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Cold-activation of the Drosophila melanogaster immune system

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COLD ACTIVATION OF THE *DROSOPHILA MELANOGASTER* IMMUNE SYSTEM

(Thesis format: Monograph)

by

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Abstract

Evidence of immune response enhancement after cold exposure suggests that cold activates the insect immune system. I investigated whether the immune response of *Drosophila melanogaster* is activated by cold exposure in the absence of pathogens. To explore if different kinds of cold affect the immune response differently, I included acute and chronic cold exposure. I cold-exposed flies, and then examined up-regulation of immune-related genes. In addition, I measured hemocyte concentration, phenoloxidase activity, and wound-induced melanization. Acute cold exposure increased hemocyte concentration and wound-induced melanization. Chronic cold did not change hemocyte concentration, phenoloxidase activity or melanization in flies. Acute and chronic cold did not affect the Toll pathway but up-regulated the JAK/STAT pathway. Acute, but not chronic cold activated the IMD pathway. I suggest a cold-immunity "cross-talk" in insects which can be affected differently by acute and chronic cold-exposure.

Keywords

Cold tolerance, Immune system, Eco-immunology, cross-tolerance

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List of Abbreviations

ANCOVA: Analysis of covariance

ANOVA: Analysis of variance

AMPs: Antimicrobial peptides

CHC: Circulating hemocyte concentration

IMD: Immune deficiency

JAK/STAT: Janus kinase/Signal Transducer and Activator of Transcription

PCR: Polymerase chain reaction

PO: Phenoloxidase

PSPs: Plasmatocyte spreading peptides

qPCR: Quantitative polymerase chain reaction

S.E.M: Standard error of the mean

TotA: turandot A

Chapter 1

1 Introduction

1.1 Overview

Insects constitute almost three-quarters of all described animal species and have a broad range of diversity, distribution and ecological functions (Gullan and Cranston, 2010; Kaya and Vega, 2012). Many ecosystem services are provided by insects, including supporting services (e.g. plant propagation via pollination), provisioning services (e.g. using insects or their products in biochemical and pharmaceutical industries), or regulating services (e.g. in control of diseases and pests) (Prather et al., 2012; Gullan and Cranston, 2010). The ecological factors that affect insect physiology can change insects' performance within the ecosystem and lead to changes in the ecosystem.

The immune system is one of the most important factors that affects insect survival. Pathogens can disrupt the normal physiology of an insect, reduce performance and cause mortality (Kaya and Vega, 2012). Improving our knowledge of the insect immune system helps to enhance survival of insects that are beneficial to humans (e.g. those that are reared for their products like honeybees or silk worm, or that are used as agricultural bio-control agents) and suppress the populations of pest species (Stanley et al.,2012; Kaya and Vega, 2012). For example, agricultural pests can be better controlled if microbial pesticides are applied during periods when pests are more susceptible to pathogens (Stanley et al., 2012).

The response of insects to pathogens can be influenced by physiological states that accompany particular developmental stages, mating or reproduction (Hurd, 2001;

Kapelnikov et al., 2008; Peng et al., 2005; Valadez-Lira et al., 2012). For example, insects are more susceptible to pathogens during molting because the digestive system and cuticle are less sclerotized and cannot prevent pathogens from entering the hemocoel (Kanost and Gorman, 2008; Stanley et al., 2012). Ecological factors, such as temperature or food availability affect the immune system (Catalán et al., 2012; Hurd, 2001; Murdock et al., 2012; Xu and James, 2012). For example, in *Anopheles stephensi* mosquitoes, the immune-related gene *defensin* was more highly expressed at low temperature (slightly lower than rearing temperature) and the immune gene *Nitric oxide synthase* was up-regulated by heat-shock but not low temperature (Murdock et al. 2012). The importance of including ecological factors in studies of immune system has led to emergence of a new field of study called ecological immunology or eco-immunology (Adamo, 2004; Martin et al., 2011).

As ectotherms, the body temperature of insects follows the temperature of the environment (Gullan and Cranston, 2010; Tattersall et al., 2012). Insects living in temperate climates experience fluctuating temperatures that necessitate activation of behavioral or physiological mechanisms to adjust to the thermal stress and gain better survival and fitness (Catalán et al., 2012; Doucet et al., 2009; Tattersall et al., 2012). At low temperatures, the physiological changes imposed by cold can lead to changes in activation of the insect immune system against pathogens (Marshall and Sinclair, 2011; Xu and James, 2012; Zhang et al., 2011). Low temperatures can negatively affect the immune response by altering immune-related reactions for example, by decreasing the rate of enzyme-mediated reactions (Catalán et al., 2012; Nakamura et al., 2011). Cold-imposed changes in insect physiology can also enhance immune function. For example,

cold-exposed *Drosophila melanogaster* flies show a higher survival from pathogen infection compared to flies that were not exposed to cold (Le Bourg et al., 2009). Any effect that cold has on insect immune system could play a key role in the insect survival during the winter, and especially at the end of the winter when higher temperatures lead to increased pathogen activity (Le Bourg et al., 2009; Mandrioli, 2012; Marshall and Sinclair, 2011).

Evidence of greater survival of cold-exposed insects after pathogen infection (Le Bourg et al., 2009; Marshall and Sinclair, 2011) and increases in the expression of immune genes after cold exposure (Xu and James, 2012; Zhang et al., 2011) suggest activation of the insect immune system in response to cold. However, it is not clear whether the enhancement of the immune response after cold exposure is caused by the direct effect of cold on the immune system or if cold indirectly affecting the immune response by decreasing the performance of pathogen. Moreover, previous studies of the effect of low temperature on the insect immune system have not included different components of the insect immune system. In this thesis, I investigated the effect of cold on the activation of different insect immune components in the absence of pathogen challenge. In addition, because different kinds of cold exposure lead to different physiological responses in insects (Lee and Denlinger, 2010; Sinclair and Roberts, 2005), I explored whether exposure to different kinds of cold (acute and chronic) can affect the immune system differently. To study the interactions between cold and immune responses, the first step was to develop a model system. I used *D. melanogaster* as a model since both cold physiology and immune mechanisms are well-known in this species and the sequenced

genome provides a powerful tool for studying activation of the immune response at the molecular level.

1.2 The insect immune response

Insects are continuously exposed to pathogens in their environment (Kaya and Vega, 2012), and this necessitates a functioning immune system to improve survival. Insects possess an immune system that allows general and rapid responses to infectious agents. Protection against pathogens begins primarily with barriers such as the cuticle that cannot be easily penetrated (Tsakas and Marmaras, 2010). If a pathogen or parasitoid overcomes the external defenses and enters the hemocoel, the insect immune system recognizes it as foreign. Recognition is followed by induction of downstream signaling pathways that eventually lead to the production of effector molecules or responses against the pathogen (González-Santoyo and Córdoba-Aguilar, 2012; Schmidt, 2008; Kaya and Vega, 2012). The immune response to pathogens can be a general response to a wide range of invaders (e.g. melanization) or the insect can respond specifically to different categories of pathogens (Pham and Schneider, 2008).

The insect immune response consists of cellular and humoral responses, classified based on the nature of the components involved. In the cellular immune response, circulating cells (hemocytes) are responsible for fighting against invaders while the humoral immune response includes synthesis and secretion of several antimicrobial proteins into the insect open circulatory system. These components consist of antimicrobial peptides produced by immune pathways, including cecropins and defensins that act against bacteria, fungi, or viruses (Pham and Schneider, 2008).

D. melanogaster is widely used as a model insect in immune studies, because compared to other model insects (e.g. *Bombyx mori* and *Anopheles gambiae*), *D. melanogaster* has a short generation time and is easy to rear. In addition, the genome of *D. melanogaster* is sequenced, which makes it a powerful tool for genomic analysis. Moreover, the immune pathways of *D. melanogaster* are well-studied, providing a good opportunity for further exploration of the immune pathways at the molecular level (Leclerc and Reichhart, 2004). The four major components of the *D. melanogaster* humoral immune response are melanization and the Toll, IMD and JAK/STAT pathways (González-Santoyo and Córdoba-Aguilar, 2012; Leclerc and Reichhart, 2004; Zeidler et al., 2000).

1.2.1 Cellular immune response

The cellular immune response in insects is mediated by hemocytes, which have several immune functions including phagocytosis, encapsulation and nodulation (Schmidt, 2008; Hoffmann, 1995; Pham and Schneider, 2008). Hemocytes are classified morphologically into plasmatocytes, lamellocytes and crystal cells (Hultmark, 2003). Plasmatocytes are the main class of hemocytes and are responsible for phagocytosis, which includes the cellular internalization and degradation of pathogen cells (Hultmark, 2003; Stanley, 2012). Invaders, such as parasitoid eggs, that are larger than plasmatocytes and cannot be phagocytosed will instead be encapsulated by lamellocytes (González-Santoyo and Córdoba-Aguilar, 2012; Hultmark, 2003). If encapsulation is accompanied by aggregation of hemocytes and adhering bacterial cells, large nodules are formed, leading to a form of encapsulation referred to as nodulation (Schmidt, 2008; Stanley and Miller, 2008). Crystal cells are a class of hemocytes that are the site for synthesis of enzymes

necessary for the melanization process (González-Santoyo and Córdoba-Aguilar, 2012; Lanot et al., 2001).

1.2.1.1 Melanization and encapsulation

Encapsulation and melanization are general cellular or humoral immune responses that act against a wide variety of pathogens and parasitoids, and also participate in repairing tissues after mechanical damage (Kaneko and Silverman 2005; Leclerc and Reichhart, 2004). When a pathogen enters the insect hemocoel, receptors in hemocytes membrane recognize the pathogens by identifying specific molecules such as lipopolysaccharides (LPS), lipoteichoic acid (LTA), peptidoglycans (PGN), and β-1,3-glucan (Ashida and Brey, 1998; Schmidt, 2008). Recognition stimulates recruitment of lamellocytes to surround the invading cell, deposit melanin and form a capsule that limits growth and reproduction of the pathogen, and eventually leads to its death (Figure 1.1; Pham and Schneider, 2008; González-Santoyo and Córdoba-Aguilar, 2012; Kaya and Vega, 2012). In infected insects, an increase in the number of circulating hemocytes or the rate of melanin formation indicates the activation of encapsulation and melanization (Lee, et al., 2006; Wilson et al., 2001). In *D. melanogaster* larvae, hemocytes are stored in hematopoetic organs (lymph glands) (Lanot et al., 2001). In adults, hematopoetic organs disappear and the increase in hemocyte number appears to occur as a result of hemocyte mitosis (Lanot et al., 2001). Moreover, sites of hemocyte reserves may exist in adult flies that have yet to be identified.

Melanin formation can be measured by simulating endoparasitism using implants that darken over time in the insect body as melanin is deposited (Lampert, 2012; Wilson et al., 2001). Because cell-mediated melanization is also associated with the repair of wounds,

another method for assessing melanization is to pierce the insect cuticle and measure changes in darkness of the pierced area over time (Gillespie and Khachatourian, 1992).

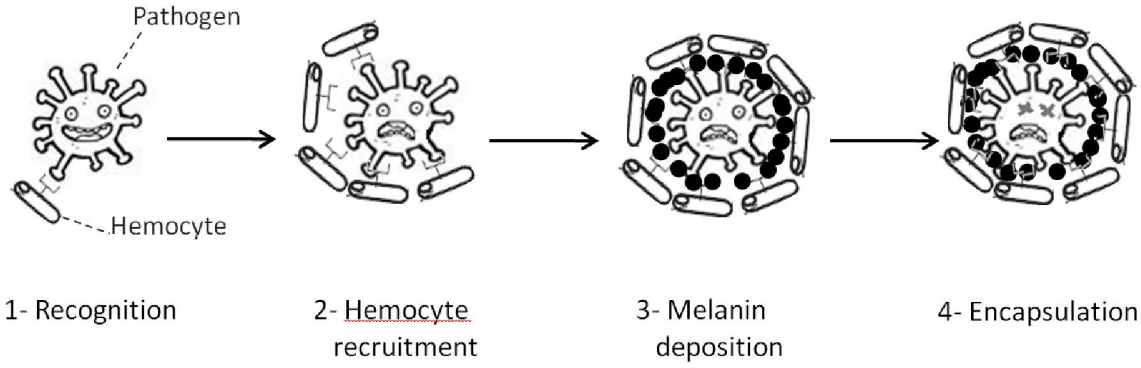

Figure 1.1 The melanization and encapsulation process. In melanization, 1) hemocytes recognize pathogens or parasitoids by their receptors and send signals for 2) recruitment of more hemocytes to 3) deposit melanin and 4) form a multicellular hemocyte wall around the pathogen (redrawn after González-Santoyo and Córdoba-Aguilar, 2012).

