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A microarray study on gene expression of Drosophila melanogaster 'm response to multiple cold exposures

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A microarray study on gene expression of *Drosophila melanogaster* in response to multiple cold exposures

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ABSTRACT

In the field, insects suffer multiple cold exposures during winter. When exposed to repeated low temperatures, *Drosophila melanogaster* females show an increase in survival, but a reduction in reproduction. In this study, microarrays were used to analyze the gene expression of female *D. melanogaster* after multiple, single sustained (or single prolonged) and single short cold treatments, which exposed the flies at 0 °C for repeated 2 h, single 10 h and single 2 h respectively. Candidate genes involved in the 6 h recovery from different types of cold exposures were identified. After repeated cold exposures, candidate genes particularly included those involved in muscle protein and muscle activity. Stress-related genes, *Turandot A*, *Turandot C*, and *Turandot M* were up-regulated in response to multiple cold exposures, and improved the cold survival in female *D. melanogaster*. This work also suggested a strong relationship between cold exposure and the immune system. I suggest that in fruit flies, chilling injuries after cold exposure may induce immune responses and contribute to recovery from cold.

Keywords:

Multiple cold exposures, microarray, cold recovery, *Drosophila melanogaster*, chilling injury, muscle proteins, stress proteins, immune response, insect cold tolerance, quantitative real time PCR

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RCH: rapid cold-hardening

Hsp: Heat shock proteins

qRT-PCR: quantitative real-time PCR

DAVID: the Database for Annotation, Visualization, and Integrated Discovery

GO: Gene ontology

CHAPTER 1: INTRODUCTION

Low temperature can be a major factor influencing insect development, reproduction, fitness and population dynamics (Simon et al., 1993; Bale & Hayward, 2010). Because insect body temperature closely tracks environmental temperature, there is a higher probability if insects freezing during cold spells (Thomas & Blanford, 2003). Hence overwintering insects have developed a broad range of responses to extremely low temperature (Chown & Nicolson, 2004). Insects have evolved two main physiological strategies to survive at low temperatures. *Freeze-tolerant* insects can survive the formation of ice in their body tissues. By contrast, *freeze-avoiding* insects can supercool their body fluids below their melting point without crystallization, avoiding ice formation in their body tissues but die once they freeze (Denlinger & Lee, 2010). However, most insect species are *chill-susceptible*: they are unable to survive long exposures at low temperatures (Storey & Storey, 1988; Bale, 2002). This pre-freeze mortality is not associated with ice formation, but rather due to chilling injuries at low temperatures (Czajka & Lee, 1990).

1.1 *The physiology of chilling injury*

Chilling injury results from accumulated damage at low temperatures and is unrelated to freezing (Lee *et al.*, 1987). Accumulated chilling injury can be induced by relatively mild temperatures and is potentially lethal for chill-susceptible species (Denlinger & Lee, 2010). For instance, *Drosophila melanogaster* (Diptera: Drosophilidae) adults and larvae are chill-susceptible: they suffer extensive mortality after a 2 h exposure at -5 °C, but freezing does not occur until -20 °C (Czajka & Lee,

1990). Usually chilling injury is divided into chronic and acute chilling effects (Sinclair & Roberts, 2005). Prolonged cold exposure or cold acclimation may induce chronic chilling injury as a result of the mismatching of metabolic pathways, membrane phospholipid composition and loss of ion homeostasis (Koštál *et al.*, 2004; Koštál *et al.*, 2006; Colinet *et al.*, 2007). Acute chilling injuries are mainly thought to be associated with membrane phase transitions (Drobnis *et al.*, 1993; Kelty & Lee, 1999; Overgaard *et al.*, 2006). The most likely target of chilling injury in insects is the muscle tissue. For example, at low temperatures, *D. melanogaster* and the honey bee *Apis mellifera* lose the resting potential in flight muscle (Hosler *et al.*, 2000) and injury in flight muscle after cold exposure has been shown to lead to apoptosis in cells (Yi *et al.*, 2007).

1.2 *Protective mechanisms against chilling injuries*

Insects have evolved adaptations to prevent chilling injuries. The synthesis of cryoprotective molecules such as glycerol, sugars and amino acids, as well as increased proportions of unsaturated phospholipid fatty acids, are believed to maintain the liquid phase of cell membranes at low temperatures, therefore increasing cold tolerance (Ohtsu *et al.*, 1998; Bale, 2002; Lee *et al.*, 2006; Clark & Worland, 2008). Stress proteins may also protect insects at low temperatures. One important set of stress proteins includes the heat shock protein families. Heat shock proteins (Hsps) are molecular chaperones that assist to re-fold or degrade denatured proteins in cells (Parsell & Lindquist, 1993). The expression of Hsps can be induced by many environmental stresses, including heat shock, cold shock, infection and desiccation stresses (Joplin *et al.*, 1990; Pockley, 2003; Hayward *et al.*, 2004), and the expression

of Hsps improves cold tolerance in many insect species. For example, after a mild heat treatment, rapid synthesis of Hsps increases the cold survival of *D. melanogaster* lavae (Burton *et al.*, 1988). When pre-diapuase larvae of *Sarcophaga crassipalpis* were injected with the double-strand RNAs of genes, the down-regulation of mRNA transcripts caused a decrease in cold tolerance during diapause (Rinehart *et al.*, 2007). Recently, an RNAi study showed that *Hsp70* may be involved in repairing chilling injury in adult bug *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) after a -5 °C cold treatment for 5 days (Koštál & Tollarová-Borovanská, 2009).

The rapid cold-hardening (RCH) response is a plastic mechanism that protects insects against chilling injury in the short term (Lee *et al.*, 1987; Czajka & Lee, 1990). The RCH response occurs in insects when they rapidly enhance their cold tolerance after a brief acute cold exposure at temperatures above the level at which their body fluids freeze. For example, a pre-cold treatment at 5°C for 30 min increased the survival rate of *D. melanogaster* at -5 °C for 2 h (Czajka & Lee, 1990). Although RCH can improve the survival of chill-susceptible insects in the short term, it may cause energetic trade-offs such as a decrease in reproductive output (Overgaard *et al.*, 2007). Also, RCH may block cold-induced cell death: the level of caspase-like protein (an initiator of apoptosis) was increased after RCH (a prior exposure to 5 °C for 2 h followed by a exposure to -5 °C or -7 °C for 2 h) and the cell survival in *D. melanogaster* was improved (Yi *et al.*, 2007).

