flex Reaction Mix (5 $\times$ ) and 1  $\mu$ l qScript Reverse Transcriptase to 1  $\mu$ g RNA of each sample. We then treated the samples at 42 °C for 75 min and 85 °C for 5 min. To amplify the cDNA, we used SYBR Green Master Mix. To normalize the data obtained from the target genes, we used *Rpl-32* as a reference gene (Zaidman et al., 2011). To determine the efficiency of primers at different cDNA concentrations, we created standard curves of target genes and the reference gene using seven different concentrations of mixed cDNA samples (0, 4, 16, 64, 256, 1024 and 4096-fold dilution). We calculated threshold cycle (Ct) values using CFX Manager Software ver. 2.1 (Bio-Rad) and we calculated and normalised the expression ratio of target genes relative to controls. We standardised all values to *Rpl-32* as a housekeeping gene, and to controls using  $\Delta\Delta$ Ct (Pfaffl, 2001).

### 2.2.3 Realised immunity

To examine the effect of cold exposure on realised immunity, We measured survival after topical application of the fungus *Metarhizium anisopliae* (Le Bourg et al., 2009), wound-induced melanisation, and the ability to clear Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria from the haemolymph (McKean and Nunney, 2001). Flies were briefly anaesthetized with CO<sub>2</sub> prior to all measures. Statistical analyses were performed in R (R Development Core Team, 2010). We used a linear mixed-effects model (fungal infections; “vial” as a covariate), a general linear model (wounding response) and a three-way ANOVA (bacterial clearance) to compare differences in realised immunity among treatments.

#### 2.2.3.1 Wounding response

Immediately after cold exposure, we pierced flies on the dorsal surface using a sterilized No. 000 insect pin (Fig. S1). We photographed the pierced area of ten flies from each treatment group at 6, 12, and 24 h post-cold-exposure. We then quantified melanisation by measuring the mean gray value of the pierced area using Image J software. To compare piercing-induced melanisation between cold-treated and control flies, we recorded the gray value of the pierced area of treated and control flies at specific time points (0, 6, and 12 h and after cold exposure).-Darker colors with lower gray values indicated more piercing-induced melanisation.

#### 2.2.3.2 Fungal infection

We obtained the broadly entomopathogenic fungus *Metarhizium anisopliae* (strain 2575, USDA, Ithaca, NY, USA) from Dr. Michael Bidochka (Brock University, St. Catharine’s, ON, Canada). *Metarhizium anisopliae* is commonly isolated from temperate soils and the overwintering habitats of insects (Bidochka et al., 1998), and we performed preliminary experiments to ensure that the fungus was lethal to flies at their rearing temperature of 21.5 °C. Following 6 h of recovery from cold exposure, we used the method of Le Bourg et al. (2009) for infecting flies by shaking them on an agar plate (30 s) with sporulating fungus. Non-infected controls of each group were shaken on a sterile plate. We returned

groups of flies to vials (n = 8-10 per vial; 5-7 vials per treatment) at 21.5 °C and monitored survival every 24 h for 16 d.

### 2.2.3.3 Bacterial clearance

To compare the ability of control and cold-exposed flies to clear bacteria from the haemolymph, we injected flies (n = 4-9 per infection, per treatment) with streptomycin-resistant Gram-positive (*B. subtilis*) and streptomycin-resistant Gram-negative (*E. coli*) bacteria suspended in PBS, following (McKean and Nunney, 2001), with some modifications. We selected flies haphazardly from each treatment group and injected 105nL ( $\sim 1 \times 10^4$  CFU) into the thorax through a glass capillary needle attached to a hydraulic manual microinjector (Sutter Instrument, Novato, CA, USA) 2 h or 6 h post-cold-exposure. We homogenized whole flies in 90  $\mu$ L PBS 30s post-injection (to confirm the concentration of injection), or after 5 h or 12 h recovery at 21.5 °C. We diluted and plated 10 $\mu$ L of each homogenate in four replicated spots on Luria broth agar containing streptomycin (25  $\mu$ g/mL), incubated the plates (37 °C, 24h) and counted the number of colony-forming units.

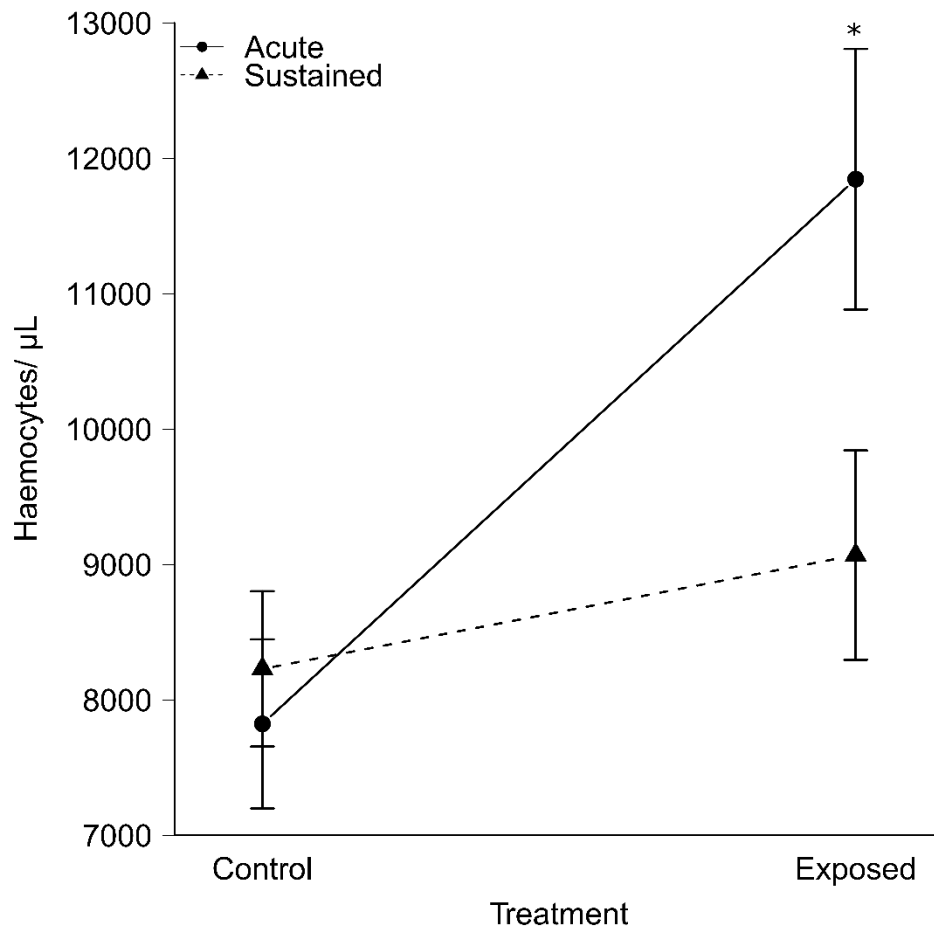
## 2.3. Results

### 2.3.1 Potential immunity

Acute, but not sustained, cold, significantly increased CHC compared to controls (Fig. 2.1; Table 2.1). I did not detect any difference in PO activity among treatments (Fig. 2.2;  $F_{2,9} = 1.09$ ,  $p = 0.38$ ). Expression of *dipteracin* increased following acute cold, and expression of *Turandot-A* increased relative to controls following both acute and sustained exposures (Fig. 2.3; Table 1). Expression of *metchnikowin* was higher after acute than sustained cold exposure (Fig. 2.3; Table 2.1). No other changes in gene expression were observed (Table S2).

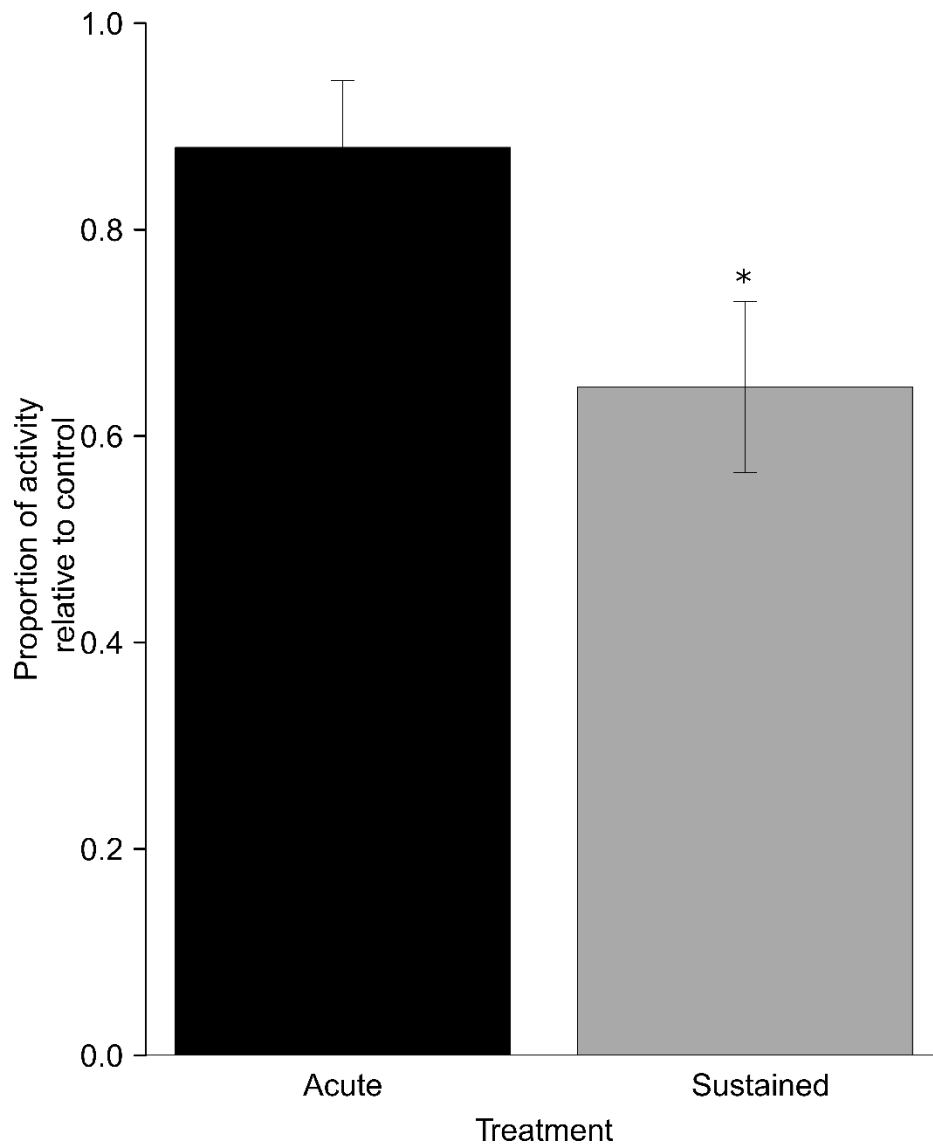
**Table 2.1 Effects of acute (2 h, -2 °C) or sustained (10 h, -0.5 °C) cold exposure on potential and realised immunity of female *Drosophila melanogaster*.** Cold refers to both acute and sustained exposures, compared to controls. P-values in bold indicate significant differences. PC = post-cold exposure; PI = post-infection

<b>Measure</b>	<b>Term(s)</b>	<b>Statistic</b>	<b>p</b>	
<b>Potential immunity</b>				
Circulating haemocyte counts	Acute vs control	$t_{15} = 3.51$	<b>&lt;0.05</b>	
	Sustained vs control	$t_{16} = 0.87$	0.39	
mRNA abundance	Acute vs control ( <i>diptericin</i> )	$t_4 = 7.10$	<b>&lt;0.01</b>	
	Acute vs control ( <i>Turandot-A</i> )	$t_4 = 3.43$	<b>&lt;0.001</b>	
	Sustained vs control ( <i>Turandot-A</i> )	$t_4 = 11.95$	<b>&lt;0.001</b>	
	Acute vs sustained ( <i>Metchnikowin</i> )	$t_4 = 8.54$	<b>0.046</b>	
<b>Realised immunity</b>				
Fungal infection	Infected vs uninfected	$t_{252} = 34.37$	<b>&lt;0.001</b>	
	Acute vs control	$t_{14} = 1.17$	0.26	
	Sustained vs control	$t_{14} = 1.72$	0.11	
Melanisation	Acute vs control	$t_{90} = 1.76$	0.08	
	Sustained vs control	$t_{90} = 1.21$	0.23	
Bacterial clearance	2 h PC; 5 h PI	Treatment	$F_{2,23} = 9.10$	<b>&lt;0.01</b>
		Bacteria	$F_{1,23} = 13.26$	<b>&lt;0.001</b>
		Treatment × Bacteria	$F_{2,23} = 3.37$	0.067
	6 h PC, 5 h PI	Treatment	$F_{2,22} = 0.50$	0.61
		Bacteria	$F_{1,22} = 7.82$	<b>0.01</b>
		Treatment × Bacteria	$F_{2,22} = 0.93$	0.93
	6 h PC; 12 h PI	Treatment	$F_{2,17} = 0.89$	0.92
		Bacteria	$F_{1,17} = 0.511$	<b>0.03</b>
		Treatment × Bacteria	$F_{2,17} = 0.892$	0.43

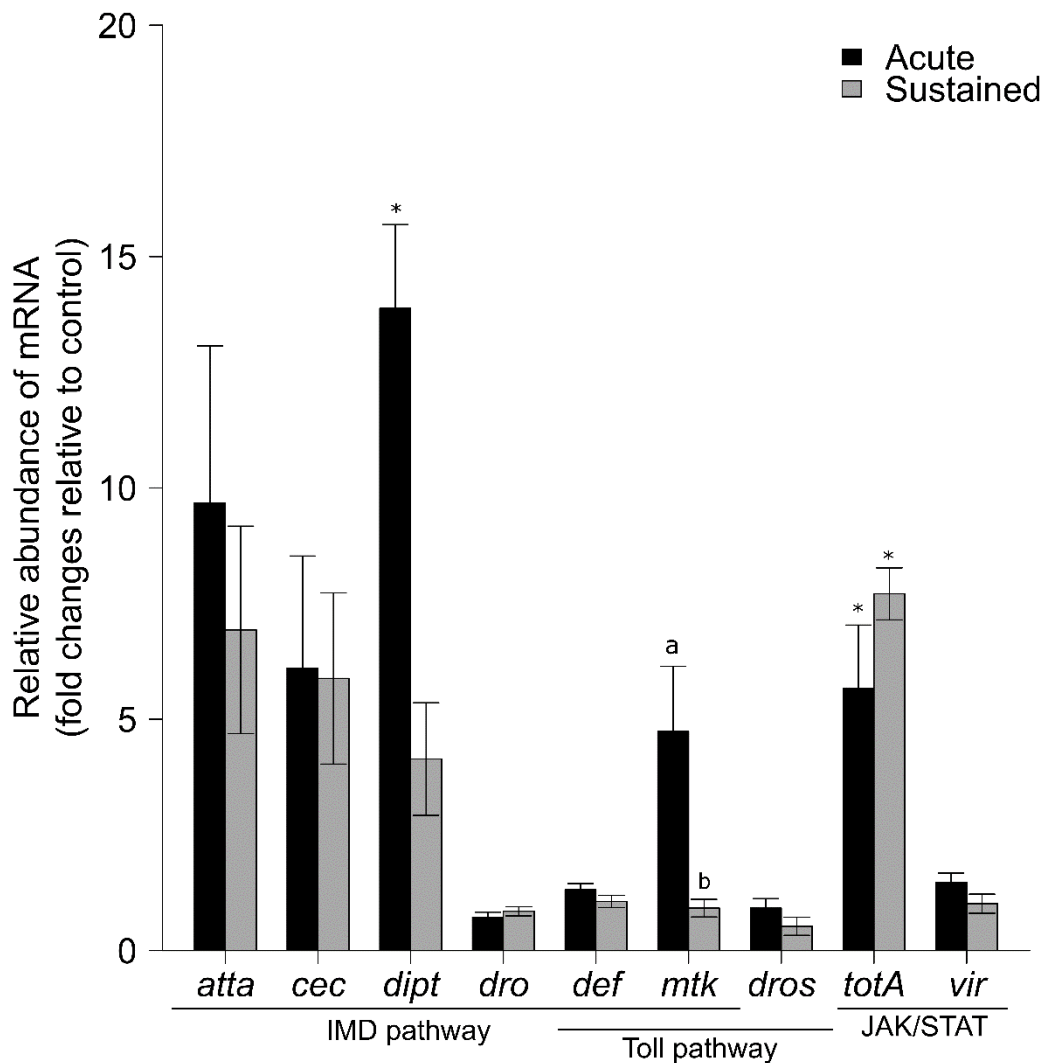


**Figure 2.1** Circulating haemocyte concentration of *Drosophila melanogaster* following cold exposure. Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold; n=10 per treatment. Mean ± SEM is shown; asterisks indicate significant differences (p<0.05) between cold-exposed and control flies (see Table 2.1 for statistics).





**Figure 2.2 Haemolymph phenoloxidase activity of *Drosophila melanogaster* following cold exposure.** Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold; n=3-5 per treatment (8-10 flies pooled per replicate). Mean  $\pm$  SEM is shown.



**Figure 2.3** Relative mRNA abundance of genes related to *Drosophila melanogaster* immune pathways measured by real-time qPCR, following cold exposure. Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold; n=5/gene/treatment (30 flies pooled per replicate). dros=drosomycin, def=defensin, mtk=metchnikowin, atta=attacin, cec=cecropin, dipt=dipteracin, dro=drosocin, TotA=Turandot A and vir=vir-1. Mean  $\pm$  SEM is shown; asterisks indicate significant differences ( $p < 0.05$ ) between cold-exposed and control flies; different lower-case letters indicate significant differences between flies exposed to acute and sustained cold (see Table 2.1 for statistics).

### 2.3.2 Realised immunity

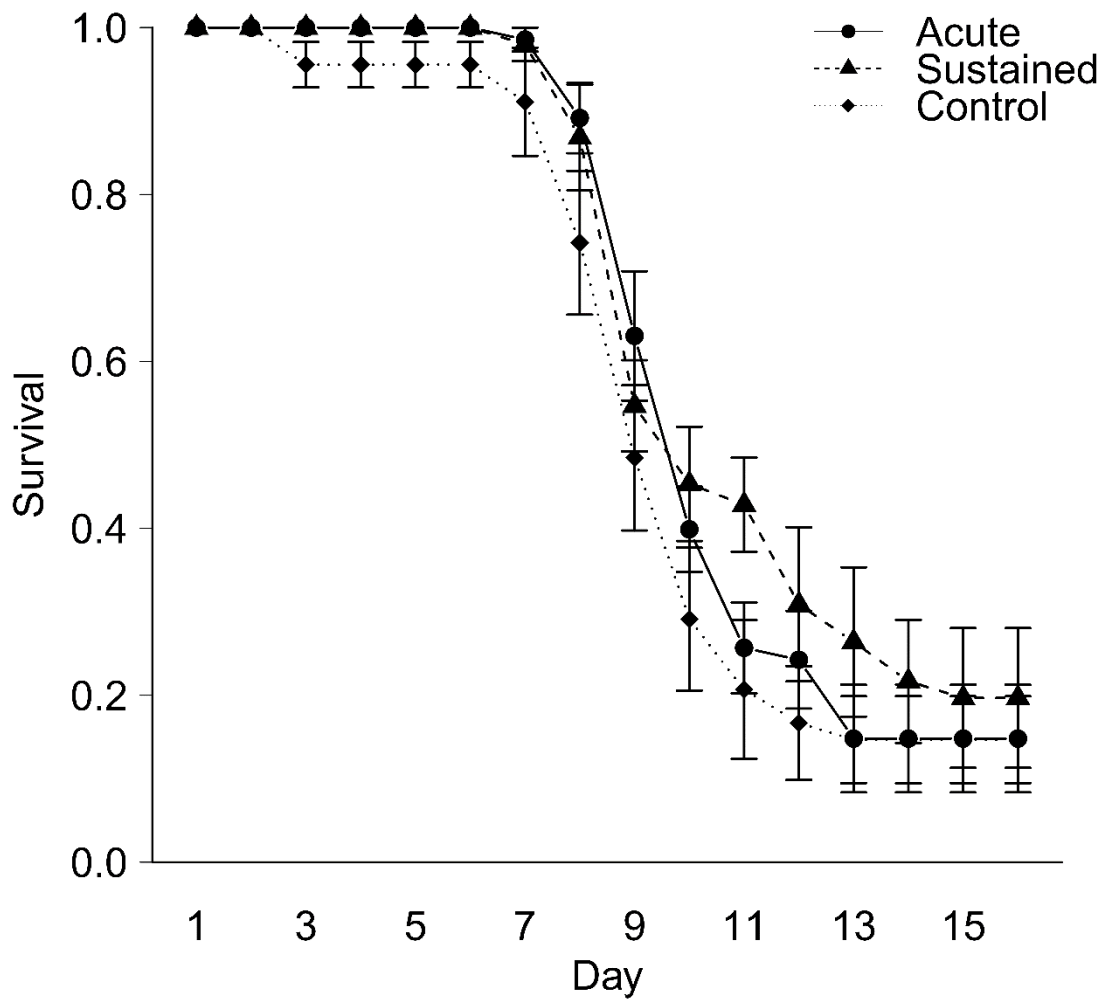
Neither acute nor sustained cold changed the susceptibility of flies to fungal infection (Fig. 2.4; Table 2.1). Only one uninfected control died over the course of the experiment in both the acute and control treatments, and no mortality occurred in uninfected flies from the sustained cold treatment; we did not detect any significant difference in survival among uninfected controls (sustained vs control:  $t_9 = 0.92$ ,  $p = 0.38$ ; acute vs control:  $t_9 = 0.11$ ,  $p = 0.91$ ). Wound-induced cuticle darkening was unchanged by either treatment (Fig. 2.5; Table 2.1). Clearance of Gram-positive bacteria decreased in flies exposed to acute cold when measured within 2 h of cold exposure (Fig. 2.6; Table 2.1), but bacterial-clearance ability recovered rapidly: following 6h of recovery from cold, treatment did not affect clearance (Fig. 2.6; Table 2.1).

## 2.4 Discussion

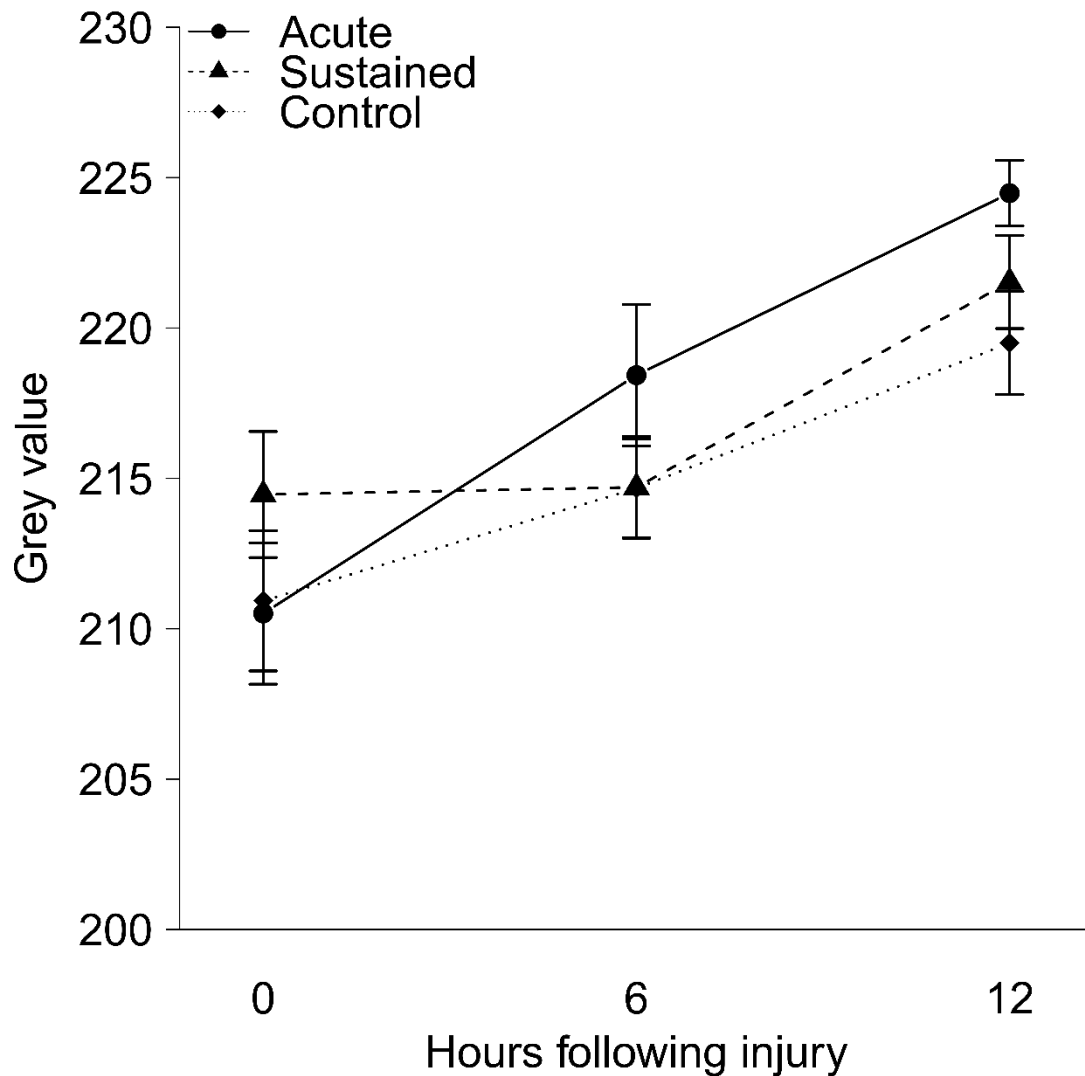
We explored the effects of acute and sustained cold exposure on immune activity in *Drosophila melanogaster*. Acute cold exposure increased potential immunity (more circulating hemocytes and increased expression of *diptericin* and *TurandotA*), but decreased one measure of realised immunity (Gram-positive bacterial clearance immediately after cold exposure). Sustained cold exposure also increased *Turandot-A* expression but did not affect realised immunity.

### 2.4.1. Chilling injury may include damage to or trade-offs with immunity

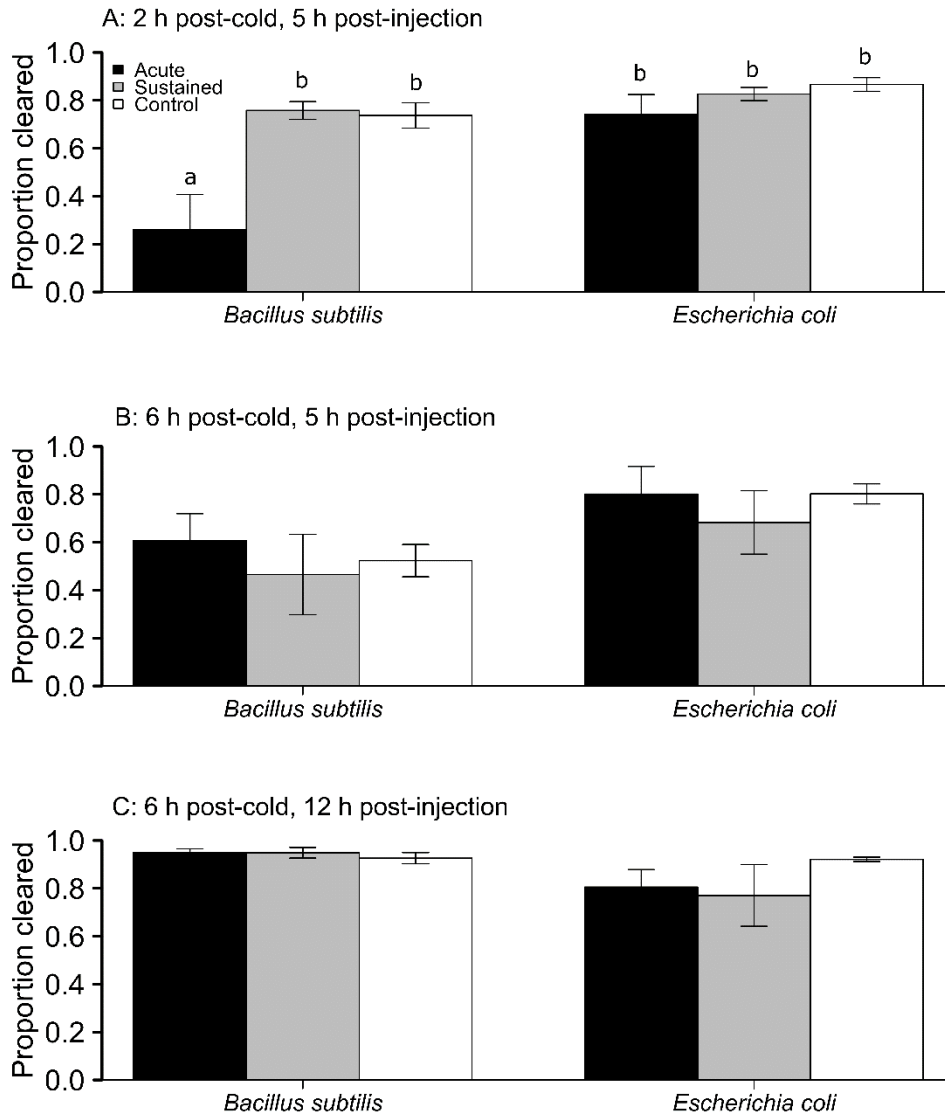
All cold-exposed flies were in chill coma (MacMillan et al., 2015), and these cold exposures have measurable non-lethal fitness impacts (Marshall and Sinclair, 2010), as well as transcriptomic profiles that suggest that they are both stressful exposures (Zhang et al., 2011). However, because sustained cold exposure did not elicit an increase in immune activity, whereas acute cold exposure increased potential immunity, it seems unlikely that cold-induced immune activity is a by-product of a general stress response.



**Figure 2.4** Survival of cold-exposed *Drosophila melanogaster* infected with *Metarhizium anisopliae*. n=8-10 flies in 5-7 vials/treatment. Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold and topically infected with *M. anisopliae* following recovery from cold. Mean  $\pm$  SEM is shown (see Table 2.1 for statistics).



**Figure 2.5 Wounding response in *Drosophila melanogaster* measured as cuticle melanisation (grey value) following cold exposure.** Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold and pierced in the thorax with an insect pin following recovery from cold. Grey value represents 255-grey value such that increasing numbers indicate darker values. n = 10 per treatment, per time point (see Table 2.1 for statistics).



**Figure 2.6 *In vivo* clearance of *Escherichia coli* and *Bacillus subtilis* from the haemolymph of *Drosophila melanogaster* following cold exposure.** Flies were exposed to acute ( $-2^{\circ}\text{C}$ , 2 h) or sustained ( $-0.5^{\circ}\text{C}$ , 10 h) cold and injected with a suspension of bacteria 2 h or 6 h following recovery from cold. The proportion of bacteria cleared from the hemolymph was assessed either 5 h or 12 h following injection; (n=4-9/treatment/infection). Lower-case letters indicate significant differences ( $p < 0.05$ ) between acute cold-exposed, sustained cold-exposed, and control flies (see Table 2.1 for statistics).

Instead, we suggest that acute cold led to increased cold shock injury [e.g. tissue damage associated with apoptosis (Yi et al., 2007) or membrane phase transitions (Lee et al., 2006)] relative to the sustained exposure, which signaled for an increase in potential immunity via direct signals from apoptotic or necrotic cells (Maltzinger, 1998). For example, repeated cold exposure both increases tissue damage as well as resistance to fungal infection in *Pyrrharctia isabella*, (Marshall and Sinclair, 2011), suggesting that cold-induced immune activity is linked to cold injury. Because hemocytes are responsible for the phagocytosis of apoptotic and damaged cells (Marmaras and Lampropoulou, 2009) and increase in circulating concentration following tissue damage (Pastor-Pareja et al., 2008), increased CHC following acute cold could represent a direct immune response to chilling injury.

Although acute cold exposure increased some measures of potential immunity, flies also displayed a reduced ability to clear Gram-positive bacteria, *B. subtilis*, shortly after acute cold exposure (2 h). This contrast between potential and realised responses suggests that the increase in CHC and AMPs is concomitant with impairment of the immune system. If chilling injury includes damage to the hemocytes themselves, the circulating hemocytes we counted may include both functional and damaged cells (Yi and Lee, 2003); thus although more cells appear to be in circulation, immune function is either impaired or unchanged. Additionally, recovery from chilling injury is metabolically costly (MacMillan et al., 2012), as are immune responses (Freitak et al., 2003), and the initial decrease in bacterial clearance after acute cold could represent a trade-off between recovery from cold exposure and immune activity. For example, infected *Drosophila* take longer to recover from cold exposure (Linderman et al., 2012), suggesting that there is a conflict between the response to cold and the response to infection. In either the case of direct damage or trade-offs between immunity and recovery from cold, cold exposure appears to have the potential to impair immune activity.

#### 2.4.2 Increased immunity is a compensatory response

Despite this initial impairment of immunity, activity appeared to have recovered by 6 h post cold exposure, we did not observe an impaired response to Gram-negative *E. coli*,

and nor was there a change in survival of fungal infection. The immune response (including rate of phagocytosis, the role of hemocytes, and the timing of expression of AMPs) varies depending on the type of pathogen encountered (Lemaitre and Hoffmann, 2007; Marmaras and Lampropoulou, 2009), thus it is possible that the mechanism for clearing *B. subtilis* is more affected by chilling injury than the mechanisms for responding to *E. coli* or *M. anisopliae*. Alternatively, up-regulation of *diptericin* through the IMD pathway (largely responsible for the response to Gram-negative bacteria) may have compensated for impaired immunity and allowed the flies to maintain clearance of Gram-negative *E. coli*. Similarly, recovery of the response to *B. subtilis* suggests that the immune response compensates for this initial, cold-induced impairment of activity.

I propose that the increase in potential immunity that we observed following acute cold exposure is a compensatory response to immune impairment through direct damage to the immune system or trades-off with the response to cold. This compensatory response may manifest as reconfiguration of the immune system (Adamo, 2014), that allows *Drosophila* to maintain responses to cold-induced tissue damage or pathogens in the environment. Overall, this suggests that cold-induced increases in immune activity are adaptive, and that it is important to maintain the ability to respond to pathogens following cold exposure. It will next be important to determine if conflicts between cold and immunity are present in other insect taxa, if they vary seasonally, and whether they affect the fitness of overwintering insects experiencing both cold and pathogen stress.

## 2.5 Conclusions

In conclusion, I suggest that *Drosophila* have evolved immune activation by cold to maintain the ability to respond to immune challenge to compensate for cold-induced damage to, or trade-offs with, the immune system, but that this activation does not result in an increase in realised activity. In the context of multiple stressors, this therefore demonstrates an evolved resilience to the direct effect of an abiotic stressor on the ability to respond to pathogen stress, and provides (another) warning about the importance of comparing multiple measures of the immunity before drawing conclusions (Adamo, 2004).



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## Chapter 3

### 3 Paradoxical acclimation responses in the thermal performance of insect immunity

In this chapter I determine that the insect immune system can acclimate to low temperatures, but does so in a paradoxical way such that activity becomes specialised to higher temperatures. This chapter is re-formatted (with permission) from the original version published in *Oecologia*<sup>2</sup> to suit the thesis style.