When melanization is activated, the enzyme phenoloxidase (PO), stored in hemocytes, catalyzes the production of melanin from phenylalanine (Figure 1.2). Phenylalanine is first hydroxilised to tyrosine and then PO leads to the conversion of tyrosine to DOPA and DOPA to dopaquinone. Through a non-enzymatic reaction, dopaquinone is immediately converted to dopachrome followed by reactions that eventually lead to melanin formation (Figure 2; González-Santoyo and Córdoba-Aguilar 2012; Lemaitre and Hoffmann, 2007). The activity of PO is thus used to detect activation of the melanization response.

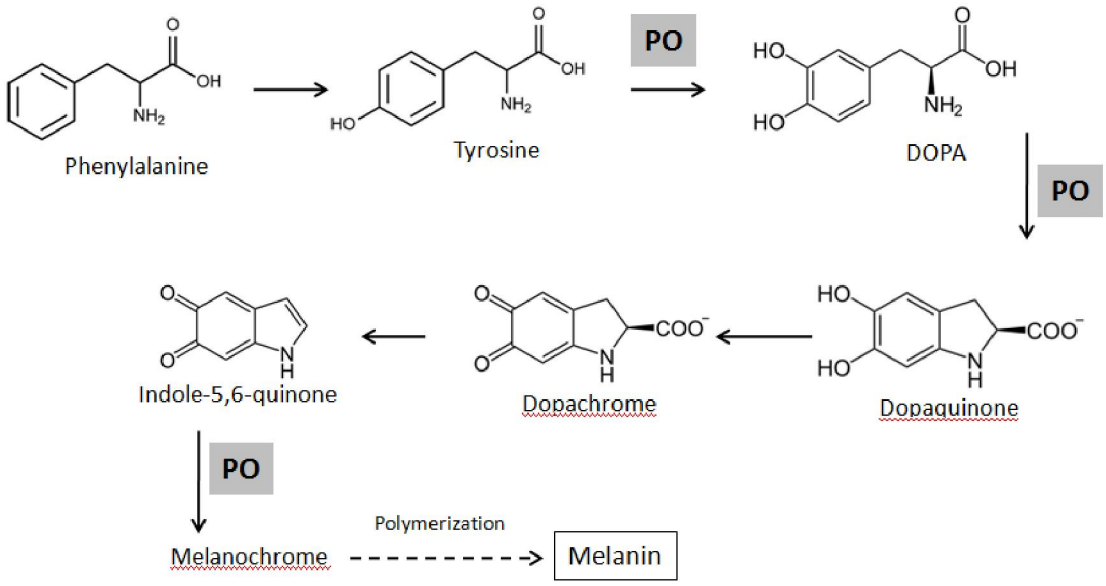

Figure 1.2 The role of phenoloxidase (PO) in reactions associated to melanin formation (redrawn after González-Santoyo and Córdoba-Aguilar, 2012).

1.2.2 Humoral immune response

Unlike the cellular immune system, in which hemocytes act against invaders, the insect humoral immune response originates from the cells of the fat body (Tsakas and Marmaras, 2010). The fat body is a diffuse tissue that is the largest organ in the insect body cavity (hemoceol), and is the functional equivalent of the vertebrate liver (Hoffmann, 1995; Tsakas and Marmaras, 2010). The three major signaling pathways of the *Drosophila* humoral immune response are the Toll, IMD and JAK/STAT pathways. These pathways initiate the synthesis of antimicrobial peptides (AMPs) that act against fungi, bacteria, viruses and parasitoids (Figure 1.3; Kaneko and Silverman 2005; Leclerc and Reichhart, 2004; Pham and Schneider, 2012; Rolff and Reynolds, 2009).

1.2.2.1 Toll and IMD pathways

The Toll pathway is activated in response to Gram-positive bacteria and fungi (Lemaitre et al., 1997; Leclerc and Reichhard, 2004). Toll is a transmembrane receptor that was first recognized for its role in development of the dorso-ventral axis in *Drosophila* embryos (Leclerc and Reichhard, 2004). Pathogen or pathogen-derived compounds are recognized by the proteins upstream of Toll, such as circulating PGRP-SA and PGRP-SD (Kaneko and Silverman, 2005). Recognition of a pathogen leads to cleavage of the Spätzle (Spz) protein and dimerization of Toll, which in turn activates a signaling pathway that sends a signal to the nucleus. This signals the transcription of genes encoding AMPs of the Toll pathway, including *drosomycin*, *metchnikowin* and *defensin* (Figure 1.3; Leclerc and Reichhard, 2004; Royet, 2004).

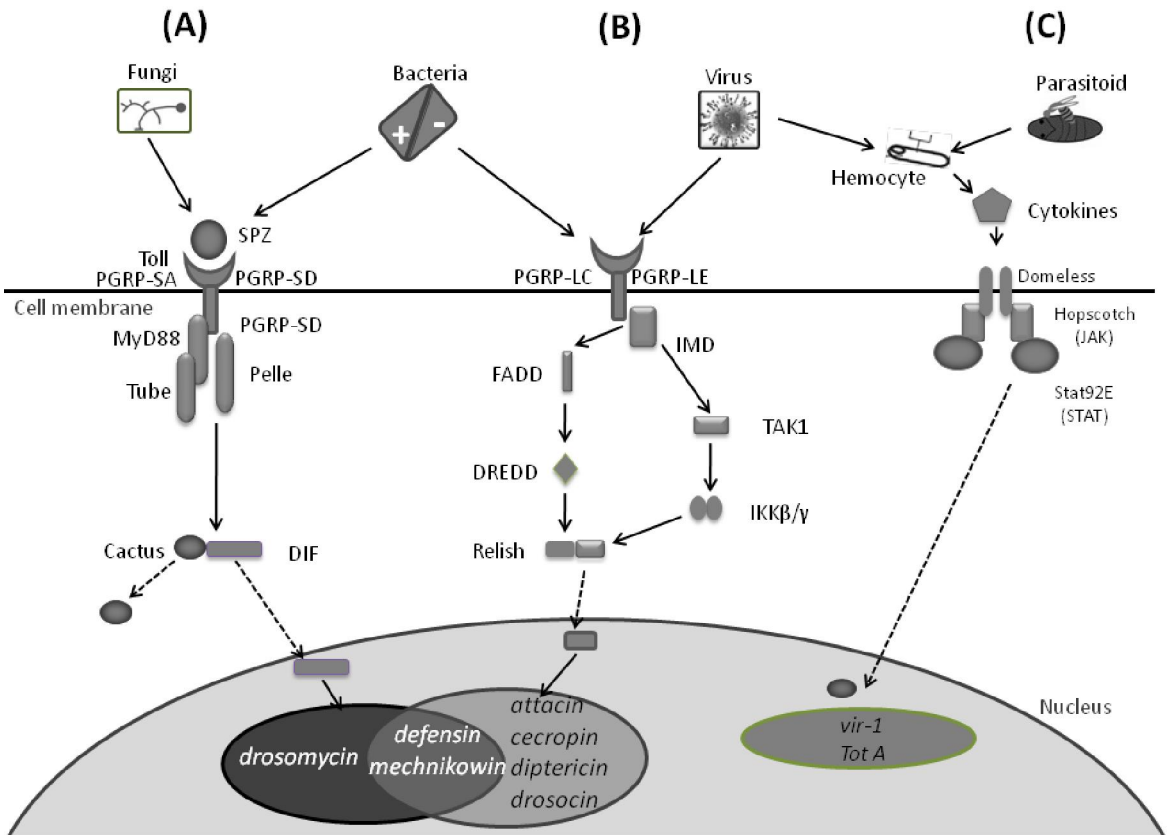

Figure 1.3 Toll, IMD and JAK/STAT pathways in *Drosophila melanogaster*. A) Toll pathway; pathogen recognition, activates Spätzle cleavage by liberation of the C-terminal of Spätzle. The cleaved Spätzle binds the Toll receptor which recruits the Tube/Myd88 complex, followed by the Pelle kinase activation. Pelle kinase triggers an intracellular signaling cascade that activates translocation of Dif in the nucleus leading to transcription of AMP genes. B) IMD pathway; the binding of bacterial PGN on the cell surface receptor PGRP-LC triggers the intracellular adaptor IMD. Signal transduction leads to Relish cleavage and the Rel domain translocates to the nucleus resulting in production of AMPs and C) JAK/STAT pathway; pathogen infections induce hemocytes to produce cytokines which are the ligand of Domeless and lead to STAT protein accumulation in the nucleus and encoding the related genes (redrawn after Royet, 2003; Zeidler et al., 2012).

The immune deficiency (IMD) pathway responds to Gram-negative bacteria and viruses. After recognition of the pathogen by transmembrane receptors, including PGRP-LC and PGRP-LE, the signal is transduced to IMD, and the downstream protein complexes of the signaling pathway (Figure 1.3). Transduction of the signal to the nucleus leads to the expression of AMP genes, such as *diptericin* (Kaneko and Silverman, 2005; Royet, 2004).

AMPs possess different structures, target organisms and modes of action, and are mainly produced by the insect fat body (Hetro et al., 1998; Shai, 1998). Antimicrobial peptides are small cationic peptides that bind to anionic membranes of bacteria or fungi and lead to disruption of the cell and death of the pathogen (Hoffmann, 1995; Rolff and Reynolds, 2009; Tsakas and Marmaras, 2010). The seven known AMPs of *D. melanogaster* are grouped into three families based on the micro-organisms that they fight against: 1) defensin acts against Gram-positive bacteria; 2) attacin, cecropin, drosocin and diptericin respond to Gram-negative bacteria; and 3) drosomycin and metchnikowin show antifungal activity (Park and Lee, 2012). The IMD pathway controls the transcription of diptericin, cercopin, attacin and drosocin whereas the Toll pathway activates drosomycin. The transcription of *defensin* and *metchnikowin* is activated by both the IMD and Toll pathways (Pham and Schneider, 2008; Romeo and Lemaitre, 2008).

Cecropins impair proliferation of Gram-positive and Gram-negative bacteria by increasing the pathogen's cell membrane permeability (Lemaitre and Hoffmann, 2007; Shai, 1998). Diptericin is found only in Diptera, and acts similar to attacins, which inhibit the synthesis of the pathogen membrane proteins by blocking the associated genes (Andreu and Rivas 1998; Shai, 1998; Tsakas and Marmaras, 2010). The modes of action

of drosocin and metchnikowin are not well-understood (Tsakas and Marmaras, 2010). Defensins break single strand DNAs or act similarly to drosomycin, forming channels in the pathogen plasma membrane that lead to cell lysis (Hoffmann, 1995; Tsakas and Marmaras, 2010).

1.2.2.2 The JAK/STAT pathway

In *D. melanogaster*, the JAK/STAT pathway contributes to the development of the embryo and adult, and is activated in response to tissue damage and invasion of parasitoids or entomtopathogens (Agaisse and Perrimon, 2004; Zeidler et al., 2000). Although JAK/STAT-deficient flies are the same as wild-type flies in bacterial resistance and the AMP profile expressed after bacterial infection, JAK/STAT-deficient flies are more sensitive to the *Drosophila* C virus (DCV), suggesting that JAK/STAT acts against viruses (Dostert et al., 2005).

The cytokines released by hemocytes in response to infection or injury, stimulate the JAK/STAT pathway receptor, Domeless (Dome) leading to activation of the intracellular cascades by Janus Kinase (JAK). The STAT protein that is the transcription factor of the pathway leads to expression of genes such as *Turandot A (TotA)* and *vir-1* (Figure 1.3; Agaisse and Perrimon, 2004; Lemaitre and Hoffmann, 2007; Park and Lee, 2012). Vir-1 is a virus-responsive product of the JAK/STAT pathway that is induced in response to DCV in the epidermis but not in the fat body of *Drosophila* (Lemaitre and Hoffmann, 2007; Zambon et al., 2005). TotA is a product of the JAK/STAT pathway in response to abiotic stressors (Lemaitre and Hoffmann, 2007). Although the function of TotA is still unknown, it is usually used as an indicator for activation of the JAK/STAT pathway (Pham and Schneider, 2008).