The mechanisms underlying RCH are poorly understood. Membrane changes, ATP generation, and in particular, cold-sensing pathways have been all hypothesized to be the mechanisms underlying the RCH responses (Denlinger & Lee, 2010).

Increasing cryoprotectants in response to RCH may contribute to stabilizing membranes at low temperatures (Lee *et al.*, 1987). In *S. crassipalpis*, RCH caused an accumulation of glycerol and increases the proportion of unsaturated fatty acids to saturated fatty acids (Lee *et al.*, 1987). Glucose concentration was also increased in *D. melanogaster* after a 4 h cooling period from 25 °C to 0 °C (Overgaard *et al.*, 2007). However, MacMillan et al. (2009) argued that 2 h cold exposure at 0 °C resulted in RCH in *D. melanogaster*, but did not decrease free glucose concentration in cell membranes, suggesting that the RCH was not associated with protection by carbohydrate cryoprotectants. Although long-term cold acclimation increases the expression of Hsp70 protein, there does not seem to be an increase in Hsp70 protein with RCH, and *Hsp70* transcripts only increased during recovery, but not during the cold exposure period in *D. melanogaster* (Nielsen *et al.*, 2005; Sinclair *et al.*, 2007).

1.3 *Changes in gene expression of insects in response to low temperatures*

Insects alter their gene transcription profiles in response to low temperatures (Clark & Worland, 2008). Although phenotypic acclimation or RCH response in insects can be reflected in the regulation of gene expression, few candidate genes have been identified in response to cold stresses (Clark & Worland, 2008). The mRNA abundance of five putative cold-related genes (*Frost*, *Hsp70*, *Hsp23*, *Desat2*, and *Smp-30*) was examined by Sinclair et al. (2007), and they found that *D. melanogaster* did not change the mRNA abundance of any of these five genes during exposure to cold, but the expression of *Frost* and *Hsp70* was upregulated during the recovery from cold exposure (Sinclair *et al.*, 2007). The authors suggested that changes in gene expression during the cold recovery period are essential to the insect

cold response. *Frost* was first discovered by Goto (2001), who found that the expression of this gene increased in *D. melanogaster* during the 2 h recovery from cold shock at 0 °C for 2 h (Goto, 2001). Characterization of *Frost* indicated that it might encode a mucin-like protein, which would be secreted into the extracellular space (Goto, 2001; Colinet *et al.*, 2010). Knockdown of *Frost* increased the mortality in *Drosophila* species after a 12 h cold treatment at 0 °C, followed by 24 h recovery at 25 °C (Colinet *et al.*, 2010).

Studies of global gene expression by insects in response to cold exposure have been limited. Qin et al. (2005) found that *D. melanogaster* increased the expression of 5 stress protein-related genes (including *Frost* and heat shock protein genes), as well as 12 membrane protein genes, when they exposed the flies to 0 °C for 2 h prior to recovery at 25 °C for 30 min (Qin *et al.*, 2005). A microarray study on the cold-adapted *Drosophila subobscura* identified differentially regulated genes that were mainly involved in carbohydrate and nucleic acid metabolism (Laayouni *et al.*, 2007). Laayouni et al. (2007) also found that *Frost*, *Hsp26*, *Hsp68* and *Turandot A* (*TotA*) were up-regulated in these cold-adapted flies. The gene expression profiles of female *D. melanogaster* from artificially selected chill coma resistant lines (first 10 % of adult insects to recover from a reversible state where motor activity was lost at low but non-lethal temperatures), and found 50 up-regulated and 44 down-regulated genes. The functions of candidate genes from this work included proteolysis, electron transport and the immune response (Telonis-Scott *et al.*, 2009).

1.4 *The immune response and cold stress*

Like other invertebrates, insects have developed a variety of defensive mechanisms to fight infection by viruses, bacteria, fungi and parasites (Hoffmann, 2003). Insects must only rely on innate immunity to against pathogens (Wilson *et al.*, 2001), of which there are two major divisions in insect innate immune response: the cell-mediated reaction is the response from insect immune cells (hemocytes), including phagocytosis, melanization and encapsulation of invading microorganisms; and the humoral reactions which result in several rapidly activated enzyme cascades, including rapid synthesis of antimicrobial peptides against pathogens (Lamberty *et al.*, 2001). When presented with a microbial challenge, the immune response is activated by receptors that recognize microbial surface peptidoglycans and challenge-induced antimicrobial peptides are rapidly produced and secreted from the fat bodies into the hemolymph (Leulier *et al.*, 2003). To kill invading microorganisms, antimicrobial peptides target invading cell membranes, bind peptides to the membranes that increase the membrane permeability (Zasloff, 2002). Different pathways regulate the expression of antimicrobial peptides. *Drosophila* species primarily utilizes the Toll pathway to resist infections by fungi and Gram-positive bacteria, and the Imd pathway for the response to Gram-negative bacterial infections (Hoffmann & Reichhart, 2002). A Janus Kinase (JAK)-dependent activation of signal transducer and activator of transcription (STAT) in the *Drosophila* fat body is also involved in the response to immune challenges and in response to septic injury (Agaisse & Perrimon, 2004).

Temperature influences the ability to defend against infection in many organisms (Padgett & Glaser, 2003; Thomas & Blanford, 2003), and there have been several studies indicating a potential link between the physiological systems involved in cold tolerance and immune function in insects. For example, cold treatment at 0 °C increased survival after fungal infection in *D. melanogaster* (Le Bourg *et al.*, 2009). In another study, exposure to 17 °C enhanced survival after infection compared with 25 °C and 29 °C, and infected flies held at cooler temperatures showed a greater up-regulation of immune genes than those held at warmer temperatures (Linder *et al.*, 2008). Stress responses such as the cold exposures described here result in the synthesis of stress proteins, which may modulate the cellular immune response and enhance elimination of invading parasites and pathogens (Moseley, 2000; Ekengren *et al.*, 2001).