#### 3.1 Introduction

Ectotherms can respond to seasonal changes by maintaining homeostasis via phenotypic or developmental plasticity. In insects, the cues that govern acclimation (in the laboratory) or acclimatisation (in the field), such as temperature or photoperiod, often coordinate diverse physiological adjustments to suit a new set of environmental conditions (Harrison et al., 2012). For example, cold-acclimation of the beetle *Dendroides canadensis* elicits antifreeze protein synthesis, removal of ice nucleators (Olsen and Duman, 1997), and modification of epicuticular waxes (Olsen et al., 1998) - all of which contribute to improved cold tolerance. In addition to cold, there are multiple abiotic and biotic stressors associated with winter (Williams et al., 2015), and these can select for thermal plasticity in multiple physiological systems. For instance, cold-acclimation often increases desiccation resistance in insects, likely because of the high water vapour deficits experienced during overwintering (Sinclair et al., 2013; Terblanche et al., 2005). Biotic stressors, such as pathogens, are also likely to affect the success of insects at low temperatures (Hokkanen, 1992; Riedel and Steenberg, 1998; Webberley and Hurst, 2002; Williams et al., 2015), yet the ability of insects to manage cold-related pathogen stress, and the role of phenotypic plasticity in this response, is unclear.

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<sup>2</sup> Ferguson, L.V., Heinrichs, D.E., Sinclair, B.J., 2016. Paradoxical acclimation responses in the thermal performance of insect immunity. *Oecologia* 181, 77-85.

### 3.1.1 The relationship between cold and immunity

Some insect pathogens are cold-active [e.g. fungi in the genera *Beauveria* and *Metarhizium* (Fernandes et al., 2008)], or have increased virulence at low temperatures [e.g. the bacterium *Yersinia enterocolitica* (Bresolin et al., 2006)]; thus there is capacity for these cold-adapted pathogens to contribute to mortality of insects at low temperatures (Hokkanen, 1992; Steenberg et al., 1995). However, cold exposure appears to upregulate the insect immune system, which may allow insects to respond to cold-associated pathogen stress (Sinclair et al., 2013). For example, cold exposure increases fungal resistance in *Pyrrharctia isabella* caterpillars (Marshall and Sinclair, 2011) and adult *Drosophila melanogaster* (Le Bourg et al., 2009), and upregulates genes encoding antimicrobial peptides in both *D. melanogaster* (Zhang et al., 2011) and the solitary bee *Megachile rotundata* (Xu and James, 2012). Further, this increased immunity appears to translate into fitness: water striders (*Aquarius najas*) with stronger immune responses have higher overwinter survival (Krams et al., 2011).

Although cold-induced upregulation of immunity may be a non-adaptive by-product of responses to cold (Fedorka et al., 2013; Sinclair et al., 2013), the potential for conflicts between the energetic costs of immune responses (Ardia et al., 2012; Freitak et al., 2003; Schmid-Hempel, 2003) and energy conservation in the cold (Sinclair, 2015) instead suggest that the immune system is upregulated as an adaptive response to low-temperature pathogen pressure (Irwin and Lee, 2003; Sinclair, 2015; Williams et al., 2012). However, most studies have been performed upon re-warming from cold exposure, and do not necessarily reflect immune activity at low temperatures. Thus, to determine the ability of insects to combat low-temperature pathogen stress, we must first explore the performance of the immune system at low temperatures, as well as the role of acclimation in shaping this performance.

I expect the insect immune system to have reduced performance in the cold because it relies on cellular and enzymatic processes that are likely temperature-sensitive (Collazos et al., 1994; Le Morvan et al., 1998; Marnila et al., 1995; Somero, 1995). Indeed, phagocytosis and encapsulation decrease at 4 °C in diapausing pupae of the giant silk

moth, *Samia cynthia pryeri*, although some immune function is maintained (Nakamura et al., 2011). However, this loss of performance has the potential to be mitigated through phenotypic plasticity, including the expression of cold-active isoforms of immune-related enzymes, or an increase in the concentration of cells and molecules necessary for an immune response (Angilletta, 2009; Somero, 1995). In either case, if there is an adaptive advantage to improved immunity at low temperatures, then cold-acclimation would be expected to modify the thermal performance of the immune system to increase activity at low temperatures (Angilletta, 2009).

### 3.1.2 Objectives

Thus, to understand the potential interactions of cold and immune stress in insects, and to understand the role of biotic interactions in shaping ectotherm performance in a changing climate, it is necessary to explore the thermal biology of the insect immune system. My objectives were to determine 1) if the immune system is thermally plastic; and 2) to determine if cold acclimation produces similar responses among physiological system (i.e. those that govern the response to temperature and the immune system). I explored the thermal sensitivity and plasticity of the insect immune system by measuring the thermal performance of immune-related enzymes *in vitro*, and immune responses *in vivo* [i.e. potential immunity and realised immunity, respectively (Fedorka et al., 2007; Gershman, 2008)] in warm- and cold-acclimated spring field crickets (*Gryllus veletis*). I used a short-term acclimation to explore the possibility of thermal plasticity in the immune system, as a first step in understanding how the thermal biology of the immune system might impact the ecology of overwintering insects. Cold acclimation differentially affected realised and potential immunity, in a direction that is not predicted by the whole-organism response to cold acclimation. I suggest that pathogen stress may be most prevalent upon re-warming from cold, and conclude that divergent thermal performance of distinct stress responses must be considered when predicting ectotherms' responses to climate change.

## 3.2 Material and Methods

I studied the thermal biology of the immune system in the spring field cricket, *Gryllus veletis*. *Gryllus veletis* overwinters as a late-instar nymph in temperate North America (Alexander, 1968), and has a cold acclimation response (Coello Alvarado et al. 2015). Our cricket colony was derived from a population collected in Lethbridge, Alberta, in 2010, and were reared from egg to nymph at 25 °C (14 L:10 D) as described by Coello Alvarado et al. (2015). I haphazardly assigned female 6th instar nymphs (the overwintering stage) into individual 180 mL clear plastic cups and provided *ad libitum* rabbit chow (Little Friends Rabbit Food, Martin Mills, Elmira, ON, Canada) and water, with cardboard shelters. I cold-acclimated (CA) individuals at 6 °C on a short light cycle (10 L: 14 D) or maintained them at rearing conditions (warm-acclimated, WA) for 7 d.

### 3.2.1 Thermal limits of locomotor activity

I measured the critical thermal minimum ( $CT_{min}$ , the temperature at which an insect enters chill coma) following MacMillan and Sinclair (2011), and chill-coma recovery (CCR) time following MacMillan et al. (2012). Briefly, I cooled crickets at 0.25 °C/min from 22 °C to the temperature at which movement ceased. Similarly, I measured the critical thermal maximum ( $CT_{max}$ ) by increasing temperature at 0.25 °C/min from 22 °C until I visually observed the onset of spasms (Lutterschmidt and Hutchison, 1997). For CCR, I cooled crickets at 0.25 °C/min from 22 °C to -2 °C and held them at -2 °C for 72 h. Crickets were returned to room temperature and I recorded the time taken to achieve a coordinated righting response.

### 3.2.2 Potential Immune Response

I collected haemolymph for measuring potential humoral immunity following Adamo (2004), and all measurements of potential immunity at different temperatures were tested on extracted hemolymph. I pierced the membrane under the pronotum and collected 2 µL of haemolymph with a micropipette. I mixed 2 µL of haemolymph with either 2 µL of anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 6.8; for lysozyme activity) or 50 µL of phosphate-buffered saline [PBS; for

phenoloxidase (PO) activity] and snap-froze it in liquid nitrogen, followed by storage at -80 °C.

To estimate the bactericidal activity of lysozyme, I followed Vilcinskas and Matha (1997), with some modifications. I added 4 µL of the haemolymph-anticoagulant-buffer suspension to 2 mm diameter wells on a petri plate containing *Micrococcus lysodeikticus* (*luteus*) agar (1 % agar; 67 mM potassium phosphate, pH 6.4, 0.1 mg/mL streptomycin sulfate; 5 mg/ml *M. lysodeikticus*). Plates were sealed with Parafilm® and incubated at one of 0, 6, 12, 18, 25 or 30 °C (MIR-153 incubators, Sanyo Scientific, Bensenville, IL, US). I then measured the area of the cleared region around each well 24 and 48 h later using NIS Elements Imaging Software (Nikon Instruments Inc, Melville, NY, USA).

Total PO activity, which indicates a potential broad-spectrum immune response, was measured spectrophotometrically (Adamo, 2004). Briefly, I mixed 25 µL of thawed hemolymph/PBS mixture with 70 µL alpha-chymotrypsin (1.3 mg/ mL in PBS) and incubated it for 20 min at room temperature (22 °C) before adding it to 0.9 mL of L-DOPA (4 mg/mL in PBS). The rate of increase in absorbance was measured over 60 min at one of 6, 12, 18, 25, or 30 °C (Carey 100 Spectrophotometer with Peltier-effect Temperature Controller, Agilent, Santa Clara, CA, USA).

Higher haemolymph protein concentration is linked to stronger immune responses in insects, especially the melanisation response (Adamo, 2004). To measure haemolymph protein concentration, I followed methods as described by Adamo (2004) with some modifications. Briefly, I used 10 µL of the haemolymph/PBS mixture in a Bicinchoninic Acid kit (BCA; Life Technologies, Carlsbad, CA, USA) and measured absorbance at 562 nm in a microplate spectrophotometer (SpectraMax, Molecular Devices, Sunnyvale, CA, USA). I then converted absorbance to concentration values using a standard curve created from bovine serum albumin.

### 3.2.3 Realised Immune Response

As an estimate of a broad-spectrum, realised, immune response, I measured melanisation by inserting a 2 mm piece of nylon filament (Krams et al., 2011) under the pronotum and



placing crickets at 0, 6, 12, 18, 25 or 30 °C for 12 h. I removed the filament after 12 h, photographed it from two different angles at 30× magnification using a Nikon DSFI1 camera (Nikon Instruments Inc. Melville, NY, USA) attached to a stereomicroscope, and determined the darkness of each filament using the average grey value calculated in ImageJ (Rasband, 1997). I calculated relative melanisation as 255 - the grey value, such that a higher number indicates more melanisation.

I measured the *in vivo* ability of crickets to clear *Staphylococcus aureus* (strain Newman with chromosomally-encoded tetracycline resistance) from haemolymph following (Haine et al., 2008). Briefly, I diluted *S. aureus* (grown overnight at 37 °C in tryptic soy broth) to  $1 \times 10^9$  CFU/mL in PBS and injected 2 µL of suspension into the thorax under the pronotum (Adamo, 2004) via a 30 G needle. Following 24 h post-challenge at 0.5, 4, 12, 18, 25, or 30 °C, I homogenised whole crickets in 900 µL of PBS to ensure that I captured all remaining bacteria (including those associated with tissue). I diluted and spotted the homogenate on tryptic soy agar (TSA) containing 2 µg/mL tetracycline and averaged the number of CFU over four replicate spots, following 24 h at 37 °C. I homogenised a subset of crickets 1 min following injection to obtain the true number of CFU injected and calculate percent clearance (Haine et al., 2008). Control crickets injected with sterile PBS did not demonstrate any bacterial growth on TSA containing tetracycline.

### 3.2.4 Statistical analyses

All analyses were performed in R v3.1.2 (R Development Core Team, 2010) and preliminary data exploration was conducted according to (Zuur et al., 2010). I compared  $CT_{min}$ ,  $CT_{max}$ , CCR, and protein concentration between CA and WA crickets using Welch's two-sample t-test. I compared the performance curves of immune activity between WA and CA crickets using ANOVA (Angilletta, 2006). Where necessary, response variables were square-root- (lysozyme, phenoloxidase), arcsine- (bacterial clearance), or log-transformed (melanisation) to satisfy the assumptions of the ANOVA. I assessed the assumptions of ANOVA by plotting residuals against fitted values to confirm homogeneity of variance, and standardised residuals against theoretical quantiles to

assess normality (Crawley, 2007). I used polynomial contrasts (Lenth, 2013) to compare means between warm and cold-acclimated crickets at each temperature.

## 3.3 Results

### 3.3.1 Thermal limits of locomotor activity

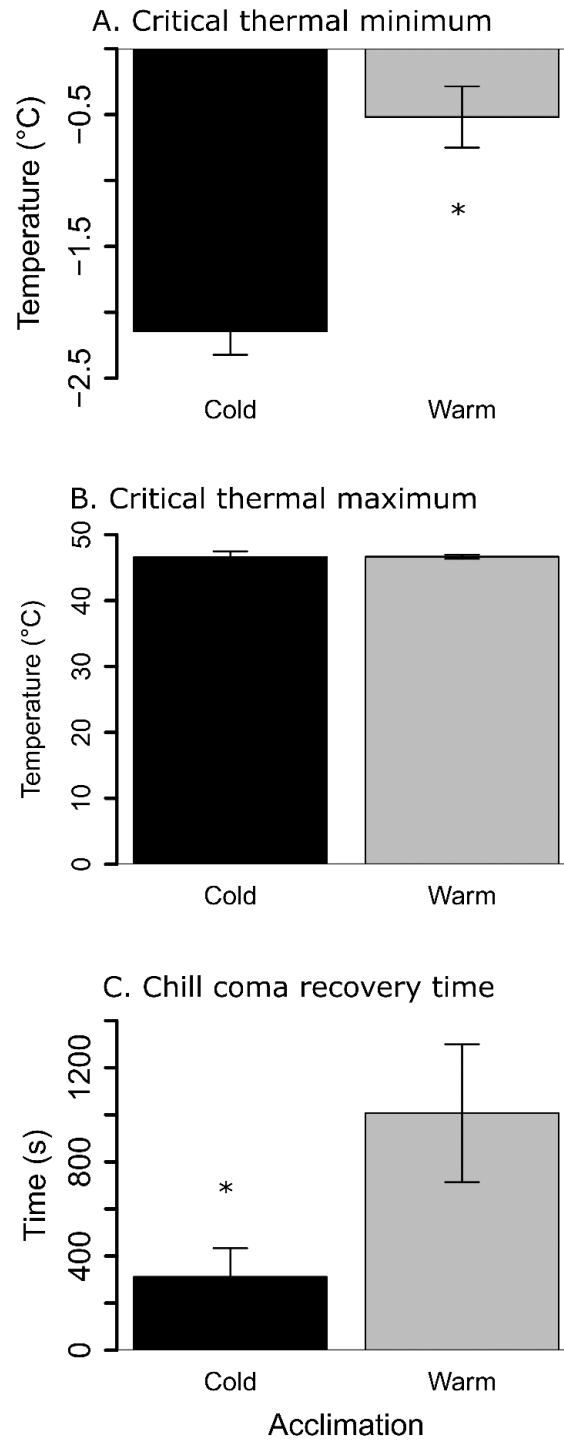
Cold-acclimation enhanced low temperature locomotor activity of *G. veletis* and shortened recovery time after cold exposure. The  $CT_{min}$  of WA crickets was significantly higher than that of CA crickets ( $t_{14.46} = 5.53$ ,  $p < 0.001$ ; Fig. 3.1A); however, there was no difference between the  $CT_{max}$  of WA and CA crickets (Fig. 3.1B;  $t_5 = 0.11$ ,  $p = 0.45$ ). Chill coma recovery time was lower in CA crickets than WA crickets (Fig. 3.1C;  $t_{5.34} = 2.19$ ,  $p = 0.04$ ).

### 3.3.2 Potential Immune Response

Potential humoral immunity was sensitive to temperature but remained unaffected by acclimation (Table 3.1). Specifically, both lysozyme and phenoloxidase activities decreased with decreasing temperature in both WA and CA crickets (Fig. 3.2); however, there was no overall difference in the activity of either enzyme in WA compared to CA crickets (Table 3.1). Haemolymph protein concentration of CA and WA crickets did not differ significantly ( $t_{22} = 0.59$ ,  $p = 0.28$ ).

### 3.3.3 Realised Immune Response

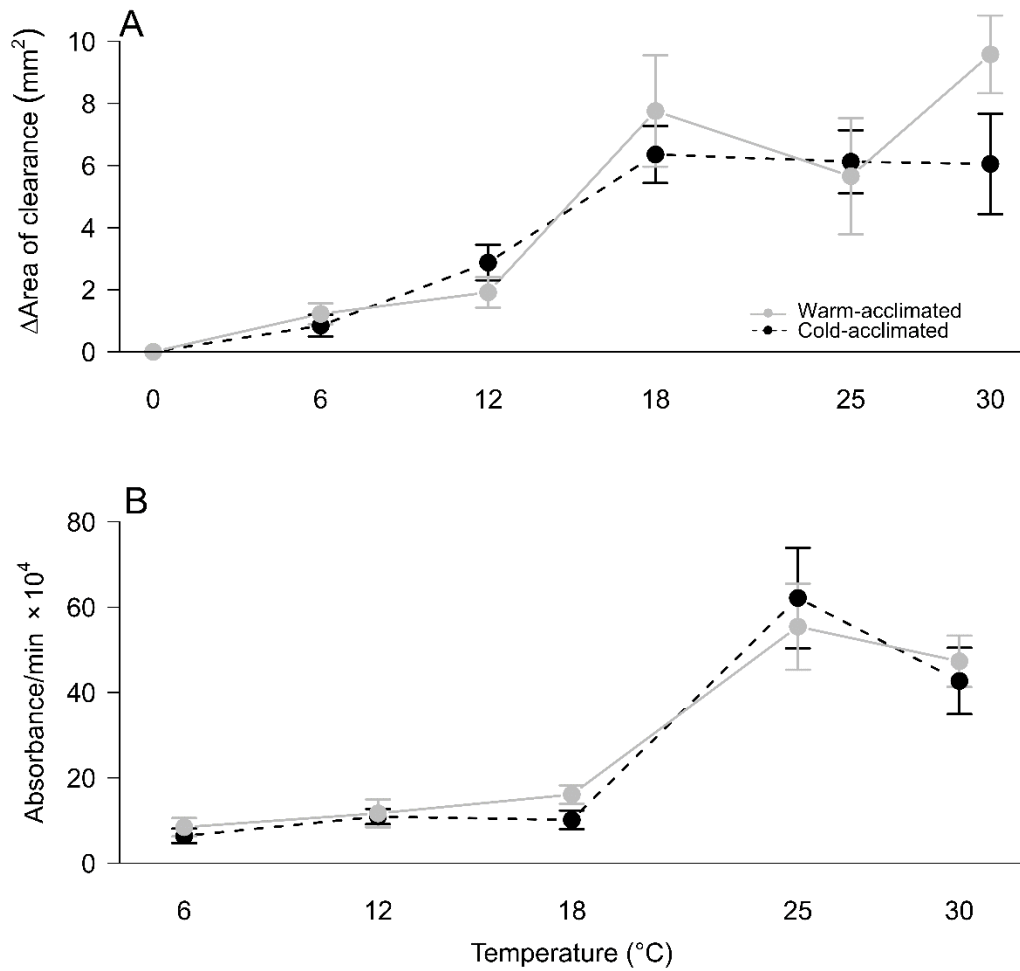
Temperature also significantly affected realised immunity, including both bacterial clearance and melanisation (Table 3.1). In addition, and in contrast to potential immunity, acclimation had a significant effect on realised immunity (Table 3.1). Specifically, melanisation and bacterial clearance were decreased in CA crickets at low temperatures, but largely unchanged at warm temperatures (Fig. 3.3).



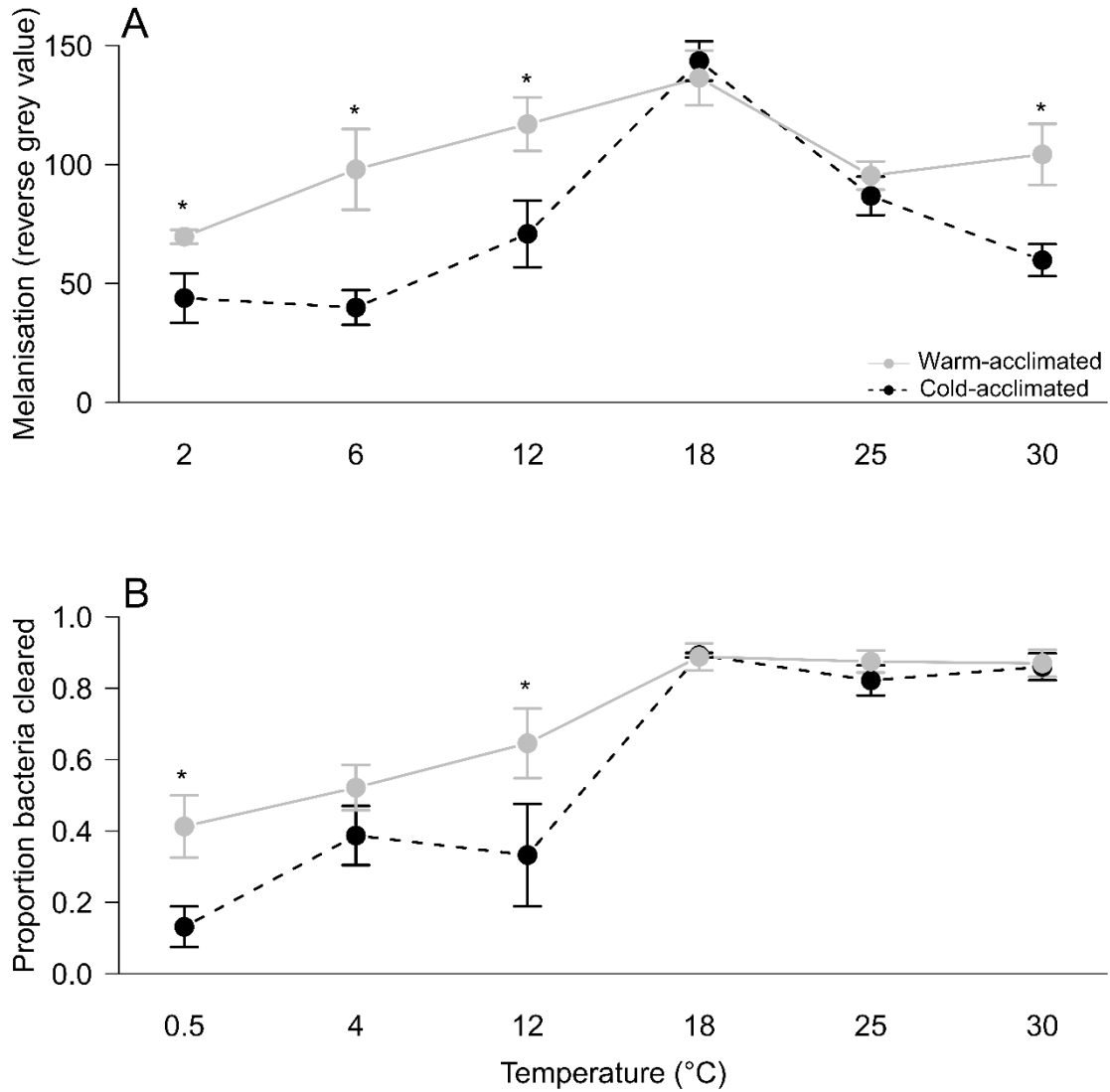
**Figure 3.1 Locomotor activity related to cold tolerance in warm- and cold-acclimated *Gryllus veletis*.** (A) The critical thermal minima, or the temperatures at which crickets entered chill coma (n = 9 WA, 8 CA). (B) The critical thermal maxima, or temperatures indicating the onset of heat spasms (n = 6 WA, 5 CA). (C) Time taken to recover from 72 h in chill coma at -2 °C (n = 5 per acclimation)

**Table 3.1 ANOVA results of the thermal performance of immune activity in warm- and cold-acclimated *Gryllus veletis*.** Bolded p-values represent significant effects of each term (acclimation or temperature) on the response variable.

<b>Immune activity</b>		<b>Term</b>	<b>df</b>	<b>F</b>	<b>P</b>
<b>Potential</b>	Lysozyme	Temperature	5	36.22	<b>&lt;0.01</b>
		Acclimation	1	0.45	0.50
		Temperature × Acclimation	5	1.24	0.30
	Phenoloxidase	Temperature	4	27.1	<b>&lt;0.01</b>
		Acclimation	1	0.50	0.48
		Temperature × Acclimation	4	0.34	0.85
<b>Realised</b>	Bacterial clearance	Temperature	5	15.49	<b>&lt;0.01</b>
		Acclimation	1	10.45	<b>&lt;0.01</b>
		Temperature × Acclimation	5	1.31	0.27
	Melanisation	Temperature	5	9.98	<b>&lt;0.01</b>
		Acclimation	1	30.6	<b>&lt;0.01</b>
		Temperature × Acclimation	5	2.62	<b>0.04</b>



**Figure 3.2 Potential immune activity in warm- and cold-acclimated *Gryllus veletis*.** (A) Lysozyme activity *in vitro*, measured as the zone of inhibition of *Micrococcus luteus* from 24 h - 48 h (n = 6-8 per acclimation, per temperature) (B) Total phenoloxidase activity measured *in vitro* as an increase in absorbance at 495 nm (n = 4-5 per acclimation, per temperature). Points represent mean  $\pm$  SEM



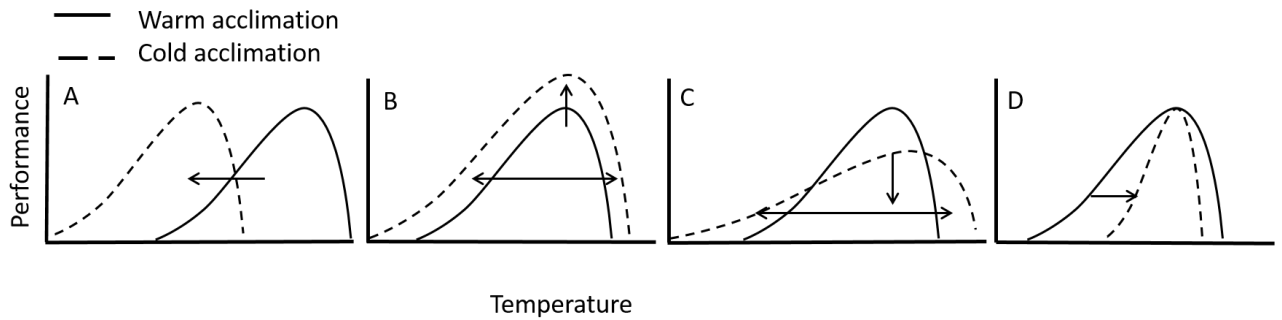
**Figure 3.3 Realised immune activity in warm- and cold-acclimated *Gryllus veletis*.** (A) Melanisation, represented as a reverse grey value, of an implanted nylon filament (n = 4-5 per acclimation, per temperature) (B) The proportion of *Staphylococcus aureus* cleared from the haemolymph *in vivo*, 24 h following inoculation (n = 4-8 per acclimation, per temperature). Points represent mean ± SEM. Asterisks indicate significant differences between warm- and cold-acclimated crickets, p < 0.05)

## 3.4 Discussion

I explored the capacity for cold-acclimation of the immune system in *G. veletis* using an acclimation regime that improved locomotor activity at low temperatures [decreased  $CT_{\min}$  and CCR, recognised proxies for cold tolerance in insects (Andersen et al., 2015)], but had no effect on heat tolerance ( $CT_{\max}$ ). Cold acclimation did not affect potential immunity nor realised immunity at higher temperatures; however, realised immunity decreased in the cold in CA crickets. I suggest that decreased activity in the cold may result from trade-off between some components of immune activity and other physiological responses initiated by cold acclimation.

### 3.4.1 Cold acclimation paradoxically narrows thermal performance of immunity

Theory suggests three ways by which the thermal performance of immunity could shift in response to acclimation, if increased low temperature performance were important in the cold: 1) a shift in the thermal performance curve (TPC) where  $T_{\text{opt}}$  decreases (i.e. beneficial acclimation; Fig. 3.4A); 2) a shift in the TPC where  $T_{\text{opt}}$  is unchanged but maximal activity is higher across all temperatures (i.e. colder is better; Fig. 3.4B); or 3) a reduction in thermal sensitivity, whereby the TPC encompasses a larger range of activity, but maximal activity at the  $T_{\text{opt}}$  is lower [i.e. generalist vs. specialist; Fig. 3.4C (Angilletta, 2009)]. However, I found that cold-acclimation resulted in a paradoxical narrowing of the TPC of realised immune responses in crickets, whereby activity was specialised to higher instead of lower temperatures, and the  $T_{\text{opt}}$  and maximal activity at the  $T_{\text{opt}}$  were unaffected (Fig. 3.3, 3.4D). Decreased performance in the cold may result from trade-offs between the increased energy demands of improving cold tolerance (e.g. production of cryoprotectants) and the immune system (Salehipour-Shirazi et al., 2016). For example, infection decreases CCR time in *Drosophila melanogaster* (Linderman et al., 2012), suggesting that immune activity conflicts with cold tolerance. Thus, cold acclimation may preferentially improve cold tolerance over whole-animal immune performance at low temperatures.



**Figure 3.4 Thermal performance curves of the potential outcomes of cold-acclimation on immune activity.** A) The Beneficial Acclimation Hypothesis; B) The Colder is Better Hypothesis; C) The Generalist-Specialist hypothesis; D) Paradoxical narrowing of the TPC, representing specialisation of activity to temperatures not predicted by acclimation temperature



### 3.4.2 Thermal performance is mismatched within the immune system

In addition to a decrease in realised immunity, cold acclimation produced mismatches between potential and realised immunity. First, acclimation appeared only to decrease realised immune responses in the cold, while potential immunity remained unchanged. Realised immune responses, such as bacterial clearance, are generally mediated by the combined activity of hemocytes, enzymes, and antimicrobial peptides (Gillespie and Kanost, 1997), while the potential immunity that I measured focused on the activity of enzymes in isolation. Decreased realised immune responses that are not paralleled in potential responses suggest that cold acclimation has a stronger effect on cellular activity than it does on the activity of enzymes or antimicrobial peptides, although I caution that I did not measure all components of the immune system. Differences in potential and realised immune activity can create a disparate estimate of overall immunocompetence (Fedorka et al., 2007), yet also hint at the relative contributions of different immune components to protection against pathogens. In this case, overall immunocompetence may decrease in the cold (realised immunity), yet a basal level of protection may persist through the activity of enzymes and antimicrobial peptides (potential immunity). I suggest that measuring multiple components of the immune system provides a more comprehensive picture of the effects of thermal acclimation on immune performance, and that both potential and realised responses should be considered when assessing the impact of the abiotic environment on immunity.

Second, although PO activity and melanisation are linked as an immune response (González-Santoyo and Córdoba-Aguilar, 2012) their thermal optima were disparate; PO activity peaked at 25 °C, whereas melanisation peaked at 18 °C, in both CA and WA crickets [similar to phagocytic capacity in mosquitoes (Murdock et al., 2012)]. The lower  $T_{opt}$  of melanisation compared to that of PO activity appears to reflect a disconnect between the  $T_{opt}$ , or thermal sensitivity, of different components of the overall melanisation response. Temperature is likely to drive the local adaptation of hosts and pathogens (Sternberg and Thomas, 2014) and may have driven the selection of *G. veletis* immune performance to a thermal optimum lower than enzyme activity would predict. Thus, using thermal performance curves, we may gain insight into the evolution of

thermal sensitivity and plasticity of immune activity, and can begin to predict the capacity for hosts to respond to pathogens under climate change scenarios.

### 3.4.3 The consequences of thermal plasticity of immunity in the cold

Growth rates of many pathogens increase as temperatures increase (Harvell et al., 2002); thus, re-warming from cold exposure is likely to lead to an increase in pathogen pressure, and require an increase in immune activity. Despite decreased immune activity in the cold, realised immune activity in CA crickets was maintained at optimal temperatures, which suggests that immune activity may be required following, but not during, cold exposure. Seasonal immune activity in other ectotherms, including fish [e.g. *Sparus aurata* (Tort et al., 1998)] and frogs [e.g. *Rana pipiens* (Maniero and Carey, 1997)], follows a pattern that reflects the effects of a short-term acclimation on immune activity in crickets; specifically, immune activity decreases during the winter but rapidly recovers, and even increases beyond control levels, upon re-warming. In hibernators, such as the golden-mantled ground squirrel (*Spermophilus lateralis*), interbout euthermia is accompanied by an increase in immune activity, thereby allowing the animal to combat pathogens that have established in the cold (Prendergast et al., 2002). The thermal performance of immune activity in *G. veletis* following a short acclimation to low temperatures may thus reflect a seasonal pattern of immune activity in insects that fluctuates with seasonal shifts in pathogen pressure.

The contrast between decreased immune activity in the cold and maintained immune activity at high temperatures suggests that fluctuating temperatures will affect the ability of cold-acclimated insects to fight cold-active pathogens and survive at low temperatures. Transient increases in environmental temperature may facilitate a response to cold-active pathogens by allowing for increased immune activity. For example, the expression of genes encoding immune peptides in *M. rotundata* increases under warming provided by fluctuating thermal regimes (Torson et al., 2015). Conversely, increased immune activity under periods of re-warming is likely to decrease the energy available for responses to

other stressors, such as cold. Further, immune activity can trade-off with components of fitness, such as growth (Rantala and Roff, 2005) and reproduction (Adamo et al., 2001; Ahmed et al., 2002); thus fluctuations in temperature may create conflict between the response to pathogens and fitness- or stress-related physiological processes. If climate change-related warming leads to an increase in both pathogen pressure and immune activity, both transient and seasonal periods of re-warming will affect the interactions between energy expenditure and pathogen response, thereby contributing to the impacts of cold and winter on insects. I do caution, however, that the acclimation used in our study does not reflect the type of long-term, seasonal acclimation that an insect would experience in preparation for overwintering (Tauber et al., 1986), and thus we are limited in using our results to predict the outcome of insect-pathogen interactions in the wild.

As global temperatures shift with climate change, it is increasingly important to understand the physiological capacity of organisms to respond to changes in their environment (Araújo and Luoto, 2007; Chown et al., 2010). Ecological physiology often quantifies this capacity of ectotherms to respond to environmental change by measuring the thermal sensitivity and plasticity of one trait or system; for example, thermal limits to activity (Terblanche et al., 2008) or reproduction (Cudmore et al., 2010). However, multiple abiotic and biotic pressures co-occur, and we must instead consider what phenotypes are produced by the simultaneous activity of multiple physiological systems in response to these pressures. Increased cold-tolerance, coupled with decreased immune activity at low temperatures in cold-acclimated *G. veletis*, demonstrates that thermal plasticity was disconnected among and within physiological systems; this suggests that plasticity in one trait does not necessarily reflect the response of the whole organism to connected shifts in its abiotic and biotic environment. Thus, to predict the phenotype of an organism that will succeed under climate change scenarios, we must begin to measure multiple physiological traits that correspond to multiple, integrated pressures in a changing environment.

### 3.5 Conclusions

I show that cold acclimation improves cold tolerance in *G. veletis*, does not affect the activity of immune enzymes *in vitro* (potential immunity), and decreases realised immune activity at low temperatures. Thus, measures of whole-animal immune performance appear to trade-off with cold tolerance, and I suggest that pathogen stress may be more prevalent upon re-warming. Climate change will result in alterations to the interactions among multiple stressors, such as between temperature and pathogens (Todgham and Stillman, 2013), and the thermal performance of the responses to these stressors will contribute to success under new environmental conditions. However, I show that thermal performance does not consistently respond to acclimation among – or even within – physiological systems. Therefore, I caution against predicting responses to climate change based on thermal performance of a single physiological system.

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## Chapter 4

### 4. Insect immunity varies idiosyncratically during overwintering

In this chapter I explore seasonal variation in immune activity in three different species of field-collected insects. This chapter is in press in the *Journal of Experimental Zoology-A* as part of a special issue on Ecoimmunology.