1.3 Low temperature and insects

Low temperature reduces insect metabolism, survival, growth and reproduction (Hutchinson and Bale, 1994; Irwin and Lee, 2002; Leather et al., 1995). In temperate areas, at the beginning of the winter, some insects use behavioral strategies to avoid freezing at sub-zero temperatures. For example, some insects, like monarch butterflies, migrate to warmer areas, and some other insects move to lower levels of soil or water below the frost line (Doucet et al. 2009; Lee and Denlinger, 2010; Stefanescu et al., 2012; Storey and Storey, 2012). However, most insects are incapable of long-distance migration, and adapt to cold conditions by adopting different strategies. Insects are grouped into freeze tolerant, freeze avoidant and chill susceptible based on their strategies in response to cold:

1) Freeze tolerant insects can tolerate internal ice formation (Lee, 2010; Sinclair and Renault, 2010; Zachariassen, 1985).
2) Freeze avoidant insects are killed by internal ice formation, but can survive sub-zero temperatures by avoiding freezing of their body water (Bale, 2002; Doucet et al., 2009; Lee, 2010).
3) Chill-susceptible insects are killed at temperatures where ice does not form in their body (Bale, 1996). Low temperatures that are above the freezing point of these insects may lead to accumulation of injuries in the insect cells referred to chilling injury (Lee, 1991; Lee, 2010).

Based on the duration and intensity of the low temperature experienced, there are two kinds of chilling injury: acute chilling injury that results from short exposure to temperatures below 0 °C that does not lead to freezing; and chronic chilling injury

induced by long exposure to relatively mild temperatures (at or near 0 °C) (Lee, 2010; Sinclair and Roberts, 2005). The underlying mechanisms of acute and chronic chilling injuries are not well-understood. Acute chilling injury appears to be caused by cell membrane damage induced by membrane phase transition from liquid-crystalline to gel and disruption of cell membrane proteins (Lee, 1991; Lee, 2010). In contrast, chronic chilling injury is associated with a loss of ion homeostasis in the insect body (Koštál et al., 2006; Lee, 2010; MacMillan and Sinclair, 2011a; MacMillan and Sinclair, 2011b).

1.3.1 Cold and *Drosophila melanogaster*

Drosophila melanogaster (Diptera: Drosophilidae) originates from tropical regions, but currently inhabits tropical and temperate areas (Demerec, 1965; Kellermann et al., 2009). Temperature and water are two main factors that limit the distribution of *D. melanogaster*, because these flies cannot survive drought or cold conditions (Demerec, 1965; Hoffmann et al., 2002; Kellermann et al., 2009). The developmental processes of flies stop at about 9-10 °C, (David et al., 1983), and at temperatures below 3.5 °C the flies start to lose muscle activity and feeding capacity and as a result enter chill coma (David et al., 1998; Hosler et al., 2000; Ransberry et al., 2011). If flies are returned to higher temperatures they can recover and resume normal activities, but the time needed for recovery depends on the intensity and duration of cold exposure and the temperature at which they recover (David et al., 1998; MacMillan and Sinclair, 2011a).

Larvae and adults of *D. melanogaster* cannot survive temperatures below approximately -5 °C, and being exposed to -8 °C for 2 hours is lethal for pupae. However, the body fluids of larvae, pupae and adults do not freeze until -17 to -20 °C (Czajka and Lee 1990). Lethal injury occurs in *D. melanogaster* in temperatures 10-15 °C higher than their body

freezing point showing that *D. melanogaster* is a chill susceptible insect (Czajka and Lee 1990; Doucet et al., 2009; Lee, 2010). In *D. melanogaster,* two hours exposure to -2 °C can lead to acute chilling injury, whereas exposure to temperatures around 0 °C for several hours can result in chronic chilling injury (Koštál et al., 2006; Lee, 2010; MacMillan et al., 2009). After cold exposure, the regulation of several genes changes presumably to rebuild the injured tissues, and refold the proteins that were denatured due to cold stress (Doucet et al., 2009; Goto, 2001; Qin et al., 2005). More than one-third of the genes expressed after recovery from two hours exposure of *D. melanogaster* adults to 0 °C are related to membrane proteins and about 16 % of the cold induced stress genes including heat shock protein genes (*Hsp83, Hsp26, Hsp23*) and *Frost* (Qin et al., 2005). Short cold exposure induces expression of different genes compared to long cold exposure in *D. melanogaster*. Microarray experiments showed that 20 genes are up-regulated after a short cold exposure (two hours at -0.5 °C), and 69 genes are expressed after long cold exposure (ten hours at -0.5 °C), while just a few of the genes are up-regulated in both the treatments, suggesting that different injuries caused by short and long cold exposure lead to differences in expression of genes for repairing cold injury (Zhang et al., 2011). Also, in *D. melanogaster* females, the glycogen content as an energy reserve was higher 24 hours after short cold exposure (two hours at -0.5 °C) compared to long cold exposure (ten hours at -0.5 °C) which could affect their further physiological responses (Marshall and Sinclair, 2010). The differences in the physiological responses induced by acute and chronic cold exposure may affect the performance of the flies in responding to other stressors that occur simultaneously with cold.

1.4 The insect response to multiple stressors

In nature, insects may experience multiple stressors that occur simultaneously (Davies et al., 2012; Sinclair et al., 2013). For example, starvation can be accompanied by cold stress because of the lack of food resources or feeding capacity during the winter (Leather et al. 1995). Desiccation is another stressor that can occur with cold since, at sub-zero temperatures, water is in the frozen form and is not available for insects, and also the environmental water vapor pressure decreases leading to disruption of vapor pressure equilibrium between insect body and the environment (Danks, 2000). Co-occurring stressors can have a negative or positive effect on the response to other concurrent stressors (Crain et al., 2008). If the effect is negative (antagonistic), up-regulation of the response to one of the stresses leads to a weaker response to the other stresses (French et al., 2011; Hoang, 2001; Hoffmann et al., 2002). A stressor might have a positive (synergistic) effect on the response to a co-occurring stress response; for instance, the soil collembolan *Folsomia candida* is more cold tolerant after exposure to desiccation stress (Bayley et al., 2001) and dehydrated larvae of *Belgica antarctica* (Diptera: Chironomidae) can better tolerate cold and heat stress (Benoit et al., 2009). Moreover, in *D. melanogaster* larvae, a combination of heat and cold treatment before a further cold exposure enhanced cold tolerance (Rajamohan and Sinclair, 2008). Synergistic effects of stresses on each other can be explained by cross-tolerance or cross-talk between the stress response pathways (Sinclair et al., 2013).

1.4.1 Antagonistic interactions among stressors

Antagonistic interactions or trade-offs occur between two physiological traits when activation of one trait leads to decline in activation of the other trait (Zera and Harshman,

2001). If two or more physiological traits use a common limited internal resource pool (e.g. energy or nutrients) that is not sufficient for keeping all of the traits activated, a trade-off happens between the physiological traits. Increase in allocation of resources to one life history trait or stress response necessitates a decrease in other traits or response to other stressors (French et al., 2011; Hoang, 2001; Zera and Harshman, 2001). For instance, butterflies, whose larvae undergo diapause to avoid unfavorable environmental conditions, obtain less energy reserves and, as a result, reduced potential fecundity (Fordyce et al., 2006). Acute cold exposure reduces fertility in *D. melanogaster* (Overgaard et al., 2007) and repeated cold exposure leads to reproduction decline in *D. melanogaster* (Marshall and Sinclair, 2010). A negative effect of concurrent stresses on the response to stressors is shown in the trade-off between high and low temperature tolerances along a latitudinal gradient in *Drosophila*, such that flies at higher latitudes recover from chill coma faster than flies at lower latitudes while more time is needed for the low latitude flies to be knocked down by heat shock compared to high latitude flies (Hoffmann et al., 2002).

The differential allocation of limited resources to different physiological traits is not the only potential reason underlying the trade-offs (Zera and Harshman, 2001). Physiological or genetic mechanisms can also lead to trade-offs between biological traits (Zera and Harshman, 2001). For example, heat shock can induce oxidative stress in yeast cells by deletion of genes regulating electron flow in the respiratory chain (Davidson and Schiestl, 2001). *Battus philenor* butterflies that do not undergo diapause have lower energy reserves, but they show a higher chemical defense against predators due to higher concentrations of unpalatable chemicals in the adult butterflies (Fordyce et al., 2006).

1.4.2 Synergistic interactions among stressors

Insects that are concurrently exposed to two or more environmental stresses may activate stress-response signaling pathways that act synergistically with other pathways (Davies et al. 2012; Sinclair et al., 2013). The synergistic interactions allow for activation of one pathway to achieve the end of other signaling pathways (MacMillan et al., 2009; Sinclair et al., 2013). Based on an organism's ability to respond to concurrent stresses with the same mechanism, two ways that synergistic stress responses can be mediated are: 1) cross tolerance, whereby activation of a shared signaling pathway results in induction of all downstream stress protection pathways; and 2) cross-talk in which the signaling pathways against different stressors are not shared but the physiological changes resulting from activation of response to one stress leads to protection against another stress (Benoit et al., 2009; Davies et al., 2012; Sinclair et al. 2013). Despite the different underlying mechanisms, cross-tolerance and cross-talk have the same phenotypes: gaining tolerance to one stressor leads to increased tolerance to another stressor (Sinclair et al., 2013).

Cross tolerance occurs when an environmental stress activates a protective mechanism that is common between other stresses, and activation of the mechanism leads to tolerance against all those stresses (Figure 1.4; Sinclair et al., 2013). For example, the sugars and polyols that prevent freeze-tolerant insects from freezing-induced cell damage decrease dehydration level in insects exposed to desiccation (Bayley and Holmstrup, 1999; Holmstrup et al., 2010). Dehydration of *Belgica antarctica* larvae (Diptera: Chironomidae) leads to increases in the synthesis of trehalose, which can in turn protect membranes and proteins from cold-induced damage and enhance cold tolerance (Benoit et al., 2009). In *D. melanogaster*, heat shock increases the cold tolerance of the flies,

which is potentially due to elicited expression of heat shock proteins (Rajamohan and Sinclair, 2008).

In cross-talk, induction of one stress response leads to activation of a shared signaling pathway that eventually activates separate protection mechanisms against different stresses (Figure 1.4; Sinclair et al., 2013). For example, in *Drosophila* the transcription factor, dFOXO, is activated in response to starvation and adapts the level of metabolism to the availability of nutrients. During starvation, activation of dFOXO leads to up-regulation of the pathways that result in over-expression of AMP genes and a cross-talk between starvation and the immune response (Becker et al., 2010; Davies et al., 2012). Another example of cross-talk is up-regulation of AMP genes in response to high concentrations of environmental oxygen, which is likely due to the effect of hyperoxia on receptors and alternative proteins of the IMD pathway (Zhao et al., 2010).

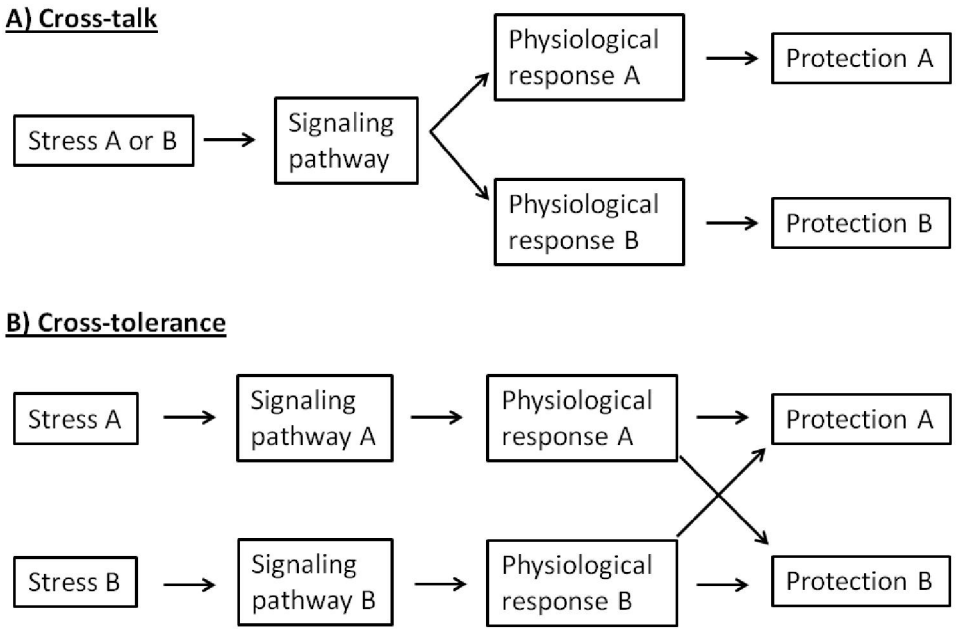

Figure 1.4 Two types of synergistic effect of co-occuring stresses on insects: A) Cross-talk in which induction of one stress response leads to activation of a shared signaling pathway that eventually activates separate protection mechanisms against different stresses; B) Cross-tolerance that occurs when an environmental stress activates a protective mechanism that is common between other stresses and activation of the mechanism leads to tolerance against all those stresses (after Sinclair et al., 2013).