1.5 *Repeated cold exposures and insect cold tolerance*

In the field, insects are generally exposed to regular and repeated cold stresses, rather than a single cold event (Sinclair, 2001). Insects exhibit different phenotypes in response to multiple cold exposures compared with single sustained (or single prolonged) cold exposures. For instance, feeding was diminished in freeze-tolerant larvae of the sub-Antarctic caterpillar, *Pringleophaga marioni* after five repeated cold cycles (Sinclair & Chown, 2005). Another freeze-tolerant species, the sub-Antarctic beetle *Hydromedion sparsutum*, showed improved survival after repeated freezing exposures (Bale *et al.*, 2001). Long-term cold exposure broken by repeated short warm temperature exposures is called a fluctuating thermal regime (FTR), and this warm temperature break is beneficial for chill-susceptible insects, enabling them to

survive cold exposure (Koštál *et al.*, 2007). Exposing the parasitic wasp *Aphidius colemani* to 4 °C interspersed with 2 h exposure to 20 °C every 24 h, caused a significant decrease in mortality compared with a constant 4 °C exposure (Colinet *et al.*, 2007). Chill-susceptible species, such as *D. melanogaster* and *D. simulans* can both maintain development at low temperatures with FTR, but cannot cope with constant cold exposures (Huey, 2001).

A recent study showed that whilst repeated cold exposures in *D. melanogaster* improved survival, this came at a cost in the flies (Marshall & Sinclair 2010). When flies were subjected to multiple cold exposures, they had lower immediate mortalities than flies subjected to either single or prolonged cold. However, the flies that were subjected to repeated cold exposures suffered a long-term negative fitness consequence: they had a lower intrinsic rate of population increase than the single cold treatment groups (Marshall & Sinclair, 2010). In addition, flies after repeated cold exposures had lower triglyceride content immediately after the second cold exposure. Flies that were repeatedly exposed to the cold also showed decreased glycogen stores in the long-term. It should be noted that the flies used in the cold treatments were collected from the field in a location that experienced regular subzero temperatures (London, Ontario, Canada). Therefore, in the natural environment, the sub-lethal effects of repeated cold exposures in *D. melanogaster* may reflect trade-offs between allocating energy to repair chilling injury (or preparation for the next exposure) and reproduction.

1.6 *Microarrays as a technique to analyze of global gene expression patterns*

Gene-expression microarrays allow the simultaneous quantitative analysis of the expression of tens of thousands of genes, even the entire transcriptome, under different biological conditions. These microarrays are manufactured using two main technologies: oligonucleotide arrays and cDNA arrays. Oligonucleotide microarrays place thousands of probes at many addressable spots on a slide (Lockhart *et al.*, 1996; Sohail & Southern, 2002). The probes are pairs of short oligonucleotides (15-70 bases) taken from either genomic or EST sequences. Experimental samples can be labeled with fluorescent dyes and hybridized to the probes. For the arrays produced by Affymetrix, oligonucleotides are directly synthesized through a process of photolithography. When hybridized with fluorescently labeled samples, the oligonucleotides probes bind complementary sequences from the spots on the array. Excess non-complementary sample is washed off and the array then consists of oligonucleotides with bound sample attached, the targets of which reflect the transcription profile of the whole animal, tissue or cell under study. Through the processes of scanning and image, the fluorescent label is excited via a laser and the amount of fluorescent emission can be detected, representing the relative amounts of the transcripts from samples bound to each complementary probe sequences. These are correlated to control probes of known concentration (Lockhart *et al.*, 1996; Sohail & Southern, 2002; McLachlan *et al.*, 2004; Allison *et al.*, 2006).

The cDNA microarray is different from oligonucleotides microarray. The cDNA microarray is generated from a library of full-length DNA clones. They use a two-color detection system with control and experimental material labeled with

different color fluorochromes. These are then hybridized onto one array slide together, and compete to bind to their complementary cDNA. Then the array slide is scanned with a laser to obtain the signal intensity from each color of dye. Three colors was result: red and green representing each of the labeled RNAs, if they are present and up-regulated and yellow, which means the target cDNA is present in both samples at a similar level. Again, the intensity of fluorescence is a measure of the relative copy number of a transcript present in the samples (Brown & Botstein, 1999; McLachlan *et al.*, 2004).

Both methodologies have their advantages and disadvantages. Oligonucleotides arrays require gene sequence information and are most frequently used for model species where the genome has been sequenced (e.g. *Drosophila*), whilst cDNA arrays are more often used for non-model species, which often have either no or very little gene information and hence have no a priori knowledge of sequence data. Compared with cDNA microarrays, oligonucleotide microarrays, with the shorter length of oligonucleotides probes, can be more accurate at detecting different regions within sequences of high similarity (McLachlan *et al.*, 2004). In addition, some oligonucleotide microarrays like the Affymetrix and NimbleGen microarrays use multiple probes at different locations to represent a single gene, therefore allowing non-specific binding of target genes to be measured (Bolstad *et al.*, 2003).

The purpose of analyzing gene expression changes in different experimental groups, or for different phenotypes, is to identify genes whose expression differs among the groups. These genes than become candidates that potentially determine the generation of specific proteins or regulate signal pathways, and differential

expression of candidate genes may lead to phenotypic differences. Although microarrays have made it possible to efficiently analyze transcription changes on a genome-wide scale, the use of microarrays is limited by the fact that cellular function in a living organism is mediated by protein quantity and function, and gene expression levels do not accurately predict the expression levels of protein: the correlation of mRNA abundance and protein production is variable (Anderson & Anderson, 1998; Gygi *et al.*, 1999).