#### 4.1 Introduction

In temperate, polar, and alpine regions, insects can spend more than half of their lives overwintering, during which they face multiple environmental stressors, including cold, desiccation, and starvation (Williams et al., 2015). However, little is known of how biotic stressors, such as pathogens, affect insects during the winter, or of how insects respond to these stressors. Pathogens are present in the overwintering environment and responsible for overwintering mortality (Mills, 1981; Steenberg et al., 1995; Steinmann et al., 2015; Webberley and Hurst, 2002), thus insects may require immune activity during the winter. However, insect immune activity may trade-off with the physiological response to cold (Ferguson et al., 2016; Linderman et al., 2012; Sinclair et al., 2013), so thermally-induced changes in immunity could modulate the strength of the immune response, potentially determining pathogen-related overwintering survival. Despite this, we know little of how insect immune activity changes across seasons, especially during overwintering, which makes it difficult to evaluate the importance of immunity in insect seasonal biology.

##### 4.1.1 The relationship between overwintering and immunity

The ways in which insects modify immune activity across seasons are likely to be mediated by interactions between temperature, pathogen prevalence, and energy reserves (Córdoba-Aguilar et al., 2009; Fedorka et al., 2013; Fedorka et al., 2012). Energy conservation is essential for successful overwintering (Sinclair, 2015) and both immune activity and the response to cold are energetically costly (Ardia et al., 2012; MacMillan et al., 2012; Sinclair, 2015). Thus, immunity may trade-off with the requirements for cold

tolerance and energy conservation, leading to decreased immune activity during the winter. Indeed, overwintering honeybees downregulate genes encoding antimicrobial peptides (Steinmann et al., 2015), and damselflies have reduced resistance to bacterial infections during the winter (Córdoba-Aguilar et al., 2009).

Conversely, both cold exposure and diapause induction can activate the immune system in some insects (Le Bourg et al., 2009; Marshall and Sinclair, 2011; Ragland et al., 2010), even in the absence of pathogen infection (Xu and James, 2012; Zhang et al., 2011), perhaps to compensate for trade-offs or cold-induced damage (Salehipour-shiraz et al., 2017). Because of the potential trade-offs associated with increased immunity, this implies that increased immune activity (even if compensatory) may be an adaptive response to overwintering pathogen pressures (Sinclair et al., 2013). To understand how immune activity fluctuates across seasons, and how activity will shift under a changing climate, we can begin by exploring immune activity in different species of insects that will experience different iterations of temperature, pathogen, and energy stress throughout the winter. Depending on the directions of shifts in activity, we can then begin to predict whether changes in immunity are conserved across species, or if immune activity changes based on the types of environmental pressures that an insect will experience across seasons.

#### **4.1.2 The relationship between immunity and temperature**

The insect immune system is largely innate and depends on a variety of cellular and humoral activities. Cellular activity is mediated by hemocytes, which circulate through the haemolymph and are primarily responsible for phagocytosis and encapsulation of pathogens and parasites (Lavine and Strand, 2002). Humoral activity includes that of enzymes - for example, phenoloxidase, whose activity ultimately culminates in the production of melanin and cytotoxic and reactive intermediate products (González-Santoyo and Córdoba-Aguilar, 2012) – as well as antimicrobial peptides (Gillespie and Kanost, 1997). These independent components of the immune system may be differentially-altered by or -responsive to environmental stress, and the immune system

has the capacity to reconfigure itself, such that cellular activity may increase to compensate for impaired humoral activity, or vice-versa (Adamo, 2014). Therefore, because of this capacity for reconfiguration, it is important to measure several components of the immune system, including constitutive or potential activity, as well as inducible defenses and resistance/tolerance to infection [e.g. realised immunity (Fedorka et al., 2007)] to capture shifts in seasonal activity.

The activity of immune cells and enzymes is largely temperature-sensitive and thus will depend directly on temperature (Catalan et al., 2012; Ferguson et al., 2016; Murdock et al., 2012). However, immune activity is also phenotypically-plastic; for example, cold-acclimation depresses immune activity at low temperatures in the cricket *Gryllus veletis*, likely as a response to, or product of trade-offs between cold-tolerance and immunity (Ferguson et al., 2016). Therefore, seasonal acclimatisation may shift the optimal temperature of immune activity, or change the breadth of temperatures at which activity can occur (Angilletta, 2009), and immune activity should be measured at both high and low temperatures in an effort to capture plasticity in the thermal performance of immunity across seasons.

### 4.1.3 Objectives

My objective in this study was to explore seasonal changes in immune activity during overwintering in a range of insects, in an effort to understand how overwintering success is mediated by immune activity. From here, we can begin to understand whether or not we can generalise changes in activity across species. I measured a variety of immune responses that incorporate humoral and/or cellular-mediated activity in both constitutive and realised responses, to determine whether immunity changes wholesale across seasons, or if the immune components are regulated differentially. Further, to capture any seasonal plasticity in the thermal sensitivity of immune activity that might increase or decrease immunocompetence at a given temperature, I measured both constitutive immune activity and realised responses to pathogens at both low (12 °C) and high (25 °C) temperatures.

## 4.2 Methods and materials

### 4.2.1 Study species

I measured immune activity of three different species of insects to detect if seasonal patterns of immunity are generalisable or species-specific. I chose three univoltine species, native to Southwestern Ontario, Canada, for which the overwintering biology (i.e. cold-tolerance strategies, microhabitat) is known: 1) The acorn weevil *Curculio* sp. (Coleoptera: Curculionidae) [derived from the same population that was misidentified as *Curculio glandium* by Udaka & Sinclair (2014)], which overwinters in the soil as freeze-avoidant larvae and emerges in late spring or summer to complete adult development (Udaka and Sinclair, 2014). 2) The goldenrod gall fly, *Eurosta solidaginis* Fitch, 1855 (Diptera: Tephritidae), which overwinters as a diapausing freeze-tolerant larva in a goldenrod stem gall. In mid-winter, *E. solidaginis* terminate diapause, remaining quiescent until temperatures rise in the spring (Irwin et al., 2001). 3) The woolly bear caterpillar, *Pyrrharctia isabella* Smith, 1979 (Lepidoptera: Arctiidae), which overwinters as a diapausing freeze-tolerant larva beneath leaf litter, before higher temperatures in the spring allow completion of development (Goettel and Philogène, 1978; Layne et al., 1999). Further, I manipulated the overwintering microhabitat of *P. isabella* as either above or below snow cover (cf. Marshall and Sinclair, 2012), to determine whether microclimate (and its resultant effects on energy use) can modify immune activity.

### 4.2.2 Insect collection and field housing

I collected red oak (*Quercus rubra*) acorns from deciduous forests on Pelee Island, Ontario, Canada (41°46'N 82°39'W) in October, 2014 and transported the acorn (containing larvae of *Curculio* sp.) to London, Ontario, Canada (42°59'N 81°14'W). I housed acorns in an urban garden over 2 × 2 cm plastic grids in 68 L plastic bins containing moistened terrarium soil (Eco Earth Coconut Fibre Substrate, San Luis Obispo, CA, USA), and collected *Curculio* sp. larvae from the bottom of the bin after they emerged from the acorns and fell through the grid. I housed *Curculio* sp. in groups of five in 35 mL plastic *Drosophila* vials filled with moistened terrarium soil (n = 70

vials). I then immersed vials in soil contained in plastic bins (68 L) buried 10 cm below the soil surface and covered the vials with mesh bags of leaves to provide the thermal barrier offered by leaf litter.

I collected approximately 200 goldenrod galls containing *Eurosta solidaginis* from fields and urban parks in London, Ontario, Canada in late September and early October of 2014, and transported them to the same urban garden as *Curculio sp.* I housed the galls in a mesh bag suspended approximately 1 m above the soil.

I collected *P. isabella* caterpillars (n = 71) from the same locations as *E. solidaginis* and housed individuals in 120 mL plastic containers with moistened terrarium soil and burdock (*Arctium sp.*) leaves as food, collected from the same area in which caterpillars were found. I grouped all containers in one of two 68 L plastic bins, covered the containers with mesh bags of fallen leaves and situated one bin approximately 50 cm above soil level (above snow cover) and one below (below snow cover), such that the caterpillars were level with the soil surface (Marshall and Sinclair, 2012).

Individual containers of insects were haphazardly chosen for collection in October (autumn), November (*P. isabella* only, representing the point at which feeding ceased; *E. solidaginis* and *Curculio sp.* ceased feeding before collection), December (early winter), February (mid-winter), and April (spring). Insects were transported to the lab and immediately used for experiments. I recorded mortality of all species at each retrieval by visually assessing movement and pathogen growth on the insect. I also monitored the temperature of the microhabitats for each species using Hobo Pro V2 temperature loggers (Onset Computers, Bourne, MA, USA).

#### 4.2.3 Constitutive levels of immune activity

I briefly surface-sterilised each insect with 70 % ethanol before haemolymph collection. I pierced the insects with a 32 G needle and collected the haemolymph that welled from the wound. I collected 1  $\mu$ L of haemolymph from individual insects and mixed it with anticoagulant (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 6.8) for haemocyte counts (final volumes: 24  $\mu$ L *P. isabella* 24  $\mu$ L *E. solidaginis*; 20  $\mu$ L

*Curculio* sp.). I collected 2  $\mu\text{L}$  (*Curculio* sp.) or 4  $\mu\text{L}$  (*P. isabella* and *E. solidaginis*) of haemolymph from individual insects and mixed it with either 50  $\mu\text{L}$  (*Curculio* sp.) or 100  $\mu\text{L}$  (*P. isabella* and *E. solidaginis*) of phosphate-buffered saline [PBS; for phenoloxidase (PO) activity] or left it unmixed for humoral antimicrobial activity. I snap-froze samples for PO and antimicrobial activity in liquid nitrogen, and stored them at  $-80\text{ }^{\circ}\text{C}$  until use.

To estimate the circulating haemocyte concentration (CHC), I counted hemocytes in fresh haemolymph in a Neubauer improved hemocytometer (Hausser Scientific, Blue Bell, PA, USA) at  $400\times$  magnification.

I measured baseline (e.g. spontaneously-activated) levels of PO activity spectrophotometrically, following Adamo (2004) with some modifications. I thawed haemolymph mixed with PBS and added 20  $\mu\text{L}$  to 900  $\mu\text{L}$  of 4 mg/mL L-DOPA (Sigma Aldrich, Oakville, ON, Canada) in a plastic cuvette. I measured absorbance over 30 min at 490 nm at both  $12\text{ }^{\circ}\text{C}$  and  $25\text{ }^{\circ}\text{C}$ , for each species and time point (Carey 100 Spectrophotometer with Peltier-effect Temperature Controller, Agilent, Santa Clara, CA, USA), and standardised the change in absorbance during the linear portion of the reaction to a control sample that did not contain hemolymph.

I quantified haemolymph antimicrobial activity, following Haine et al. (2008). Briefly, I diluted 1  $\mu\text{L}$  (*Curculio* sp.) or 2  $\mu\text{L}$  (*P. isabella*, *E. solidaginis*) of haemolymph in 24 or 23  $\mu\text{L}$  of PBS, respectively, and added 1  $\mu\text{L}$  of a suspension (for a final volume of 25  $\mu\text{L}$ ) of streptomycin-resistant *Escherichia coli* (Gram-negative) or *Bacillus subtilis* (Gram-positive) at approximately  $5\times 10^6$  colony forming units (CFU)/mL in PBS. I incubated the haemolymph-bacteria suspensions for 2 h at either  $12\text{ }^{\circ}\text{C}$  or  $25\text{ }^{\circ}\text{C}$  on a shaking plate at 150 rpm. I diluted and spotted the suspensions on lysogeny broth (LB) agar containing 25  $\mu\text{g}/\text{mL}$  streptomycin and averaged the number of CFU over three replicate spots, following 24 h at  $37\text{ }^{\circ}\text{C}$ . I plated a control suspension containing no haemolymph to obtain the average number of CFU added to each suspension, and calculated the proportion of bacteria cleared from each suspension as:  $(\text{CFU remaining in sample})/(\text{CFU added to sample})$ .



#### 4.2.4 Realised immune responses

In *Curculio* sp. I measured the strength of the melanisation response against an imitation parasite by inserting a 2 mm length of nylon filament (0.25 mm diameter) behind the head capsule, towards the posterior end. Due to sample size and logistic constraints, I did not measure melanisation in *P. isabella* or *E. solidaginis*. Following 24 h incubation at either 0 °C, 12 °C, or 25 °C, I removed the filament, allowed it to dry, and photographed two sides of the length of the filament (Krams et al., 2011) at 30 × magnification using a Nikon DSFI1 camera (Nikon Instruments Inc. Melville, NY, USA) attached to a stereomicroscope. The strength of the melanisation response was calculated as the average darkness (grey value, GV), of each filament, using ImageJ (Rasband, 1997), and expressed as 255-GV, such that a higher value indicates increased melanisation.

I infected *E. solidaginis* and *Curculio* sp. with a cold-active strain of *Metarhizium brunneum* (provided by Michael Bidochka, Brock University, ON, Canada). I was unable to obtain enough individuals of *P. isabella* to include them in the fungal infections. I diluted *M. brunneum* spores to  $5 \times 10^7$  spores/mL in 0.01% Tween 80 and briefly dipped each insect in the suspension (De La Rosa et al., 2002). Control insects were dipped in 0.01% Tween 80. I housed insects on moist filter paper in plastic 6-well plates at either 12 °C or 25 °C and assessed mortality daily. Mortality due to *M. brunneum* infection was confirmed by growth of characteristic green spores on the exterior of the insect.

#### 4.2.5 Statistical Analysis

All analyses were performed in R v3.1.2 (R Development Core Team, 2010) and preliminary data exploration was conducted according to Zuur et al. (2010). I compared CHC, PO activity, melanisation, and humoral antimicrobial activity against each species of bacteria across season using ANOVA and detected differences between season or temperatures using Tukey's HSD. To satisfy the assumptions of the ANOVA, I square-root- (CHC for all insects and PO activity of *P. isabella*), log- (PO activity of *Curculio* sp.) or arcsine-square-root-transformed (humoral antimicrobial activity of all insects) response variables. I assessed the assumptions of ANOVA by plotting residuals against fitted values to confirm homogeneity of variance, and standardised residuals against

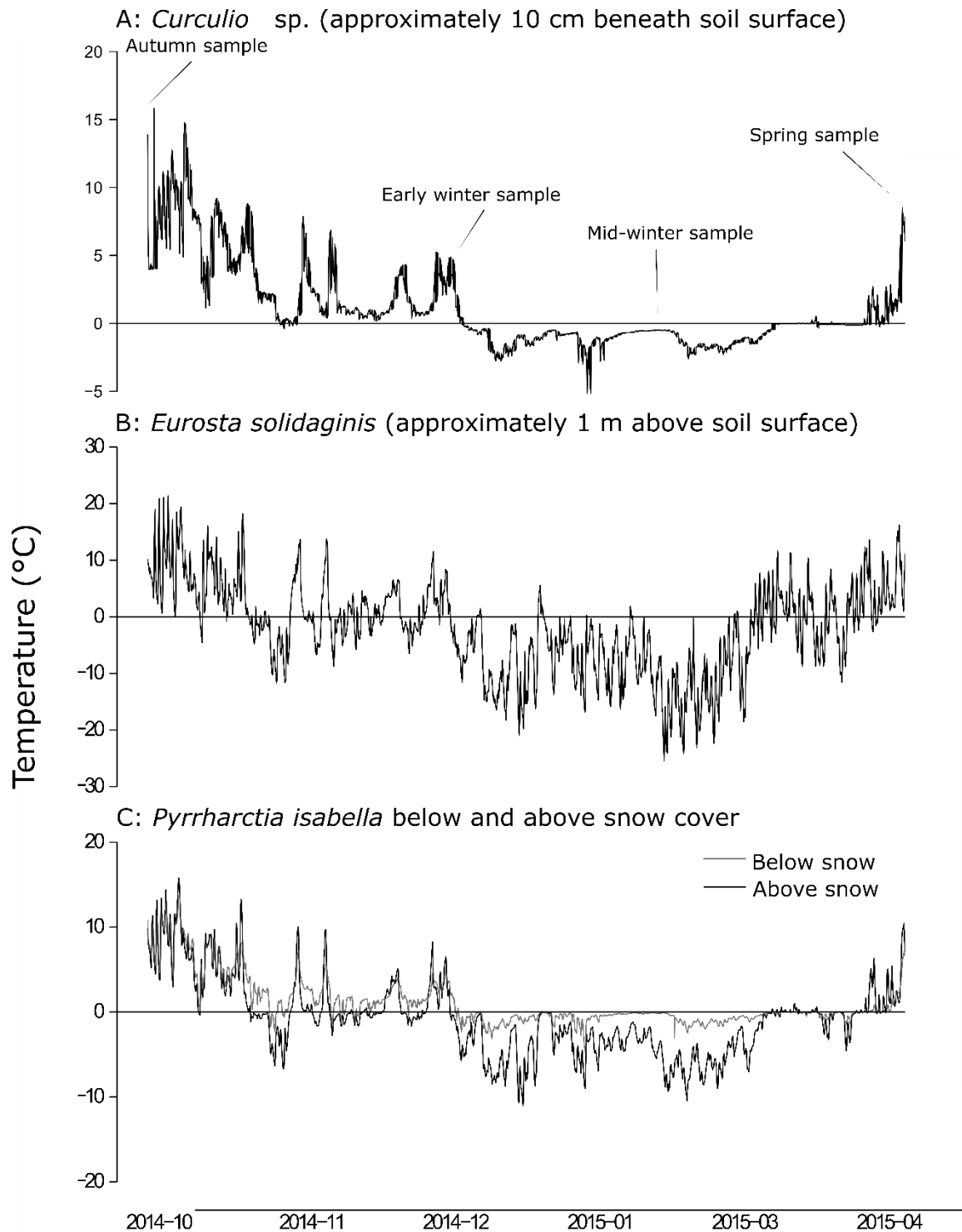
theoretical quantiles to assess normality (Crawley, 2007). I used the ‘survival’ package in R (Therneau and Grambsch, 2015) and a lognormal model (chosen based on log-likelihood values compared among models) to detect differences between survival curves of fungal infections. Due to constraints on survival models when no “event” occurs, when no mortality occurred in a group (e.g. uninfected controls) I did not include these groups in the analysis. For *P. isabella*, November was considered a separate season.

## 4.3 Results

### 4.3.1 Overwintering mortality and temperatures in the field

*Curculio* sp. had high winter mortality: 51 %  $\pm$  5 % (SEM; n = 29 vials) of *Curculio* sp. retrieved in early winter and 74 %,  $\pm$  5 % (SEM; n = 25 vials) retrieved in mid-winter, were dead. Further, due to unforeseen, localised flooding, the majority of weevils were dead (presumably drowned) in the spring. I did not observe any mortality in *P. isabella* during winter; however, 26 % (5/19) of the caterpillars housed above snow cover were dead at the time of the spring collection, compared to 58 % (5/12) of those housed below snow cover. When opening galls throughout the study, I did not observe any dead *E. solidaginis*.

Temperatures below soil (i.e. experienced by *Curculio* sp.) remained buffered throughout the period of snow cover and did not reach the average supercooling point of these insects, thus it is unlikely that they experienced temperatures at which they would freeze (Fig. 4.1A; Udaka and Sinclair, 2014). Temperatures 1 m above soil level were highly variable throughout the winter and *E. solidaginis* were likely to experience both prolonged as well as repeated freezing and thawing (Fig. 4.1B; Baust and Lee, 1981). *Pyrrharctia isabella* below snow cover remained buffered from extreme temperatures and were potentially unfrozen for the winter, based on their average supercooling points in previous years; however, *P. isabella* above snow cover experienced greater variation in temperature and were likely to freeze (possibly repeatedly) throughout the winter (Fig. 4.1C; Marshall and Sinclair, 2011).



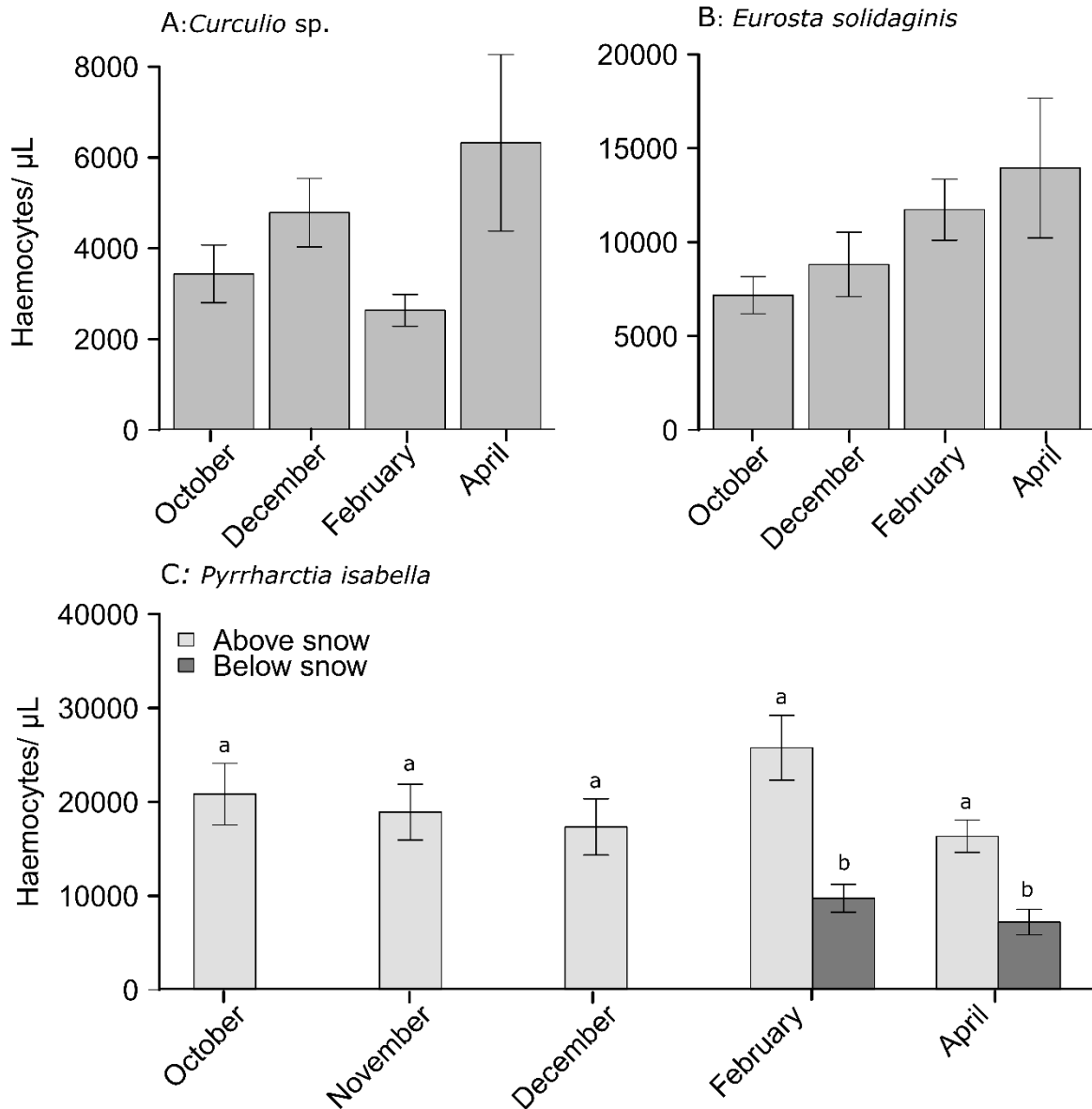
**Figure 4.1 Microhabitat temperatures.** **A.** Temperatures experienced by *Curculio* sp., situated approximately 10 cm beneath soil surface. **B.** Temperatures experienced by *Eurosta solidaginis*, situated approximately 1 m above the soil surface. **C.** Temperatures experienced by *Pyrrharctia isabella* either below (black line) or above (grey line) snow cover.

### 4.3.2 Baseline immune activity

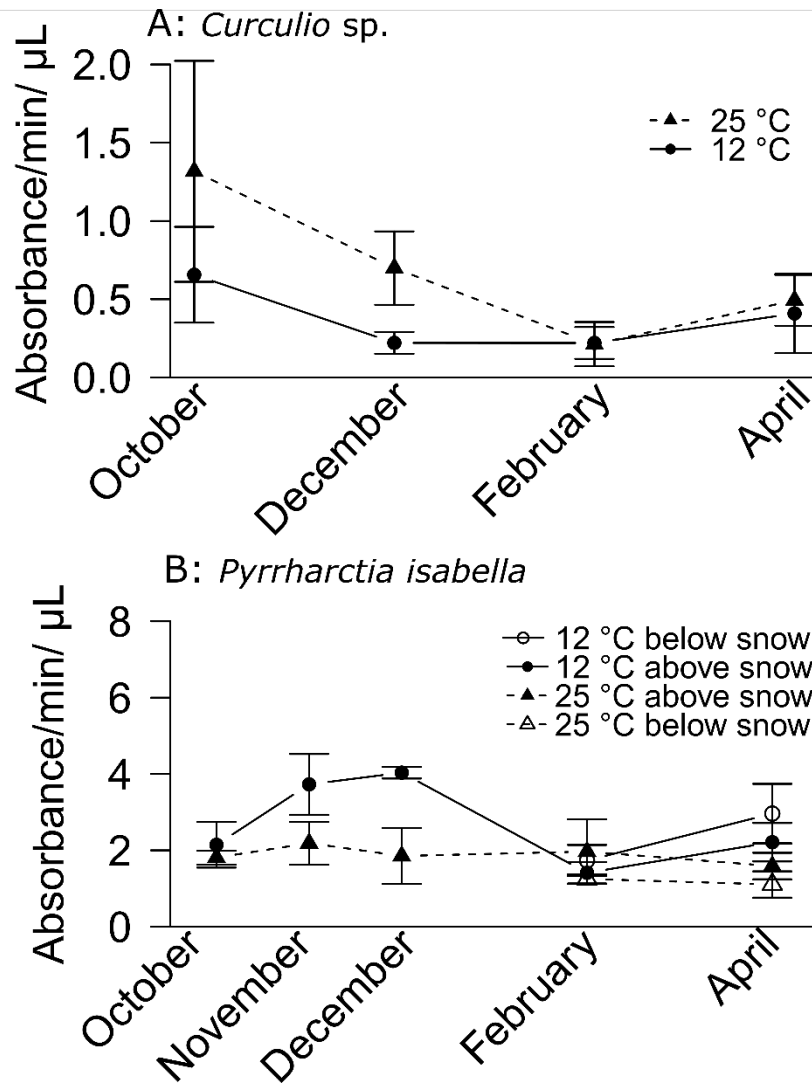
In *Curculio* sp., I did not detect any changes in CHC or PO activity among seasons (Fig. 4.2A:  $F_{3, 17} = 2.32$ ,  $p = 0.11$ ; Fig. 4.3A;  $F_{3, 29} = 0.68$ ,  $p = 0.57$ ) or measurement temperatures (Fig. 4.3A;  $F_{1, 29} = 0.89$ ,  $p = 0.35$ ). Humoral antimicrobial activity was higher at 12 °C than 25 °C against the Gram-negative bacterium, *E. coli* (Fig. 4.4A;  $F_{1,32} = 4.40$ ,  $p = 0.04$ ), although activity remained unchanged across season ( $F_{3,32} = 2.21$ ,  $p = 0.10$ ) and there was no significant interaction between season and temperature ( $F_{3, 32} = 1.11$ ,  $p = 0.36$ ). Conversely, humoral activity against the Gram-positive bacterium, *B. subtilis*, was lowest in autumn (Fig. 4.4A;  $F_{3, 32} = 4.05$ ,  $p = 0.01$ ); however, temperature did not affect clearance ( $F_{1,32} = 0.53$ ,  $p = 0.47$ ). There was no significant interaction between season and temperature ( $F_{3, 32} = 0.19$ ,  $p = 0.90$ ).

I did not detect any seasonal changes in CHC in *E. solidaginis* (Fig. 4.2B;  $F_{3, 16} = 1.56$ ,  $p = 0.24$ ) and was unable to detect any baseline PO activity. Further, I could not detect PO using  $\alpha$ -chymotrypsin to activate the zymogen form of PO (proPO) to PO. Humoral antimicrobial activity by *E. solidaginis* against *E. coli* did not change across seasons (Fig. 4.4B;  $F_{3,31} = 2.01$ ;  $p = 0.13$ ); however, humoral activity against *B. subtilis* was highest in mid-winter and early spring (Fig. 4.4B;  $F_{3,31} = 18.97$ ,  $p < 0.001$ ), and was significantly higher at 12 °C than 25 °C (Fig. 4.4B;  $F_{1,31} = 20.34$ ,  $p < 0.001$ ), although there was no significant interaction between season and temperature ( $F_{1, 31} = 1.92$ ;  $p = 0.15$ ).

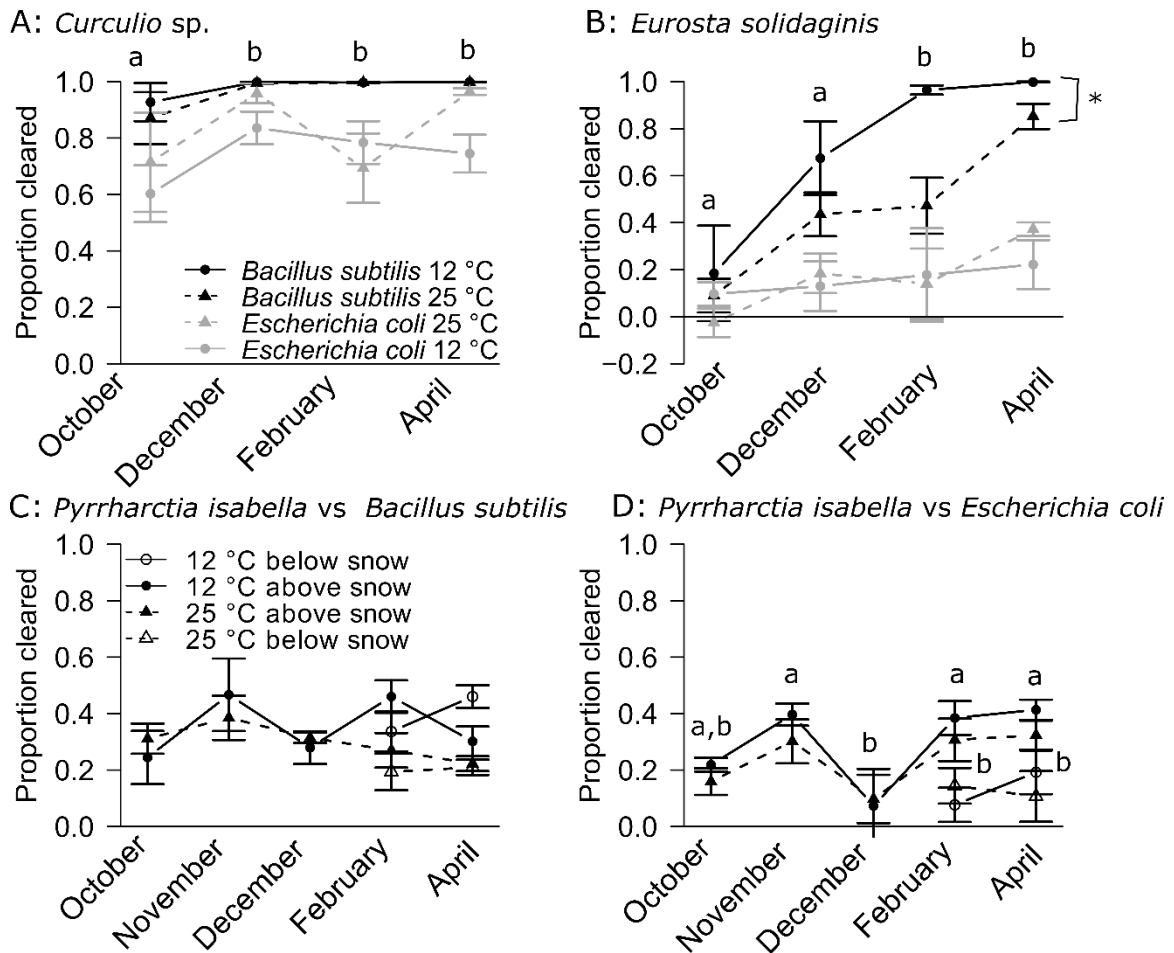
In *P. isabella*, CHC decreased in mid-winter and spring, but only in caterpillars housed beneath snow cover (Fig. 4.2C;  $F_{4, 28} = 6.51$ ,  $p < 0.001$ ). Phenoloxidase activity was higher at 12 °C compared to 25 °C (Fig. 4.3B;  $F_{1,55} = 8.73$ ,  $p < 0.01$ ) but I did not detect a seasonal change in PO activity (Fig. 4.3B;  $F_{4,55} = 2.27$ ,  $p = 0.05$ ). Humoral activity against *B. subtilis* remained unchanged with season ( $F_{4,55} = 1.87$ ,  $p = 0.10$ ), and although I detected a significant effect of temperature on activity ( $F_{1,55} = 7.10$ ,  $p = 0.01$ ) I was unable to find specific significant differences between groups using Tukey's HSD (Fig. 4.4C). However, humoral antimicrobial activity against Gram-negative *E. coli* was lower in *P. isabella* housed beneath snow cover than in those housed above snow cover (Fig. 4.4D;  $F_{4,57} = 6.94$ ,  $p < 0.0001$ ). Antimicrobial activity by *P. isabella* against *E. coli* was unaffected by temperature ( $F_{1,57} = 1.68$ ,  $p = 0.20$ ).



**Figure 4.2 Circulating haemocyte counts across season.** Haemocytes were counted in standardised volumes of haemolymph extracted from three different insects in months throughout autumn (October; November for *Pyrrharctia isabella* only), early winter (December), mid-winter (February), and spring (April). **A.** *Curculio* sp. (n = 5 per month). **B.** *Eurosta solidaginis* (n = 5 per month). **C.** *Pyrrharctia isabella* above and below snow cover (n = 5 per month, per microhabitat). Error bars represent the SEM.



**Figure 4.3 Baseline phenoloxidase activity across seasons** Activity was measured at either 12 °C or 25 °C **A.** *Curculio* sp. (n = 5 per month, per temperature, except n = 4 for October, February, and April at 25 °C) **B.** *Pyrrharctia isabella* (n = 5 per month, per temperature, except n = 6 December, 12 °C; n = 4 October 12 °C & 25 °C, April 12 °C & 25 °C). Error bars represent the SEM.