1.4.3 The interplay between cold and immune responses

Activation of the insect immune system in response to environmental pathogens is energetically-costly (French et al., 2011; Lochmiller and Deerenberg, 2000). Due to the high cost of activation of the immune system, an antagonistic interaction between response to immune challenge and other environmental stresses including low temperature is expected (Lochmiller and Deerenberg, 2000; Marshall and Sinclair, 2010; Sinclair et al., 2013). However, recent evidence of cross-talk among cold and immune responses in insects suggests that cold exposure can activate or prime the immune

response of insects even in the absence of pathogens. For instance, repeatedly-frozen larvae of *Pyrrharctia isabella* (Lepidoptera: Arctiidae), survive fungal infection better than caterpillars not exposed to cold (Marshall and Sinclair, 2011). In *Aquarius najas* (Heteroptera: Gerridae), enhanced encapsulation response was associated with higher winter survival suggesting that cold-immune cross-talk can go towards the both ends (Krams et al., 2011). In *Megachile rotundata* (Hymenoptera: Megachilidae), there was an increase in the expression of genes related to immune response after exposure to temperatures lower than the normal nest temperature (Xu and James, 2012). Moreover, cold-exposed *D. melanogaster* adults survived better from fungal infection compared to flies that were not exposed to cold (Le Bourg et al., 2009). Finally, cold exposure leads to up-regulation of several immune-related genes in *D. melanogaster* including *immune induced molecule 1*, *immune induced molecule 23*, *attacinA*, *attacinB*, *attacinC* and *metchnikowin* (Zhang et al., 2011).

The evidence of immune response enhancement after cold exposure suggests that there are links between cold response and immunity. However, the mechanisms of the interactions and the evolutionary importance of the links between responses to cold and pathogens are not well explored. Sinclair et al. (2013) suggest four hypotheses for the evolution of interactions between cold response and immunity in insects. First, after cold exposure immune system could be activated as a general response to stress. Second, eicosanoids that play roles in both thermoregulatory mechanisms and immune response of insects could underlie a link between cold and immune responses. Third, the potential cold-induced tissue damage could lead to activation of the insect immune system. Physical damage to tissues could directly activate the immune system, or indirectly lead

to immune activation by entrance of gut symbionts to hemocoel as a result of cold-induced gut tissue damage. Fourth, pathogens and parasitoids that are active while the overwintering insects are in chill coma or diapauses could lead to selection for higher immune activity during the cold seasons. To determine the nature and significance of the potential positive interaction between cold response and immunity, first the immune components that could be influenced by low temperature should be recognized.

Despite the evidence suggesting cold-activation of the immune response, the effect of cold on activation of the insect immune system has not been systematically studied in the absence of pathogens. Most studies either include pathogens, or use immune assays that focus on only a small fraction of the insect immune system. However, studies including pathogens in immune assays cannot explain whether the enhancement of immune response after cold exposure is due to the direct effect of cold on the insect immune response or results from the thermal impacts on pathogen performance. Moreover different specificity of immune pathways to different pathogens necessitates a systematic study of immune system in response to cold. Moreover, no study has been performed on the potentially different effects of acute and chronic cold exposure on activation of the insect immune system while it appears that acute and chronic cold cause different physiological responses in insects.

1.5 Objectives

Several lines of evidence suggest that exposure to low temperature can enhance the insect immune response. However, it is not clear if cold exposure activates a general immune response or if the effect of low temperature is limited to some specific immune pathways. My first objective was to determine whether each of the major components of the

immune system in *D. melanogaster* is up-regulated in response to cold, in the absence of pathogens. Absence of pathogens indicates that the immune response is activated not in response to pathogens but rather by cold as a stressor. After exposing the flies to cold, I compared the activation of four major immune components of *D. melanogaster* (e.g. encapsulation and melanization, Toll, IMD and JAK/STAT pathways) between cold-exposed and control flies.

Overwintering insects are exposed to different kinds of cold that might differentially affect the insect physiological traits. My second objective was to investigate whether the type of cold exposure affects activation of the immune system. I measured the up-regulation of each of the immune pathways after acute and chronic cold exposure. This would indicate whether different gene expression and cold injury caused by acute and chronic cold exposure could differently affect the insect immune response.

This study provided evidence of cold-induced activation of different immune components in *D. melanogaster*, and different effects of acute and chronic cold exposure on activation of the flies' immune response. My findings could allow future research on the potential mechanistic links between cold exposure and immune response of overwintering insects.

Chapter 2

2 Methods

2.1 Fly rearing

Drosophila melanogaster flies were collected in London, Ontario and Niagara-on-the-Lake, Ontario, Canada (43°00'N, 81°15'W) in 2008, and subsequently maintained in large, outbred populations in the lab (Marshall and Sinclair, 2010). Flies were maintained in a Percival I36VL incubator (Percival Scientific Inc., Perry, IA) at 21.5 °C, 60 ± 5% RH, under a 14L:10D light cycle and mass-reared as described previously by Nyamukondiwa et al. (2011). Flies were reared on banana food (Containing 825 g banana, 180 g malt, 285 g corn syrup, 165 g yeast and 39 g agar, balance water per liter; Markow and O'Grady 2006). To prevent microbial contamination and to remove *Wolbachia* spp. (a bacterial endosymbiont that affects the immune system), I added the antibacterial tetracycline and the antifungal methylparaben to the banana food media (Carrington et al., 2010). I found no evidence of *Wolbachia* using standard PCR assay (Pourali et al., 2009). To collect eggs for founding each new generation of flies, I placed approximately 1000 flies in a 3.8 L clear plastic population cage (23 cm × 15 cm × 13 cm) and attached a piece of medical stockinette wrapped with a paper clip at the opening of the cage to provide easy access. I put a Petri dish containing 35 mL of banana food in the cage with a small amount of active yeast paste (active yeast, *Saccharomyces cerevisiae*, mixed with distilled water) on the food to stimulate oviposition. After 16 hours I collected the eggs laid on the food by cutting the food in pieces containing 80-100 eggs and transferring them to 35 ml vials containing approximately 10 mL banana food. I

kept pre-adult flies in the 35 mL vials and used the adults that emerged from each vial for founding the next generation or for experiments.

To collect virgin female flies, I removed all adults that appeared in the 35 ml vials and after eight hours collected any newly emerged adults. I anesthetized the flies by CO_2 for less than 10 min to identify and separate the females under a dissecting microscope. I then transferred the virgin females to new 35 ml vials containing approximately 10 ml of banana food. I placed the virgin females in rearing condition of 21.5 °C and 60 ± 5% RH for seven days before starting the experiments to minimize the risk of anesthesia effects on immune response and cold tolerance (Nilson et al., 2006).

2.2 Experimental design

To investigate the potential effect of cold exposure on the immune response, I exposed seven-day old adult virgin females to low temperature and examined activation of melanization, Toll, IMD and JAK/STAT pathways. I divided the population of flies into four experimental groups (Figure 2.1): 1) acute cold exposure group that was exposed to -2 °C for two hours; 2) chronic cold exposure group that was exposed to -0.5 °C for ten hours; 3) control group of the acute cold exposure, in which flies were handled the same as flies in treatment 1 but at rearing temperature; and 4) control flies for chronic cold exposure that were handled the same as group 2 but without exposure to cold (they were kept at 21.5 °C for the duration of the experiment). After the cold treatment, I returned the flies to the normal rearing temperature for four to six hours (four hours for PO activity and six hours for counting hemocytes and mRNA measurements) to let them recover from the cold and to allow for activation of any immune response. All four groups had access to food during treatment and recovery.

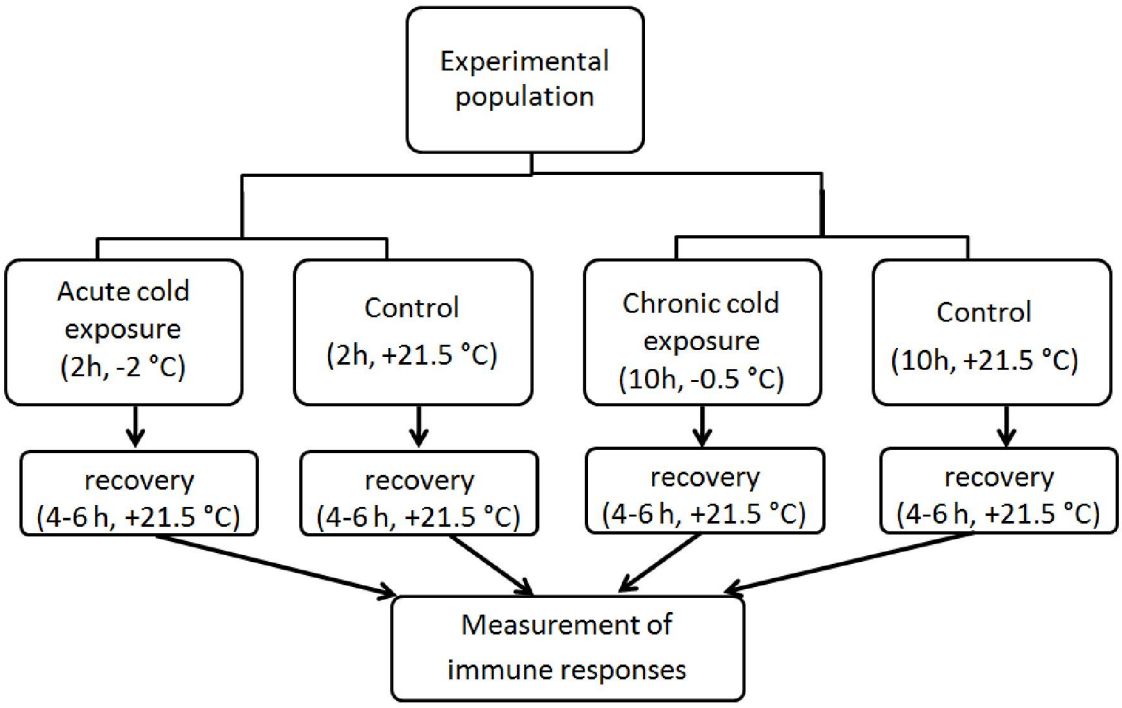

Figure 2.1 Experimental design to explore the effect of cold exposure on the *Drosophila melanogaster* immune system. Seven day old virgin females were divided into four groups of acute cold, control for acute cold, chronic cold, and control for chronic cold.

2.3 Melanization

To investigate the activation of melanization in flies following cold exposure, I measured the hemocyte concentration in the hemolymph, phenoloxidase activity in the whole body fluid and also melanization in response to wounding.