Another issue is that lists of candidate genes arising from published microarray studies often do not overlap among similar studies. This is largely due to the limitations of which genes were originally on each array in the first place and also to variation in experimental conditions. This issue is exacerbated by incomplete data annotation and incomplete descriptions of the individual study's microarray processing and data analysis when the studies are published (Ioannidis *et al.*, 2009). A disadvantage of the microarray technology itself is that the microarray data become less reliable because of background noise when examining genes at low expression levels, a particular problem when examining natural populations, which have an inherently wide variability in gene expression. To improve the reliability of microarrays, general suggestions include performing independent biological replicates, and technical replicates, to account for manufacturing and hybridization defects on the array (Lee *et al.*, 2000; Piper *et al.*, 2002; Butte, 2002).

1.7 *Analysis of candidate gene functions*

After identifying candidate genes through microarray data analysis, the next step is to understand the biological functions of the candidate genes. To explore and

extract biological meaning from large gene list, genes are first annotated with any feature attached to the genomic sequence (Misra *et al.*, 2002). Ideally, the annotation of a gene is based directly on experimental studies, but can also be automatically predicted based on sequence features and sequence similarity to orthologous genes in other organisms where gene function has been experimentally determined (Ashburner *et al.*, 2000). The list of candidate genes identified from a microarray is usually large, and for this reason, a database and bioinformatics tool for the functional analysis of a large list of genes is required (Huang *et al.*, 2009). For *D. melanogaster*, FlyBase (http://flybase.org/) is the standard database providing traceable evidence of gene annotations and gene functions (Misra *et al.*, 2002).

Gene ontology (GO) (http://www. geneontology.org/) provides an additional resource to broadly classify the annotated candidates from microarrays (Ashburner *et al.*, 2000). GO is a set of terms indexing gene information. This index is built with a hierarchical relationship among the GO terms (Doniger *et al.*, 2003). Once the GO terms have been established for the candidate genes, GO allows the identification of all the gene products that are involved in a particular biological function (Ashburner *et al.*, 2000), and contains three categories of GO terms: biological process, referring to a biological objective of gene or gene product, such as "signal pathway" or "translation"; molecular function, referring to the biochemical activity of the gene product, such as "enzyme" or "transporter"; cellular component, referring to the active location of the gene product in the cell, such as "ribosome" or "nucleus" (Ashburner *et al.*, 2000). After the annotation of interested genes, statistically functional analysis highlights the most over-represented (enriched) GO terms, thus

the most relevant biological processes underlying biological phenomena can be identified (Huang *et al.*, 2009).

1.8 *Objectives*

Insects have successfully developed a range of physiological and molecular adaptations to low temperatures (Chown & Nicolson, 2004). The physiology of *D. melanogaster* is very tractable in laboratory and the availability of the whole annotated genome sequence makes it a good model system to study responses to low temperature at the molecular level. The phenotype of *D. melanogaster* after repeated cold exposures indicates that gene expression patterns might be different to the flies after sustained (or prolonged) and single short cold exposures (Marshall & Sinclair, 2010).

The objective of this study was to analyze changes of gene expression in the mechanisms underlying the recoveries from repeated cold exposures, a single sustained (or prolonged) cold exposure, and a single short cold exposure using microarrays. The cold treatments used were adapted from the experimental design of Marshall and Sinclair (2010). To identify candidate genes that were responsible for responses to 5-day repeated cold exposures, changes in gene expression were examined in virgin female *D. melanogaster* after a prolonged (6 h) recovery from the cold treatments. Identified candidate genes were validated by quantitative real-time PCR. Gene Ontology analysis was performed to identify the enriched functions of candidate genes that were differentially regulated in response to multiple cold exposures.

CHAPER 2: METHODS

2.1 *Study animals*

Wild *Drosophila melanogaster* (Diptera: Drosophilidae) were collected from the London, Ontario area in 2007 and mass-reared in 35 ml plastic vials in the lab on Tucson food (yeast : yellow cornmeal 1:1.8), at 22 °C, 50% RH, light : dark 12 h:12 h. Approximately 1000 flies were placed into each population cage, and after 8 hours, eggs were obtained from a Petri dish of food and transferred into new 35 ml plastic vials (approximately 100 eggs per vial). After 10-11 days, virgin females were collected under CO_2 anesthesia, transferred in groups of 15 to new 35 ml vials and maintained at 22 °C. Cold treatments started 96 hours after CO_2 anesthesia.

The experimental design was a simplified version of the experiment conducted by Marshall & Sinclair (2010). Five days old adult virgin female *D. melanogaster* were divided into four groups: a control group maintained at 22 °C; a multiple cold group which was exposed to -0.5 °C ± 0.25 °C for 2 hours every 24 h for five days; a sustained (or prolonged) cold group exposed to -0.5 °C ± 0.25 °C for 10 h on the fifth experimental day; and a single short cold group exposed to -0.5 °C ± 0.25 °C for 2 hour the fifth experimental day (Figure 2.1). All cold treatments were performed in a low temperature incubator (MIR153, Sanyo, Bensenville, IL, USA). For each cold treatment, twenty 35 ml plastic vials containing food (15 flies per vial) were placed upside down in the incubator. All flies were maintained at normal rearing temperatures unless undergoing cold exposures. After the cold treatment, the flies were returned to rearing temperatures for 6 hours to allow them to recover from the

cold, and then snap-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. To obtain biological replicates, this experiment was repeated three times on separate generations to control for any rearing effects.

2.2 *RNA extraction and quality control*

Total RNA samples used for microarrays were isolated from groups of 30-35 whole, frozen, 5 day-old virgin female *D. melanogaster* using the TRIzol Reagent (Invitrogen, Burlington, ON, Canada), and purified by MEGAclear (Ambion, Streetsville, ON, CA), according to the manufacturer's instructions. Extracted RNA was re-suspended in RNase-free water. The purity of RNA was assessed by the ratio of absorbance at 260 and 280 nm. A260/A280 ratios from 1.8 to 2.0 indicated no protein contamination.