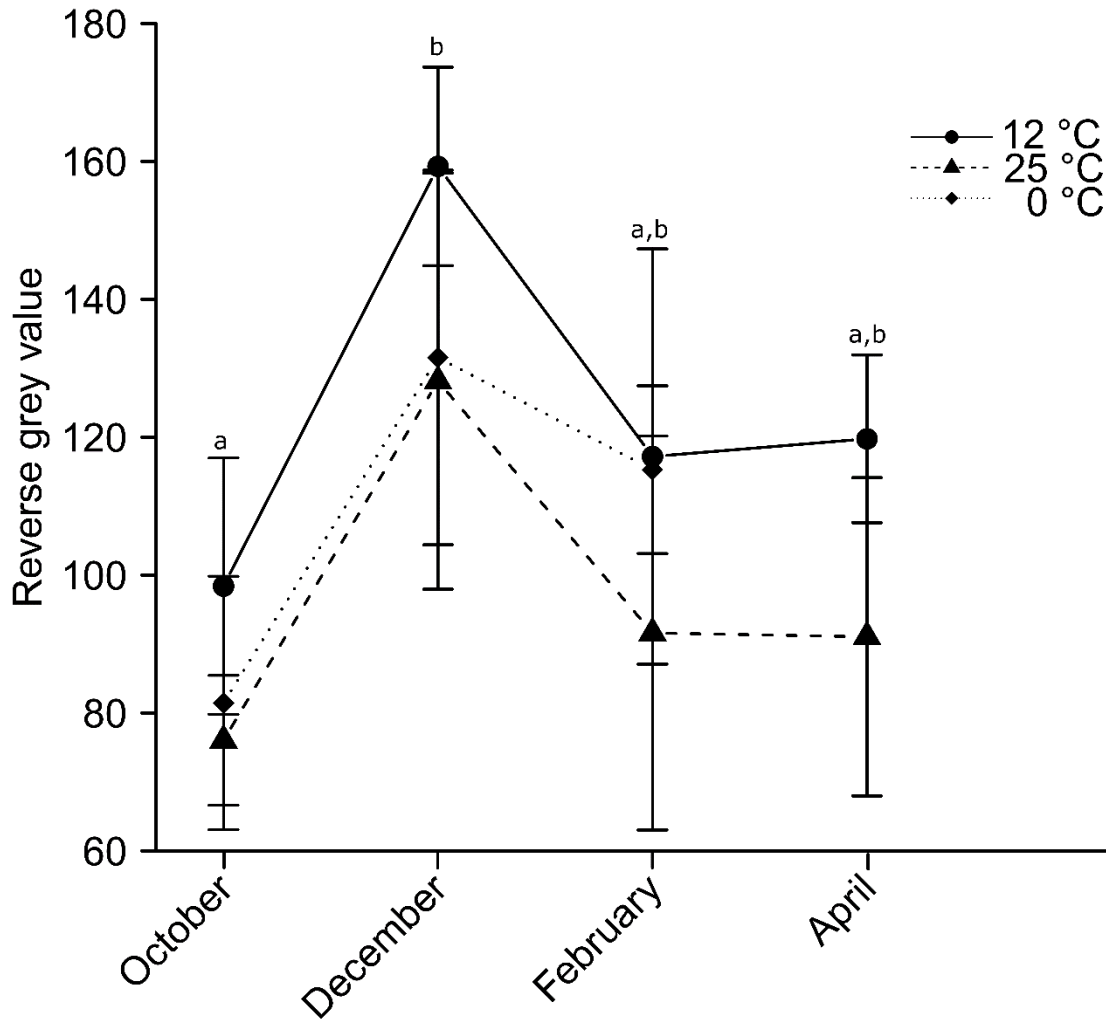
**Figure 4.4 Humoral antimicrobial activity across seasons.** Baseline levels of humoral antimicrobial activity were measured *in vitro* with standardised volumes of haemolymph extracted from three species of insects in months throughout autumn, winter, and spring. Activity was measured at either 12 °C or 25 °C against a Gram-positive (*Bacillus subtilis*) or Gram-negative (*Escherichia coli*) bacteria. Negative activity indicates samples in which bacteria grew, suggesting that any antimicrobial activity present was not sufficient to overcome infection. **A.** *Curculio* sp. (n = 5 per month, per temperature, per bacteria). **B.** *Eurosta solidaginis* (n = 5 per month, per temperature, per bacteria). **C.** Activity of *Pyrrharctia isabella* housed above and below snow cover against *Bacillus subtilis* (n = 5 per month, per temperature, per microhabitat). **D.** Activity of *Pyrrharctia isabella* housed above and below snow cover against *Escherichia coli* (n = 5 per month, per temperature, per microhabitat). Error bars represent the SEM.

### 4.3.3 Realised immune responses

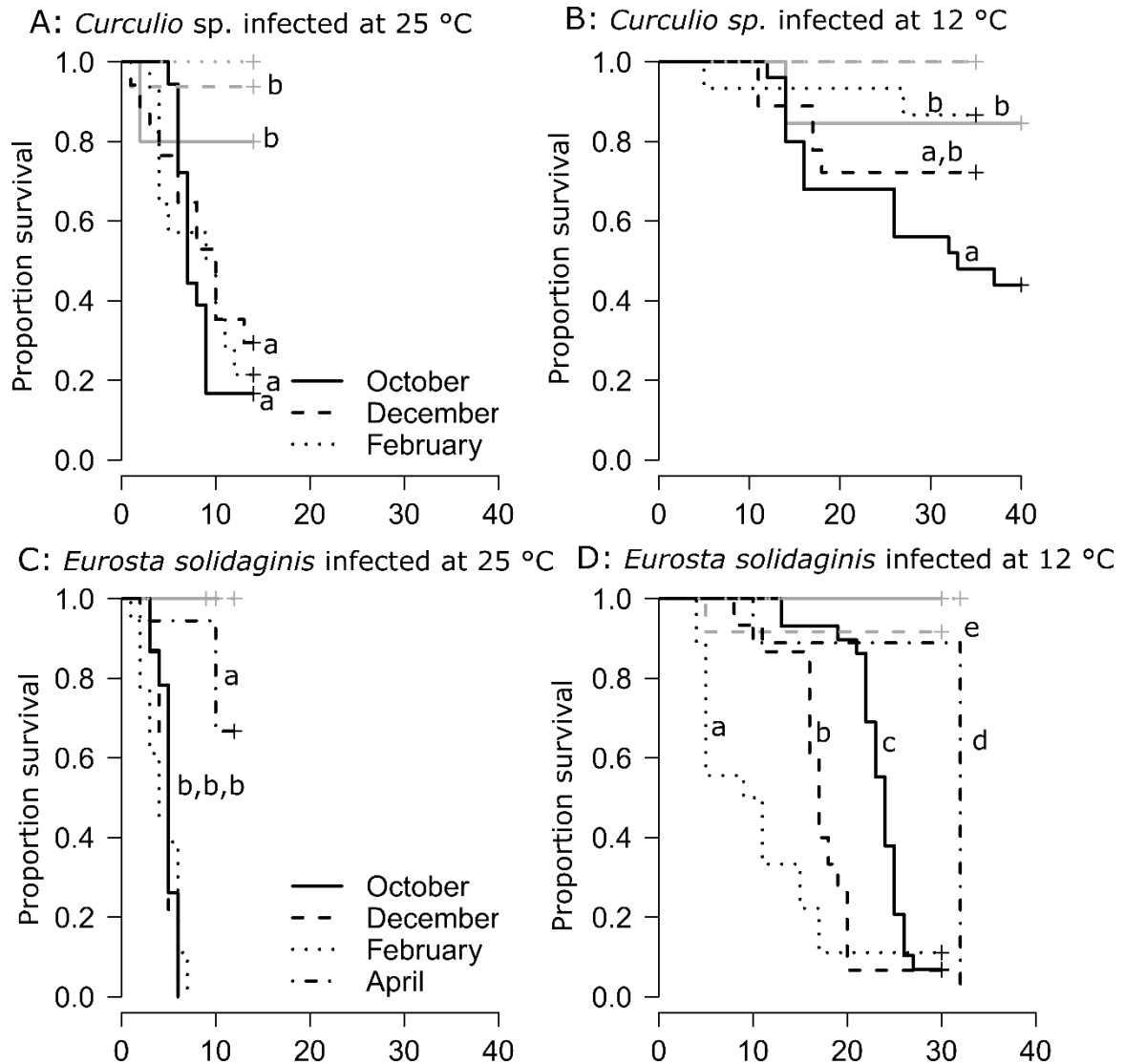
Melanisation in *Curculio* sp. was lower in autumn compared to early winter (Fig. 4.5;  $F_{3, 39} = 3.78$ ,  $p = 0.018$ ) but was unaffected by temperature ( $F_{2, 39} = 1.66$ ,  $p = 0.20$ ). The ability of *Curculio* sp. to survive fungal infection was lowest in autumn compared to mid-winter, and trended towards lower survival when compared to early winter, although these differences were only detectable at 12 °C (Fig. 4.6A,B; Table 4.1).

At 12 °C, *E. solidaginis* were more likely to survive fungal infection in autumn and spring, and least likely to survive in early and mid-winter (Fig. 4.6D; Table 4.1); however, at 25 °C, survival was higher only in the spring (Fig. 4.6C; Table 4.1). At 25 °C in April, all *E. solidaginis* pupated within 48 h of inoculation (including controls) and survival was measured as survival to eclosion. At 12 °C in April, all *E. solidaginis* pupated, but did not emerge; however, controls emerged when returned to 25 °C, whereas infected flies did not, and fungus was observed growing on the outside of the infected pupae.





**Figure 4.5 Melanisation response in *Curculio* sp. across seasons.** Melanisation was measured as the darkness (grey value) of melanin deposited on a nylon filament (2 mm) introduced into the body cavity of the insect for 24 h at either 0 °C (except for in April), 12 °C, or 25 °C (n = 5 per month, per temperature except: n = 7 October 12 °C; n = 6 October 25 °C; n = 4 December, all temperatures; n = 3 February and April, all temperatures). Error bars represent the SEM. Letters represent differences among seasons.



**Figure 4.6 Survival of infection with *Metarhizium brunneum* across season** Insects were infected topically with spores of *M. brunneum* suspended in 0.01% Tween80, and infection progressed at either 12 °C or 25 °C. Controls (indicated in grey) were inoculated with sterile 0.01% Tween80 **A.** *Curculio* sp. infected at 25 °C; **B.** *Curculio* sp. infected at 12 °C (n = 18 October 25 °C, December 12 °C; n = 25 October 12 °C; n = 17 December 25 °C; n = 14 February 25 °C; n = 15 February 12 °C; controls: n = 12-15). **C.** *Eurosta solidaginis* infected at 25 °C; **D.** *Eurosta solidaginis* infected at 12 °C (n = 29 October 12 °C, n = 24 October 25 °C; n = 15 December; n = 18 February & April; controls: n = 15-18). In April, pupal cases formed rapidly at 25 °C and the majority of flies survived to eclosion as adults. At 12 °C, mortality was difficult to detect after the pupal case was formed; however, these flies did not eclose, even after transfer to 25 °C, and were thus determined dead at the end of the study. Different letters represent curves that are significantly different from one another.

**Table 4.1 Statistical results from lognormal models comparing survival curves.** Survival was monitored at 12 °C and 25 °C for *Eurosta solidaginis* and *Curculio* sp. infected with the entomopathogenic fungus, *Metarhizium brunneum*. Bolded p-values represent significant differences between curves.

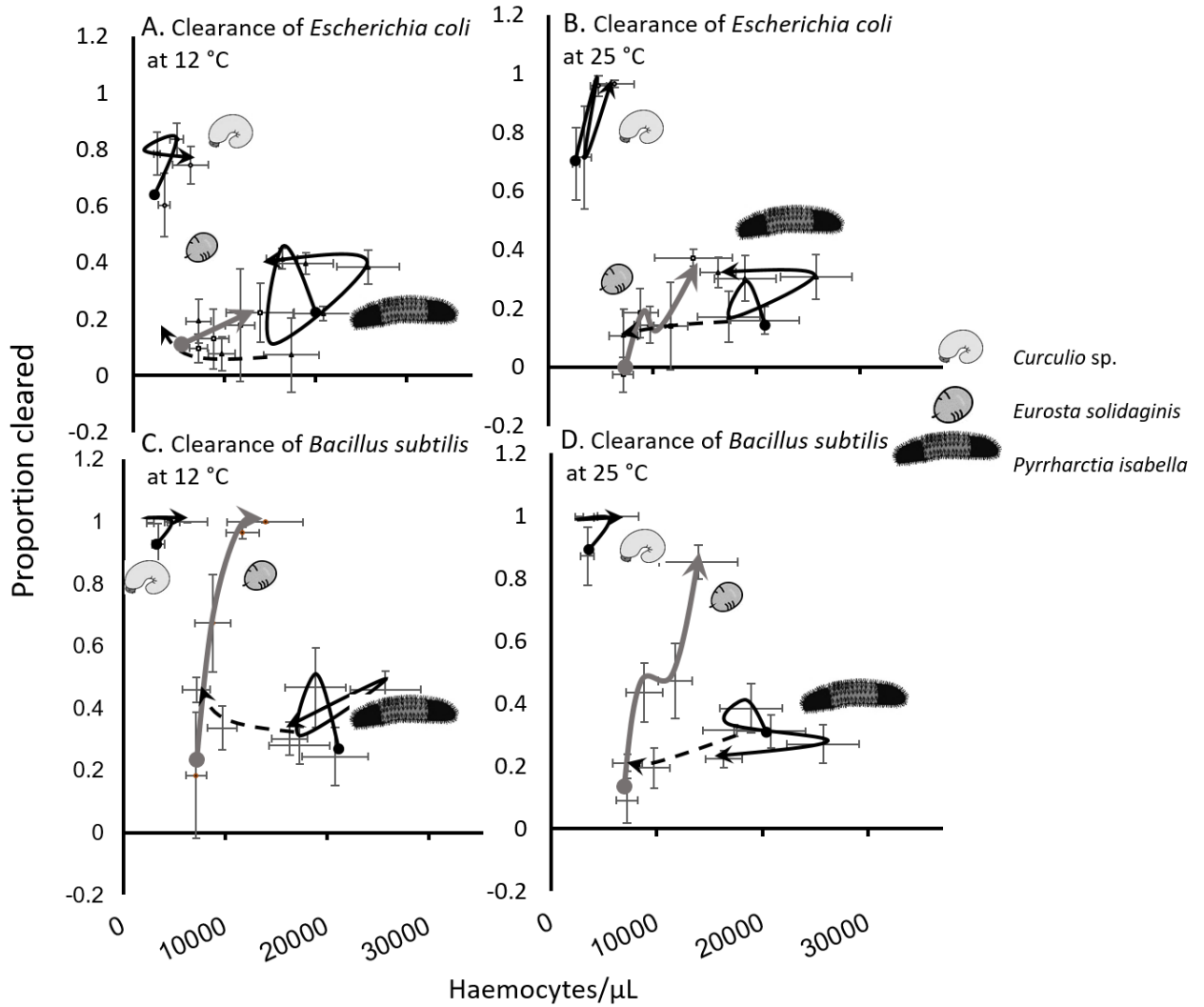
Species	Temperature (°C)	Comparison	Z	P
<i>E. solidaginis</i>	12	Autumn vs Early winter	2.19	<b>0.03</b>
		Autumn vs Mid-winter	6.73	<b>&lt;0.001</b>
		Autumn vs Spring	1.30	<b>0.02</b>
		Early winter vs Mid-winter	3.79	<b>&lt;0.001</b>
		Early winter vs Spring	3.12	<b>&lt;0.01</b>
		Mid-winter vs Spring	7.25	<b>&lt;0.001</b>
	25	Autumn vs Early winter	0.29	0.77
		Autumn vs Mid-winter	1.91	0.06
		Autumn vs Spring	7.12	<b>&lt;0.001</b>
		Early winter vs Mid-winter	1.44	0.15
		Early winter vs Spring	6.79	<b>&lt;0.001</b>
		Mid-winter vs Spring	8.43	<b>&lt;0.001</b>
<i>Curculio</i> sp.	12	Autumn vs Early winter	1.88	0.06
		Autumn vs Mid-winter	1.01	<b>0.03</b>
		Early winter vs Mid-winter	0.96	0.3
	25	Autumn vs Early winter	0.04	0.97
		Autumn vs Mid-winter	0.14	0.88
		Early winter vs Mid-winter	0.11	0.91

## 4.4 Discussion

I found that *Curculio* sp., *E. solidaginis* and *P. isabella* all shift their immune activity across seasons; however, changes in immunity were inconsistent among species (summarised in Fig. 4.7), and even among measures of immune activity. Overall, this suggests that the ways in which insect immunity responds to changes in season will be species-specific with respect to both the host and the pathogen, and that different measures of immune activity can reflect different seasonal patterns. Thus, it may be difficult to generalize about seasonal shifts in insect immunity.

### 4.4.1 Trade-offs likely drive species-specific changes in seasonal immunity

The patterns of immune activity among and within species may reflect trade-offs between the demand on different physiological responses to multiple, seasonal pressures. For example, the response to cold may trade-off with immune activity in insects (Ferguson et al., 2016; Linderman et al., 2012), leading to decreased immune activity during times in which insects are physiologically preparing for overwintering. Both *E. solidaginis* and *Curculio* sp. displayed low antimicrobial activity in autumn, the timing of which coincides with increased production of cryoprotectants (Storey and Storey, 1986) and transition from acorn to an overwintering state in the soil, respectively. In the face of responding to multiple stressors and potential trade-offs, the immune system may reconfigure activity to maintain some level of protection (Adamo, 2014), leading to differences in activity among measures of immunity, such as those I observed in this study. For example, in mid-winter when *E. solidaginis* was least likely to survive fungal infection, they simultaneously displayed increased humoral activity, compared to autumn. Overall, it appears that seasonal shifts in immune activity are, at least in part, governed by the relationship between the immune system and the physiological response to concurrent stressors, such as cold.



**Figure 4.7 Summary of humoral immunity across season to highlight idiosyncrasy of activity among species.** Arrows depict the change in season from autumn (October) through spring (April). Autumn is shown with a circle at the start of the arrow. Each panel represents humoral activity against bacteria, plotted against circulating haemocyte concentrations. Each species demonstrates a different direction of change across season. Dashed lines represent the direction of change in *P. isabella* beneath snow cover. **A.** Humoral activity against *Escherichia coli* at 12 °C **B.** Humoral activity against *Escherichia coli* at 25 °C. **C.** Humoral activity against *Bacillus subtilis* at 12 °C **D.** Humoral activity against *Bacillus subtilis* at 25 °C.

*Eurosta solidaginis* displayed increased bacterial clearance and survival of fungal infection in the spring, and *Curculio* sp. increased bacterial clearance after autumn. This increase in activity could indicate a (potentially prophylactic) response to an increase in pathogen stress that may either act to increase immunocompetence overall, or compensate for any damage to, or trade-offs experienced by, the immune system (Salehipour-Shirazi et al., 2016). As temperatures increase, infection by new pathogens, or growth of pathogens overwintering in the insect, may increase (Altizer et al., 2006; Harvell et al., 2002), thereby initiating increased immune activity in response to, or in preparation for, increased pathogen stress (Sinclair et al., 2013). Conversely, increases in immune activity from autumn to spring may represent selection for individuals with stronger immune responses (Krams et al., 2011); this explanation is supported by high overwintering mortality in *Curculio* sp. as a potential source of selection, but is unlikely in *E. solidaginis* for which I observed no mortality. In either case, increased immune activity in spring, coupled with high overwintering mortality in *Curculio* sp., indicates that overwintering is likely an important period of pathogen exposure and/or selection on immunocompetence.

Species-specific patterns of seasonal immune activity may be explained by overwintering habitat, wherein the temperatures experienced in these microhabitats determine both the amount of temperature stress experienced by the insect, as well as the probability of pathogen encounter. *Pyrrharctia isabella* that overwintered below the snow cover had reduced numbers of circulating hemocytes and humoral antibacterial activity, compared to those that overwintered above snow cover, and experienced more severe and variable low temperatures (Fig. 4.1C). Warmer conditions beneath snow cover, or during a milder winter, may increase energy consumption (Irwin and Lee, 2003; Marshall and Sinclair, 2012), and consequently decrease energy available for immune activity; insects exposed to higher temperatures through the winter may thus be immunocompromised in the spring and vulnerable to infection. Indeed, a higher proportion of *P. isabella* died in the spring, with some evidence of fungal infection, when housed beneath snow cover for the winter. Therefore, microhabitat is likely to act as an important driver of protection against pathogens, overwintering success, and fitness in the spring.

#### 4.4.2 Novel findings for species-specific immune responses

In addition to species-specific responses in the context of season, it is interesting to note overall, species-specific, differences in immune activity. For example, I was unable to detect PO activity in the haemolymph of *E. solidaginis* – neither spontaneous activity, nor activity when I added the activator  $\alpha$ -chymotrypsin. Although another activator [e.g. cetylpyridinium chloride (Adamo et al., 2016)] may have allowed us to detect PO, I also observed that the substrate for PO activity, L-DOPA, as well as haemolymph samples from *Curculio sp.* and *P. isabella*, would darken (e.g. auto-oxidation) over time (within two hours at room temperature). However, samples of L-DOPA containing haemolymph, as well as pure haemolymph, from *E. solidaginis* remained clear, potentially from a lack of PO activity due to inhibitors [e.g. serine protease inhibitors (Sugumaran et al., 1985)]. To our knowledge, this is the first investigation of immune activity in *E. solidaginis*. *Eurosta solidaginis* larvae do have genes coding the PO enzyme (H. Udaka, A.B. Dennis & B.J. Sinclair, unpublished data), which suggests that *E. solidaginis* larvae suppress PO activity in winter, possibly to avoid toxic by-products of the melanisation response (González-Santoyo and Córdoba-Aguilar, 2012), or as a trade-off between immunity and other physiological systems. The gall environment likely provides some protection from pathogens, and I did not observe fungus-killed larvae in field collections for this or other studies (L.V. Ferguson & B.J. Sinclair, unpublished observations). Further, *E. solidaginis* contain high concentrations of free fatty acids (Marshall et al., 2014), which may play a role in immunity (Hwang, 2000). Thus, these larvae may not require PO activity while overwintering in the gall.

#### 4.4.3 Seasonal patterns in immunity interact with temperature

Temperature also governed the response to pathogens across seasons, in part through the thermal performance of the immune system. I detected seasonal differences in the ability to survive fungal infection at 12 °C, which indicates that the interaction between host and parasite changes depending on temperature. This may result from shifts in the thermal performance of the immune system across season, and highlights the importance of considering temperature when predicting the outcome of infection (Thomas and Blanford, 2003). Despite this, most measures of immune activity did not change with measurement

temperature, which suggests that these insects have a broad thermal range of immune performance, and that their immune systems may be well-suited to function over the large fluctuations in temperature that insects experience across seasons (Fig. 4.1). However, the interaction between temperature and phenology may override immune activity when development coincides with pathogen challenge. In spring, high survival of *E. solidaginis* against fungal infection coincided with rapid pupation (within 48 h of inoculation) at 25 °C, which indicates that the pre-pupal cuticle and/or puparium may have formed before the fungus was able to penetrate the cuticle and establish in the haemolymph [e.g. *M. anispoliae* reaches the haemolymph by 48 h post-inoculation at 28 °C in *Schistocerca gregaria* (Gillespie et al., 2000)].

## 4.5 Conclusions

Here I show that immune activity in three species of overwintering insects fluctuates by season, but that these changes in activity largely vary by species (summarised in Fig. 4.7). These variations in activity may depend on the interaction between the physiological responses to multiple stressors, and are also governed by the thermal performance of the immune system. Further, immune activity is affected by overwintering microhabitat, in which the relationship between multiple, seasonal pressures may shift. Based on the idiosyncratic nature of our results, I cannot generalise the effects of season on insect immunity; however, I suggest that further understanding of the mechanisms underlying these species-specific shifts in immune activity (e.g. trade-offs between cold tolerance and immunity) will allow us to make broader predictions of the effects of season and climate change on overwintering success.



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## Chapter 5

### 5. Thermal variability and plasticity determine the outcome of host-pathogen interactions

In this chapter I explore the relative influence of both host and pathogen acclimation on the outcome of infection. Further, I determine why the outcome of infection under fluctuating temperatures is different from the outcome under constant temperatures. This chapter has been prepared for submission to *American Naturalist*.

#### 5.1 Introduction

The success or failure of ectotherms in the face of climate change will depend on their ability to moderate physiological responses through phenotypic plasticity (Deutsch et al., 2008). Under heterogeneous environments, phenotypic plasticity can allow ectotherms, such as insects, to maximise fitness by compensating for the effects of the environment on performance (Angilletta et al., 2002; Angilletta et al., 2006). Because insects in temperate areas spend more than half of their lives overwintering (Williams et al., 2015), thermal plasticity is of particular importance in allowing insects to respond to pressures associated with exposure to low temperatures. For example, cold acclimatisation in the emerald ash borer, *Agrilus planipennis*, decreases their lower lethal temperature by 10 °C, which allows them to survive low sub-zero temperatures in the winter (Crosthwaite et al., 2011).

Because temperature directly affects the thermal performance of ectotherms, biotic interactions are also likely to shift with changes in temperature (Thomas and Blanford, 2003; Van der Putten et al., 2010). To compensate for shifts in these interactions, we might expect that thermal acclimation will also modify physiology relevant to the interaction (Ferguson et al., 2016; Sinclair et al., 2013). For instance, in host-pathogen interactions, we might expect plasticity in immunity and pathogenicity in anticipation of temperature-driven shifts in the outcome of infection. Therefore, thermal plasticity in the physiology of both players should contribute to modifying the outcome of a biotic interaction. However, in the case of host-pathogen interactions, we know very little of

how the independent thermal responses of host and pathogen combine to affect the outcome of infection (Altman et al., 2016; Raffel et al., 2012).

### 5.1.1 the role of host and pathogen thermal plasticity in the outcome of infection

There is some evidence that both insect immunity and pathogen infectivity or virulence are thermally plastic. For example, cold-acclimation narrows the thermal performance curve of the immune system in the spring field cricket, *Gryllus veletis* (Chapter 3; Ferguson et al., 2016). In this case, plasticity may be driven by trade-offs between immunity and the response to cold. Cold exposure can also activate the immune system in the absence of pathogen infection (Chapter 2; Le Bourg et al., 2009; Salehipour-Shirazi et al., 2016). In this case, plasticity may occur as a prophylactic response to temperature-mediated changes in pathogen pressure (Sinclair et al., 2013). Parasites and pathogens are likely to have a similar capacity for thermal plasticity. For example, virulence is activated by low temperatures in species of bacteria (Smirnova et al., 2001), such as the insecticidal bacterium, *Yersinia enterocolitica* (Bresolin et al., 2006). Conversely, acclimation to low temperatures can decrease infectivity of the amphibian trematode parasite, *Ribeiroia ondatrae* (Altman et al., 2016). In this case, cold acclimation may injure the parasite (Altman et al., 2016). Thus, acclimation (i.e. the physiological response to temperature) and thermal history (i.e. the recent temperature experience) can influence both host and pathogen physiology in myriad ways that are likely to influence the outcome of their interaction. However, we are generally ignorant of the contribution of both host and pathogen thermal plasticity to the outcome of biotic interactions under different thermal environments (Altman et al., 2016; Paull et al., 2015; Raffel et al., 2012; Sinclair et al., 2016).

The influence of thermal history and plasticity on host-pathogen interactions can result in several different outcomes depending on how performance of each player is matched or mismatched (Fig. 5.1., Thomas and Blanford, 2003). If hosts and pathogens respond similarly to temperature, such that immunity and pathogenicity shift synchronously, the effects of plasticity on the outcome of the biotic interaction are likely to be negligible (Fig. 5.1). However, if the magnitude of shifts in performance or the direction and extent

of displacement of thermal performance across temperature differ between each party, we are likely to see substantial mismatches in performance which will alter the outcome of the interaction, depending on the thermal environment (Fig. 5.1). Thus, to understand how acclimation of both parties will determine the outcome of infection, we must understand the underlying thermal performance of each.

### 5.1.2 The influence of thermal variation on the outcome of biotic interactions

Exposure to variation in environmental temperature is also likely to contribute to the outcome of biotic interactions, as the physiological response to fluctuating temperatures (FTs) produce different phenotypes than exposure to constant temperatures (Colinet et al., 2015; Dillon and Woods, 2016; Dillon et al., 2016; Kingsolver et al., 2015; Paaijmans et al., 2013; Pamminger et al., 2016). Much of the discrepancies between performance at constant vs fluctuating temperatures can be explained by the non-linear relationship of performance with temperature and Jensen's inequality (Colinet et al., 2015). When temperatures fluctuate across the accelerating portion of a thermal performance curve (TPC; e.g. Fig. 5.2B), the mean of the rate of performance will increase as variation in temperature increases (Williams et al., 2012). Thus, the total output of performance at a mean temperature will not reflect that under fluctuating conditions (Colinet et al., 2015). Consequently, because terrestrial insects and other animals live in thermally heterogeneous environments, we may not be able to create accurate predictions about the effects of temperature on performance based on experiments under constant temperatures (Colinet et al., 2015; Sinclair et al., 2016).

The difference in performance under FTs compared to CTs may instead be the sum of performance at each of the single temperatures that comprise the fluctuating regime (Colinet et al., 2015). By contrast, most evidence suggests that fluctuating temperatures fundamentally alter thermal performance. In this case, FTs may cross physiological thresholds (e.g. injury, signaling) or act *via* unknown mechanisms, such that performance cannot be explained as the sum of performance under single temperatures (Colinet et al., 2015; Kingsolver et al., 2015; Paaijmans et al., 2013). Further, the effects of fluctuating



temperatures on performance depend on whether variations in temperature are random or predictable (Raffel et al., 2012), or lead to acclimation (Kingsolver et al., 2015). Overall, we are only beginning to understand the role of thermal variation in determining performance within an individual, much less how FTs will modify biotic interactions.

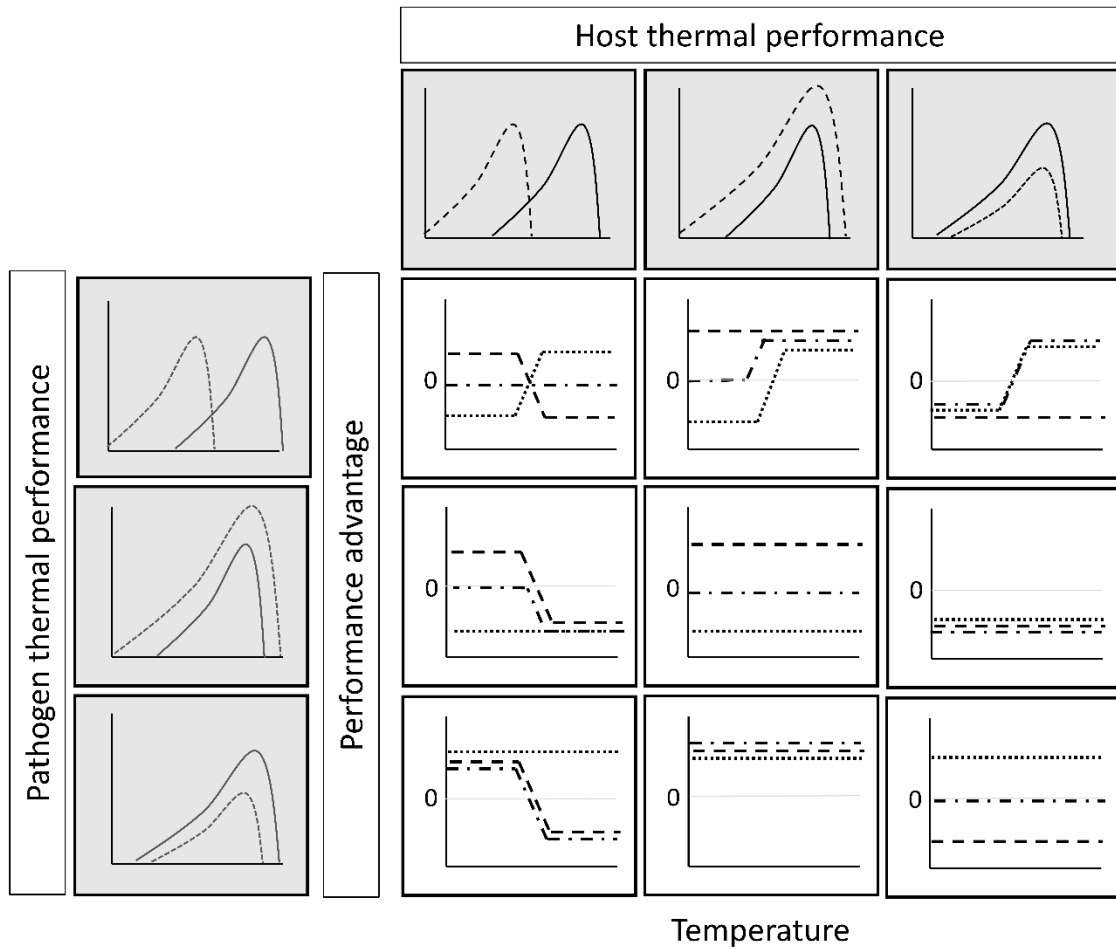
The complexity of FTs may extend to host-pathogen interactions and alter the outcome of infection by modifying the thermal performance of one or both parties (Murdock et al., 2012; Paaijmans et al., 2013; Pamminger et al., 2016; Paull et al., 2012; Rohr and Raffel, 2010; Terrell et al., 2013). For example, FTs can either increase or decrease susceptibility of aphids to the fungus *Erynia neoaphidis* compared to susceptibility at constant temperatures. In this example, the outcome of infection under FTs depended on the magnitude of variation in temperature, and whether the host or parasite cross physiological thresholds (Blanford et al., 2003). Thus, the outcome of infection under FTs is likely to be complex and may complicate our capacity to predict the outcome of infection (Murdock et al., 2013; Paaijmans et al., 2013). However, the outcome of biotic interactions should rely on the interplay in the thermal performance of host and pathogen [e.g. Fig. 5.1; (Sinclair et al., 2016; Thomas and Blanford, 2003)]. Thus, the outcome of these interactions may simply be the sum product of which party wins or loses at each temperature (Fig. 5.2). If we can determine why FTRs produce different outcomes of infection than CTs, we will then understand how we need to design experiments with results that allow us to make more accurate predictions about the effects of temperature on the dynamics of infection and disease.

### 5.1.3 Objectives

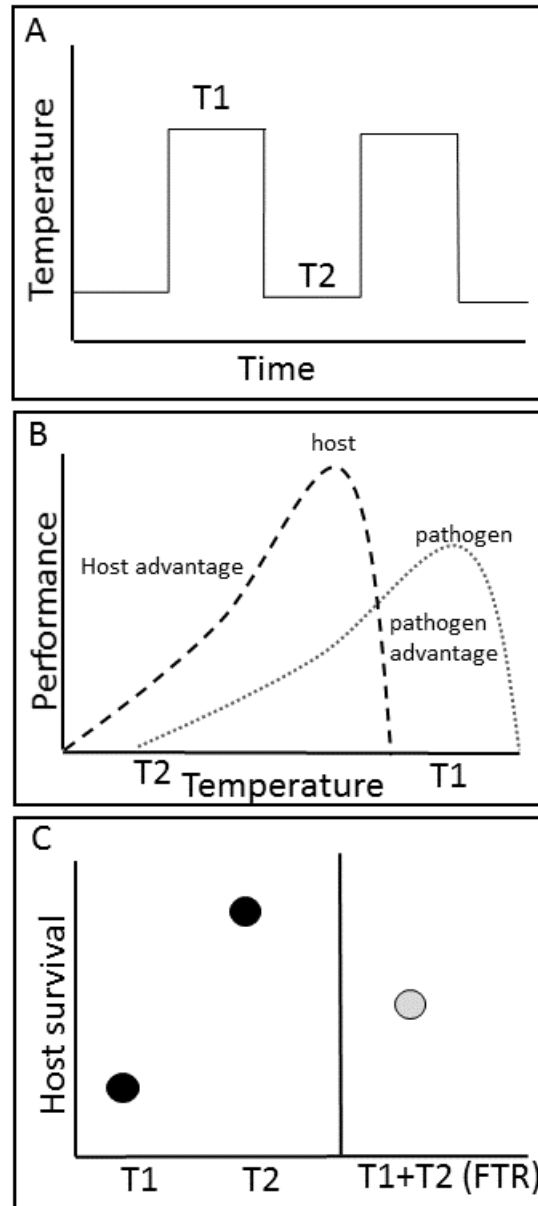
My first objective was to understand how thermal history and plasticity influence the outcome of infection. I tested the hypothesis that if host and pathogen match their acclimation, the effects of plasticity will be masked (Fig. 5.1); however, if their responses to temperature are dissonant, then the outcome of infection will vary depending on the thermal history of each party and the temperature under which they interact. For example, cold acclimation may differentially affect both host and pathogen such that the host shifts the thermal performance of immunity to favour low temperatures, whereas the pathogen

increases its pathogenicity overall (e.g. Fig. 5.1). In this scenario, the host may lose against the pathogen at high temperatures (e.g. where pathogen performance dominates; Fig. 5.1). In contrast, at low temperatures the host may either match or exceed the performance of the pathogen and lead to different outcome of infection. Depending on how the outcome of infection shifts when acclimation regimes are matched (e.g. warm-acclimated *vs* warm-acclimated) or mismatched (cold-acclimated *vs* warm-acclimated) under different thermal regimes, I am then able to determine whether or not thermal history/plasticity is an important contributor to predicting the outcome of infection.