2.3.1 Circulating hemocyte concentration

For estimating the circulating hemocyte concentration (CHC), six hours after cold exposure I anesthetized flies by exposing them to CO_2 for 30 seconds. I collected hemolymph from the flies following the method used by MacMillan (2013). I placed the

fly into a 10 µl pipette tip, and then connected a plastic tube to the base of the tip. By blowing into the tube I forced the fly towards the end of the tip which was cut so that just the antennal junction of the fly was exposed. I removed one of the antennae using forceps. The pressure produced by blowing into the plastic tube forced a droplet of hemolymph from the antennal junction. Using a glass capillary tube (0.5 µl total volume with 0.142 mm inner diameter; Cat. No. P1299. Sigma-Aldrich) I collected the hemolymph drop and immediately measured the length of the hemolymph in the capillary using a digital camera installed on a dissecting microscope (Nikon SMZ 1500, Nikon, Tokyo, Japan). Using the inner diameter of the capillary tube and the length of the hemolymph in the tube, I could estimate the hemolymph volume taken from each fly using equation 2.1:

$$V = \pi(\frac{\mathrm{ID}}{2})^2 \times \mathrm{L}$$

(Equation 2.1)

Where:

V= Volume of hemolymph taken from a fly

ID= the internal diameter of the capillary tube

L= Length of hemolymphcolumn in the tube

I diluted the hemolymph taken from each fly in phosphate buffered saline (PBS; Cat. No. P-3813, Sigma-Aldrich) containing 0.55 % (W/V) cresyl violet (for staining the hemocytes) and 0.5 % ethylene-diamine-tetraacetic acid (EDTA; Cat. No. E6758, Sigma-Aldrich) as an anticoagulant to obtain a 10 µl solution. I applied the solution to a

hemocytometer (Cat. No. 100; Hausser Scientific) and counted the number of hemocytes using a compound microscope with 400× magnification. Using the number of circulating hemocytes, I calculated the concentration of hemocytes (number of hemocytes per microliter hemolymph, CHC). I compared the CHC of acute and chronic cold exposure treatments and the control flies by two-way ANOVA using SPSS software (SPSS Inc, 2004, Version 13).

2.3.2 Phenoloxidase activity

Four hours after cold exposure, I transferred flies to a -80 ° C freezer for storage until measuring PO activity. I placed approximately 20 mg (16-19 individuals) of frozen flies in a 1.7 ml microcentrifuge tube that was pre-cooled on ice. I then homogenized the flies using a polyethylene plastic pestle for 30 s and added 1 ml of PBS containing protease inhibitor (Cat. No. S8820, Sigma-Aldrich) to the homogenized flies. I homogenized the flies again for 3 min and then centrifuged at 18,000 × g at 10 ° C for 10 min. I transferred 0.5 ml of the clear supernatant to each of two 1.7 ml microcentrifuge tubes. Again, I added 1 mL PBS to the pellet and homogenized and centrifuged at the same condition that was described above. I repeated homogenizing and centrifuging the pellet one more time and each time transferred the supernatant to two 1.7 ml tubes so that, at the end, a total of 1.5 ml of homogenate could be collected in both of the tubes. I kept the body fluid samples in a -80 ° C freezer for further use. For each treatment (control, acute or chronic cold exposure) I prepared ten samples of body fluids and measured the PO activity spectrophotometrically using a SpectraMax M2 (Molecular Devices, Sunnyvale, CA) and L-dihydroxyphenylalanine (L-DOPA; Cat. No. 333786, Sigma-Aldrich) as the substrate (Wilson et al., 2001).

To treat hemolymph with L-DOPA for measuring PO activity, in each well of a 96-well flat-bottom plate I mixed 50 µl of body fluid (supernatant) with 50 µl of L-DOPA (3mg/ml of PBS) and 50 µl of PBS using a multi-channel micropipette. Phenoloxidase converts colorless L-DOPA into orange dopachrome (Figure 1.2; Harisha, 2006). I measured the absorbance of the dopachrome produced in each sample at 492 nm against an L-DOPA control with no body fluid at 10 minutes intervals for one hour at 25 ° C.

I used the Bradford assay (Bradford, 1976) to determine the protein concentration of the body fluid. Bovine serum albumin (BSA) (Cat No. P5369, Sigma) was used as the standard protein. I loaded 5 µl of each of the eight concentrations of BSA (0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0) in triplicate and 5 µl of each body fluid sample in individual wells of a 96-well plate. I added 200 µl PBS and 40 µl of Bradford reagent to each well and mixed them using a multi-channel pipette. After 15 minutes the absorbance was recorded at 595 nm using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). I estimated the protein content of the samples by comparison with the standard curve run on each plate.

I expressed phenoloxidase enzyme activity as changes in absorbance at 492 nm per minute per mg body mass (Wilson et al., 2001). I compared absorbance per minute (i.e. PO activity values excluding mg body mass) among treatments using two-way ANCOVA with body mass as a covariate using SPSS software.

2.3.3 Melanization

Immediately after cold exposure, I anesthetized 40 females from each of the experimental groups (control, acute and chronic cold exposure) by exposing them to CO_2 for one

minute. Using a sterilized No. 000 insect pin with 0.0254 cm diameter (Cat. No. 128B000, Bioquip), I pierced each fly in the right thoracic vertical cleft along the notopleural suture until it reached the humeral callus (Figure 2.2). Immediately after piercing, I photographed the pierced area with a digital camera installed on a dissecting microscope at 10× magnification. The 40 flies of each treatment were divided into four groups of ten, to take pictures of the pierced area after 6, 12, 24 or 48 hours. I quantified melanization by measuring the mean gray value of reflected light from the pierced area using Image J software (National Institutes of Health, http://rsbweb.nih.gov/ij).

To compare piercing-induced melanization between cold-treated and control flies, I recorded the gray value of the pierced area of treated and control flies at specific time points (0, 6, 12, 24 and 48 h after cold exposure). Darker colors with lower gray values indicated more pierce-induced melanization. I compared the gray value of the pierced area at the time of piercing to make sure that none of the groups were darker from the beginning. Then I compared the mean gray value after 12 hours from piercing among treatments by two-way ANOVA using SPSS.

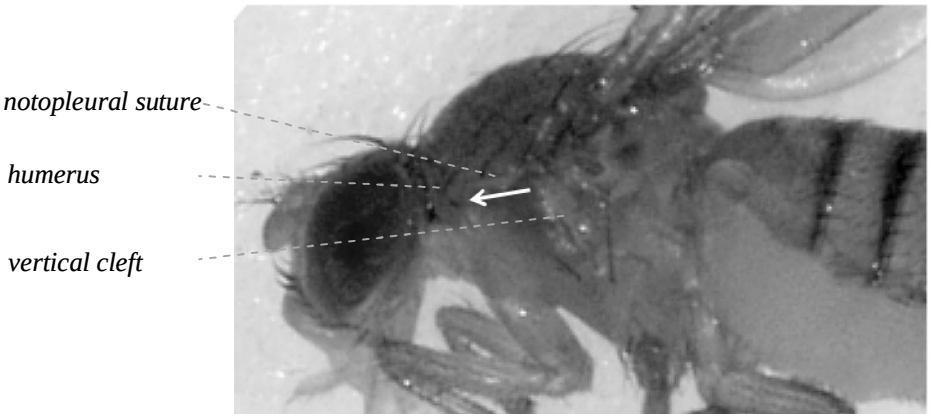

Figure 2.2 The lateral aspect of the *Drosophila melanogaster* thorax. The white arrow shows the direction of piercing with a needle to measure melanization (Adapted from Demerec, 1965).

2.3.4 Toll, IMD and JAK/STAT pathways

To investigate the expression of genes associated with Toll, IMD, and JAK/STAT pathways, expression of genes related to products or intermediate components of pathways was quantified by quantitative real-time polymerase chain reaction (RT-qPCR). For each cold exposure treatment, I extracted RNA from six samples of 30 frozen seven-day-old virgin females using Trizol Reagent according to protocol (Cat No. 15596018, Invitrogen, Burlington, Canada). I dissolved the extracted RNA pellet in 60 µl RNase-free water and determined the total RNA concentration and purity ratio at 260 and 280 nm absorbance using a NanoDrop 2000 spectrophotometer and associated software (Thermo Fisher Scientific Inc., Wilmington, DE). I used only samples with a 260/280 ratio of 1.98 or more.

To remove DNA contaminants, I treated the RNA samples with DNase. One microgram RNA from each sample was mixed with 1 µl of DNase I Amp Grade (Cat No. 18068015, Invitrogen, Burlington, Canada) and 1 µl of 10X DNase I Reaction Buffer (Cat. No.

Y02340, Invitrogen). After incubation at room temperature for 15 min I added 1 µl of 25 mM EDTA (Cat. No. Y02353, Invitrogen) and incubated the samples at 65 °C for 5 min to inactivate the DNase. The samples were cooled on ice before being used for cDNA synthesis.

To synthesize cDNA, I added 2 µl of oligodT, 4 µl qScript Flex Reaction Mix (5×) and 1 µl qScript Reverse Transcriptase (Cat. No. 95049025, Quanta Bioscience) to 1 µg RNA of each sample. The samples were then treated at 42 °C for 75 min and 85 °C for 5 min. I used SYBR Green Master Mix (Invitrogen, Burlington, Canada) to amplify the cDNA.

I used the primers shown in Table 2-1 for RT-qPCR analysis and used *Rpl*-32 as a reference gene to normalize the data obtained from the target genes (Zaidman et al., 2011). I chose the primer sequences from the literature (Tsai et al., 2008) and checked them in Primer3 (http://primer3.sourceforge.net) to ensure a length of 19-23 nucleotides, G-C content of 35-65%, and T_m of 60-68 °C. To determine the efficiency of primers at different cDNA concentrations, I drew 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). I calculated threshold cycle (Ct) values by CFX Manager Software ver. 2.1 (Bio-Rad) and calculated and normalized the expression ratio of target genes relative to controls. I determined differences in abundance of mRNA between cold-treated and control groups using *t*-tests. Moreover, I used *t*-test to compare the level of changes in expression of each gene between acute and chronic cold treatments.

Table 2.1 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 melting temperature. References are noted for primers derived from literature.

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

Chapter 3

3 Results

3.1 Cellular immune response

3.1.1 Circulating hemocyte concentration

The circulating hemocyte concentration (CHC) of flies exposed to acute cold was significantly higher than that of control flies (Figure 3.1, Table 3.1). There was no difference between the CHC of chronic cold-exposed and control flies. I observed a significant interaction between the effect of cold (cold exposed vs. control flies) and duration (acute vs. chronic cold exposure) on the CHC of *D. melanogaster* females indicating the greater effect of acute cold on the CHC increase compared to chronic cold exposure (Figure 3.1, Table 3.1, two-way ANOVA).

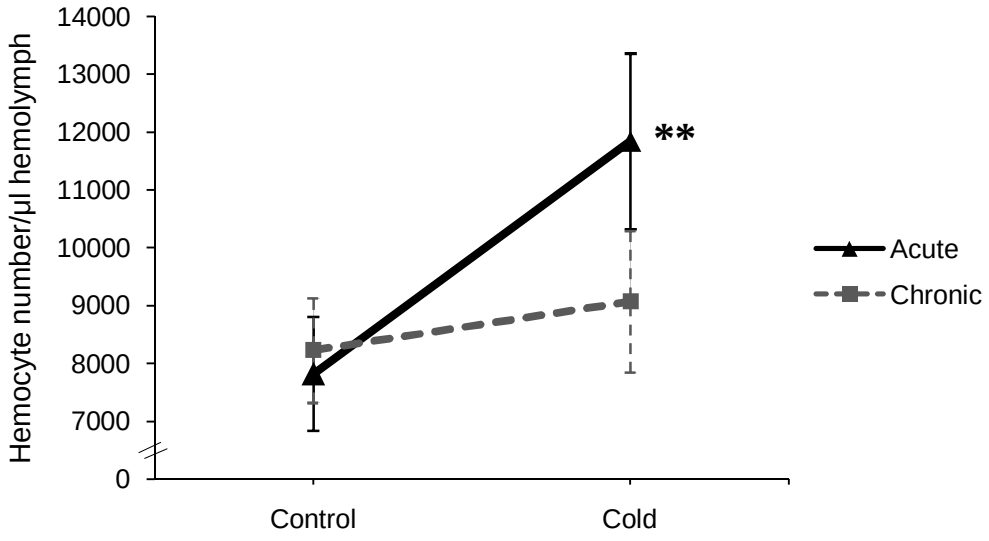

Figure 3.1 Circulating hemocyte concentration (CHC) of *Drosophila melanogaster* females compared to control flies. The asterisk indicates a significant change in CHC of cold-exposed compared to control flies in two-way ANOVA, ** $P<0.01$. Data are presented as mean ± SEM, n=10.

3.1.2 Phenoloxidase activity

Phenoloxidase (PO) activity after acute cold exposure did not change significantly from untreated controls. By contrast, chronic cold exposure decreased PO activity but the difference between cold exposed and control flies was not significant (Figure 3.2 and Table 3.1). The significant difference in slopes of acute and chronic cold exposure ($F_{1,35}$= 5.271, *P*= 0.028) implies that acute and chronic cold exposure elicit different responses by PO to cold.