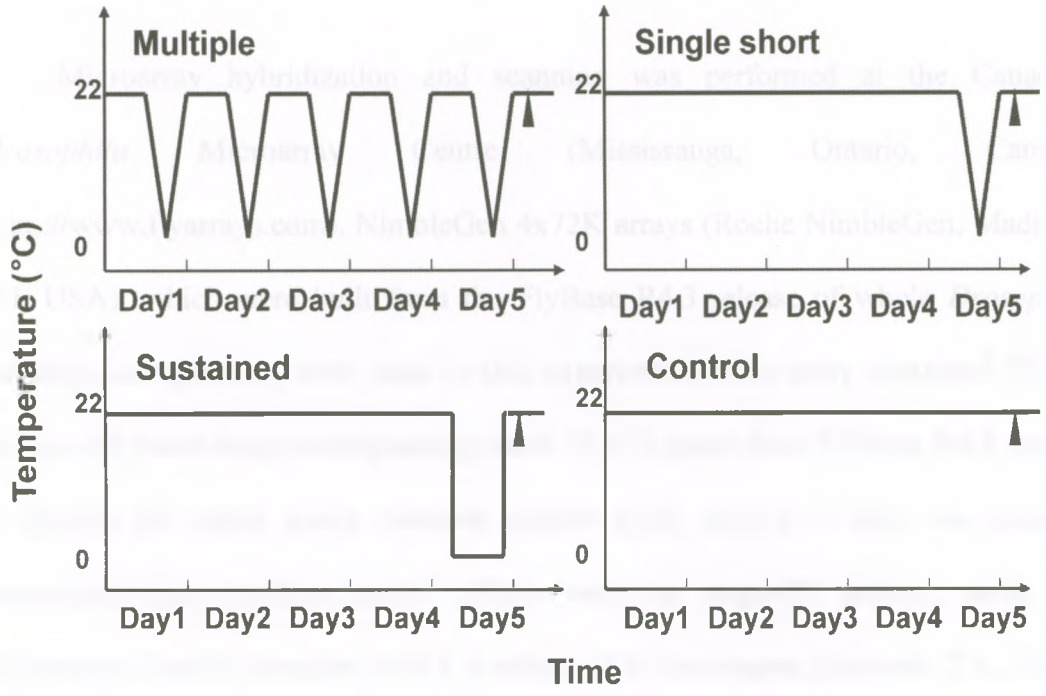


Figure 2.1: Treatment design. All experiments were performed on adult, virgin female *Drosophila melanogaster*. Each triangle represents a 2 h exposure to -0.5 °C, while rectangle represents a 10 h exposure to -0.5 °C. Flies were reared at 22 °C when not being exposed to cold. Arrowheads indicate sampling points. All samples were collected 6 h after final treatments.

2.3 *Microarray hybridization and data analysis*

Microarray hybridization and scanning was performed at the Canadian *Drosophila* Microarray Centre (Mississauga, Ontario, Canada) (http://www.flyarrays.com). NimbleGen 4x72K arrays (Roche NimbleGen, Madison, WI, USA), which were built from the FlyBase R4.3 release of whole *Drosophila melanogaster* genome, were used in this experiment. Each array contained 72,000 probes (60 bases long) corresponding to all 15,473 genes from FlyBase R4.3 release (4 probes per target gene). Isolated mRNA (with poly-A 3' tail) was reverse-transcribed into double-stranded cDNA with an oligo-dT primer, using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), and was labeled with either Cy3 dye or Cy5 dye (NimbleGen One-Color DNA Labeling Kit, Madison, USA). Each cDNA sample was hybridized to a single array. The cDNA samples from multiple, sustained and single short cold exposures and control with three biological replicates each were hybridized on three slides (four arrays on one slide). All the microarray hybridization, washing, and scanning steps were performed according to the microarray manufacturer's instructions.

Images scanned from the microarrays were quantified using NimbleScan v2.5 software (Roche NimbleGen, USA). Microarray data were extracted from the raw images and the expression value of each gene was calculated as the $\log_2$ ratio of relative signal intensities to exacted background. The Robust Multi-Array Average (RMA) algorithm was used to normalize data from three slides (Irizarry *et al.*, 2003). NimbleScan v2.5 assessed the quality of microarray hybridization according to the software default setting: signal range standing for the uniformity of signal intensity

over the array should be < 0.5; mean empty representing the mean signal intensity of empty probes or the background should be < 400; mean experimental representing the mean signal intensity of experimental probes should be in the range of 1000-8200; mean random measuring the non-specific hybridization from the mean signal intensity of random probes should be < 450.

2.4 *Candidate gene selection*

ArrayStar (DNAStar, Madison, WI, USA) software version 3.0.2 was used for analysis of the microarray data. To identify differentially expressed genes, normalized expression ratios ($\log_2$) of transcripts from the *D. melanogaster* transcriptome after multiple, sustained and single short cold exposures were each compared with control animals, and the fold-change was used to describe the degree of up- or down-regulation of each gene. A moderated t-test was performed to confirm the significance of differential expressed transcripts from three biological replicates (Mutch *et al.*, 2002). In this experiment, genes were designated candidates if they were up- or down-regulated by more than 2-fold in cold-exposed *D. melanogaster* relative to animals under control conditions, with a p-value < 0.05. In addition, a F-test (ANOVA) was performed to confirm if expression of the candidate genes differed significantly across the treatment groups rather than just compared to controls. Some candidate genes did not differ among treatment groups, but they were still considered candidates because they were significantly up- or down-regulated in response to cold exposure when compared with the control. A hierarchical clustering method was performed to cluster the expression ratio of all the transcripts after three cold treatments and control in order to build an experimental tree to represent the

relationship of gene expression patterns among cold treatments and control (Jinwook & Shneiderman, 2002). A k-mean clustering method was used to cluster the genes after cold treatments based on their expression differences to the control (Hartigan & Wong, 1979).

2.5 *Gene ontology and pathway analysis*

Gene ontology and pathway analysis were investigated with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 (http://david.abcc. ncifcrf.gov/). Gene annotation was built using the FlyBase R4.3 release, and then converted into DAVID IDs. The whole *D. melanogaster* genome was set as background, and a modified Fisher's Exact Test (EASE score) was used to examine the significance of candidate gene enrichment in annotation terms. A p-value (EASE score) < 0.05 indicates genes that were more greatly associated with certain annotation terms in particular treatment groups rather than by random chance. For the pathway analysis, DAVID was used to identify candidate genes involved in pathways from the KEGG_PATHWAY (http://www.kegg.com/kegg/pathway.html) and PANTHER_PATHWAY databases (http://www.pantherdb.org/pathway/).