My second objective was to determine why fluctuating thermal regimes can produce different outcomes of infection compared to constant temperature regimes. I hypothesised that the outcome of infection under FTs would be the sum of the relative performance of host and pathogen under CTs. Therefore, I predicted that I should be able to predict the outcome of infection based on the outcome under CTs. To test this hypothesis, I deconstructed the temperatures that comprise an FTR into two constant temperature components and allowed infections to progress under both constant and fluctuating conditions (Fig. 5.2B). This design allowed me to determine if there was a putative mismatch in thermal performance between host and pathogen that would switch between an advantage to the host and an advantage to the pathogen (e.g. Fig. 5.2B); in this case, relative performance should lead to an aggregate outcome under FTRs that represents the sum of the thermal performance of host and pathogen under CTs (e.g. Fig. 5.2C). Further, I compared the survival of hosts of two different acclimation regimes under each of these conditions, because differential acclimation may produce different thermal performance curves. If acclimation changes thermal performance and affects the outcome of infection, this may provide additional information about the role of FTRs. For example, if the outcome of infection for both acclimation groups of crickets is similar under constant temperatures but different under FTRs, then it would be unlikely that the outcome under FTRs is the sum of performance. Rather, there is more likely to be an acclimation  $\times$  environment interaction that is specific to FTRs.



**Figure 5.1** Examples of potential plasticity in response to low temperatures in both host and pathogen thermal performance leading to matches or mismatches in their interaction. Grey panels show the thermal performance curves of unacclimated (solid lines) and acclimated hosts (dashed lines) or pathogens (dotted lines). Middle panels include the possible outcome of the host-pathogen interaction, based on the sum of performance of each player. The zero mark indicates matched performance; above the zero indicates advantage to the host (e.g. host wins), below the zero indicates advantage to the pathogen (e.g. pathogen wins). Dashed lines represent the outcome if only the host acclimates; dotted lines represent the outcome if only the pathogen acclimates; dashed and dotted lines represent the outcome when both parties acclimate to the thermal conditions.



**Figure 5.2 Possible additive effects of host and pathogen performance under fluctuating thermal regimes (FTRs).** **A.** A simple FTR with equal amount of time spent at each of two temperatures (T1 = temperature 1; T2 = Temperature 2). **B.** Relative thermal performance of host (e.g. immunity) and pathogen (e.g. infectivity); performance is mismatched such that at T1 the pathogen wins, whereas at T2 the host wins. **C.** An additive outcome of infection under FTRs predicted by outcome of infection under constant temperatures (T1 and T2).

My third objective was to confirm whether or not the outcome under FTRs is the sum of relative performance under CTs, as opposed to a signal provided by the FTR itself. If FTRs are the sum of relative performance under CTs, I predict that a single switch in temperature during infection (as opposed to repeated fluctuations) should switch the outcome of infection to mimic the outcome under the latter constant temperature. In contrast, if FTs do not operate as the sum of host-pathogen performance under CTs, then I hypothesize that fluctuating to a high or low temperature crosses a physiological threshold in host or pathogen during infection that modifies the outcome of their interaction. In this case, I predict that a single switch in temperature during infection should prompt such a signal, and should modify the outcome of infection to match that under FTs.

## 5.2 Methods

### 5.2.1 Culture, acclimation, and infection of crickets and fungi

*Gryllus veletis* overwinter as late instar nymphs and, based on their occurrence in field habitats, are likely to encounter species of *Metarhizium* fungus that occupy the overwintering habitats of insects (Bidochka et al., 1998). Further, *Metarhizium* species are likely to require hosts in which to overwinter (Bidochka et al., 1998). Therefore, it is probable that these two players would interact at low temperatures.

*Gryllus veletis* were originally collected in Lethbridge, Alberta, in 2010 and our colony was established from this collection. I reared *G. veletis* as described by Coello Alvarado et al. (2015) under constant 25 °C and 14 L: 10 D photoperiod with *ad libitum* water and rabbit chow (Little Friends Rabbit Food, Martin Mills, Elmira, ON, Canada). When crickets reached the 6<sup>th</sup> instar, I haphazardly assigned females to individual, horizontally-oriented *Drosophila* vials (46 mL) with paper shelters and *ad libitum* rabbit chow and water, provided in 1.5 mL tubes with cotton stoppers. I then acclimated crickets in individual vials to one of three thermal regimes for seven days in a temperature-controlled incubator: warm (WA; 25 °C; 14 L: 10 D), cold (CA; 6 °C; 10 L: 14 D) (cf. Ferguson et al., 2016), or a regime that fluctuated between 18 °C (day) and 6 °C (night) on a 12:12 photoperiod (FTR1-acclimated).

I grew cold-active *M. brunneum* (Clavicipitaceae: Hypocreales, strain 43a2ii; provided by Michael Bidochka, Brock University, ON, Canada) on potato dextrose agar (PDA) (Kamp and Bidochka, 2002) contained in Parafilm-sealed petri plates under darkness at 25 °C in a temperature-controlled incubator. When I observed characteristic green sporulation, I directly transferred and streaked spores using a metal spatula under sterile conditions in a biological safety cabinet onto new PDA plates. I then allowed *M. brunneum* to germinate and sporulate on sealed plates of PDA under one of three thermal regimes (Fig. 1A): warm (25 °C; 14 d), cold (12 °C; 28 d), and a fluctuating regime (FTR1; 20 d) from 18 °C (12 h) to 6 °C (12 h) under darkness until I observed sporulation of the fungus. Following sporulation, I prepared a suspension of spores for injection into crickets following Gao et al. (2011); briefly, under sterile conditions in a biological safety cabinet, I added approximately 2 mL of 0.01 % Tween 80 directly to a plate of sporulating fungus and gently scraped fungal spores into suspension using a sterile metal spatula. I then strained the fungal suspension through cheesecloth to remove any large agglutinations, and determined the concentration of *M. brunneum* spores using a Neubauer improved hemocytometer under light microscopy at 400 × magnification. Subsequently, I diluted the suspension in 0.01 % Tween 80 to  $5 \times 10^7$  spores/mL.

To infect crickets, I injected 1.5 µL of fungal spore suspension (i.e.  $7.5 \times 10^4$  spores) from one acclimation regime into the membrane under the pronotum, using a 10 µL Hamilton syringe and 32-gauge needle. During injection, I took care to not let haemolymph or fungal suspension leak out of the wound. I injected control crickets with 1.5 µL of Tween 80. Following injections crickets were returned to their *Drosophila* vials and maintained under different thermal environments in either Experiment 1, 2 or 3. I monitored crickets each day and declared a cricket dead if it was immobile. If a cricket moulted following infection, I discarded it from the trial. Upon death, I surface-sterilised crickets with 70 % ethanol, placed them on moist filter paper in individual, sterile petri plates, sealed the plates with Parafilm and incubated them at 25 °C to confirm characteristic green spore growth of *M. brunneum*.

## 5.2.2 Experiment 1: Role of thermal plasticity in hosts and pathogens on the outcome of infection

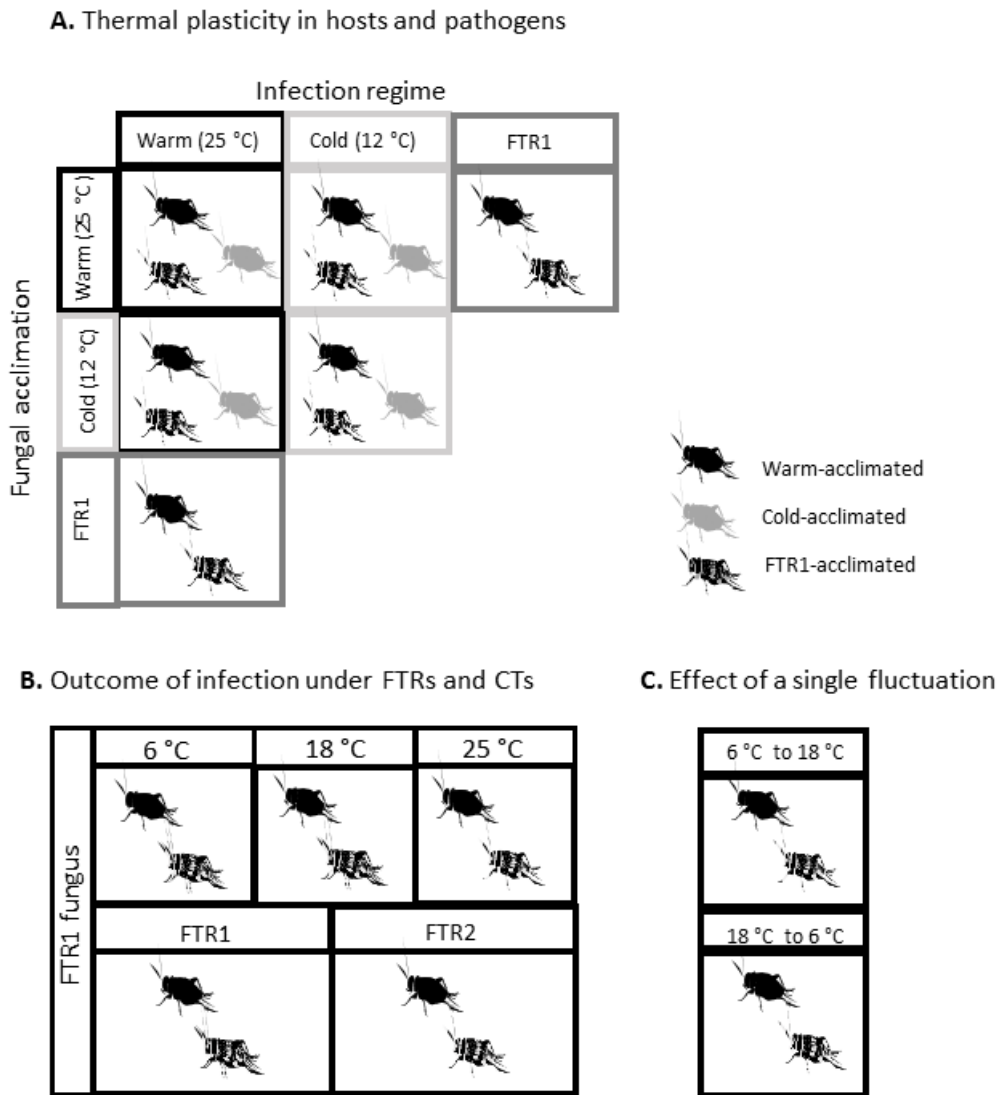
To understand how thermal history and plasticity influence the outcome of infection, I allowed both host and parasite to acclimate to low, high, or fluctuating temperatures (as described in Section 5.2.1) and matched them together under low, high, or fluctuating thermal environments in a factorial design (Fig. 5.3). This design allowed us to determine if and when plasticity in each party would influence the outcome of infection (e.g. Fig. 5.1). To achieve these infection conditions, I haphazardly selected crickets from each acclimation regime and paired them with fungi from each acclimation regime.

Immediately following injections, I transferred crickets to either constant warm (25 °C; 14:10 L:D), constant cold (12 °C, 10:14 L:D), or conditions that fluctuated from 6 °C (day) to 18 °C (night) on 12:12 photoperiod (Fig. 5.3A). Each infection pairing was repeated two or three times with 10-23 crickets infected in each repetition.

## 5.2.3 Experiment 2: Comparing outcomes of infection under FTRs to CTs

To determine whether or not I could explain the outcome of infection under fluctuating temperatures by the sum of performance under constant temperatures, I allowed infections to progress under constant temperatures that represented the components of two different fluctuating thermal regimes. Specifically, I infected WA and FTR1A crickets with FTR1 fungus, and immediately transferred infected crickets to conditions corresponding to FTR1, conditions that fluctuated from 25 °C (day) to 6 °C (night) on a 12:12 photoperiod (FTR2), as well constant 6 °C, 18 °C, and 25 °C (Fig. 5.3B). Each infection was repeated twice (n = 5-10 crickets per repetition).

To determine if I could predict the outcome of infection under fluctuating temperatures from the outcome under constant temperatures, I calculated expected survival under FTs as a product of survival under CTs. I expressed the outcome under CTs as the final proportion of host survival. Then, I averaged the outcome of infection under CTs to provide a predicted proportion of survival under FTs.



**Figure 5.3 Experimental design of interaction between acclimated crickets and fungi under different thermal infection regimes.** Crickets were acclimated for 7 d at 25 °C (Warm-acclimated; rearing temperature), 6 °C (cold-acclimated) or under FTR1. **A.** Experiment 1: The effects of thermal plasticity in host and pathogen on the outcome of infection. **B.** Experiment 2: Relative outcome of infections under fluctuating conditions and constant conditions that comprise an FTR. **C.** Experiment 3: Outcome of infection under a single switch in temperature. FTR1 = 6 °C 12 h, 18 °C 12 h; FTR2 = 6 °C 12 h, 25 °C 12h.



### 5.2.4 Experiment 3: The effect of a single temperature switch on the outcome of infection

To determine if a single switch in temperature during infection would mimic the outcome of infection under FTRs, I infected both WA and FTR1A crickets with FTR1A fungus and transferred crickets to either a constant 6 °C or 18 °C (Fig. 5.3C) approximately when mortality began (as determined by a preliminary experiment). I then compared survival under the final temperatures to survival under the same constant conditions without a switch in temperature.

### 5.2.4 Statistical analysis

Using the *survival* package in R (Brant, 2007), I generated Kaplan-Meier survival curves for each pairing of host and pathogen under each environmental temperature. I then created generalised linear models of survival and compared the log-likelihood values between models to determine which provided the best fit for our data. To help determine which model would be the best fit for my data, I assessed the shape of the hazard rate over time for each pairing of host and pathogen under each environmental condition. To do so, I generated hazard plots and determined the shape of the distributions based on Cox et al. (2007). Based on log-likelihood values compared among models and the shape of plotted hazard rates, I used a lognormal model to test for differences between curves (Klein et al., 2014; Cox et al., 2007). There were no significant differences in the curves from replicates; thus, I pooled replicates for further analysis.

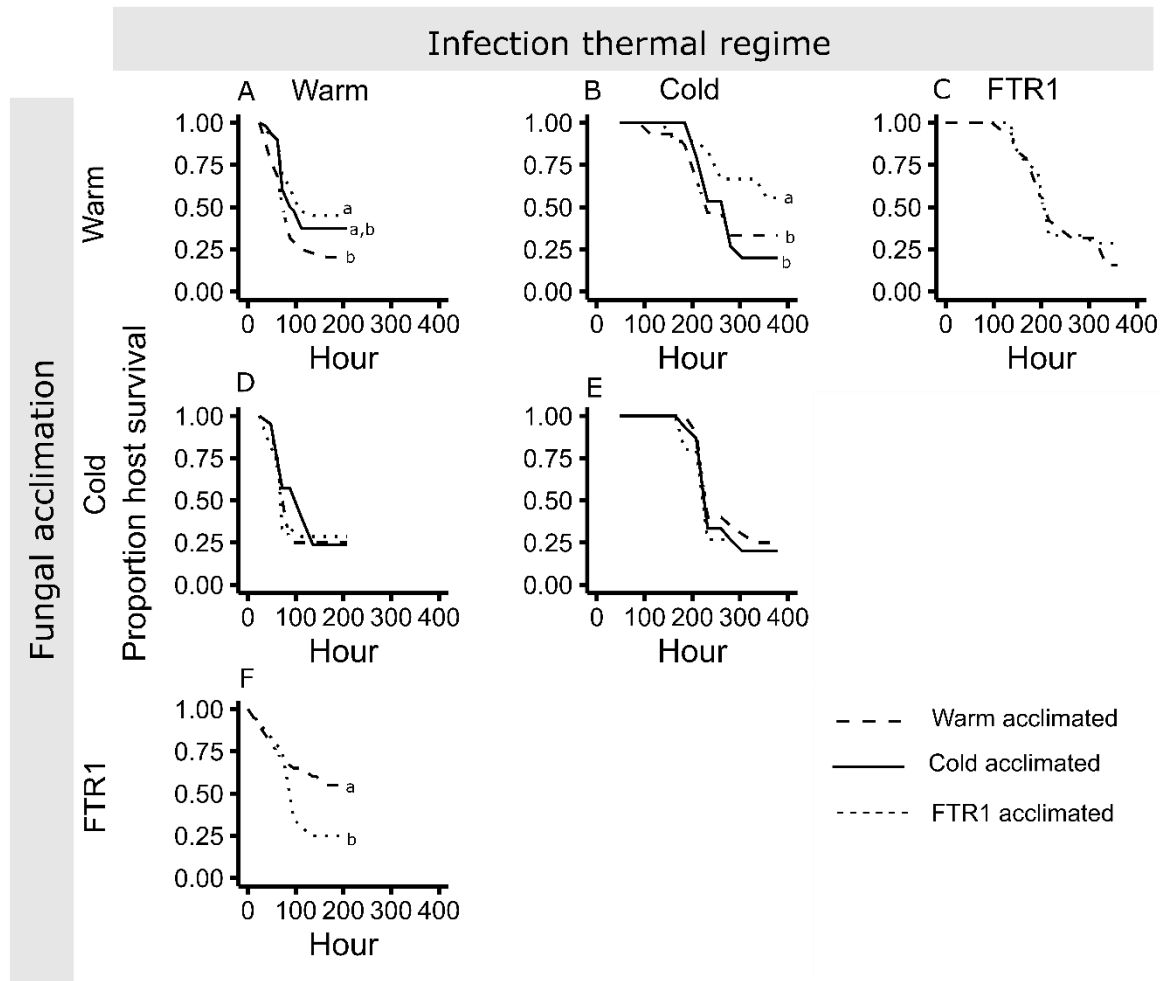
## 5.3 Results

### 5.3.1 Experiment 1: Thermal history in both host and pathogen influences the outcome of infection under different thermal regimes

The outcome of infection changed depending on whether host and pathogen were matched or mismatched in their acclimation regimes, and depending on the temperature under which infection progressed. Acclimation to FTR was beneficial to crickets, as survival was higher in FTR1 acclimated crickets under constant infection temperatures (12 °C and 25 °C), compared to WA crickets (Table 5.1; Fig. 5.4A, B, F). However, this advantage was lost when crickets were infected with a cold-grown fungus (Table 5.1;

**Table 5.1 Experiment 1: Statistical comparisons of survival among groups of differentially-acclimated *Gryllus veletis* infected with acclimated *Metarhizium brunneum*.** Using a lognormal model, I compared survival of crickets acclimated to warm, (25 °C, 7 d), cold (6 °C 7d) and fluctuating thermal regimes (FTR1; 18 °C 12 h; 6 °C 12 h). Infections progressed at constant or fluctuating temperatures. P-values in bold type represent significant differences. FTR1A = acclimated to FTR1. Sample sizes of crickets are included at first mention of each acclimation group.

Acclimation	Comparison	Fungal growth temperature	Infection temperature	z	df	P
Warm (n = 42)	FTR1A (n = 40)	Warm	25 °C	2.84	1	<b>&lt;0.01</b>
Cold (n = 46)	Warm			1.71	1	0.08
FTR1A	Cold			-1.21	1	0.23
Warm (n = 21)	FTR1A (n = 20)	Cold	25 °C	-0.11	1	0.91
Cold (n = 21)	Warm			0.58	1	0.56
FTR1A	Cold			-0.69	1	0.49
Warm (n = 26)	FTR1A (n = 28)	Warm	12 °C	3.28	1	<b>&lt;0.01</b>
Cold (n = 15)	Warm			0.77	1	0.44
Cold ×FTR1A				-2.11	1	<b>0.03</b>
FTR1A (n = 20)	Warm (n = 15)	Cold	12 °C	0.95	1	0.34
Cold (n = 15)	Warm			0.07	1	0.94
Cold	FTR1A			-0.87	1	0.38
FTR1A	Warm	Warm	FTR1	-0.08	1	0.94
FTR1A	Warm	FTR1	25 °C	2.44	1	<b>0.02</b>



**Figure 5.4 Experiment 1: Thermal history of host and pathogen modify outcome of infection under different thermal regimes.** *Gryllus veletis* were acclimated to cold (6 °C), warm (25 °C) and fluctuating temperatures (FTR1; 6 °C 12 h; 18 °C 12h) for 7 d and then challenged with the entomopathogenic fungus *Metarhizium brunneum* acclimated to cold (12 °C), warm (25 °C) or fluctuating temperatures (FTR1, as for crickets). Infections then progressed under cold (12 °C), warm (25 °C), or fluctuating conditions (FTR1). Survival was compared among cricket acclimations under each thermal environment; letters that are different from each other (and asterisks) indicate survival curves that differ significantly.

Fig. 5.4 D,E), or when the infection temperature fluctuated from 6 °C to 18 °C (Table 5.1; Fig. 5.4C). When thermal history of both host and pathogen matched infection temperature, the outcome of infection (defined as overall survival) was roughly the same: WA cricket + WA fungus + Warm infection = 20 % survival, CA cricket + CA fungus + cold infection = 25% survival, FTR1-A cricket + FTR1-A fungus + FTR1 infection = 23% survival), thereby masking both host and pathogen plasticity.

### 5.3.2 Experiment 2: The outcome of infection under fluctuating temperatures is the sum of matches or mismatches in thermal performance

At a constant infection temperature of 6 °C (Fig. 5.5A) or 25 °C (Fig. 5.5C), FTR-acclimated hosts performed better than WA hosts; at 18 °C this advantage was lost (Fig. 5.5B). Further, FTR1 acclimated crickets regained their advantage when the infection temperature fluctuated from 25 °C to 6 °C – both constant temperatures at which FTR-acclimated crickets perform well (Fig. 5.5E). In contrast, this host advantage was again lost when temperatures fluctuated between an advantageous and disadvantageous temperature (Fig. 5.5D). Mortality began several days earlier at 6 °C (mortality commenced at approximately 100 h) compared to 12 °C (mortality commenced at approximately 200 h) (Fig. 5.4 B & E; 5.5A).

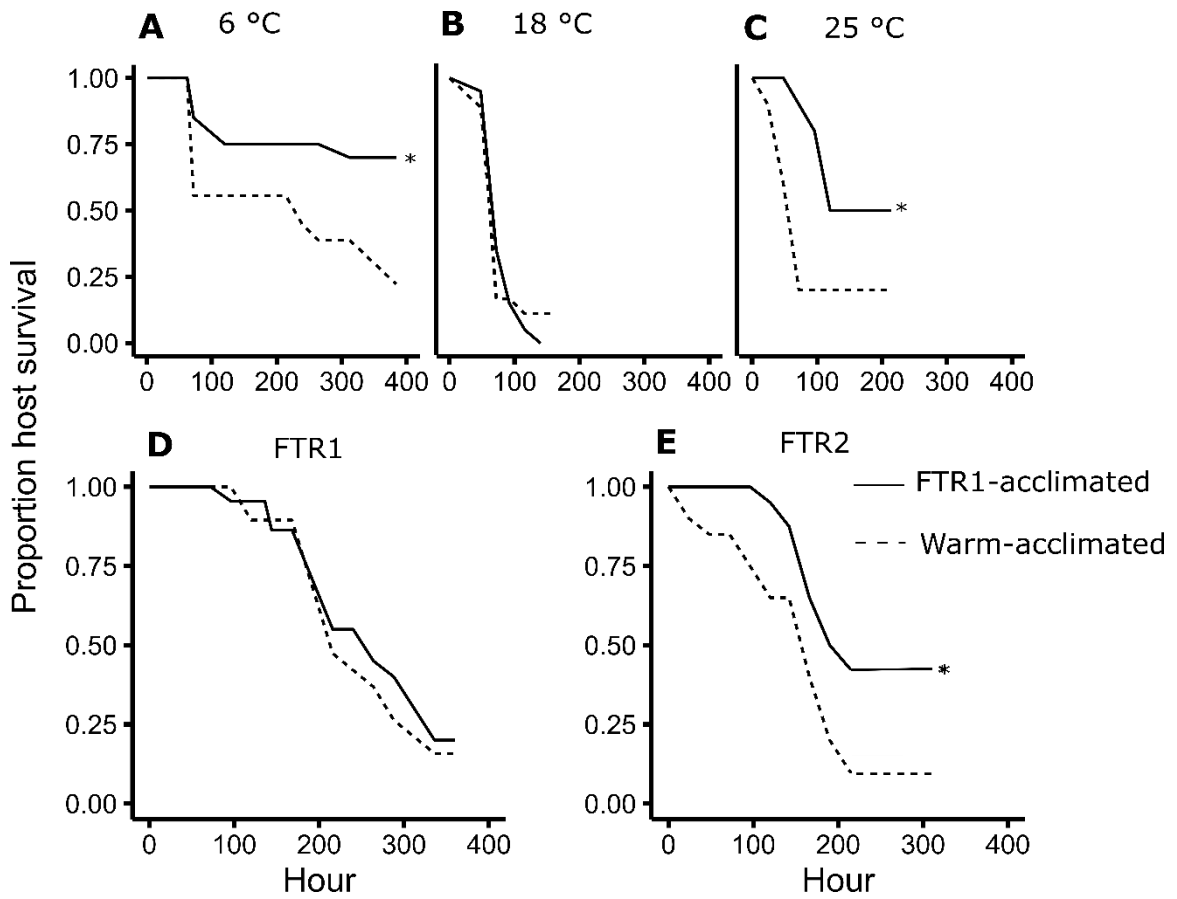
When I calculated an average of the final proportion of host survival under constant 6 °C and 18 °C I predicted 35 % survival of FTR1A hosts (compared to 20 % observed) and 13% survival (compared to 11 % observed) of WA hosts under a FTR1 conditions during infection. When I averaged the final proportion of host survival under constant 6 °C and 25 °C, I predicted 60 % survival of FTR1A hosts (compared to 50 % observed) and 19% survival of WA hosts (compared to 20 % observed) under FTR2 conditions during infection (Fig. 5.7).

### 5.3.4 Experiment 3: Outcome under a single switched temperature mimics outcome under final constant temperature

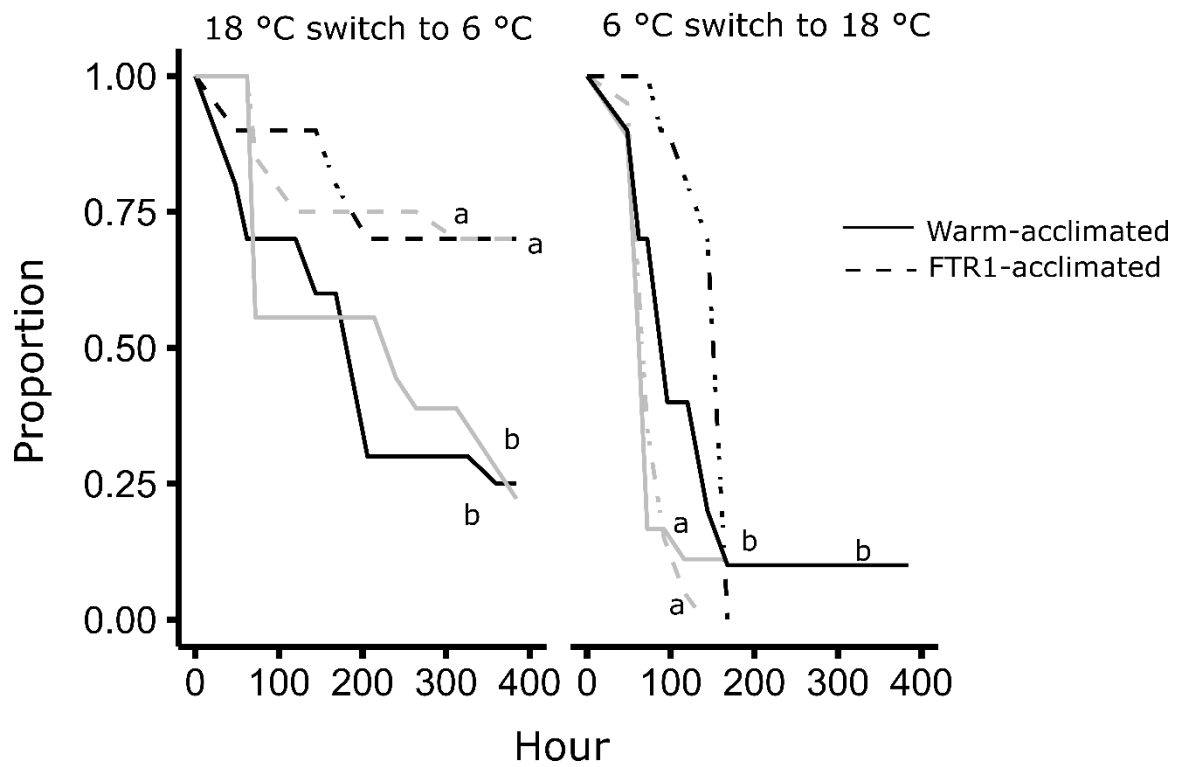
When crickets were switched from 6 °C to 18 °C, or from 18 °C to 6 °C at the point at which mortality began, the outcome of infection mimicked the outcome under constant

**Table 5.2. Experiment 2 and 3: Statistical comparison of survival among acclimated *Gryllus veletis* under constant and fluctuating thermal regimes.** *Gryllus veletis* were acclimated to either warm (25 °C 7 d) or fluctuating (FTR1; 6 °C 12h; 18 °C 12h; 7 d) conditions and infected with *Metarhizium brunneum* acclimated to FTR1 conditions. Infections then progressed under constant or fluctuating conditions. I compared survival between warm and FTR1 using a lognormal model. Under switch conditions, infections began at one constant temperature and were switched to another at the point at which mortality began. FTR2 = 25 °C 12 h; 6 °C 12 h. Values in bold type face represent significant differences between groups.

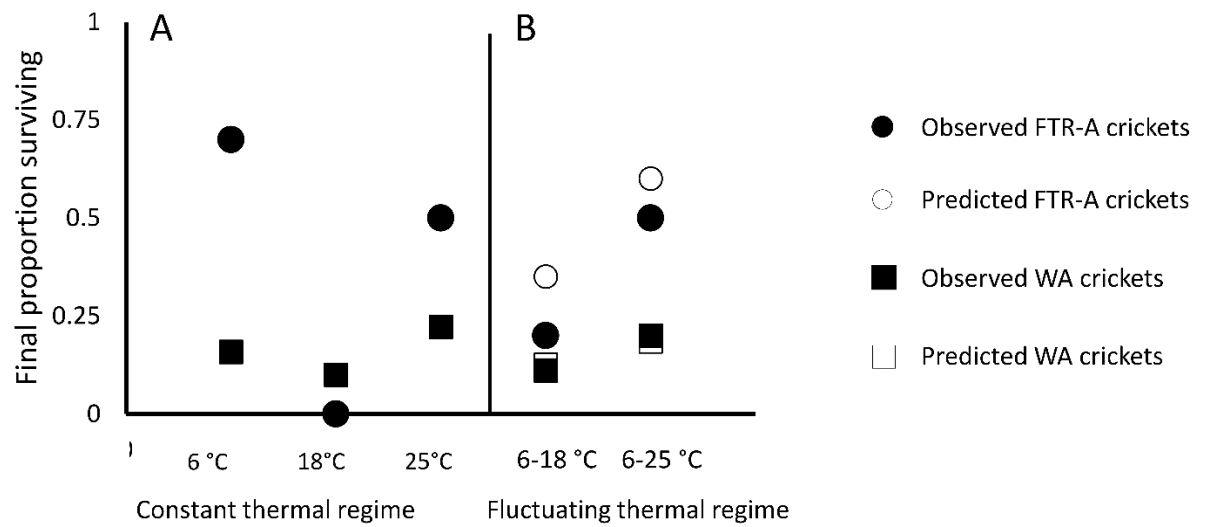
<b>Acclimation</b>	<b>Comparison</b>	<b>Infection conditions</b>	<b>z</b>	<b>df</b>	<b>P</b>
FTR1(n = 20)	Warm (n = 18)	6 °C	2.32	1	<b>0.02</b>
FTR1 (n = 20)	Warm (n = 18)	18 °C	0.30	1	0.76
FTR1 (n = 10)	Warm (n = 10)	25 °C	-2.82	1	<b>&lt; 0.01</b>
FTR1 (n = 22)	Warm (n = 19)	FTR1	0.72	1	0.47
FTR1 (n = 20)	Warm (n = 20)	FTR2	-3.28	1	<b>0.001</b>
FTR (n = 10)	Warm (n = 10)	6 °C to 18 °C switch	2.31	1	<b>0.02</b>
FTR (n = 10)	Warm (n = 10)	18 °C to 6 °C switch	1.94	1	0.05



**Figure 5.5. Experiment 2: The outcome of infection under constant versus fluctuating conditions.** *Gryllus veletis* were acclimated to warm (25 °C) or fluctuating temperatures (FTR1; 6 °C 12 h; 18 °C 12h) for 7 d and then challenged with the entomopathogenic fungus *Metarhizium brunneum* acclimated to warm (25 °C) or fluctuating temperatures (FTR1, as for crickets). Infections progressed at constant (A, B, C), or fluctuating conditions (D,E). Asterisks indicate survival curves that differ significantly.



**Figure 5.6 Experiment 3: The outcome of infection under a switched thermal regime.** *Gryllus veletis* were acclimated to warm (25 °C) or fluctuating temperatures (FTR1; 6 °C 12 h; 18 °C 12h) for 7 d and then challenged with the entomopathogenic fungus *Metarhizium brunneum* acclimated to warm (25 °C) or fluctuating temperatures (FTR1, as for crickets). Infections began at either 6 °C (A) or 18 °C (B) and then switched to 18 °C and 6 °C, respectively, when mortality began. Grey lines represent outcomes of infection at constant 6 °C (first panel) or 18 °C (second panel) from Figure 5. Letters denote significant differences among curves within a panel.



**Figure 5.7 Experiment 2: Prediction of outcome of infection under fluctuating temperatures based on outcomes under constant temperatures. A.** Outcome of infection under constant temperatures. **B.** Outcome of infection under fluctuating temperatures. Predicted values under FTs are derived from the average of the proportion of host survival under the two constant temperatures that comprise the FTR. Final survival was assessed at 384 h post-infection (PI) (6 °C; FTR1), 220 h PI (25 C); 320 h PI (FTR2) and 150 h PI (18 °C).



conditions of the final temperature (i.e. a switch from 18 °C to 6 °C most resembled the outcome of infection under constant 6 °C in both WA and FTR1-A crickets; Fig. 5.6). Lastly, all uninfected control crickets remained alive under all experimental conditions.