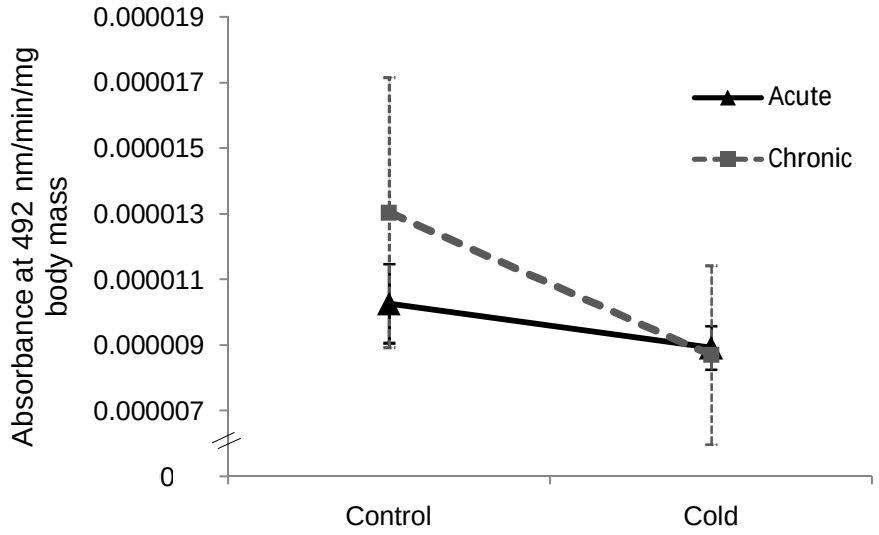

Figure 3.2 Changes in phenoloxidase (PO) activity of *Drosophila melanogaster* females in response to acute and chronic cold exposure compared to control females that were not cold-exposed. PO activity after acute cold (2h, -2 °C) is represented by the black solid line and after chronic cold exposure (10h, -0.5 °C) by the gray dashed line. Data (absorbance per minute) were analyzed using two-way ANCOVA with body mass as a covariate, *P*<0.05. PO activity data are presented as mean (absorbance per minute per mg body mass) ± SEM, n=10.

3.1.3 Melanization

Melanization in the pierced area of the flies' thorax increased over time in all of the experimental groups (Figures 3.3 and 3.4). I did not detect any significant difference in darkness of the pierced area among treatments at the time of piercing. All the treatments showed an increase in the darkness by time that plateaued around 12 h after piercing (Figure 3.4), therefore I made a comparison in darkness of the pierced area at 12 h after piercing among treatments. Acute cold exposure led to a significant increase in darkening after 12 hours compared to control flies (Figure 3.5; Table 3.1), but the difference between piercing-induced darkening of chronic cold-exposed and control flies was not statistically significant. There was no significant interaction between the effect of cold (cold exposed vs. control flies) and duration (acute vs. chronic cold exposure) on the piercing-induced melanization of *D. melanogaster* females indicating that the effect of acute cold on the melanization response was not significantly different from that of chronic cold exposure (Figure 3.5, Table 3.1, two-way ANOVA).

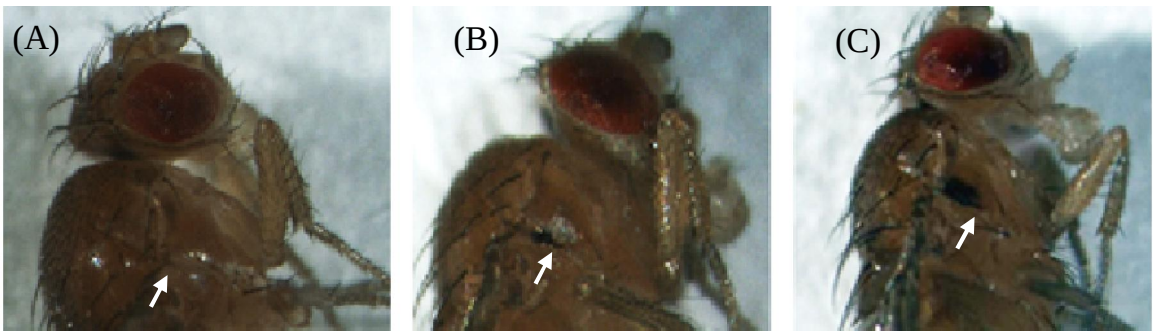

Figure 3.3 Melanization in the pierced area of *Drosophila melanogaster* females' thorax. The white arrows indicate the melanization in the pierced area of flies: A) at the time of piercing, B) six hours after piercing and C) 48 hours after piercing.

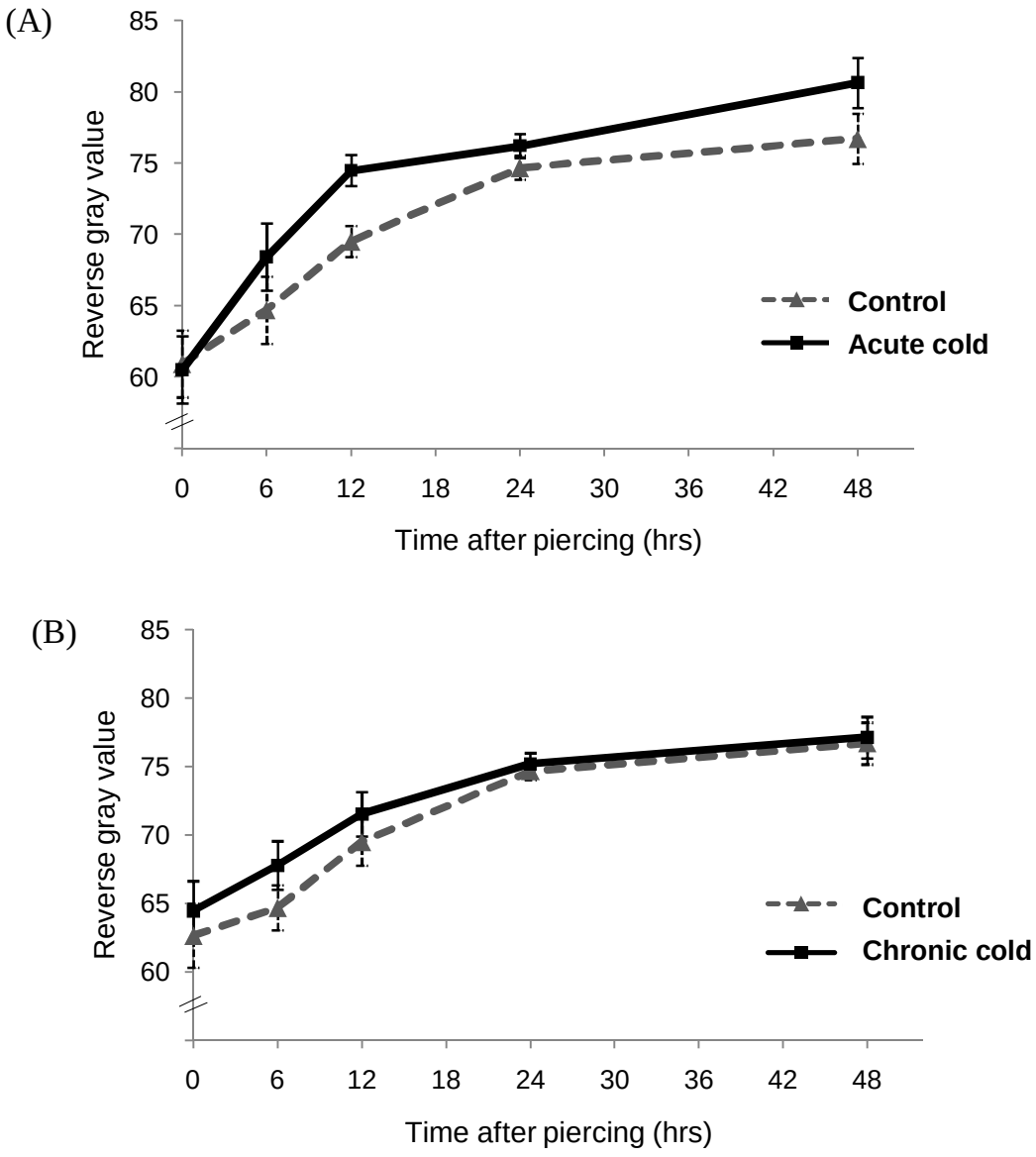

Figure 3.4 Changes in melanization of cold-exposed and control *Drosophila melanogaster* flies over a period of 48h. A) Melanization in acute cold-exposed (2h, -2 °C) flies (solid line) compared to control (dashed line) over 48 h. B) Effect of chronic cold (10h, -0.5 °C) on melanization of flies. Darkness of the pierced area is an indicator of melanization that is represented as reverse gray value. Data for each time point are presented as mean ± SEM, n=10.

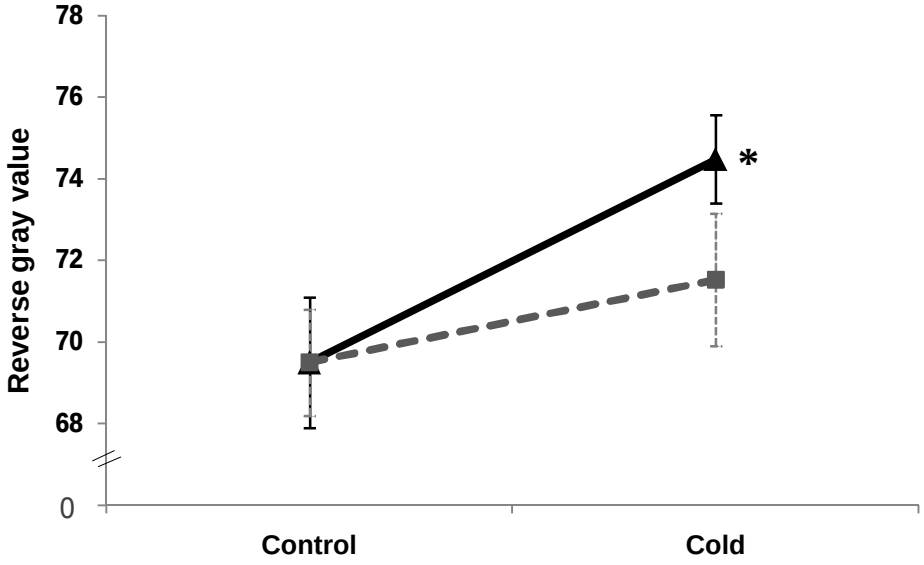

Figure 3.5 Wound-induced melanization in acute and chronic cold-exposed *Drosophila melanogaster* females compared to untreated females after 12 hours from piercing. Each point represents reverse gray value of the flies' pierced area after 12 hours from piercing. Black solid line indicates acute cold (2h, -2 °C) and gray dashed line indicates chronic cold exposure (10h, -0.5 °C). Two-way ANOVA was used to detect significant changes between treatments. Asterisks indicate the sigificant effect of cold on darkening, $P<0.05$. Data for each time point indicates mean ± SEM, n=10.

Table 3.1 Statistical analyses of the effect of cold on CHC, PO activity and melanization on *Drosophila melanogaster* females. The table shows the effect of cold (cold-exposed vs. control flies) and the duration of cold (acute vs. chronic cold exposure) on the CHC and melanization after 12 h (two-way ANOVA), and phenoloxidase activity (two-way ANCOVA). Asterisks indicate the statistically significant differences in results (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$).

Factor	Degrees of freedom	F	*P*-value
Circulating hemocyte concentration			
Cold	1	10.555	0.003**
Duration	1	2.508	0.122
Cold×Duration	1	4.520	0.040*
Error	36		
Phenoloxidase (PO) activity			
Cold	1	0.49	0.827
Duration	1	3.707	0.062
Cold×Duration	1	2.756	0.106
Body mass (covariate)	1	0.476	0.495
Error	35		
Melanization			
Cold	1	6.028	0.025*
Duration	1	2.751	0.295
Cold×Duration	1	1.729	0.163
Error	36		

3.2 Humoral immune response

3.2.1 End products of Toll, IMD and JAK/STAT pathways

There was no difference in expression of *drosomycin, defensin* and *metchnikowin* after either acute or chronic cold exposure (Figure 3.6). Among the genes related to the IMD pathway (*attacin*, *cecropin*, *diptericin* and *drosocin)*, only the mRNA abundance of *diptericin* was significantly higher in acute cold exposed rather than control flies (Figure 3.5; Table 3.2). Abundance of *TotA* transcripts increased significantly after both acute and chronic cold exposure whereas the expression level of *vir-1* did not change after either acute or chronic cold (Table 3.2). The only difference in expression of AMP genes between acute and chronic cold exposure was observed in *metchnikowin* (t_4= 8.536, P<0.05) that showed a higher expression after acute compared to chronic cold exposure.