2.6 *Quantitative real-time PCR validation*

To validate the changes in gene regulation detected by the microarrays, the mRNA abundance of selected candidate genes with quantified with three new biological samples by quantitative real-time PCR (qRT-PCR). The 11 most significantly up- and down-regulated genes that were differentially expressed after multiple cold exposures in comparison to the control flies, but not in the flies after

sustained and single short cold exposures, were selected for qRT-PCR validation. *TotA,* the gene that was up-regulated in all three cold treatments, was also selected for validation. The *Actin79B* gene was used as a reference to normalize data of target genes. Primers for the 12 genes were designed with the online tool NCBI primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/), and checked with the online tool, Primer3 (http://primer3.Sourceforge.net/). Primers are presented in Table 2.1. Primer pairs were predicted to produce unique amplicons no more than 300 bp in length, with G/C content higher than 50% and avoiding self-complementarity. The cDNAs were created by reverse-transcription from a total of 5 μg RNA (Invitrogen, Carlsbad, CA, USA), and then amplified in qRT-PCR using the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK).

The qRT - PCR reactions were cycled up to 45 times as follows: 95°C for 15 min, 55 °C for 30s, 72 °C for 30s in a Rotor-Gene 6000 cycler (Corbett, San Francisco, CA, USA). Standard curves of target genes were generated using five different concentrations of mixed samples, and Ct values were calculated from Corbett Rotor-Gene 6000 Application Software version 1.7 (Build 87). For all the tested genes, the correlation coefficient of Ct values (r) > 0.99 and reaction efficiencies were close to 1 (indicating that each cycle results in a doubling of cDNA) from each gene. The relative expression ratio of target genes was calculated and normalized by REST software version 2009 using equation 1. The relative expression ratio from qRT-PCR and normalized expression value from the microarray was regressed to test if the microarray data were reliable.

$$\text{Expression Ratio} = \frac{(\text{Efficiency}_{\text{target gene}})\Delta Ct_{\text{target gene}}}{(\text{Efficiency}_{\text{housekeeping gene}})\Delta Ct_{\text{housekeeping gene}}} \quad \text{(Equation 1)}$$

Table 2.1: Primer pairs used for candidate genes in qRT-PCR validation.

Primer		Sequence	Source
Actin79B	F	5'-TTGGAGATCCACATCTGCTG-3'	Sinclair *et al.* 2007
	R	5'-CCAGGTATCGCTGACCGTAT-3'	
TotA	F	5'-GCACCCAGGAACTACTTGACATCT-3'	Ekengren *et al.*2001
	R	5'-GACCTCCCTGAATCGGAACTC-3'	
TotM	F	5'-CGGACTTGAACACACGCGCC-3'	
	R	5'-CCAGAATCCGCCTTGTGCGGG-3'	
Cp16	F	5'-GAAAGTGACTGGTCATCGAG-3'	
	R	5'-AATTAACCAGAGCGTGTGTC-3'	
Upheld	F	5'-GAGACAAAACCACCTCAGAC-3'	
	R	5'-GGACGAGAGTCCCAACAC-3'	
CG6300	F	5'-TCTCGGGCTGCCTTGGAGGT-3'	
	R	5'-TCCGATCTCATCGGGTCCCAGG-3'	
CG33669	F	5'-ACTCCGAAGCGAGAATGAGC-3'	
	R	5'-AACGTCGCCGTGGAGCATGT-3'	
Tm2	F	5'-AGTGTGCGTGTGCGTTGGATCT-3'	
	R	5'-CGTTTCAAACACACTATACGCTGTCG-3'	
Lsp1 beta	F	5'-AGCGCCTGTCGCACGGATTC-3'	
	R	5'-TCGATGGCCTCGGGTTTGCG-3'	
CG14995	F	5'-ATTACGCGCCCTGCCGAACC-3'	
	R	5'-GGCAACTGGGGCGGCTCTTT-3'	
CG6188	F	5'-TGACCAGTGAGTTCCGTCTG-3'	
	R	5'-ATGTAGAAGGCGGGGTTCTT-3'	
CG13921	F	5'-TGGCTCCATAGCCCATCGCCA-3'	
	R	5'-AGGGTGGCGGGCACAAAGGT-3'	
CG16711	F	5'-GGCAGTGGACCACGGTGACG-3'	
	R	5'-GCAGCGGCCGCCATCTGATT-3'	

CHAPTER 3: RESULTS

3.1 *Candidate gene identification*

In this work, 15,403 protein-encoding genes in 5-day old virgin female *D. melanogaster* were examined by microarrays. RNA integrity, assessed by agarose gel electrophoresis, showed no degradation of the ribosomal RNA band (Figure 3.1). The quality check results of the microarray hybridizations were as follows: signal range was lower than 0.45; mean empty was lower than 400; mean experimental was in the range of 2000-3000; and mean random was lower than 350 in all 12 images. The quality assessment results indicate good quality hybridization, with a low spatial bias of signal intensity and low non-specific binding during hybridization. The $\log_2$ ratios of expression ranged from 5.8 to 15.3 (Figure 3.2). Heat maps were used to visualize the overall patterns of expression ratios of genes across treatments (Figure 3.2). The differential expression ratio of genes from treatments compared with the control ranged from -2.4 to 3.9 (Figure 3.3). Heat maps were used to visualize differential expression ratio of genes across treatments after k-mean clustering (Figure 3.3). The similarity of the gene expression patterns among three treatments was calculated by hierarchical clustering and represented by an experimental tree. This tree indicates that the expression patterns of genes from multiple and sustained cold exposures cluster together, and the single short group clusters with the control (Figure 3.2).