## 5.4 Discussion

Here I explored two relatively neglected aspects of the thermal interactions between hosts and pathogens 1) the relative contribution of thermal plasticity in both hosts and pathogens, and 2) how and why FTs affect the outcome of these interactions. I determined that the thermal history of both host and pathogen could sway the outcome of infection, and that the influence of thermal history depended on the thermal environment during infection. Further, the effects of FTs on the outcome of infection were two-fold: FTs during acclimation were generally beneficial to hosts, possibly indicating that FTs provide a signal to broaden the TPC of immunity. Further, FTs during infection appear to oscillate between advantageous conditions to host or pathogen, such that the outcome under FTs is an additive product of host and pathogen performance under CTs.

### 5.4.1 Thermal history of pathogen and host both contribute to the outcome of infection

Host acclimation to constant high or low temperatures did little to modify the outcome of infection; however, crickets gained an advantage from acclimation to fluctuating temperatures that improved their survival under constant temperatures. This advantage was maintained even when infected with an FTR-acclimated pathogen (e.g. Fig. 5.4F), which suggests that hosts and pathogens do not necessarily match their performance when acclimated to the same conditions (i.e. Fig. 5.1). Further, under constant temperatures, the host lost its advantage of FTR-acclimation when infected with a cold-acclimated fungus. This suggests that constant cold acclimation was beneficial to the pathogen, but did little to modify the performance of the host.

This lack of matched acclimation may be the norm, at least among generalist pathogen-host systems. For example cold-acclimation improves immunity in tadpoles of *Lithobates clamitans*, but induces injury or dormancy in its trematode parasite, *Ribeiroia ondatrae*

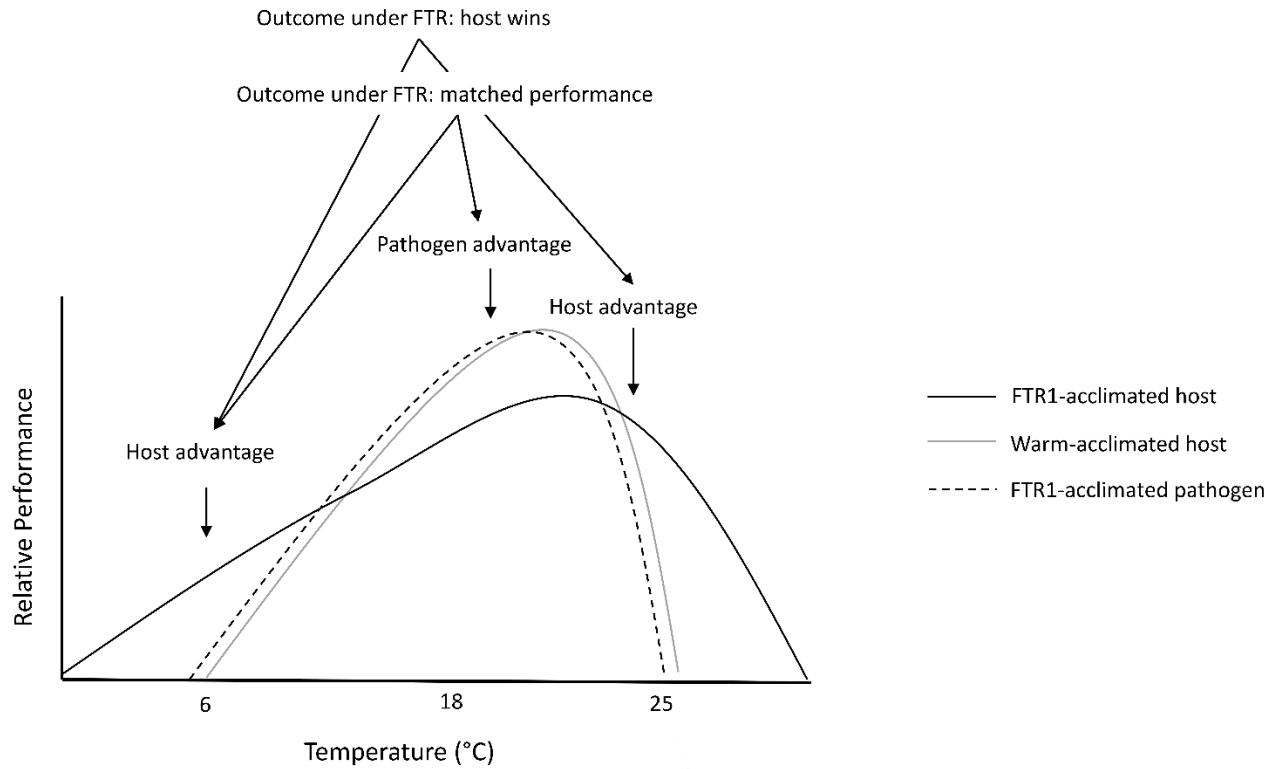
(Altman et al., 2016). In this case, it appears that acclimation to the same environmental conditions can place different selective pressures on both host and parasites, thereby creating mismatches in thermal performance. Consequently, we cannot expect that acclimation to the same conditions will result in matched performance. Therefore, both the thermal plasticity of host and pathogen will contribute to differing outcomes of infection under different thermal environments.

The exception to the importance of thermal history and plasticity occurs when the host and pathogen are acclimated to the same conditions and then interact under the same infection environment (e.g. warm acclimated host + warm acclimated pathogen + warm infection temperature or FTR + FTR + FTR). Under these conditions we achieve the same outcome of infection (i.e. the final % of crickets surviving; Fig. 5.7) and the effects of acclimation are masked. In this case, so long as we expect that the thermal history of the host and pathogen are matched, and also match the thermal environment under which they will interact, we are relieved of the burden of considering plasticity. These matched conditions may be achievable in buffered microhabitats that are co-habited by host and pathogen (e.g. aquatic, soil, or subnivean habitats). However, because we cannot assume that matched thermal history leads to matched thermal performance we cannot use the outcome of infection under matched conditions to predict outcomes under different temperatures. Further, there are several scenarios under which host and pathogen will have disparate thermal histories. For example, migrating insects will have disparate thermal histories from pathogens at their destination (Altizer et al., 2011) which are likely to modify their relative thermal performance. Thus, in variable environments – or in situations where it is unlikely for thermal histories to match - it is essential to understand the relative performance of each party to predict the outcome of infection.

#### 5.4.2 Acclimation to fluctuating conditions broadens the thermal performance of the host

Improved survival following acclimation to FTRs may be an adaptive response by the host to pathogen stress associated with exposure to low temperatures (Sinclair et al., 2013; Torson et al., 2015). For example, both *Megachile rotundata* and *Anopheles stephensi* increase expression of genes coding for antimicrobial peptides following exposure to fluctuating temperatures (Murdock et al., 2013); a similar response in *G. veletis* could account for improved survival. The transition from low to high temperatures (or vice-versa) may act as a prophylactic signal for changes in pathogen stress (Torson et al., 2015) that is lacking under constant temperatures. To understand why acclimation to fluctuating conditions increases host resistance/tolerance of infection, it will be important to determine if changes in pathogen pressure have selected for this plasticity. Overall, not only is it important to consider thermal plasticity of both host and pathogen, but also that this plasticity is modified by constant *vs* fluctuating conditions.

Based on overall proportion of survival as a metric of the outcome of infection under constant temperatures (Fig. 5.7), we can extrapolate and contrast relative thermal performance of the host and the pathogen as thermal performance curves (TPCs) (Fig. 5.8). In this case, TPCs show that acclimation to FTRs broaden thermal performance of *G. veletis* and produces a mismatch in performance of the host and the pathogen. This mismatch explains why FTRs improve host survival under all CTs but 18 °C and suggests that TPCs can be used to help predict the outcome of biotic interactions (Sinclair et al., 2016). It will next be important to tease apart the mechanisms underlying these thermal responses and the selective pressures driving plasticity in the host and pathogens to move towards quantitative predictions of the effects of the thermal environment on the outcome of biotic interactions.



**Figure 5.8 Relative thermal performance of host and pathogen at constant temperatures determines the outcome of infection under fluctuating conditions.** Putative thermal performance curves of WA and FTR1-A hosts and FTR1-A pathogen derived from results under constant temperatures in Figure 5.7. Arrows point to the temperature under which an interaction occurs, with fluctuating temperature regimes (FTRs) represented by connected arrows. The putative outcome of the interaction under FTRs is noted above the arrows.

### 5.4.2 Performance under constant conditions predicts outcome under fluctuating conditions

Based on the mismatch in performance that explains the differences in the outcome of infection under CTs (Fig. 5.8), we are also able to predict the outcome of infection under FTRs. Hosts performed poorly under an FTR that fluctuated between a constant temperature that was advantageous to the host and one that was advantageous to the pathogen. Conversely, *G. veletis* performed well under an FTR that was comprised of constant temperatures that should be advantageous to the host. Indeed, if we simply take an average of overall survival of the host at both of the constant temperatures that comprise an FTR, our predictions roughly match our observed outcomes (Fig. 5.7B). Further, when the constant temperature of infection was switched (e.g. from 6 °C to 18 °C), the outcome of infection mimics the outcome under the latter temperature, indicating that the outcome under FTRs is not a product of a signal to either host or pathogen during infection. Rather, it appears that we can predict the outcome of infection under fluctuating conditions based on the outcome under constant conditions.

Because of the complexity and non-additive nature of fluctuating temperatures on individual performance (Colinet et al., 2015; Sinclair et al., 2016), one of the grand challenges of predicting ectotherm performance under climate change has been the inability to predict fitness under fluctuating temperatures. Further, this complexity of individual performance suggested that predicting the outcomes of biotic interactions would be increasingly complex (Sinclair et al., 2016). However, I demonstrate that the effects of the thermal environment on biotic interactions actually represent a case of reduced complexity. The outcome of infection is based on how the relative performance of host and pathogen produces a winner under a particular temperature. Under fluctuating conditions, we can simply sum the wins and losses under constant temperatures to determine which player will be favoured under fluctuating conditions. Thus, regardless of the complex effects of temperature and plasticity on individual performance, the outcomes of biotic interactions under fluctuating temperatures can be reduced to the sum of relative performance between players.

I note that the experimental conditions I used in this study were step changes in temperature that held for the same amount of time at each temperature, which may limit our interpretations (Kingsolver et al., 2015). If the fluctuating conditions during infection are uneven in timescale, then we would need to weight the outcome of infection by time spent at each temperature, which might challenge our ability to simply sum performance. Further, pathogens and parasites may be able to rapidly acclimate during infection (Raffel et al., 2012), which would shift their thermal performance in a different way under fluctuating conditions that we cannot predict from constant conditions. Similarly, random fluctuations in temperature appear to differentially affect the outcome of infection compared to predictable fluctuations in temperature (Raffel et al., 2012). Thus, it will be important to determine if additive performance also accounts for these effects, or if there is an inherent difference between random and predictable (i.e. diurnal) fluctuations in temperature. Overall, moving forward it will be important to explore the mechanisms underlying variation in the outcome of infection under different types of fluctuating thermal regimes.

## 5.5 Conclusions

Here I demonstrate that acclimation to FTs appear to provide a signal to *G. veletis* to increase resistance or tolerance to infection, and growth at low temperatures modifies pathogen performance, confirming that both host and pathogen thermal history contribute to the outcome of infection under different thermal environments. Further, I provide a framework for understanding biotic interactions under fluctuating temperatures. I demonstrate that the outcome of the interaction between *G. veletis* and *M. brunneum* under FTs is a product of the sum of these interactions under constant temperatures. As such, we can use the outcome of the interaction under CTs to extrapolate relative performance, and then predict the outcome of interactions under FTs.

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## Chapter 6

### 6 Seasonal shifts in the insect gut microbiome are concomitant with changes in cold tolerance and immunity

In this chapter I explore seasonal shifts in the composition of the gut microbiome and host physiology of the spring field cricket, *Gryllus veletis*. This chapter has been prepared for submission to *Proceedings of the Royal Society B*.

#### 6.1 Introduction

The insect gut microbiome is composed of bacteria, archaea, and protozoa that may colonise the epithelial tissue of the gut or exist transiently in the food as it passes through the digestive tract (Douglas, 2015; Engel and Moran, 2013). These microbes contribute to digestion of food and provide essential nutrients, protect the host from colonisation by pathogenic microbes, and communicate with the host through neuroendocrine signaling to regulate host physiology (Douglas, 2015; Engel and Moran, 2013; Shin et al., 2011). Thus, the insect microbiome is an important contributor to the physiology of its host, and regulates insect success and survival (Douglas, 2011, 2015).

To understand how the microbiome influences host physiology, we can begin by characterising the species of bacteria present. Using sequencing of 16S rRNA, we can determine the community composition of the microbiome. Based on the characteristics of dominant microbes, we can then infer the functional significance of these communities with the host. For example, the gut microbiome of the mosquito *Anopheles gambiae* shifts to favour *Enterobacteriaceae* and *Klebsiella* following a blood meal. These bacteria have high redox capacity and, consequently, are likely to facilitate the response to oxidative stress in the gut associated with digesting blood (Wang et al., 2011). Thus, by employing sequencing techniques, we can capture any plasticity in the composition and function of the insect microbiome.

Animals live in fluctuating environments under which they must continually adjust their physiology to survive multiple, dynamic environmental pressures and challenges (Colinet

et al., 2015). Because animal phenotypes are a product of their interaction with microbes, their ability to survive and thrive also depends on the stability and function of the microbiome (Douglas, 2011, 2015; Engel and Moran, 2013). Microbes can be exposed to many of the same environmental pressures as their hosts. In ectotherms, the stability of the microbiome is likely to be challenged by changes in temperature, thereby influencing its functional relationship with the host, and the fitness of both (i.e. the holobiont; Lokmer and Mathias Wegner, 2015; Webster et al., 2008). In temperate areas, overwintering dominates the life cycle of ectotherms, such as insects (Williams et al., 2015), thus prolonged exposure to low temperatures and seasonal physiochemical rhythms (e.g. changes in gut structure, nutrient availability) are likely to influence the composition of the microbiome (Carey and Duddleston, 2014). However, we know little of how changes in environmental pressures, such as a shift in season, may influence or disrupt the microbiome and its functional relationship with insect hosts.

### 6.1.1 The influence of overwintering on the insect microbiome

Because overwintering includes multiple challenges associated with low temperatures (Williams et al., 2015), the composition and function of the microbiome within an ectotherm may be influenced by overwintering in four, non-mutually exclusive ways: 1) low temperatures select for the growth of psychrophilic microbes that outcompete others; 2) cold directly kills those microbes intolerant of low temperatures, thereby increasing the relative abundance of those tolerant of cold; 3) seasonal changes in physiochemical conditions in the gut select for particular microbes; or 4) the host actively manipulates the composition of the microbiome. For example, the host can regulate the composition of the microbiome through the immune system (Carey et al., 2013) or *via* microRNAs (Hussain et al., 2011). To understand the functional significance of shifts in the microbiome, we need to distinguish between these driving factors, as they each have different implications for the fitness of the holobiont under scenarios such as climate change. For instance, if the microbiome is directly regulated by the external environmental temperature, then warmer or more variable winters predicted under climate change are likely to play a large role in shaping the microbiome. By contrast, if the microbiome is directly regulated by the host, there are two possible consequences

under climate change. First, the host continues to regulate the microbiome which allows them to maintain and maximise performance under novel conditions, or second, the programmed regulation of the microbiome under novel conditions leads to an inability to shift physiology to suit new environments.

Changes in the community composition of the gut microbiota are likely to reflect functional shifts in its relationship with the host that contribute to the overwintering success or failure of the animal. Seasonal shifts in the microbiome could represent a stressful dysbiosis (Carr et al., 1976), or plasticity and seasonal rhythms in the holobiont that allows for dynamic function (Carey et al., 2013; Wier et al., 2010). For example, overwintering appears to favour pathogens in the gut microbiome of bullfrogs, *Lithobates (Rana) catesbiana*, creating dysbiosis that directly leads to increased infection and overwintering mortality (Carr et al., 1976). In this case, we might expect that the host immune system also shifts seasonally (Maniero and Carey, 1997), and its interaction with the microbiome affects host survival. Conversely, freeze-avoidant insects, such as *Dendroides canadensis*, appear to regulate their gut microbiota by voiding or masking bacteria that contribute to ice nucleation. This regulation of bacteria then protects their ability to remain unfrozen and survive the winter (Olsen and Duman, 1997). In this case, host-regulation of the microbiome directly affects the ability to survive exposure to low temperatures. Thus, the composition and function of the microbiome is related to survival of multiple overwintering pressures (e.g. cold and pathogens). Thus, by understanding concomitant shifts in both the microbial community as well as host physiology (e.g. immunity, cold tolerance), we can begin to understand the functional links between them.

### 6.1.2 Variation in the gut microbiome between lab and field

Many species of insects carry and maintain a conserved subset of bacteria across geographic areas (Sudakaran et al., 2012), habitats (Cariveau et al., 2014), or even between lab and field populations (Wang et al., 2011). For example, almost all of the taxa found in lab-reared adults of the mosquito *Anopheles gambiae*, are also found in adults in the field, and these taxa represent the majority of bacteria in both groups (Wang et al.,

2011). In these cases, the gut is likely a selective ecosystem for these microbes (Wang et al., 2011), leading to a conserved community within the microbiome despite putative exposure to different microbes in different foods and habitats.

Conversely, the community composition of the insect microbiome can be highly variable among individuals (Wong et al., 2013) or vary among habitats, such as between the lab and the field (Belda et al., 2011). Conceivably, these differences could infer functional shifts in the relationship between the microbiome and the host. For example, the gut microbiota of the European Corn Borer, *Ostrinia nubilalis* are mainly Gram-negative and function to degrade cellulose, whereas in lab *O. nubilalis* the composition shifts to favour Gram-positive bacteria and the functional relationship is lost. In this case, these differences likely reflect different selection pressures in the gut based on diet (Belda et al., 2011). Because there can be variability in the microbes that insects are exposed to between lab and field, it is important to understand variability in the microbiome among populations, and whether or not we can generalise the functional significance of microbiome plasticity across populations of the same species.

### 6.1.3 Objectives

To unravel the role of the microbiome in determining host success under fluctuating environments we aimed to: 1) to determine if the insect microbiome varies across seasons; 2) to determine if these patterns vary between a lab and field microcosm; and 3) if the microbiome does shift, to determine if those shifts are concomitant with changes in host physiology that could help explain the possible functional significance of shifts in the microbiome. To address these objectives, we exposed the overwintering stage of a temperate species of field cricket native to Ontario, *Gryllus veletis* to simulated overwintering conditions in either a lab or field microcosm. Throughout the overwintering period, we characterised the composition of the gut microbiome. Further, we measured seasonal shifts in both immune activity and cold tolerance. We show that the community of gut microbes does shift with season, and that these shifts are conserved across both microcosms. Further, the microbiome does not reset in the spring, suggesting permanent perturbation or dynamic function of the microbiome. Concomitant to changes in the community composition of the microbiome, immune activity decreases while cold

tolerance increases in *G. veletis*. Overall, we highlight that the microbiome of insects is dynamic in its response to changes in the environment, which allows us to progress in untangling its functional significance in regulating host success under fluctuating environments.

## 6.2 Methods

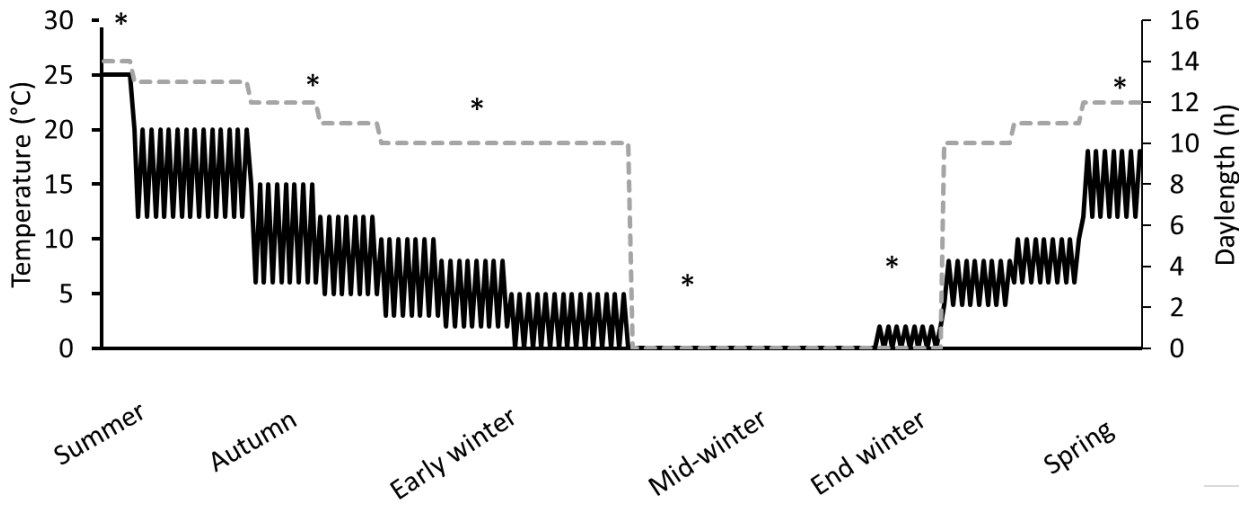
### 6.2.1 Cricket housing and overwintering conditions

*Gryllus veletis* were derived from a population collected in Lethbridge, Alberta, Canada in 2010. In 2014-2015, we reared *G. veletis* from egg to 6<sup>th</sup> instar nymph at 25 °C (14 L:10 D) as described by Coello Alvarado (2012). Rearing conditions represented summer conditions. We maintained crickets in densities of approximately 100 individuals in plastic bins (28 × 17 × 15 cm) on *ad libitum* rabbit chow (Little Friends Rabbit Food, Martin Mills, Elmira, ON, Canada) and water with cardboard shelters.

To determine if habitat would influence the composition and seasonal plasticity of the microbiome, I divided crickets into two bins (28 × 17 × 15 cm): a field microcosm (FM) and a lab microcosm (LM). We exposed all crickets to a gradual, fluctuating decline in temperature and photoperiod in a temperature-controlled incubator until they reached 0 °C (Fig. 6.1), to mimic autumn temperatures and photoperiods in London, ON, Canada. During autumn, we introduced soil and humus collected from local areas where I had previously heard *G. veletis* males calling, thereby creating the field microcosm. To mimic conditions under snow cover where *G. veletis* overwinter, we maintained crickets at 0 °C and under darkness for four weeks. we then gradually increased temperature and photoperiod to mimic conditions in the spring (Fig. 6.1).

### 6.2.2 Gut dissection, DNA extraction, amplification and sequencing

In summer, autumn, early winter, mid-winter and spring (Fig. 6.1) I haphazardly selected ten crickets from each microcosm for gut extraction. In a laminar-flow clean bench under sterile conditions, we surface-sterilised crickets with 70 % ethanol and removed the hindgut as described by MacMillan and Sinclair (2011). we immediately



**Figure 6.1** Temperature and photoperiod of simulated overwintering conditions of *Gryllus veletis*. Grey dashed lines line represents hours of daylight; black lines represent temperature. Asterisks represent sampling points.



placed the hindgut in a sterile plastic tube and snap froze specimens in liquid nitrogen. Samples were then stored at -80 °C until DNA extraction.

To extract total genomic DNA from cricket guts, we pooled two guts for each replicate and used the DNeasy blood and tissue DNA extraction kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. We analysed the quality of the extracted DNA spectrophotometrically at 260 and 280 nm using a 260/280 ratio >2 to indicate sufficient quality of the DNA. We diluted the samples in sterile water and confirmed the presence of bacterial DNA using PCR and the universal bacterial DNA primers 27F BacU and 534R BacU (Integrated DNA Technologies, Coralville, Iowa, USA).

We pooled extracted genomic DNA (gDNA) from each sampling point for a total of one sample per season. We shipped the extracted gDNA to a Molecular Research Laboratory in Shallowater, Texas (MR DNA, 503 Clovis Road, Shallowater, Texas, USA 79363) and analyzed by Illumina MiSeq sequencing (Illumina) at 300 bp reads and >20,000 reads per sample following the manufacturers standard protocols.

A fragment of the Bacterial 16S rRNA gene, spanning the V1 – V2 hypervariable regions, was amplified using universal bacteria primers that were modified by adding ligation adaptors and/or MID barcodes (sample identification sequences) to the 5' - ends. PCR was performed using a high fidelity polymerase (HotStarTaq Plus Master Mix Kit, Qiagen, Valencia, CA, USA) and with the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds; 53 °C for 40 seconds and 72 °C for 1 minute; after which a final elongation step at 72 °C for 5 minutes was performed. PCR products were further examined in a 2% agarose gel to determine the success of amplification and the relative intensity of bands. Amplicons were mixed in equal concentrations and purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA) before sequencing.

Following sequencing, we obtained a mapping file from MR DNA and verified the file for errors. We filtered the sequences using Qiime software for removal of unique barcodes and primers, low quality reads (< quality score 25), short and long sequences (< 200 nt; >1000 nt), zero ambiguous base calls, gaps, zero primer sequence mismatch, and sequences with homopolymer runs exceeding 6nt. We then checked the filtered sequences for chimeras for removal (Edgar, 2010; uchime as part of usearch v5.2.236). We then picked OTU (Operational Taxonomical Units) from the sequences using USEARCH v5.2.236 based on a 97% or greater similarity threshold against the GreenGenes database through the Qiime software pipeline using a closed-reference OTU picking protocol (Caporaso et al., 2010a; Caporaso et al., 2010b; Caporaso et al., 2011; Kuczynski et al., 2012). We picked and aligned representative set of sequences using PyNAST (Caporaso et al., 2010a) and the Green genes core set template file. All unassigned species (not binned to the kingdom level) were removed from the data set and a Newick formatted tree using FastTree (Price et al., 2009) and an OTU Biom table (McDonald et al., 2012) were respectively generated from the representative filtered OTUs for downstream analysis. We then rarefied each of the treatments to 58669 sequences per sample, the lowest number of sequences detected in a sample, to eliminate uneven sampling depth for diversity analysis.

We then used Qiime software to generate rarefaction curves for Chao1, observed species, and Shannon Index for alpha diversity as parameters for measuring abundance, frequency, richness and diversity in the microbiome communities within the samples. We then examined the statistical significance of beta diversity (between sample diversity) using Qiime by creating weighted and unweighted Unifrac matrices [based on normalized abundance data; (Lozupone et al., 2011)]. We then used these matrices to create a jackknifed beta diversity analysis (Caporaso et al., 2010b; Caporaso et al., 2011), along with Principal Coordinate Analysis (PCoA) (Vázquez-Baeza et al., 2013) to compare the gut microbiome communities between samples. We constructed a bootstrap consensus tree from the jackknifed iterations using UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering. We transformed OTUs as presence/absence data of individual OTUs, averaged across samples, and used the software package Krona (Ondov et al., 2011) to create non-metric multidimensional interactive scaling visualization of gut

OTUs from each species (i.e. interactive pie charts of the relative abundance of bacterial OTUs).

### 6.2.3 Seasonal shifts in cold tolerance and immunity of *G. veletis*

To determine if cold tolerance of *Gryllus veletis* shifted seasonally, we compared the ability of summer and mid-winter crickets to survive low temperatures in the laboratory microcosm only. One measure of plasticity in cold tolerance is to determine if an insect increases its ability to survive low temperatures (Sinclair et al., 2015). To determine if crickets were plastic in their cold tolerance, we haphazardly selected a subset of crickets in the summer and winter and placed them in 1.5 mL plastic tubes with the body of the cricket in contact with the tip of a 36 AWG (American Wire Gauge) copper-constantan thermocouple (Omega, Laval, QC, Canada). We then placed tubes containing crickets into an aluminum block cooled by propylene glycol circulated by a programmable proline 3530C refrigerated bath (Lauda, Wurzburg, Germany) and decreased the temperature from 0 °C to -10 °C at a rate of 0.25 °C/min. We recorded the temperature as described by Marshall and Sinclair (2011) using PicoLog software (Pico Technology, Pico, UK) and detected freezing from exotherms (Sinclair et al., 2015). The freezing exotherm represents the supercooling point (SCP) of the insect. Following 4 h at -10 °C, we rewarmed crickets to 0 °C at 0.25 °C/min and recorded survival 24 h following cold exposure. We compared SCPs using a t-test in R (R Development Core Team, 2010).

To determine if constitutive and realised immunity change from summer through to spring, we haphazardly selected female nymphs in the laboratory microcosm in the summer, mid-winter, and spring and measured circulating haemocyte concentrations, melanisation, *in vivo* bacterial clearance, and survival of fungal infection. Due to sample size constraints following mortality during overwintering, we were unable to measure bacterial clearance in the spring. To understand whether or not substrate availability might limit the melanisation response, we also measured hemolymph protein concentration. All statistical analysis was performed in R (R Development Core Team, 2010).

We collected 1  $\mu\text{L}$  of haemolymph following Adamo (2004) and diluted it in 21  $\mu\text{L}$  of phosphate buffered saline. We counted the total number of circulating hemocytes (CHC) in a Neubauer improved hemocytometer (Hausser Scientific, Blue Bell, PA, USA) at 400  $\times$  magnification following Wilson et al. (2002). We compared CHC among groups (summer, winter, & spring) using ANOVA.

We assessed the strength of the melanisation response as described by Ferguson et al. (2016); briefly, we inserted a nylon filament into the hemocoel of the cricket. We removed the filament after 24 h, photographed the filament from two angles, and measured the grey value, where lower grey values indicated a darker area and thus a stronger melanisation response. We expressed melanisation at 255-grey value such that higher values would then indicate a stronger response. We compared the melanisation response among groups using ANOVA with a log-transformation of the grey value.

We measured clearance of the Gram-positive bacteria, *Staphylococcus aureus*, following Ferguson et al. (2016). Briefly, we injected *G. veletis* with a suspension of streptomycin-resistant *S. aureus* [ $1 \times 10^7$  colony forming units (CFU)/mL] in the membrane under the pronotum. Either 1 min (to capture the number of bacteria injected) or 24 h later, we homogenised whole crickets in PBS, spot-plated an agar plate containing streptomycin (25  $\mu\text{g}/\text{mL}$ ), incubated the plates at 37  $^{\circ}\text{C}$  for 24 h, and counted the number of colony-forming units. We used the number of CFU present at 1 min post-injection as our true number of CFU injected, and subtracted the remaining CFU to obtain the number of CFU cleared from the cricket. We compared clearance in summer and winter samples using a t-test.

We infected *G. veletis* with the entomopathogenic fungus *Metarhizium anisopliae* by injecting 1  $\mu\text{L}$  of a spore suspension of *M. anisopliae* diluted in 0.01 % Tween 80 in the membrane under the pronotum using a Hamilton syringe and a 32-gauge needle. We injected control crickets with the same volume of 0.01 % Tween 80. We housed infected and control crickets individually in *Drosophila* vials with *ad libitum* rabbit chow and water and paper shelters at 25  $^{\circ}\text{C}$ . Upon death, we surface-sterilised crickets and maintained them on moist paper towel at 25  $^{\circ}\text{C}$  to confirm the characteristic growth of

green spores. We used the *survival* package in R to generate Kaplan-Meier survival curves and compared the fit of generalised linear models to the data using log-likelihood. Based on log-likelihood, we chose a lognormal model to test for differences among survival curves.

We measured haemolymph protein as described by Ferguson et al. (2016). Briefly, we diluted 1  $\mu$ L of haemolymph in 50  $\mu$ L of PBS in a microcentrifuge tube. We then plated 10  $\mu$ L in triplicate in a microplate and used a Bicinchoninic Acid kit (BCA; Life Technologies, Carlsbad, CA, USA). We measured absorbance at 562 nm in a microplate spectrophotometer (SpectraMax, Molecular Devices, Sunnyvale, CA, USA). We then converted absorbance to concentration values using a standard curve created from bovine serum albumin. We compared protein in summer, winter, and spring using ANOVA in R.

## 6.3 Results

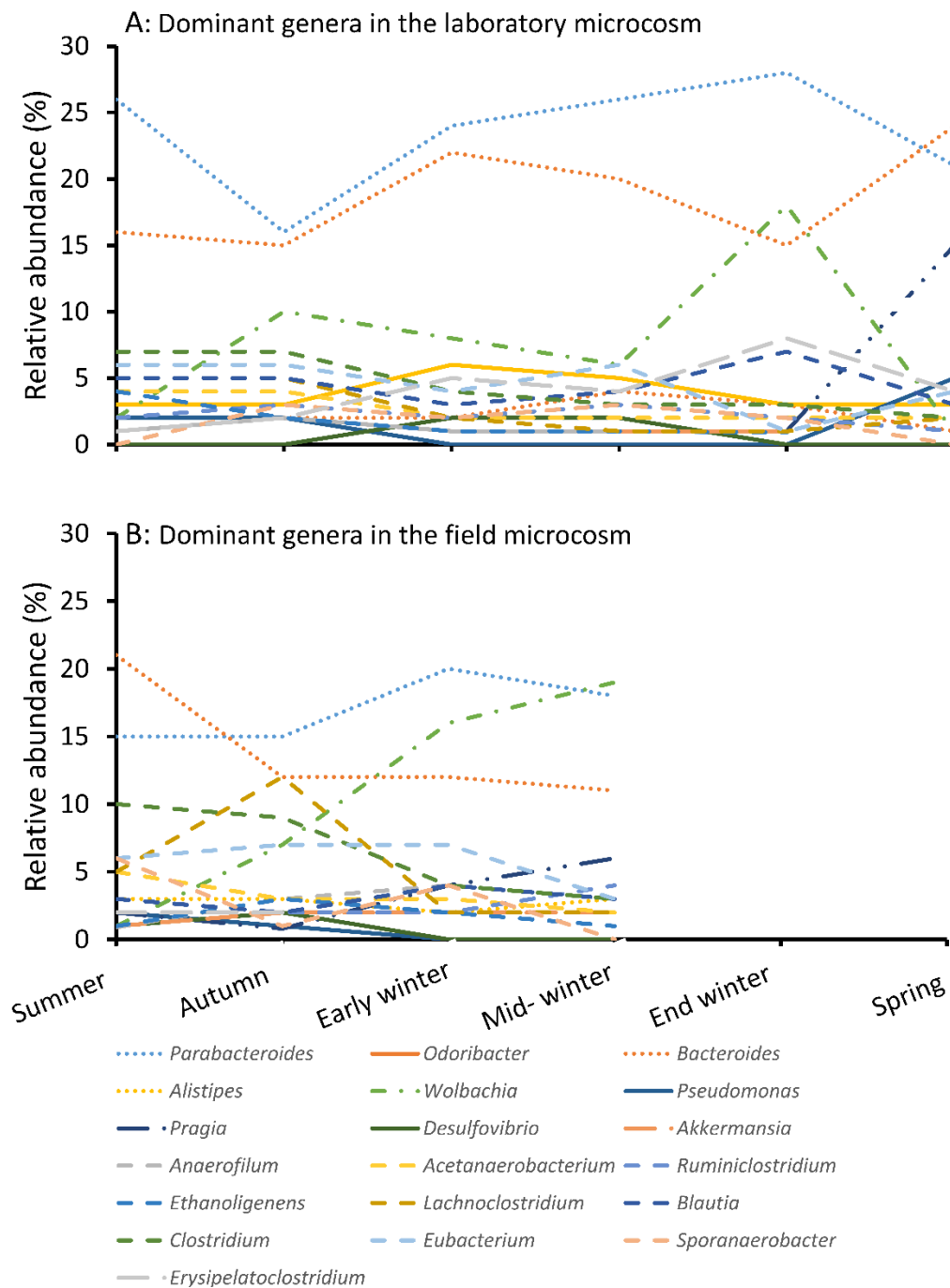
### 6.3.1 Composition of the gut microbiome shifts with changes in season

Sequencing of microbial gDNA produced an average of  $79026 \pm 10\,211$  reads per sample, ranging from 58669 to 90453 (Table 6.1). I assigned reads to 1377 OTUs at 97% sequence identity threshold ranging from 906 to 1127 (Table 6.1) per sample with an average of  $991 \pm 59$  OTU/sample. The gut microbiome was dominated by Bacteroidetes (majority in the genera *Bacteroides* and *Parabacteroides*; Fig. 6.1A,B), Firmicutes, and Proteobacteria across all seasons and environmental treatments (Fig. 6.2 A,B). However, within the Bacteroidetes, *Bacteroides* dominated over *Parabacteroides* in the field microcosm, whereas this dominance was switched in the lab microcosm (Fig. 6.1A,B). Due to high overwintering mortality in the field microcosm, I was unable to complete sampling in that microcosm beyond the mid-winter timepoint.