Table 3.2 Statistical analyses of the effect of acute and chronic 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 after chronic cold exposure (10h, -0.5 °C). Asterisks represent a significant change in mRNA abundance of cold-exposed compared to control flies in *t*-test (* $P<0.05$, ** $P<0.01$, *** $P<0.001$), n=5.

Gene	Treatment	Test value	*P*-value
attacin-B	Acute	2.56	0.063
	Chronic	2.65	0.057
cecropin-A	Acute	2.11	0.102
	Chronic	2.64	0.058
defensin	Acute	2.35	0.078
	Chronic	0.46	0.67
diptericin-A	Acute	7.10	$P<0.01$**
	Chronic	2.58	0.061
drosocin	Acute	2.66	0.056
	Chronic	1.66	0.172
drosomycin-B	Acute	0.41	0.701
	Chronic	2.42	0.072
metchnikowin	Acute	2.66	0.056
	Chronic	0.33	0.756
PGRP-LB	Acute	2.47	0.069
	Chronic	2.47	0.070
PGRP-LC	Acute	2.60	0.060
	Chronic	1.69	0.166
PGRP-SB	Acute	2.26	0.086
	Chronic	2.59	0.060
PGRP-SD	Acute	1.46	0.22
	Chronic	0.32	0.76
relish	Acute	2.44	0.071
	Chronic	2.57	0.061
toll	Acute	1.05	0.352
	Chronic	1.45	0.220
TotA-1	Acute	3.43	0.026 *
	Chronic	11.95	$P<0.001$***
vir-1	Acute	2.40	0.074
	Chronic	0.06	0.956

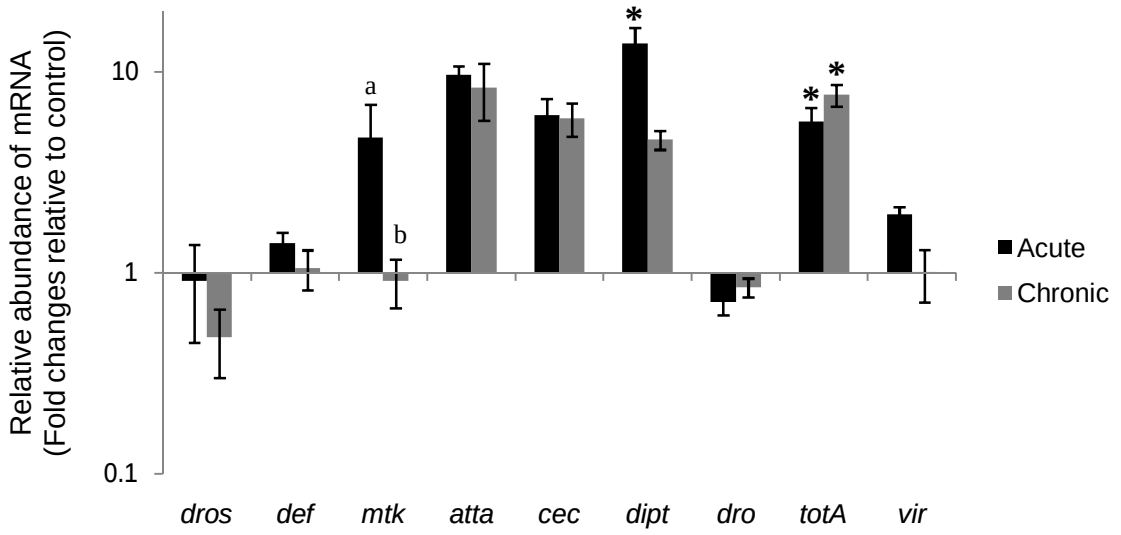

Figure 3.6 The effect of acute (2 h, -2 °C) and chronic (10 h, -0.5 °C) cold exposure on relative mRNA abundance of genes related to *Drosophila melanogaster* immune pathways measured by real-time qPCR. Black bars and gray bars represent the relative abundance of the immune genes mRNA (normalized to Rpl-32) after acute cold exposure and after chronic cold exposure, respectively. Error bars show the standard error from five biological replicates. Asterisks are indicators of a significant change in mRNA abundance of cold-exposed compared to control flies in t-test. Different lower case letters show a significant difference in relative mRNA abundance between acute and chronic cold-exposed flies.*dros=drosomycin, def= defensin, mtk= metchnikowin, atta= attacin, cec= cecropin, dipt= diptericin, dro= drosocin, TotA= turandot A and vir= vir-1.*

3.2.2 Receptors and alternate proteins of Toll, IMD and JAK/STAT pathways

Neither the relative mRNA abundance of the genes related to the Toll pathway (including *PGRP-SB, PGRP-SD* and *toll*) nor the ones related to the IMD pathway (*PGRP-LB, PGRP-LC* and *relish*) were changed compared to control after acute or chronic cold exposure. Moreover, neither acute nor chronic cold exposure significantly affected the expression level of any of the genes related to receptors or alternate proteins of AMP pathways (Figure 3.7).

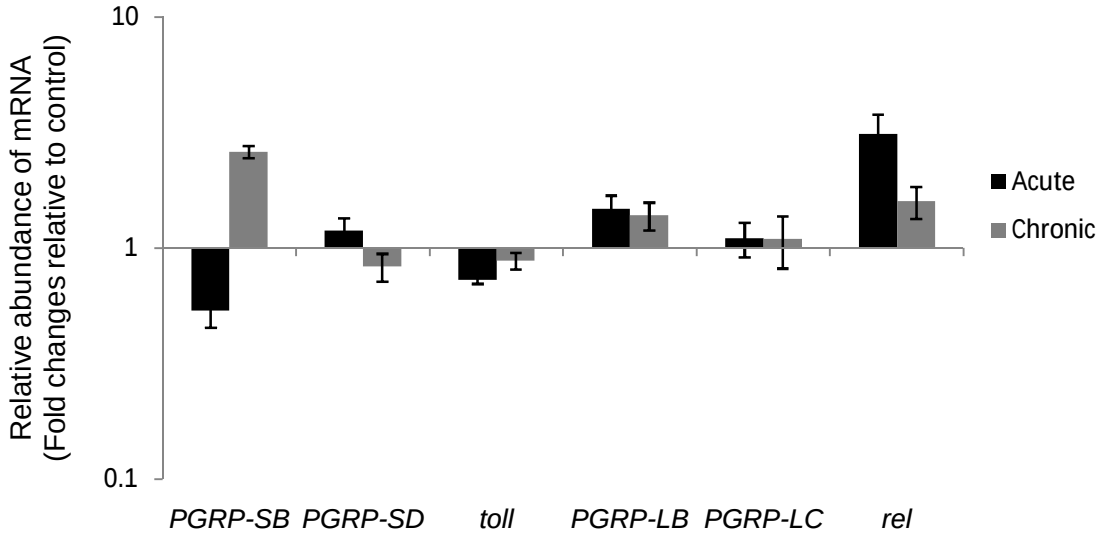

Figure 3.7 The effect of acute and chronic cold exposure on relative mRNA abundance of genes related to receptors or alternate proteins of *Drosophila melanogaster* immune pathways measured by real-time qPCR. Black and grey bars are representative of fold changes in expression of genes relative to control (normalized to *Rpl-32*) after acute (2h, -2 °C) and chronic (10h, -0.5 °C) cold exposure, respectively. Error bars show the standard error from five biological replicates. *rel=relish*.

Chapter 4

4 Discussion

Overwintering insects are exposed to multiple environmental stresses that can occur simultaneously and might affect one another positively or negatively (Holmstrup et al., 2008; Sinclair et al., 2013). There is evidence of an increase in immune activity after cold exposure in insects, suggesting an interaction between cold and immune responses (Le Bourg et al., 2009; Marshall and Sinclair, 2011; Xu and James, 2012; Zhang et al., 2011). The overall objective of my thesis was to investigate whether cold exposure can activate the insect immune response in the absence of pathogens, and whether different kinds of cold exposure could affect the immune response differently.

I found that low temperature affected some components of the immune system of *D. melanogaster*. Cold exposure in general enhanced the melanization response, and activated the IMD and JAK/STAT pathways. Acute cold exposure increased hemocyte concentration and wound-induced melanization, while PO activity of acute cold-exposed flies did not change. Chronic cold did not change the CHC, PO activity or melanization in *D. melanogaster* flies. Acute cold did not affect the Toll and JAK/STAT pathways but activated the IMD pathway. By contrast, chronic cold up-regulated the JAK/STAT pathway but did not lead to any effect on Toll and IMD pathways. I suggest that low temperature could induce activation of some of the *D. melanogaster* immune components. However different kinds of cold do not affect the immune system similarly and the impact of acute cold on the immune response is different from chronic cold exposure.

4.1 Impact of cold on the *Drosophila melanogaster* immune response

4.1.1 Melanization pathways

Acute cold exposure increased the CHC and wounding response significantly but did not change PO activity. By contrast, chronic cold exposure did not significantly change CHC, PO activity or the wounding response. Therefore, it appears that acute but not chronic cold exposure activates the melanization response which implies that the potential different physiological mechanisms induced by acute and chronic cold exposure could affect the melanization differently.

An increase in CHC after acute cold exposure could raise melanization in *D. melanogaster* because an increase in the number of circulating hemocytes may translate to an increase in the source of melanin (Fellowes and Godfray, 2000; Lanot at al., 2001; Wilson et al., 2002). Parasitoid attack or pathogen infection leads to an increase in the number of hemocytes (Elsin and prevost, 1995; González-Santoyo and Córdoba-Aguilar, 2012; Zibaee et al., 2011). In my study, CHC increase could explain the increase in melanization of acute cold-exposed flies, which is consistent with the hypothesis that cold activates the melanization in *D. melanogaster*. Therefore, if an increase in CHC is due to chilling injury induced by cold exposure, I suggest that there is a synergistic interaction between the acute cold response and melanization response.

The increase in CHC after acute cold could be due to cell damage induced by cold. Acute cold exposure can cause disruption in cell membrane and as a result cell damage in insects (Lee and Denlinger, 2010). Cell lysis triggers secretion of Plasmatocyte spreading peptides (PSPs) in hemolymph and, as a result, increases the hemocyte concentration

(González-Santoyo and Córdoba-Aguilar, 2012; Moreau et al., 2003). In adult flies that do not possess hematopoetic organs, the CHC increase after acute cold exposure could be a result of hemocyte mitosis (Holz et al., 2003; Lanot et al., 2001). A possible reason for CHC increase after acute but not chronic cold exposure could be different kinds of injury caused by acute and chronic cold. Cell membrane disruption caused by acute cold exposure might activate a response similar to cell lysis and act as a trigger for CHC increase. Chronic cold leads to chilling injury by imposing ion imbalance (not similar to cell lysis) in cold-exposed cells (Kostal, 2006; Lee and Denlinger, 2010; MacMillan and Sinclair, 2011a; MacMillan and Sinclair, 2011b) that is less likely to initiate CHC increase.

Although the decrease in PO activity after cold exposure was not significant, it could indicate a trade-off between cold response and immunity (Fedorka et al., 2004; Rantala and Roff, 2005). Exposure to cold could possibly lead to degradation of PO or could negatively affect PO transcription. Trade-offs can also occur between two immune components, for example, activation of encapsulation leads to decreased levels of antibacterial proteins (lysozymes) (Ardia et al., 2012). In my study, a shift of resource allocation towards activation of other immune components (e.g. JAK/STAT pathway) could lead to a decrease in PO activity. Another possible explanation for the observed decrease in PO activity after cold exposure might be the time elapsed after cold-induction of PO activity. Optimal PO activity occurs a few hours after induction of the PO activity (Catalan et al., 2012; Gillespie et al., 1997). The long cold exposure and recovery time may provide enough time for proteases to rebalance the hemolymph PO level and accumulate during cold exposure and recovery time, leading to PO degradation to a level

lower than control flies. I suggest exploring PO activity in flies after shorter time points from the beginning of cold exposure. I predict that the peak of PO activity might occur earlier than the time that I detected it.