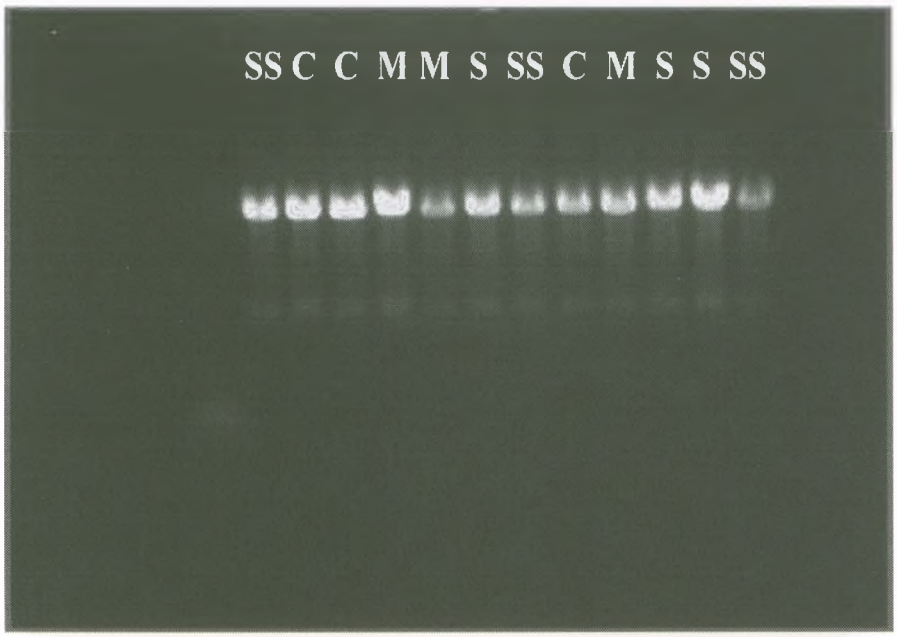


Figure 3.1: Agarose gel electrophoresis to confirm the integrity of RNA from samples of 5-day old female virgin *D. melanogaster* after multiple, sustained and single short cold exposures with three biological replicates (M = multiple; S = sustained; SS = single short; C = control). The single bright band indicates the rRNA from samples.

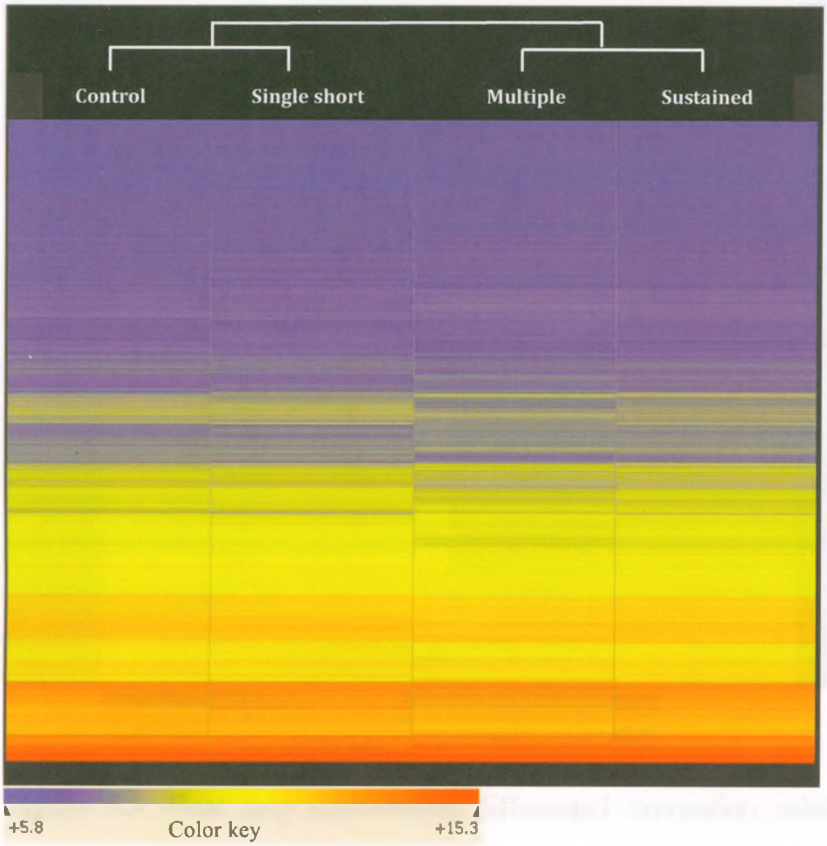


Figure 3.2: Heat map representing the expression values of all the examined transcripts in female *D. melanogaster* after multiple, sustained, single short cold exposures and control. The experimental tree of the three treatments and control were calculated by hierarchical clustering and represented on the top of the figure. The color key corresponds to the scale of transcript expression ratio on the heat map. Blue indicates lower expression values yellow indicates intermediately expressed genes, and red represents highly expressed genes.