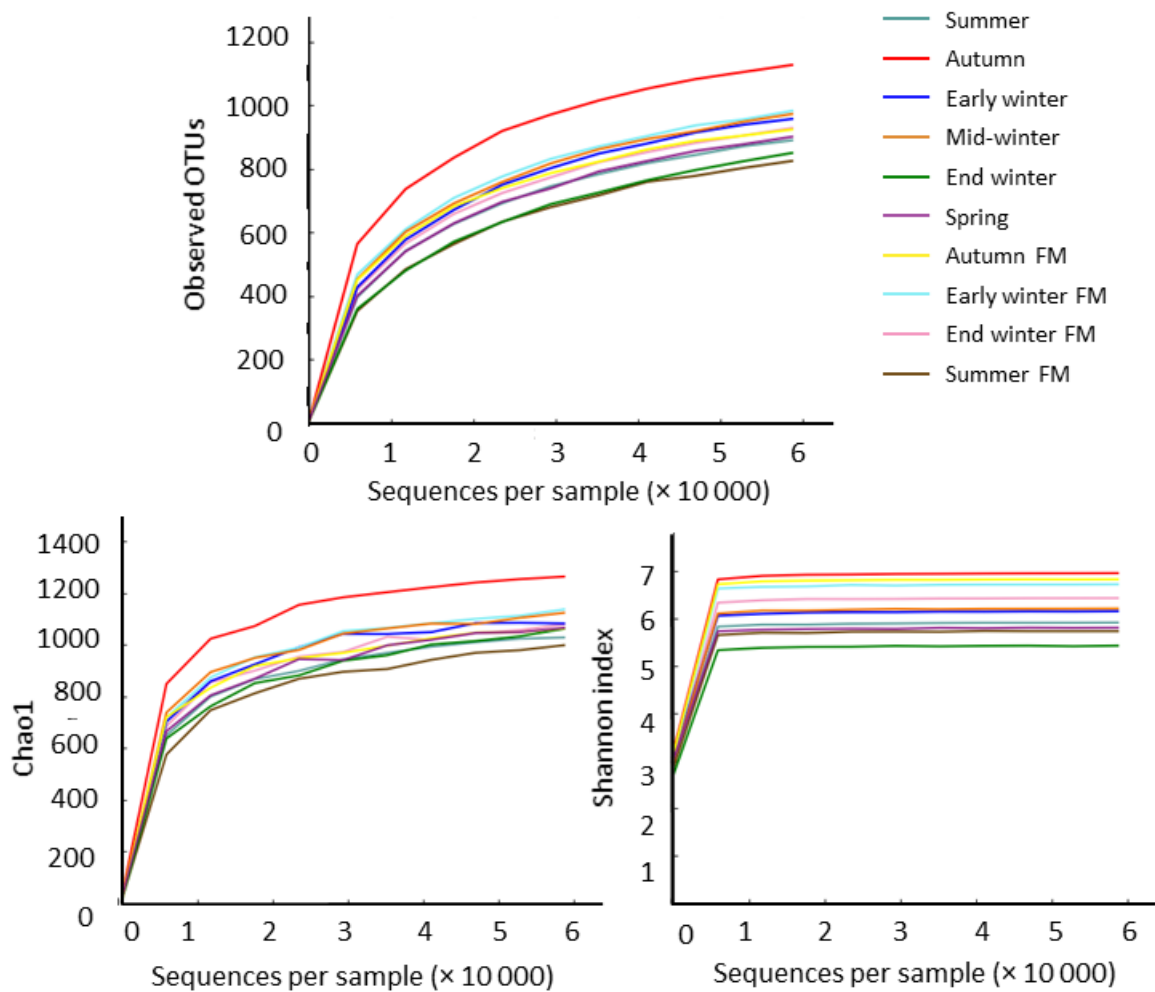
The alpha rarefaction curve of OTU approached saturation (Fig. 6.3A), suggesting that I captured the majority of diversity in each sample; however, it is possible that there remain rare taxa that I was unable to capture. Each sample of bacterial gDNA was sampled to the same sequencing depth of reads (Fig. 6.3 B). Species richness of the gut

**Table 6.1 Summary of sequence number and operational taxonomic units (OTUs) in the microbiome of *Gryllus veletis*.** Crickets were sampled at different time points across season in two different microcosms. Both microcosms underwent simulated overwintering in the lab; however, the field microcosm contained soil, leaves and humus from a field site inhabited by *Gryllus veletis*.

	<b>Sample</b>	<b>Sequence number</b>	<b>OTUs</b>
<b>Lab microcosm</b>	Summer	69 254	955
	Autumn	58 669	1127
	Early winter	75 560	1005
	Mid winter	90 453	1047
	End winter	90 061	945
	Spring	88 525	979
	<b>Field microcosm</b>	Summer	68 979
Autumn		80 391	988
Early winter		80 848	947
Mid winter		87 524	906



**Figure 6.2** Relative abundance of dominant genera in the microbiome of the hindgut of *Gryllus veletis* exposed to overwintering conditions in a laboratory or simulated field microcosm. Solid lines represent genera within the Bacteroidetes; dashed lines represent genera within Firmicutes, dashed lines represent those within gammaproteobacteria, and dashed-dotted lines represent genera within the alphaproteobacteria,



**Figure 6.3 Rarefaction plots for bacterial microbiomes in the hindgut of *Gryllus veletis* across season in a lab microcosm (LM) and simulated field microcosm (FM).** **A.** Observed operational taxonomic units (OTUs) plotted against the number of sequences per sample; curves approach saturation suggesting that I captured most taxa present. **B.** Chao1 as a measure of species richness plotted against the number of sequences per sample (Lozupone et al., 2011); species richness is highest in autumn in the LM, but is largely similar among seasons. **C.** Shannon index of species diversity plotted against the number of sequences per sample (Lozupone et al., 2011). Again, diversity is similar among seasons.

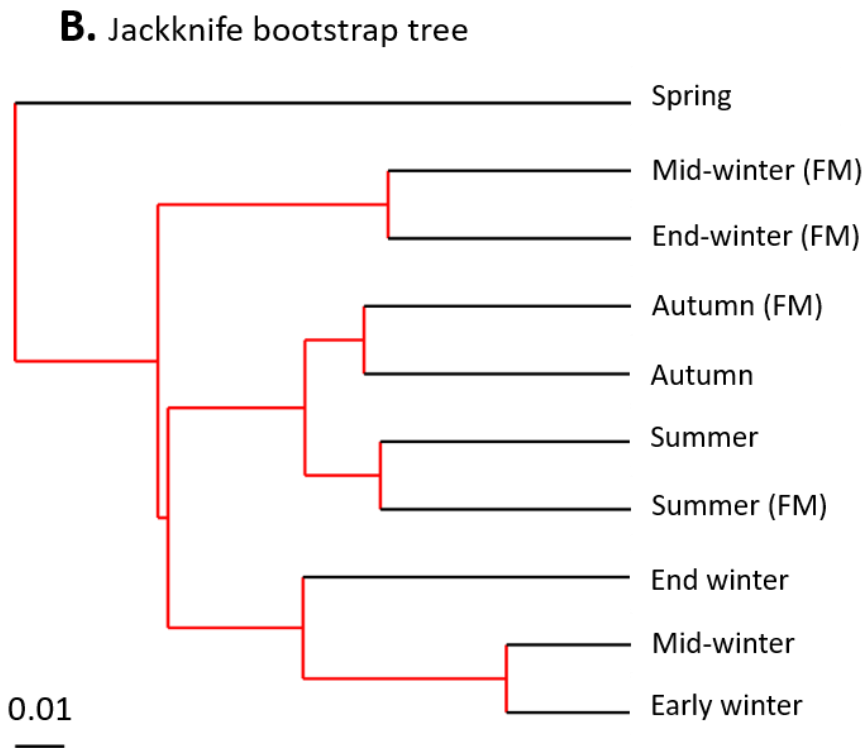
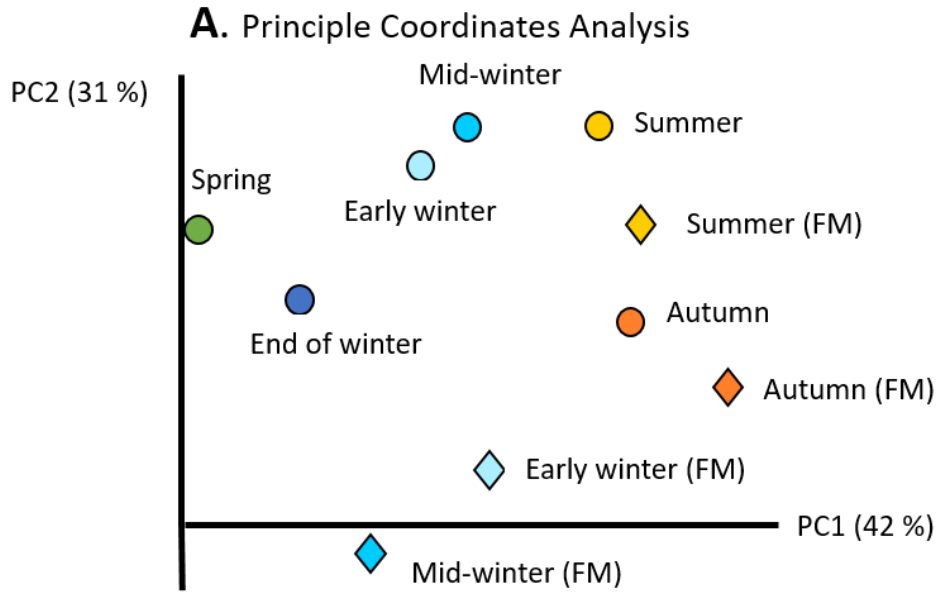


microbiome was highest in the autumn (LM) and lowest in early and mid-winter (FM); however, species richness was similar among seasonal time points and between microcosms (Fig. 6.3 B). Autumn and summer (LM) microbiomes had the most diverse bacterial communities, whereas early- and mid-winter (FM) had the lowest diversity (Fig. 6.3 C).

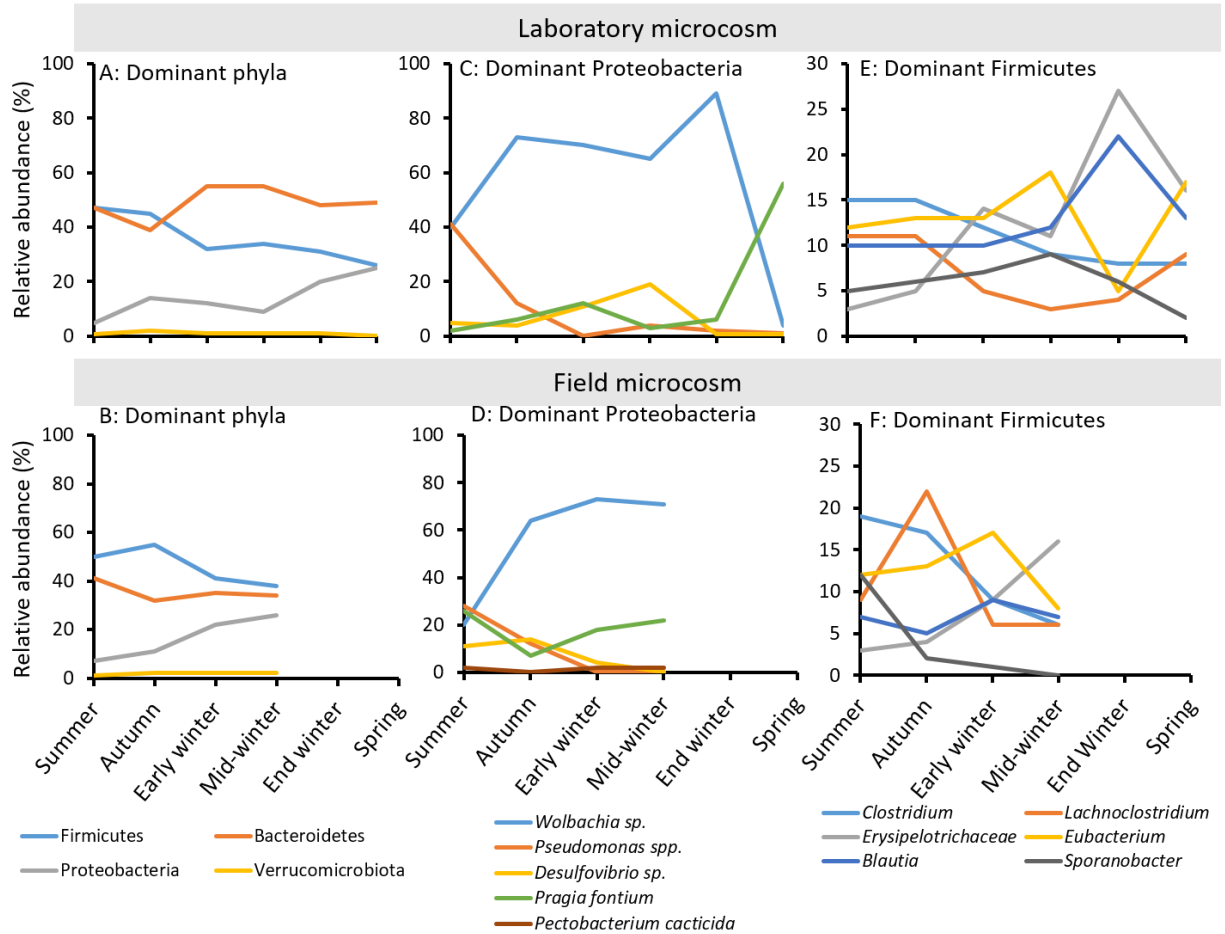
Overall, the community composition of gut microbiota cluster by season, as well as by microcosm (i.e. by the addition of soil and leaves; Fig. 6.4A). The first axis of the principle coordinated analysis (PCoA; Fig. 6.4) describes 42 % of the variation among samples, and is driven largely by season. The second axis describes 31 % of the variation among samples and appears to be driven by microcosm. Jackknifed bootstrap trees (Fig. 6.4B) support the clustering of the samples at a confidence level of 75 – 100%, such that spring clusters away from all other samples, autumn samples cluster together and then most closely with summer samples, and winter samples cluster together based on whether or not soil and leaves were present in the microcosm (Fig. 6.4B).

The trends between the two microcosms were similar across season. As season progressed, the relative abundance of Proteobacteria increased from 5 % to 26 % of the relative abundance of the microbiome from summer to spring in the LM, or 7 % to 26 % from summer to mid-winter in the FM (Fig. 6.5A,B), whereas the relative abundance of Firmicutes decreased from 47 % to 25 % from summer to spring in the LM and 50 % to 38 % from summer to mid-winter in the FM (Fig. 6.5A,B).

At the species level, *Wolbachia* sp. displayed the greatest change across season, increasing to almost 90 % of the Proteobacteria, and 20 % of the total bacterial abundance in mid- and late-winter (Fig. 6.5 C,D); however, the relative abundance of *Wolbachia* decreased back to summer levels in the spring (Fig. 6.5C). By contrast, *Pseudomonas* spp. decreased from summer through winter, and remained at low relative abundance in the spring (Fig. 6.5C). Additionally, *Pragia fontium* appeared to increase in abundance in the spring (Fig. 6.5C). Within the Firmicutes, *Blautia* sp. and *Clostridium spiroforme* (Erysipelatoclostrichaceae) peaked in relative abundance in the winter, although this trend was most apparent for *C. spiroforme* in the FM. In both microcosms,



**Figure 6.4. Measures of beta-diversity in the microbiome of *Gryllus veletis* across season and between two microcosms. A.** Principle coordinates analysis (PCoA) of the composition of the gut microbiome. **B.** Jackknife bootstrap tree as a measure of validation for the PcoA. Red lines indicate a confidence level of 75 – 100 %.



**Figure 6.5. Relative abundance of the dominant phyla and species or genera within phyla that show the most variation across season in the microbiome of the hindgut in *Gryllus veletis*.** Top panels represent crickets in a lab microcosm and bottom panels represent crickets in a simulated field microcosm. **A.** Dominant phyla; **B.** Dominant species within the Proteobacteria; **C.** Dominant genera within the Firmicutes.

the relative abundance of species in the genus *Clostridium* decreased (Fig. 6.5 E,F). Finally, I detected genera of facultative pathogens in the gut (Lysenko, 1985), as well as potential ice nucleators [e.g. *Pseudomonas* spp. (Lee et al., 1993)]. In particular, *Serratia marcescens* represented >1 % of the composition of Proteobacteria in spring and summer, but falls below detectable levels in the winter. Similarly, *Pseudomonas* spp. decreased in relative abundance in both microcosms

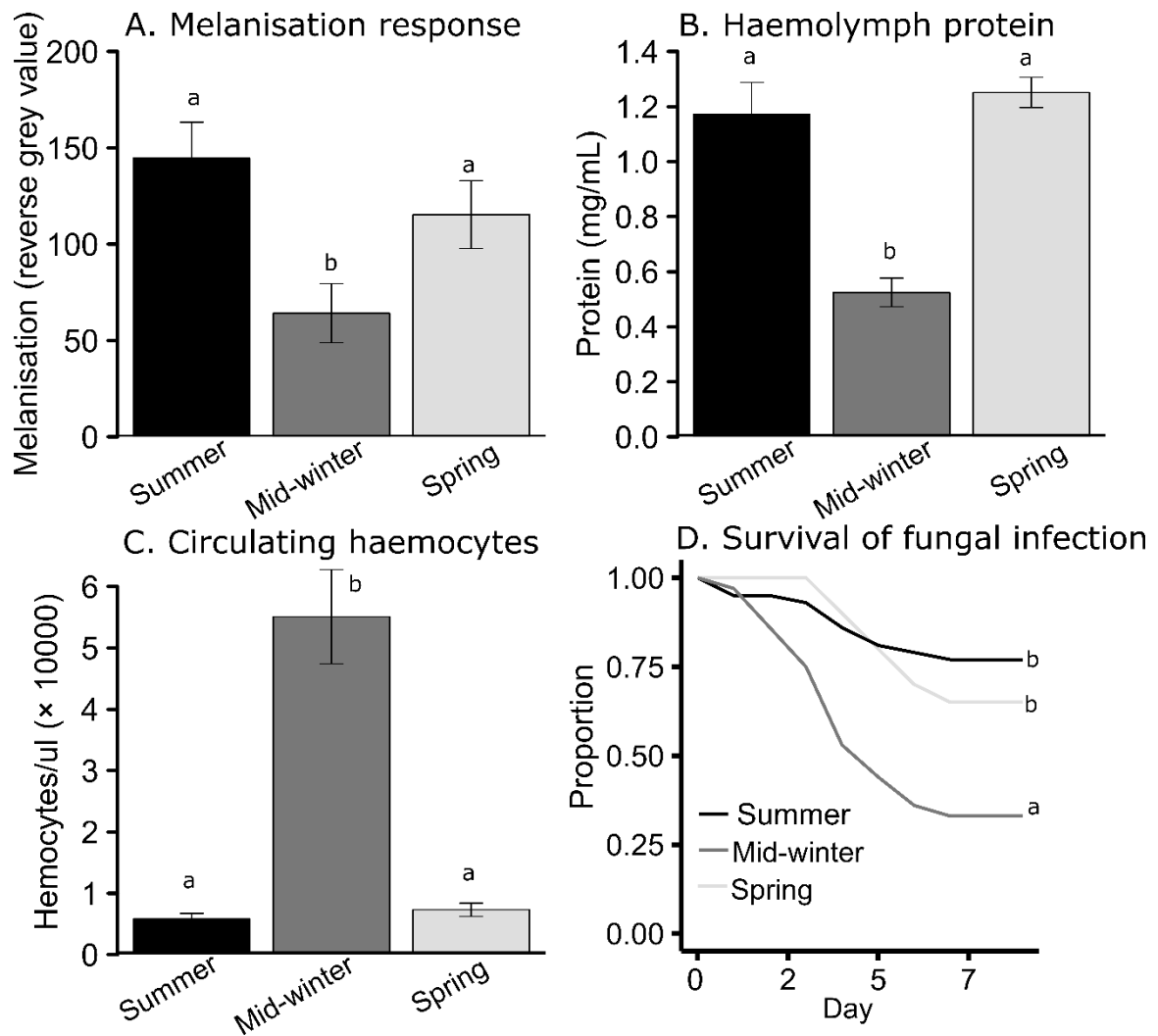
### 6.3.2 Cold tolerance increases in the winter

Winter-acclimated crickets were more cold-tolerant than summer-acclimated crickets: 4/7 winter-acclimated crickets survived exposure to  $-10\text{ }^{\circ}\text{C}$  for 4 h, whereas 0/7 summer-acclimated crickets survived this exposure. However, there was no significant change in the supercooling point (winter:  $-8.7 \pm 0.4\text{ }^{\circ}\text{C}$ ; summer:  $-8.1 \pm 1.8\text{ }^{\circ}\text{C}$ ;  $t_7 = 0.78$ ,  $p = 0.23$ ).

### 6.3.2 Immune activity is differentially affected by overwintering

Crickets displayed a weaker melanisation response (Fig. 6.6A,  $F_{2,11} = 5.46$ ,  $p = 0.02$ ;  $n = 5-10$  per season), coupled with decreased total hemolymph protein (Fig. 6.6B;  $F_{2,21} = 24.91$ ,  $p < 0.001$ ;  $n = 5-10$  per season) and decreased survival when infected with a fungal pathogen (Fig. 6.6D; winter vs summer:  $z = -3.41$ ,  $p < 0.001$ ;  $n = 10$  per season).

However, circulating haemocyte counts increased (Fig. 6.6C;  $F_{2,24} = 51.66$ ,  $p < 0.001$ ;  $n = 5-16$  per season), and the ability to clear bacteria from the haemolymph remained unchanged ( $F_{1,11} = 0.8$ ,  $p = 0.39$ ;  $n = 5-10$  per season). Further, CHC decreased back to summer levels in the spring (Fig. 6.6C), and melanisation (Fig. 6.6A) and survival of fungal infection (Fig. 6.6D; winter vs spring:  $z = -2.40$ ,  $p = 0.02$ ; summer vs. spring:  $z = 0.45$ ,  $p = 0.65$ ) also returned to similar levels as seen in the summer.



**Figure 6.6. Measures of immune activity and haemolymph protein in summer, mid-winter and spring in *Gryllus veletis* in a laboratory microcosm. A.** the strength of the melanisation response against a simulated pathogen (n = 5-10 per season) **B.** Concentration of haemolymph protein as a correlate for substrate available for the melanisation response (n = 5-10 per season). **C.** Concentration of hemocytes in the haemolymph (n = 5-16 per season). **D.** Survival against the fungal entomopathogen, *Metarhizium anisopliae* (n = 10 per season). Different letters indicate seasons that differ significantly from each other.

## 6.4 Discussion

Here I show that overwintering affects both the composition of the gut microbiome and host physiology and in the spring field cricket, *Gryllus veletis*. Regardless of the microbial conditions in the external environment (i.e. presence or absence of soil and leaves), the gut microbiome is similar in summer and autumn, but shifts in winter to favour an increase in the relative abundance of Proteobacteria through winter and spring. Immune activity decreases during the winter, while crickets also increase their cold-tolerance; however, immune activity recovers in the spring. Thus, a change in season appears to provoke a conserved shift in the microbiome, which could represent seasonal shifts in the functional relationship between microbiome and host. Further, shifts in the microbiome are likely to be driven by seasonal changes in host physiology. These concomitant shifts in the microbiome and host physiology highlight the importance of considering multiple aspects of host function to understand how an animal responds to changes in its environment.

### 6.4.1 The microbiome shifts seasonally

Apart from the conserved dominance of *Parabacteroides* and *Bacteroides*, there was seasonal variation in a variety of taxa. In particular, overwintering appeared to favour increased abundance of the endosymbiont *Wolbachia*, as well as *Clostridium symbiosum* and *Pragia fontium*. Conversely, other *Clostridium* species, as well as *Pseudomonas* spp. decreased in relative abundance. Upon recovery from overwintering conditions in the spring, the composition of the microbiome did not return to its original state. Although the increase in *Wolbachia* spp. is reversed in the spring, others, such as *Pseudomonas* spp., and the overall relative abundance of Proteobacteria (potentially driven by *Pragia fontium*) remains elevated in spring compared to summer. Because the microbiome is an ecosystem of cooperative and competitive interactions, disruption of these interactions can permanently shift communities (Coyte et al., 2015). I suggest that, unlike the reversible changes in host physiology, the microbiome of *G. veletis* is highly sensitive to disruption via environmental pressures. These disruptions appear to permanently shift the microbiome following overwintering, likely due to either competitive or cooperative

interactions. I suggest that variation in season can provoke a non-reversible, functional shift in the relationship between the microbiome and the host.

### 6.4.3. The microbiome is dominated by anaerobes and represents a conserved core of dominant species

Overall, the microbiome in both microcosms was dominated by anaerobic bacteria across seasons (e.g. *Bacteroides* and *Parabacteroides*). This is in concordance with a previous study on the hindgut bacteria of crickets (*Acheta domesticus*) in which the microbiome is dominated by *Bacteroides* spp. (Santo Domingo et al., 1998b). Indeed, the cricket hindgut appears to be fermentative (Santo Domingo et al., 1998a), and the bacteria present are essential for the digestion of complex plant polysaccharides (Kaufman and Klug, 1991). Although the specific function of *Bacteroides* has not been determined in crickets, *Bacteroides* spp. are dominant in the human gut microbiome as well, and play an essential role in carbohydrate fermentation (Wexler, 2007). Thus, the predominantly anaerobic nature and dominance of *Bacteroides* and *Parabacteroides* across season indicates that the overarching dietary function of the microbiome is likely conserved across season and between microcosms.

The seasonal shifts in *Wolbachia*, *Clostridium* spp., *Clostridium symbiosum*, and *Pseudomonas* spp. were similar in both microcosms, and each shifted towards increasing relative abundance of Proteobacteria overall. We expected that the introduction of soil, leaves, and humus would change the composition of microbes in the external environment and food of the crickets, thereby introducing new microbes into the gut; however, because the overall composition of the microbiome shifts similarly across season, the dominant bacterial taxa do not appear to have been disturbed by a change in microcosm. Changes in diet can provoke shifts in the composition of the cricket microbiome (Santo Domingo et al., 1998a); however, these shifts are likely to be driven by a switch in nutrients available to the microbial community. Further, core microbiota are less likely to be perturbed by changes in habitat (Cariveau et al., 2014; Sudakaran et al., 2012). Thus it appears that the hindgut of *Gryllus veletis* houses a core group of resident microbiota. Further, the conserved shifts between microcosms suggests that we

will be able to predict generalisable shifts in core microbiota following environmental perturbations.

#### 6.4.4. What drives the seasonal shifts in the microbiome?

Because the microbiome appears to respond in a conserved way to variation in season, our task becomes to determine the driving force behind these patterns. In the introduction to this chapter, I suggested four hypotheses and I will address the likelihood of each as the driver of changes in the microbiome of *G. veletis*.

*1) Low temperatures may directly select for psychrophilic bacteria:*

*Pseudomonas* spp. include ice-nucleating bacteria (Lee et al., 1993) and psychrophiles that are selected for in the gut of overwintering bullfrogs (Carr et al., 1976). However, one of the prominent shifts in bacterial abundance is a decline in *Pseudomonas* spp. decline in the winter. Thus, it seems unlikely that low temperatures are favouring psychrophilic bacteria, leading to changes in the gut microbiome.

*2) Low temperatures directly kill or select against bacteria that are intolerant of cold.*

*Pseudomonas* spp. are likely to be cold-tolerant as ice-nucleators, yet decline during the winter, which suggests that low temperatures are not driving the composition of the microbiome. Further, the increase in abundance of *Wolbachia* represents one of the largest shifts during the winter, but there is no current evidence to suggest that they are psychrophilic; rather, relatively low temperatures (18 °C) appear to decrease their abundance in other insects such as the wasp *Nasonia vitripennis* (Bordenstein and Bordenstein, 2011). Lastly, the abundance of *Clostridium* spp. appears to decline as winter progresses; *Clostridium* spp. are often found in soil in temperate areas (Wobeser et al., 1987), thus it seems unlikely that they would be intolerant of cold. However, as I was unable to determine species, I cannot make any conclusions about their cold tolerance. Overall, to further understand the consequences of these shifts it would be beneficial to perform functional analyses of the bacteria that dominate these seasonal changes.



3) *Low temperatures indirectly mediate the gut microbiome by modifying the physiochemical environment of the gut.*

*G. veletis* do not void their guts during overwintering although they do cease feeding (personal observation), which may alter the substrates available to microbes and thereby select for different communities of bacteria capable of using these nutrients. For example, the gut microbiome of the thirteen-lined ground squirrel *Ictidomys tridecemlineatus* in torpor shifts to favour bacteria that can survive on host-derived mucins in the gut as external food sources are lost (Carey et al., 2013). Similarly, it is possible that the gut becomes increasingly anaerobic throughout the winter if insects close their spiracles to reduce water loss (Danks, 2000) or maintain a barrier against pathogens (Hajek and Leger, 1994) and ice-nucleating bacteria (Olsen et al., 1998) thereby selecting against aerobes. Indeed, I observed a marked decrease in *Pseudomonas* spp. which are the only dominant, obligate aerobes I observed in the microbiome (excluding *Wolbachia*, which are intracellular). Conversely facultative and obligate anaerobes continue to dominate the gut throughout the winter.

4) *The host may directly regulate the microbiome.*

Increased abundance of *Wolbachia* was concomitant with a decrease in immunity and, although it is currently unclear whether or not host immunity controls the proliferation of *Wolbachia* (Siozios et al., 2008) the immune system clearly plays a role in modulating the microbiome (Login and Heddi, 2013) and other endosymbionts (Douglas et al., 2011; Login et al., 2011). Thus, it is possible that the overwintering declines in immune activity in *G. veletis* could contribute to the proliferation of endosymbionts and gut microbiota that are otherwise kept in check by the host.

The host may also regulate gut bacteria to control ice nucleation. *Pseudomonas* spp. are known ice nucleators whose populations can be regulated during overwintering via voiding of the gut or masking by antifreeze proteins (Olsen and Duman, 1997) and I report a decline in their relative abundance in the gut of *G. veletis* during the winter. This loss is accompanied by an increase in cold tolerance, but no change in the supercooling

point, which could suggest that the host controls *Pseudomonas* spp. populations to prevent ice nucleation. However, *Gryllus veletis* become freeze-tolerant when acclimated to winter conditions and ice nucleators are located in the haemolymph (McKinnon, 2015). Further, ice-nucleation is largely beneficial to freeze-tolerant insects because it initiates slow and controlled ice formation at a higher temperature (Lee and Costanzo, 1998). Thus, it may be unlikely that *G. veletis* would actively void ice-nucleators from their guts.

However, some species of *Pseudomonas* are also pathogenic to insects, which may suggest that hosts regulate potential pathogens prior to overwintering. Cold can cause tissue damage that would allow gut microbes access to the hemocoel (MacMillan and Sinclair, 2011; Marshall and Sinclair, 2011), and it appears that host immunity is largely compromised in the winter, which suggests that the host is vulnerable to infection; thus, it would benefit the host to reduce populations of potential pathogens in the gut, such as *Pseudomonas* spp. and *Serratia marcescens*. I largely report a decrease in immune activity associated with overwintering (although I did not measure activity directly in the gut); however, hosts did maintain their ability to clear bacteria from the haemolymph, suggesting that they are capable of maintaining anti-bacterial responses which could participate in controlling pathogens in the gut.

## 6.5 Conclusions

Overall, the gut microbiome of *Gryllus veletis* shifts during overwintering, and these shifts are likely to be driven by changes in host physiology as opposed to direct effects of low temperatures on the composition of the microbiome. Because I observed concomitant shifts in the microbiome and cold tolerance, these microbial shifts may drive the ability for insects to survive low temperatures during the winter. Similarly, if changes in the microbiome are also linked to changes in immunity, these microbial shifts may drive the relationship between host and pathogen pressure during overwintering. It will next be important to move towards a functional understanding of these shifts in the microbiome, as their role may be an important contribution to insect overwintering success that we have previously ignored.

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## Chapter 7

### 7. General Discussion

One of the most pressing challenges to life on our planet is the climate-change-driven shift in multiple environmental pressures whose interactions dictate organismal survival (Kaunisto et al., 2016; Todgham and Stillman, 2013). Because these pressures may shift either concurrently or in dissonance, climate change is creating new interactions or modifying the timing and intensity of others (Sinclair et al., 2013), thereby presenting new physiological challenges. Changes in the abiotic environment, such as temperature, are likely to shift biotic interactions through modifications to phenotype and phenology (Sinclair et al., 2013; Sinclair et al., 2016). In particular, the response to climate change rests in part on environmentally-driven shifts in the relationships between animals and the microbial world, including both pathogenic and symbiotic microorganisms (Araújo and Luoto, 2007; Carey and Duddlestone, 2014; Parmesan, 2006; Tylianakis et al., 2008; Van der Putten et al., 2010). To predict how animals will cope with shifts in these interacting biotic and abiotic pressures, we must begin by understanding the ecological and evolutionary physiology underlying their ability to respond to their complex environments (Sinclair et al., 2013).

In ectothermic animals, such as insects, temperature is one of the most influential determinants of physiology, through thermodynamics, trade-offs, signaling, and injury (Angilletta, 2009; Tattersall et al., 2012); thus, species interactions among ectothermic animals or between animals and microbes will shift with changes in the thermal environment (Sinclair et al., 2016). In this thesis I have demonstrated that temperature shapes the outcome of host-pathogen interactions by modifying the thermal performance both host and pathogen. In *Drosophila melanogaster*, injurious exposure to low temperatures activates the immune system to compensate for cold-induced damage or trade-offs that threaten immune activity (Chapter 2). In the spring field cricket, *Gryllus veletis*, acclimation to low temperatures paradoxically decreases immune activity in the cold, potentially as a trade-off with increased cold tolerance (Chapter 3). Similarly, trade-offs with the response to multiple environmental pressures are likely to drive the seasonal



patterns of immunity in insects, which appear to be largely idiosyncratic among species (Chapter 4). Pathogens are also responsive to temperature, and thermal plasticity in both *G. veletis* and a fungal entomopathogen, *Metarhizium brunneum*, shape the outcome of infection under different thermal environments (Chapter 5). Further, we can predict the outcome of infection under thermally variable environments from the sum of the relative performance of host vs pathogen under constant temperatures (Chapter 5). Lastly, as temperature and season shift host physiology (e.g. both immunity and cold tolerance), the community composition of the gut microbiome also shifts. Thus, we must also begin to consider how environmentally-induced shifts in symbiotic microbiomes affect their functional relationship with their hosts and overall success of the holobiont in new environments (Chapter 6). In this chapter I aim to provide synthesis across the five experimental chapters in this thesis and produce a framework for future directions in research into how the thermal environment shapes host-pathogen and other biotic interactions.