4.1.2 Toll, IMD and JAK/STAT pathways

Expression of *drosomycin,* an antimicrobial gene at the end of the Toll pathway or the genes up-regulated by activation of both Toll and IMD pathways (*defensin* and *metchnikowin*) did not change after either acute or chronic cold exposure. From the antimicrobial genes related to the IMD pathway, only *diptericin* was up-regulated significantly after acute cold exposure. Since diptericin acts against Gram-negative bacteria, expression of *diptericin* after acute cold could result in enhancement of the *D. melanogaster* immune system against Gram-negative bacteria.

Although only *diptericin*, among all the detected genes, was up-regulated significantly, the changes in expression of some of the other genes are close to significant (Table 3.2): *attacin*, *drosocin* and *metchnikowin* after acute cold, and the IMD pathway genes *attacin*, *cecropin*, and *diptericin* after chronic cold. The near-significant up-regulation of the IMD pathway genes could support the effect of cold on activation of the IMD pathway and consequently enhancement of the cold-exposed insects' response to Gram-negative bacteria and viruses. By increasing the sample size, I could possibly observe significant cold-induced up-regulation of more immune genes.

In my study, the increase in expression of *diptericin* that I observed after acute cold exposure occurred in the flies that were not pathogen-infected. Activation of the immune system in the absence of pathogens indicated that immune response is activated not in

response to pathogens but rather by cold as a stressor. Up-regulation of AMP genes induced by stress in the absence of an immune challenge has been reported in some studies (Overend et al., 2012; Zhang et al., 2011; Zhao et al., 2010). For example, in *D. melanogaster*, cold exposure and hyperoxia (high levels of environmental oxygen) lead to up-regulation of AMP genes even in the absence of pathogens (Zhang et al., 2011; Zhao et al., 2010). To investigate if cold exposure can make the immune pathways more sensitive to immune challenge, I measured expression of receptors and alternate proteins of each pathway (*PGRP-LB, PGRP-LC* and *relish* related to IMD pathway, and *PGRP-SB, PGRP-SD* and *toll* related to Toll pathway). Expression of none of these genes differed statistically between cold-exposed and control flies. Therefore, it appears that the observed cold activation of IMD and JAK/STAT pathways are not related to expression level of associated receptors and alternate proteins. However, near-significant increase in gene expression of some of the receptors and alternate proteins (table 3.2) necessitates more profound studies on cold-induced expression of these genes and exploring changes in the level of these proteins after cold exposure. To determine the up-regulation of promoters of the immune pathways in response to cold, I could further explore which of the proteins and enzymes involving in immune pathways (including transcription factors and protein kinases) are affected by cold. For example I could block the receptors or alternate proteins of each pathway using pharmacological inhibitors, and determine if the pathway can still be activated by cold (Engstrom, 1998). If blocking a protein leads to a loss of immune response to cold, the tested protein or the ones upstream of that would be the point of cold effect on the immune pathway and as a result the cross talk among cold and immune responses. Moreover, I could explore whether activity of any of the enzymes

affecting an immune pathway (e.g. protein kinases) is changed after cold and consequently accounts for a cold-immune responses cross-talk.

Activation of the IMD pathway after acute cold exposure could be due to a cross-talk between IMD pathway and the cold tolerance pathways. Studies on *Eurosta solidaginis* and *D. melanogaster* indicate that cold-induced increase in calcium enhances cold tolerance (Takeuchi et al., 2009; Teets et al., 2013) and raises the level of calcineurin (a calcium-dependent phosphatase enzyme) which in turn contributes to immune responses and activation of AMPs (Davies and Dow, 2008; Dijkers and O'Farrell, 2007). To detect the potential role of calcium in cold-activation of immune response, the first step is to induce an increase in concentration of calcium in the absence of pathogens and explore if the IMD pathway response is similar to that observed after cold exposure. If calcium could activate the IMD pathway in the same manner as that observed in my study, the next step is to investigate how calcium could activate the IMD pathway. For example, I could inject pharmacological inhibitors of calcineurin (e.g. cyclosporin; Dijkers and O'Farrell; 2007) and then explore the effect of cold on up-regulation of AMPs. If inhibition of calcineurin leads to decrease in cold-activation of the AMPs that were up-regulated in my study, it would suggest that calcineurin mediates cross-talk between cold and immune responses.

Expression of *TotA* is used as an indicator of JAK/STAT pathway activation (Pham and Schneider, 2008). In my study, *vir-1* that is involved in antiviral responses did not change in expression after acute or chronic cold exposure. The other end product of the JAK/STAT pathway, *TotA,* was up-regulated in response to acute and chronic cold exposure. *TotA* is up-regulated in response to many stressors including cold (Lemaitre

and Hoffmann, 2007; Zhang et al., 2011), which makes it difficult to explain if up-regulation of *TotA* in response to cold is caused by cold-activation of the immune response or it is part of a general stress response. To investigate whether activation of JAK/STAT pathway is a specific response to cold exposure and not a general response to stress, I could also measure the up-regulation of the pathway after exposure to other stressors (e.g. starvation or desiccation). If *TotA* is also up-regulated in the flies that are exposed to stresses other than cold, I could suggest that activation of JAK/STAT pathway after cold exposure is due to a general response to stresses. I would not reject the potential cross-talk between cold and immune responses. However, I would suggest that the mechanism underlying the cold-activation of immune response is not cold specific and cross talk occurs due to a general response to environmental stress.

4.2 The interaction between cold and immune responses

Based on my findings, I conclude that low temperature activates some of the immune components of *D. melanogaster*, and that acute and chronic cold injury affected the immune system differently. I did not observe any trade-off between cold response and activation of Toll, IMD and JAK/STAT pathway. Thus, I conclude that cold exposure activates the *D. melanogaster* immune system. Acute cold exposure increased activation of melanization and IMD pathway and both acute and chronic cold exposure led to activation of JAK/STAT pathway.

Cross-talk between activation of cold and immune responses in *D. melanogaster* happens when a physiological response to cold leads to activation of immune response in flies. Non-adaptive or adaptive mechanisms can be responsible for the potential cross-talk between cold and immune responses (Sinclair et al., 2013). I suggest four main

hypotheses for a cross-talk or cross-tolerance between cold and immune responses concerning the potential role of cold induced increases in calcium concentration, cell damage, release of gut symbionts in the hemoceol and expression of heat-shock genes on activation of immune system.

First, increases in calcium and consequently calcineurin can enhance both cold tolerance and activation of IMD pathway (Dijkers and O'Farrel, 2007). If cold exposure increases the concentration of calcium, then the resulting activation of both cold and immune responses could underlie a cross-talk between protection responses to these two stressors (Takeuchi et al., 2009; Teets et al., 2013).

Second, cold activation of the immune response could be due to a general response to stresses (Sinclair et al., 2013). For example, physical stressors such as shaking of *Galleria mellonella* and sterile wounding of *Bombus terrestris* larvae induce up-regulation of AMPs and hemocyte increase (Erler et al., 2011; Mowlds et al., 2008). I suggest that acute cold-induced cell damage could lead to a general stress response which in turn activates the immune system. However, this hypothesis should be investigated by identification of the underlying mechanisms of cell damage induced by cold exposure, and by further exploring the activation of immune system in response to artificial induction of the same kinds of cell damage. Specifically, the difference in the cell damage posed by acute and chronic cold exposure should be determined to clarify the difference in activation of immune system after these two kinds of cold exposure. If the precise mechanism of cold-induced damage in the insect cell membrane is recognized, then it might be possible to induce the same damage to the flies' cell membrane (e.g. using chemicals that induce the same injury in cell membrane) and detect the potential

changes in the insect immune response. Activation of the immune system in response to the induced injury could support a potential synergistic interaction between cold and immune responses.

My third hypothesis for the mechanisms underlying a synergistic interaction between cold and immune responses is entrance of the insect gut symbionts to the hemoceol due to cold-induced gut damage. Exposure to cold could cause damage to cells of gut and Malpighian tubes (MacMillan and Sinclair, 2011; Marshall and Sinclair, 2011) leading to the entrance of gut flora to the hemoceol (MacMillan and Sinclair, 2011). The gut symbionts would be recognized as foreign elements, activating the immune system and leading to a cross-talk between cold and immune responses. In my study, up-regulation of melanization could be due to the gut cell damage imposed by acute cold exposure and the subsequent release of gut flora into the hemocoel. The symbionts that are escaping from the gut may match the pathogens targeted by more specific pathways (e.g. IMD pathway) and activate the pathways. I suggest detecting release of the symbionts into the *D. melanogaster* hemocoel after cold exposure and then selectively remove specific symbionts to determine which microbes have activated the immune system. Physical induction of gut damage can also show if it could be a reason for cold-activation of immune system (Laura V. Ferguson, pers. comm.).

My fourth hypothesis for the mechanisms leading to synergistic interaction between cold and immune responses is that heat shock proteins might be responsible for a cross-tolerance between cold and immune responses. The increase in expression of heat shock proteins (that appear to be responsive to multiple stresses) happens after both cold exposure and immune challenge (Guedes et al., 2005; Qin et al., 2005; Zhang et al., 2011;

Zhao and Jones, 2012). Beside the important role of heat-shock proteins in thermo-tolerance of insects, different studies show the association of these proteins with enhancement of the insect immune response (Zhao and Jones, 2012). For example, a study on honeycomb larvae, *Galleria mellonella*, showed an increase in immune response in heat-shocked larvae. The authors suggested that heat-shock proteins might be a shared point in the converging heat-shock and immune responses (Wojda and Jakubowicz, 2007). Moreover, the flies exposed to heat shock accompanied with further cold exposure, indicated an increased tolerance to low temperature (Rajamohan and Sinclair, 2008). To investigate whether expression of heat-shock proteins activates the immune response, I could use mutant flies unable of expressing the heat-shock proteins (specifically the ones that are expressed after cold exposure). If cold exposure induced immune response in normal flies but not in the mutant ones, it would suggest that heat-shock proteins account for cold-activation of the immune system in flies.

Activation of melanization and up-regulation of the IMD pathway in response to acute cold exposure suggests that immunity of *D. melanogaster* adults should be enhanced in response to pathogens. Because my study showed an increase in expression levels of *diptericin,* which acts against gram negative bacteria, I predict that cold-exposed flies might better survive infection with gram negative bacteria. Moreover, increases in melanization response after cold exposure in my study leads to the prediction that cold-exposed flies would be less susceptible to parasitoid attack. CHC increase after cold may lead to enhancement of encapsulation in cold-exposed flies and consequently better survival from infection with large pathogens (e.g. fungi).

Finally, cold-activation of the insect immune system should be studied in field situation as well. Although I included two kinds of cold exposure in my studies, the fluctuating temperatures in natural environment of overwintering insects are not necessarily the same as that in my experiments (Irwin and Lee, 2003; Stenseth and Mysterud, 2005). Different patterns of fluctuating temperature may lead to activation of physiological mechanisms different to those that occurred in my study.

4.3 Conclusions

Based on my results, cold does activate the immune system of adult female *D. melanogaster* even in the absence of pathogens, and duration and intensity of cold exposure can affect the cold-activation of immune system differently. Acute cold exposure imposes a stronger impact on the *D. melanogaster* immune response. Melanization and encapsulation are stimulated in acute cold-exposed flies by an increase in the concentration of hemocytes. Higher hemocyte concentration could potentially underlie the increased formation of melanin (the end product of melanization) that was observed in acute cold-exposed flies compared to controls. Chronic cold exposure does not affect the hemocyte concentration and consequently its effect on the final step of the melanization process is not significant. In addition, the up-regulation of the IMD pathway that happened after acute cold exposure did not occur after chronic cold exposure.

Regarding the increase in melanization and up-regulation of the IMD pathway that I observed after cold exposure, I suggest that cross-talk or cross-tolerance may exist between cold and immune responses. Studies at the cellular and molecular level should be performed to determine the mechanisms underlying the cold or immune response to be able to determine the mechanistic links between cold and immune responses.

In conclusion, I predict that overwintering insects can enhance their immunity as a result of cold exposure, which could possibly lead to gaining tolerance to two stressors with less energy cost and a better fitness of the insect in its environment. However, changes in the intensity and duration of cold experience would result in different interactions between cold and immune responses. Therefore, to make predictions about performance of immune system in overwintering insects, the type of cold that the insect is exposed to and the potential induced chilling injury should be considered.

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