## 7.1 Biotic interactions in the context of thermal biology

The discipline of thermal biology has an extensive set of tools and hypotheses for understanding the influence of temperature on physiology and the ability for an ectotherm to respond to its thermal environment (Angilletta, 2006; Angilletta, 2009; Angilletta et al., 2002; Angilletta et al., 2003; Kingsolver, 2009); however, we are only beginning to apply these tools and concepts to biotic interactions (Sinclair et al., 2016), or understand how biotic interactions shape thermal adaptation. In this section I explore how the data presented in this thesis informs the study of biotic interactions in the context of thermal biology theory and practices (e.g. using thermal performance curves), as well as how biotic interactions may contribute to thermal adaptation and survival under different thermal environments.

### 7.1.1 The value of understanding measures of thermal performance in the context of biotic interactions

Thermal performance curves (TPCs) can be used to understand how physiological processes within an individual respond to temperature (Angilletta, 2006; Sinclair et al., 2016); however, thermal performance of an individual may not reflect the outcome of a biotic interaction (Raffel et al., 2012); for example, although whole-organism immune activity peaks at 18 °C in *G. veletis* (Chapter 3), this also represented the temperature at which crickets were most susceptible to fungal infection (Chapter 5). Further, because the ability to survive fungal infection was dependent on the interaction between the thermal performance of *G. veletis* and *Metarhizium brunneum*, I would not have been able to predict host survival simply based on plasticity in the thermal performance of the host (Chapter 5). Therefore, if we are to use TPCs as a heuristic predictor of performance (Sinclair et al., 2016) and the outcome of biotic interactions (Chapter 5; Thomas and Blanford, 2003) we must be cautious with which measures of physiology and fitness (e.g. survival) we choose to base these predictions.

Further, if individual physiological performance can be masked during an interaction (e.g. Chapter 5), we must ask ourselves: what is the value of individual measures of performance in predicting the outcome of biotic interactions? Why not only measure the fitness outcome of biotic interactions (e.g. survival)? I suggest that the value of understanding individual performance lies in understanding *why* the interaction between organisms results in a particular outcome, and also *how* the underlying physiology of these interactions will affect the response to multiple stressors. In *G. veletis*, although the TPCs of immunity in Chapter 3 do not necessarily predict the outcome of infection with a fungal pathogen in Chapter 5, they do provide information about how the immune system might be interacting with other physiological systems, such as those that govern the response to cold (Ferguson et al., 2016); both will be important in determining fitness.

If we are to only measure the final outcome of a biotic interaction (e.g. survival of infection), we neglect to place this interaction in the context of a multiple stressor

environment. For example, if in Chapter 2 we had only measured survival against fungal infection following cold exposure in *Drosophila melanogaster*, we might conclude that cold does not affect the immune system and thus does not interact with low temperatures. However, by measuring both individual performance as well as the outcome of an interaction, we can instead conclude that the immune system may be damaged by cold, or trade-off with the response to cold (Chapter 2; Chapter 3); as such, the potential trade-offs between these physiological systems will have important consequences for energy use and overall survival of low temperatures (Linderman et al., 2012; Salehipour-Shirazi et al., 2016; Williams et al., 2015). Therefore, the importance of understanding biotic interactions in the context of the thermal environment is two-fold: I suggest that to understand 1) *the influence of temperature on the outcome of a biotic interaction*, individual measures of performance are unlikely to provide an accurate prediction and it is best to measure the interactions themselves. By contrast, to understand 2) *the influence of the interaction between multiple pressures, such as biotic interactions and temperature, on animal phenotype* it is useful to understand multiple, individual measures of physiology that underlie the responses to each pressure.

### 7.1.2 The contribution of biotic interactions to thermal adaptation and success under new thermal environments

Thermal sensitivity and plasticity differ both among and between physiological systems in ectotherms (Chapters 3 & 4; (Sinclair et al., 2016; Stevenson et al., 1985); for example, in *Eurosta solidaginis* (Chapter 4), the ability to clear bacteria from the haemolymph is stronger at 12 °C than 25 °C, although the rate of development is faster at 25 °C (as suggested by the rate of pupation at 25 °C relative to 12 °C). These differences in thermal performance among traits within a species suggest that adaptation to the thermal environment is driven by selection on multiple traits and the potential interactions between them (Angilletta et al., 2002; Angilletta et al., 2003). Therefore, to understand the functional importance of differences in thermal performance among or within systems, we must first understand the selective pressures that have influenced the evolution of these traits.

Thus, the question becomes: *what drives the evolution of the thermal performance of a physiological system?* In the case of the immune system, thermal adaptation of this physiological system may be driven by its interactions with pathogen, as a form of co-evolution driven by “arms races” between host and pathogen. For example, in Chapter 5, the fungal pathogen *M. brunneum* appears to perform very well at 18 °C; in Chapter 3, realised immune activity of *G. veletis* peaks at 18 °C, which could represent an example of thermal co-adaptation between host and parasite. In this case, the thermal performance of the immune system may be highly labile and under rapid selection to combat the ability for pathogens to evolve more rapidly than their hosts (Raffel et al., 2012). Additionally, in Chapter 5, acclimation to fluctuating conditions appears to broaden the thermal performance of the host. I hypothesize that fluctuating environments provide information about changes in pathogen stress that may signal to the host to change its thermal performance. Overall, if changes in biotic pressures drive the thermal performance of a physiological system (e.g. immunity) then these changes are likely to be adaptive and allow ectotherms to cope with shifts in the thermal environment.

Conversely, but not exclusive from the previous hypothesis, the thermal performance of a physiological system, such as the immune system, may have evolved because of trade-offs between systems [(Angilletta et al., 2003); e.g. Chapter 2, 3]. For example, in Chapters 2 and 3, I suggest that temperature-driven changes in immune activity result from trade-offs between immune activity and the response to cold (Ferguson et al., 2016; Salehipour-Shirazi et al., 2016). Thus, the thermal performance and plasticity of the immune system may have evolved as constrained by the physiological response to temperatures.

In either case of pathogen-driven or trade-off-driven evolution of thermal performance, I suggest that the thermal adaptation of the immune system is likely to be an important contributor to insect success in a changing climate, especially in the context of novel and invasive species. For example, *Drosophila melanogaster* from African climates possess immune systems that are maladapted to cold; therefore, they are unlikely to be able to

effectively combat a pathogen that is adapted to lower temperatures (Lazzaro et al., 2008). Further, this suggests that even if they are able to physiologically tolerate low temperatures, they will be unable to survive other physiological pressures associated with a new thermal environment (e.g. cold-adapted pathogens). In Canada, we currently base our ability to predict the survival of novel and invasive insect species in a new environment on the ability to survive low temperatures or other winter-associated abiotic stressors (e.g. Crosthwaite et al., 2011; Sinclair et al., 2015). However, I suggest that we must move towards considering the thermal response to multiple environmental pressures, and especially biotic interactions, if we are to fully understand whether or not an ectotherm is adapted for a particular thermal environment.

The importance of considering thermal adaptation in the context of biotic interactions also extends beyond host-pathogen interactions to those between host and the microbiome. For example, in Chapter 6 the microbiome shifts across season; however, we do not yet know the functional significance of the relationship between the host and the microbiome, and whether or not temperature changes this relationship. For example, the obligate bacterial symbiont of the green stinkbug *Nezara viridula* is heat-intolerant, and loss of this symbiont under experimental climate change conditions leads to decreased host fitness. Thus, whether or not an organism will survive or be mal-adapted to new thermal conditions does not rely on solely its own physiological response to temperatures, but on how temperature affects the thermal performance of its associated microbes.

## 7.2 Implications for the field of eco-immunology

In concordance with the literature (Adamo, 2004, 2014, 2016; Adamo and Lovett, 2011; Fedorka et al., 2007; Shikano and Cory, 2015), one of the main themes that emerges across my thesis is the discord in responses between different branches of the immune system – whether it be in thermal performance, the contribution of each component to surviving infection, or patterns of response to environmental pressures. However, despite the importance of temperature on the outcome of infections (Thomas and Blanford,

2003), the simplicity of only measuring one or few components of immune activity, or only activity at one temperature, to assess immunocompetence and draw conclusions about the ability for insects to survive infection under shifting environments remains active in the literature (e.g. Eggert et al., 2015; Gherlenda et al., 2016; Karl et al., 2011; Krams et al., 2016). In the following section I outline the importance of considering the immune system as dynamic in its response to thermal and other environmental pressures.

### 7.2.1 Thermal dynamics of the insect immune system

Across this thesis I demonstrate that the insect immune system is dynamic in its response to temperature, with some of the first examples of thermal plasticity of insect immune function (Chapter 3, 5). In chapter 3, cold-acclimation suppresses immune activity at low temperatures; in contrast, acclimation to fluctuating temperatures (Chapter 5) appears to broaden the thermal performance of the immune system, improving survival against a pathogen at both high and low temperatures. Thus, our interpretation of how well an insect is able to survive infection under different thermal environments is completely dependent on the thermal history of the host and resultant state of immunity.

I was also able to explicitly demonstrate that the temperature at which immunity is measured in insects can markedly change our interpretation of the effect of environmental shifts on host success. For example, in Chapter 4, the ability to survive fungal infection in *Eurosta solidaginis* and *Curculio* sp. shifts across season; however, I was only able to detect this effect at 12 °C, whereas there was no difference in survival at 25 °C. Similarly, I was only able to detect the effects of acclimation on immunity at particular temperatures, whereas these effects were masked under other temperatures (Chapters 3, 5). Despite the importance of temperature on the outcome of infection (Thomas and Blanford, 2003) there are few examples of studies that measure insect immunity at more than one temperature, or at least at temperatures that might be ecologically relevant (e.g. Adamo and Lovett, 2011; Fedorka et al., 2016; Murdock et al., 2013; Murdock et al., 2012a; Nakamura et al., 2011). And, to include myself in this critique, my interpretation of immune activity in both Chapters 2 and 6 are limited, as I only measured immune

activity at one temperature (e.g. room temperature) that is unlikely to be a relevant temperature for an overwintering insect. Thus, I stress that the field of eco-immunology must move towards interpreting ectotherm data in the context of the thermal environment, and it behooves us to consider and incorporate the ecological conditions under which we expect host-pathogen interactions to occur into our experimental designs.

I demonstrate that the response to the thermal environment is not conserved within the immune system, and differs depending on the response I measured. For example,  $T_{opt}$  of phenoloxidase activity does not match the  $T_{opt}$  of the melanisation response (Chapter 3), increased haemocyte counts rarely correlate with increased survival of infection (Chapters 2, 6), and evidence of acclimation to low temperatures is only detectable in measures of realised, but not potential, immunity (Chapter 3). Thus, our understanding of the state of the immune system and its response to the environment relies on multiple measures of multiple components of the immune system.

Despite the importance of considering multiple branches of immune activity (Adamo, 2004; Adamo and Lovett, 2011), or activity across multiple temperatures (Adamo and Lovett, 2011; Murdock et al., 2012a), there must be limits to what we can achieve experimentally, and it is intractable to suggest that every ecoimmunological study should measure as many possible measures of immune activity at all possible temperatures. Thus, we can construct appropriate measures of immune activity depending on the types of questions we wish to address. For example, if our goal is to understand and predict disease resistance in an insect under different thermal environments, the most useful information will arise from measuring and interpreting the interaction between host and parasite (e.g. survival of a pest against a biocontrol agent), and including the potential for thermal plasticity. By contrast, simply measuring multiple components of immune activity, such as circulating haemocyte concentrations and phenoloxidase activity, is unlikely to yield an accurate picture of how well the insect will resist disease.

By contrast, if our goal is to understand the relationship between the immune system and other physiological systems, then multiple measures of both potential and realised

immunity will provide the most useful information. Throughout my thesis, I suggest that simultaneous increases and decreases in immune activity signify that the immune system has reconfigured itself, such that particular branches compensate for others and allow for the animal to maintain some protection against pathogens and parasites (Adamo, 2014, 2016; Adamo et al., 2016). Further, an increase in survival that is not accompanied by an increase or change in other measures of immune activity can indicate that the animal has reconfigured its physiology to favour tolerance of a pathogen, as opposed to resistance (Moreno-Garcia et al., 2014). These measures and interpretations lend themselves well to understanding how the immune system interacts with other physiological systems [e.g. reconfiguration may be prompted by trade-offs between immunity and the response to other environmental pressures; Chapters 2,6; (Adamo, 2016; Adamo et al., 2016)] and thus how these interactions will affect the fitness of the animal under different thermal environments.

Our ability to interpret resistance *versus* tolerance is also important for understanding pathogen load and transmission potential in disease vectors (Moreno-Garcia et al., 2014); thus, eco-immunological studies in vector biology should maintain measures of multiple components of the immune system. Overall, although the dynamic nature of the immune system increases the potential complexity of experimental design and interpretation, I suggest that, much like in multiple stressors studies (Kaunisto et al., 2016), if we choose measures of immunity based on the ecological context of those measures, this will allow us to exploit this complexity as opposed to being stymied and suffocated by it.

### 7.2.2 The importance of understanding microbial physiology and ecology

The other important theme that emerges from my thesis is the idea that the microbes – whether pathogenic, parasitic, or symbiotic- matter too when considering the influence of the environment on biotic interactions. Temperature plays an important role in both the rate of growth and development of pathogens, as well as their infectivity (Murdock et al.,



2012b); however, despite the importance of pathogen ecology and thermal biology in determining the outcome of infection (Murdock et al., 2012b; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003), few studies consider the physiological effects of temperature on parasites and pathogens, especially beyond the simple context of growth rate (Murdock et al., 2012b), or within the context of thermal plasticity (Altman et al., 2016; Raffel et al., 2012).

Some of the most pressing questions in eco-immunology surround medically-important vectors, such as mosquitoes and the human pathogens that they harbour. In these cases, where the pathogen or parasite develops in a mammalian host before reaching an insect, thermal history should simply be a consistent 37 °C. However, it is common practice to feed mosquitoes through a membrane on animal or human blood obtained from blood banks (Rampersad and Ammons, 2007), where blood is stored at low temperatures until use; thus, the pathogen is actually exposed to a different thermal history in the lab than in the field. Further, vectors are often incubated at a standard rearing temperature that does not necessarily reflect the thermal environment for the pathogen when infecting its insect vector (Murdock et al., 2012a; Murdock et al., 2012b). I demonstrate in Chapter 5 that cold-acclimation of the pathogen can change the outcome of infection; therefore, thermal history is an important contributor to the outcome of infection and if we fail to consider thermal responses and plasticity of our pathogens, we are likely to incorrectly interpret the outcomes of infection (Altman et al., 2016; Raffel et al., 2012; Schmid-Hempel, 2003).

Due to the physiological relationships of the microbiome with the host, the insect microbiome has important ecological and anthropogenic impacts. For example, changes in the gut microbiome of mosquitoes can alter their vectorial capacity of medically-important pathogens and parasites (Dennison et al., 2014; Weiss and Aksoy, 2011). Further, dysbiosis in beneficial insects, such as honeybees, may be contributing to the global problem of colony collapse disorder. In Chapter 6 I demonstrate that the microbiome varies seasonally, and that seasonal shifts may represent permanent perturbations of the microbiome. I suggest that the gut microbiome will be dynamic in

heterogeneous environments with cyclical variation in environmental pressures. Thus, if we are to further understand how the microbiome will change the ecological and anthropogenic impacts of their insect hosts, we must incorporate this capacity for plasticity.

### 7.3 Implications for multiple stressor studies

Understanding how animals respond to changes in their environment and environmental stress is experiencing a paradigm shift towards integrating multiple pressures and stressors as opposed to studying each in isolation (Kaunisto et al., 2016). In this group of studies, I study a subset of the wider set of multiple stressor problems by studying the influence of the thermal environment on the immune system as a means of addressing the interaction between temperature and pathogen stresses. One of the biggest challenges facing the study of multiple stressors is the discrepancy between generalised and idiosyncratic responses (Kaunisto et al., 2016), such that our ability to make broad-stroke predictions may be compromised. One approach to take is a phylogenetically-cogent approach, such as with using multiple species of *Drosophila* as models for responses to multiple stressors to begin to understand the mechanisms underlying the responses to these stressors (Kaunisto et al., 2016; MacMillan et al., 2015).

An alternative method may be to approach the study of multiple stressors from a trait-based perspective, as opposed to a species-based perspective. In the overwintering insects in Chapter 4, immune activity varied idiosyncratically among species, which suggests that the connection between temperature, pathogen pressure, energy use, and immunity do not follow conserved seasonal patterns. Rather, the species-specific changes in immune activity that we observed may arise from the differential effects of different combinations of overwintering pressures on immune activity. For example, both the temperature and pathogen stressors that an acorn weevil experiences 10 cm under the soil surface, compared to a maggot in a gall 1 m above the soil, are likely to be vastly different (Chapter 4) and thus their physiological responses to their environments will be

different (Irwin and Lee, 2003; Udaka and Sinclair, 2014), as will the relationships between these physiological responses (Chapter 4).

If we approach multiple stressor studies by considering physiological groups of organisms – e.g. those that are freeze-tolerant and experience similar microhabitats - are potentially more likely to experience similar combinations of stressors, and potentially evolved similar responses to these sets of stressors (e.g. Chapter 4). Such an approach may also help us to create hypotheses for the mechanisms underlying connected responses to multiple stressors: for example, if soil-dwelling insects that are both freeze-avoidant and freeze-tolerant activate their immune activity in response to low temperatures, whereas insects above snow cover down-regulate immune activity, this might suggest that the link between temperature and immunity is regulated via increased pathogen pressure in the soil. Conversely, if freeze-tolerant insects respond similarly to each other, but differently than freeze-avoidant insects within a shared habitat, this suggests that the link may rest not on pathogen pressure in the environment, but instead the physiological response to cold via trade-offs. Thus, our understanding of the pressures underlying these responses may allow us to create more generalizable predictions (Kaunisto et al., 2016).

Finally, the relationship between multiple stressors may manifest in several different ways. In Chapter 2, cold may damage the immune system, but also trade-off with the physiological response to cold, as is suggested in Chapter 3. Further, cold may act as a prophylactic signal for changes in pathogen stress, but the ability to respond to pathogens in the cold will also be mediated through energy reserves, or damage induced by cold exposure (Chapters 2, 4). I suggest that to understand the functional importance of the relationship between multiple pressures we must move towards a mechanistic understanding of how animals respond physiologically to their environments, and how these physiologies might then interact (Kaunisto et al., 2016).

## 7.5 What is the relationship between cold and immunity?

The work I present in this thesis does not yet provide a definitive answer to the questions of why cold might activate the immune system, nor what the general relationship between cold and immunity might be. However, we have begun to narrow our hypotheses for the functional relationship between the response to these two environmental pressures. In Chapter 1, I present four, non-mutually exclusive hypotheses for why cold might activate the immune system. Based on the work in this thesis, I suggest overall that cold-activation of the immune system actually represents a reconfiguration of the immune system, such that certain components are activated while others are down-regulated or suppressed (i.e. not activated) (e.g. Chapters 2, 4, 6). I propose four modified hypotheses to explain this reconfiguration that arise as a means to both balance conflicts in energy use between the response to cold and the immune system (e.g. as suggested in Chapters 2-6), and maintenance of immune activity to pathogens present in the environment. Each of these new hypotheses are, once again, not mutually exclusive, and it is likely that cold will affect the immune system in more ways than one.

- 1) *Immunity is reconfigured in response to cold-induced damage from stressful, injurious exposures*

As suggested in Chapter 2, an injurious cold exposure could directly damage the immune system, such that it must compensate by activating particular branches of immunity and thereby maintaining some protection against pathogens. To understand if damage occurs to the immune system or other tissues, I would measure damage to hemocytes, fat body, and other immune-responsive organs, such as the Malpighian tubules (Marshall and Sinclair, 2011; Yi and Lee, 2003, 2004; Yi et al., 2007) following different types of cold exposure. I would predict that damage should coincide with a decrease in activity of some components of immunity, while others are up-regulated.

- 2) *Immunity is reconfigured to minimise the energetic demands of immune activity during cold exposure*

Cold exposure may elicit a reconfiguration of available resources and energy to maintain a state of energy conservation that is associated with overwintering (Williams et al., 2015), and this has selected for a reconfiguration of immunity such that energetic costs of immune function are minimised while maintaining a baseline level of function (Adamo, 2016). This may also include a switch from resistance to tolerance of infection. To test this hypothesis, I would measure the ability of genetic mutants of *Drosophila melanogaster* deficient in different branches of the immune system to survive prolonged exposure to low temperatures. Concomitantly, I would measure their energy reserves throughout the winter to understand if immunodeficient flies expend less energy. I would predict that if immunity is reconfigured to conserve energy, immunodeficient flies should live longer at low temperatures and expend less energy throughout the overwintering period. Further, I would predict that infection should increase energy use and decrease survival.

### 3) *Immunity is reconfigured in response to trade-offs between the response to cold and the response to pathogens*

Cold exposure can signal for many physiological changes that lead to increased cold tolerance (Tattersall et al., 2012; Zachariassen, 1985) and re-warming can allow for recovery and repair of cold-induced injury (Colinet et al., 2015). This is energetically costly (MacMillan et al., 2012) and thus may directly trade-off with the immune system such that immunity is compromised. If the responses to cold and immunity trade-off with each other, then wherein do these trade-offs lie? For example, hemocytes seem to be consistently up-regulated in response to cold (Chapters 2,5,6), and one could envision that perhaps hemocytes play a yet unknown role in both immunity and the response to cold exposure, thereby necessitating increased numbers to partition between each physiological response. To understand if there are shared mechanisms between immunity and cold exposure, I would begin by comparing transcriptomic and/or proteomic responses of specific tissues (e.g. fat body, hemocytes) to both cold and immune challenge in an insect to determine if there is overlap in their transcriptional responses. If there are shared responses, this would suggest that each response competes for these resources. Further, if these responses are tissue-specific, I would predict that

transplanting tissues after cold exposure should elicit reconfiguration of the immune response. For example, if hemocytes, fat body, or Malpighian tubules signal produce signals to respond to cold exposure, then these transplanted signals should elicit the same immune reconfiguration in a non cold-exposed insect.

#### 4) *Immunity is reconfigured in response to temperature-mediated pathogen exposure*

In Chapter 5, fluctuations to low temperatures increased survival against a fungal pathogen, suggesting that fluctuating temperatures may signal for changes in pathogen pressures. Cold exposure may change pathogen pressure in the environment [both externally or within the microbiome] by modifying the presence, abundance, and infectivity/virulence (Altizer et al., 2006; Carr et al., 1976; Smirnova et al., 2001). These changes in the environment may have selected for a reconfiguration of the immune system such that insects respond optimally to these changes: insects may benefit from tolerance over resistance to pathogens at low temperatures as a way of mitigating energetic costs of immunity (Moreno-Garcia et al., 2014), or may instead reconfigure immunity to maximize responses to pathogens more likely to be present at low temperatures. To understand if pathogen pressure might drive these responses, I would next measure pathogen pressure over season and at different temperatures. I would set up field microcosms with insects as bait for pathogens and use metagenomics analysis to compare the communities of potential pathogens across season. Further, I would culture pathogens (e.g. fungi) from insects that died of disease, and test their thermal performance and tolerance. Concurrently, I would arrange microcosms at different temperatures in the lab, inoculate each with different pathogens with different thermal tolerances, and bait the microcosms with insect. Again, I would use metagenomics analysis coupled with qPCR to determine the abundance of pathogens present at each temperature, as well as survival of the hosts. I would predict that different thermal tolerances in pathogens would dictate their prevalence and success in infecting hosts in different thermal habitats. Further, I would measure the thermal performance of different components of the immune system in my “bait” insects acclimated to different temperatures. I would predict that any reconfiguration of the immune system should correspond with changes in pathogen pressure. For example, if pathogenic fungi become

more prevalent at low temperatures, then the immune system should reconfigure to increase antifungals while down-regulating other components.

## 7.7 Conclusions

Overall, the ability for an ectotherm to respond to changes in the thermal environment, and thus their ability to cope with climate change, will depend on the phenotype produced by its connected responses to multiple physiological pressures. In this thesis, I demonstrate that a subset of biotic interactions – those between insects and both pathogenic and symbiotic microbes – are directly influenced by changes in temperature, as well as indirectly through trade-offs with other physiological systems that are also responding to temperature. Taken together, the studies in this thesis suggest that 1) our ability to understand how an ectotherm responds to its thermal environment must be considered in the context of multiple environmental pressures and, especially, biotic interactions; thus, animal fitness is a product of Genotype  $\times$  Microbe  $\times$  Environment interactions; and 2) when applying the influence of temperature to biotic interactions, we must consider that both players may respond to temperature and that the outcome of these interactions cannot be predicted based on the physiological performance of one player. By integrating temperature and biotic interactions in the context of multiple pressures, the conclusions from this thesis should influence both how we interpret and understand the importance of ectotherm thermal biology, as well as host pathogen interactions in a changing climate.

## 7.8 References

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## Appendix A Chapter 2 Supplementary methods and results

**Table S1: Primers used to measure expression of genes related to Toll, IMD, and Jak/STAT pathways in *Drosophila melanogaster* using q-PCR.** The reference gene is Rpl32.  $T_m$  indicates the melting temperature. References are noted for primers derived from literature. When designed in-house, Primer3 was used to design primers with amplicons of less than 250 bp long and GC content of 45-65% with no self-complementarity. To determine the efficiency of primers at different cDNA concentrations, we created standard curves of target genes and the reference gene using seven different concentrations of mixed cDNA samples (0, 4, 16, 64, 256, 1024 and 4096-fold dilution).

Gene	Nucleotide sequence (5' to 3')	$T_m$	Reference
<i>Rpl32</i>	5'-GACGCTTCAAGGGACAGTATCTG-3'	62	(Zaidman-Remy et al., 2011)
	5'-AAACGCGGTTCTGCATGAG-3'	62	
<i>attacin-B</i>	5'-GGCCATGCCAATTTATTCA-3'	63	(Tsai et al., 2008)
	5'-CATTGCGCTGGAACCTCGAA-3'	63	
<i>cecropin-A</i>	5'-TCTTCGTTTTTCGTCGCTCTC-3'	61	(Tsai et al., 2008)
	5'-CTTGTTGAGCGATTCCCAGT-3'	60	
<i>defensin</i>	5'-GCCAGAAGCGAGCCACAT-3'	63	(Tsai et al., 2008)
	5'-CGGTGTGGTTCCAGTTCCA-3'	63	
<i>diptericin-A</i>	5'-AGGTGTGGACCAGCGACAA-3'	63	(Tsai et al., 2008)
	5'-TGCTGTCCATATCCTCCATTCA-3'	63	
<i>drosocin</i>	5'-CCACCACTCCAAGCACAATG-3'	60	
	5'-TGAGTCAGGTGATCCTCGATGG-3'	58	
<i>drosomycin-B</i>	5'-CTCCGTGAGAACCTTTTCCA-3'	60	(Tsai et al., 2008)
	5'-GTATCTTCCGGACAGGCAGT-3'	59	
<i>metchnikowin</i>	5'-CTACATCAGTGCTGGCAGAG-3'	60	
	5'-CGGTCTTGTTGGTTAGGATTG-3'	58	
<i>PGRP-LB</i>	5'-TGTGGCCGCTTTAGTGCTT-3'	62	(Tsai et al., 2008)
	5'-TCAATCTGCAGGGCATTGG-3'	63	
<i>PGRP-LC</i>	5'-ACGGAATCCAAGCGTATCAG-3'	60	(Tsai et al., 2008)
	5'-GGCCTCCGAATCACTATCAA-3'	60	
<i>PGRP-SB</i>	5'-CTGCGGCTGTTATCAGTGAA-3'	60	(Tsai et al., 2008)
	5'-TGATGGAATTTCCGCTTTTC-3'	60	
<i>PGRP-SD</i>	5'-CCTTGCCACGTGCTGTGA-3'	63	(Tsai et al., 2008)
	5'-TGTAACATCATCCGCACAAGCT-3'	63	
<i>relish</i>	5'-GTGGAGTTGGACCTAAGTAGTGG-3'	55	
	5'-TGATTCAGCAGCGAACAGAGC-3'	59	
<i>toll</i>	5'-AACTTGGGCAACCTTGTGAC-3'	60	(Tsai et al., 2008)
	5'-GTAACCAAACGGGGAGTTGA-3'	60	
<i>TotA-1</i>	5'-TGAGGAACGGGAGAGTATCG-3'	60	(Tsai et al., 2008)
	5'-GCCCTTACACCTGGAGATA-3'	60	
<i>vir-1</i>	5'-TGTGCCCATGACCTATCCA-3'	62	(Tsai et al., 2008)
	5'-GATTACAGCTGGGTGCACAA-3'	60	

**Table S2. Statistical analyses of the effect of acute and sustained cold on expression level of immune genes of *Drosophila melanogaster* females.** The data shows the relative abundance of the immune genes mRNA (normalized to Rpl-32) after acute cold exposure (2h, -2 °C) and sustained cold exposure (10h, -0.5 °C).

Gene	Treatment	Test value	P-value	Adjusted P
<i>attacin-B</i>	Acute	2.56	0.063	0.13
	Sustained	2.65	0.057	0.13
<i>cecropin-A</i>	Acute	2.11	0.102	0.13
	Sustained	2.64	0.058	0.14
<i>defensin</i>	Acute	2.35	0.078	0.13
	Sustained	0.46	0.67	0.75
<i>dipterocin-A</i>	Acute	7.10	<0.01	0.09
	Sustained	2.578	0.061	0.13
<i>drosocin</i>	Acute	2.66	0.056	0.13
	Sustained	1.66	0.172	0.22
<i>drosomycin-B</i>	Acute	0.41	0.701	0.75
	Sustained	2.42	0.072	0.13
<i>metchnikowin</i>	Acute	2.66	0.046	0.13
	Sustained	0.33	0.756	0.76
<i>PGRP-LB</i>	Acute	2.47	0.069	0.13
	Sustained	2.47	0.070	0.13
<i>PGRP-LC</i>	Acute	2.60	0.060	0.13
	Sustained	1.69	0.166	0.22
<i>PGRP-SB</i>	Acute	2.26	0.086	0.13
	Sustained	2.59	0.060	0.13
<i>PGRP-SD</i>	Acute	1.46	0.22	0.26
	Sustained	0.32	0.76	0.76
<i>relish</i>	Acute	2.44	0.071	0.13
	Sustained	2.57	0.061	0.13
<i>toll</i>	Acute	1.05	0.352	0.40
	Sustained	1.45	0.220	0.26
<i>TotA-1</i>	Acute	3.43	<0.001	0.01
	Sustained	11.59	<0.001	0.01
<i>vir-1</i>	Acute	2.40	0.074	0.13
	Sustained	0.06	0.0956	0.14

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**Figure S1. Location of piercing for wound-induced melanisation.** Flies were pierced in the right thoracic vertical cleft along the notopleural suture until it reached the humeral callus, as indicated by the black arrow.

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*Master of Science*. 2012.

“Fecundity reduction and modification of behaviour in mosquitoes of the genus *Culex* infected with blood parasites of the genus *Hepatozoon*.”

Department of Biology, Acadia University, Wolfville, Nova Scotia, Canada.

Supervisor: Todd Smith, PhD.

*Bachelor of Science (Honours)*. 2010.

“Host seeking behaviour of *Culex* mosquitoes parasitized with *Hepatozoon clamatae*.”

Department of Biology, Acadia University, Wolfville, Nova Scotia, Canada.

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## Selected awards and scholarships

- 2015 *Best student presentation: Division of Ecoimmunology and Disease Ecology*  
Society for Integrative and Comparative Biology  
*Malcom Ferguson award in Life Sciences*  
The University of Western Ontario
- 2014 *Helen Battle Award for Best Student Poster*  
Canadian Society of Zoologists Annual Meeting
- 2013 *Ruth Horner Arnold Fellowship (1 year)*  
Awarded for academic excellence and research potential by the University of Western Ontario  
*NSERC Post-Graduate Doctoral Scholarship (3 years)*  
Natural Sciences and Engineering Research Council of Canada
- 2012 *Murray Fallis Award (Best Oral Presentation)*  
Parasitism, Immunity and Environment section of the Canadian Society of Zoologists

## Publications

*Undergraduate mentees denoted with a <sup>U</sup>*

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13) Salehipour-shirazi G\*, Ferguson LV\*, Sinclair BJ. 2017. Does cold activate the *Drosophila melanogaster* immune system? *Journal of Insect Physiology*. 96: 29-34

12) Kaunisto S, Ferguson LV, Sinclair BJ. 2016. Can we predict the effects of multiple stressors on insects in a changing climate? *Current Opinion in Insect Science* 17:55-61.

- 11) Toxopeus J, Jakobs R, Ferguson LV, Garipey T, Sinclair BJ. 2016. Reproductive arrest and stress resistance in winter-acclimated *Drosophila suzukii*. *Journal of Insect Physiology* 89: 37-51.
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- 9) Sinclair BJ, Coello-Alvarado, LE, Ferguson LV. 2015. An invitation to measure insect cold tolerance: Methods, approaches and workflow. *Journal of Thermal Biology* 53: 180-197.
- 8) MacMillan HA, Ferguson LV, Nicolai A, Staples JF, Donini A, Sinclair BJ. 2015. Parallel ionoregulatory adjustments underlie phenotypic plasticity and evolution of *Drosophila* cold tolerance. *The Journal of Experimental Biology* 218: 423-432.
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- 6) Forzà MJ, Ferguson LV, Smith, TG. 2014. Calcium oxalate nephrolithiasis and tubular necrosis in recent metamorphs of *Rana sylvatica* (*Lithobates sylvaticus*) fed spinach during the premetamorphic (tadpole) stage. *Veterinary Pathology* DOI: 10.1177/0300985814535607.
- 5) Ferguson LV, Smith TG. 2014. Fecundity reduction in the second gonotrophic cycle of *Culex pipiens* infected with the apicomplexan blood parasite *Hepatozoon sipedon*. *Journal of Parasitology* 100: 442-446.
- 4) Sinclair BJ, Ferguson LV, Salehipour-shirazi G, MacMillan HA. 2013. Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and immune stress in insects. *Integrative and Comparative Biology* 53: 545-556.
- 3) Ferguson LV, Hillier NK, Smith TG. 2013. Influence of *Hepatozoon* parasites on host-seeking and host-choice behaviour of the mosquitoes *Culex territans* and *Culex pipiens*. *International Journal of Parasitology: Parasites and Wildlife* 2: 69-76.
- 2) Trites MJ<sup>U</sup>, Ferguson LV, Ogbuah CT, Dickson CM, Smith TG. 2013. Factors determining the in vitro emergence of sexual stages of *Hepatozoon clamatae* from erythrocytes of the Green Frog (*Rana clamitans*). *Canadian Journal of Zoology* 91: 219-226.
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