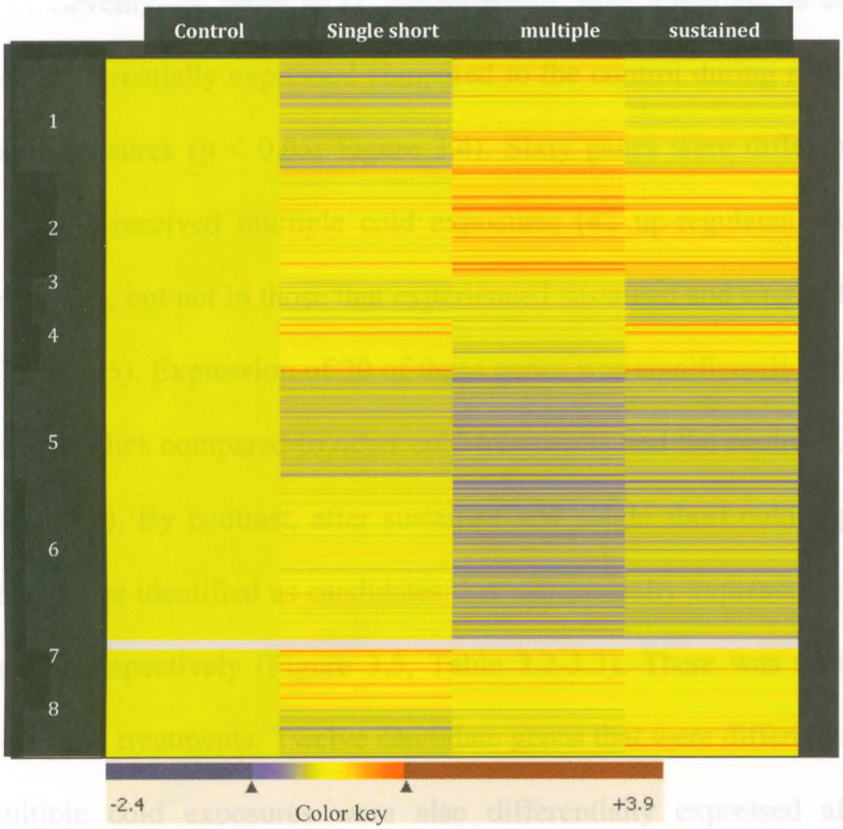


Figure 3.3: Heat map representing differential expression value of the examined transcripts in female *D. melanogaster* after multiple, sustained, single short cold exposures and control, and clustered by the k-mean method. The color key corresponds to the scale of transcript expression ratio on the heat map. Blue indicates genes down-regulated after treatments, yellow color indicates genes with no expression changes, and red represents genes up- regulated after treatments, grey indicates empty probes or missing data.

Seventy-six genes in *D. melanogaster* were identified as candidate genes that were differentially expressed compared to the control during recovery from multiple cold exposures ($p < 0.05$; Figure 3.4). Sixty genes were differentially expressed in flies that received multiple cold exposures (42 up-regulated and 18 genes down-regulated), but not in those that experienced sustained and single short cold exposures (Figure 3.5). Expression of 20 of these genes was significantly different in repeatedly exposed flies compared to other cold treatments and the control (ANOVA: $p < 0.05$; Table 3.1). By contrast, after sustained and single short cold exposures, 69 and 20 genes were identified as candidates that differentially expressed in comparison to the control respectively (Figure 3.5, Table 3.2-3.3). There was some overlap between three cold treatments. Twelve candidate genes that were differentially expressed after multiple cold exposures were also differentially expressed after sustained cold exposure; seven candidate genes differentially expressed after multiple cold exposures were also differentially expressed after single short exposure; and eleven candidate genes after sustained cold exposure were also differentially expressed in response to single short cold exposure. There were three genes that were differentially expressed after all three treatments (Figure 3.5, Table 3.4).

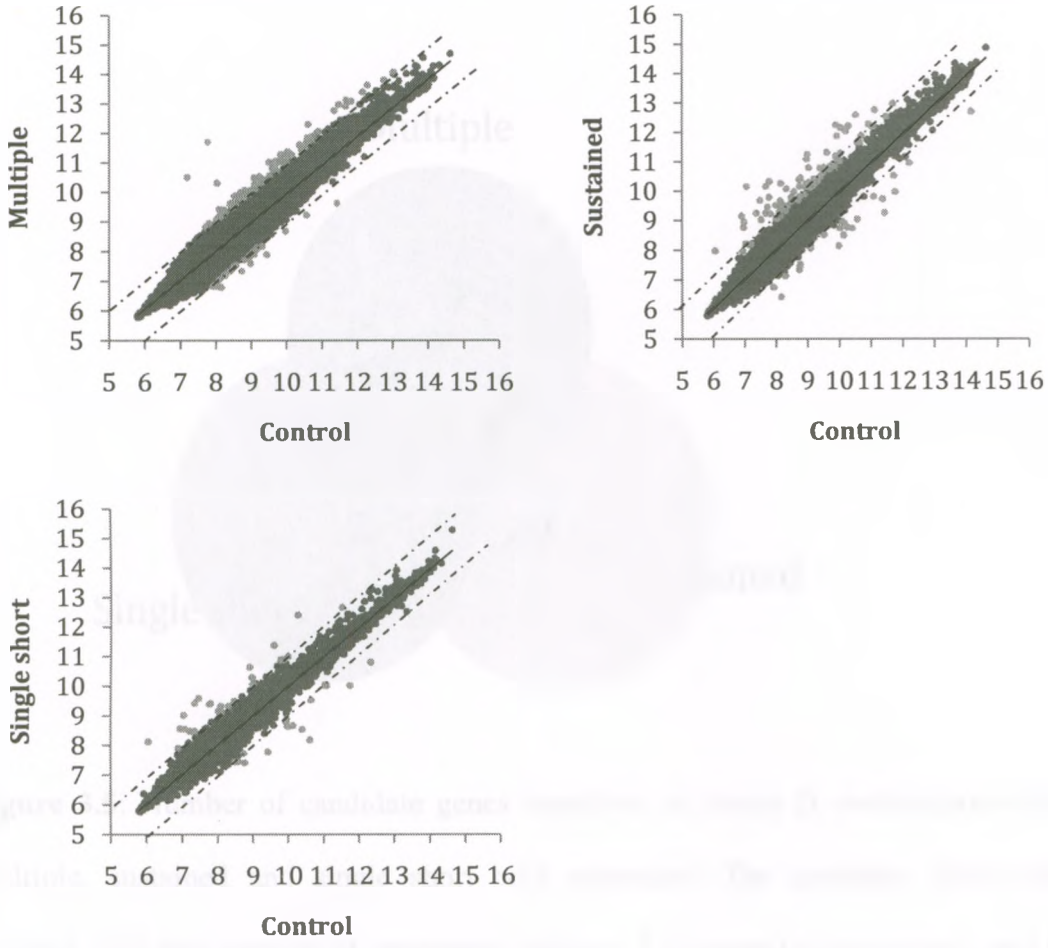


Figure 3.4: Expression value (log_2) of 16,944 transcripts in female *D. melanogaster* after multiple, sustained, single short cold exposures vs. control. The solid line is the linear regression (Multiple: r = 0.997; Sustained: r = 0.99; Single short: r = 0.99). Two dashed lines delineate genes with at least a two-fold change in intensity value in one of the datasets. Light grey dots indicate genes have both fold change > 2 and $p < 0.05$.

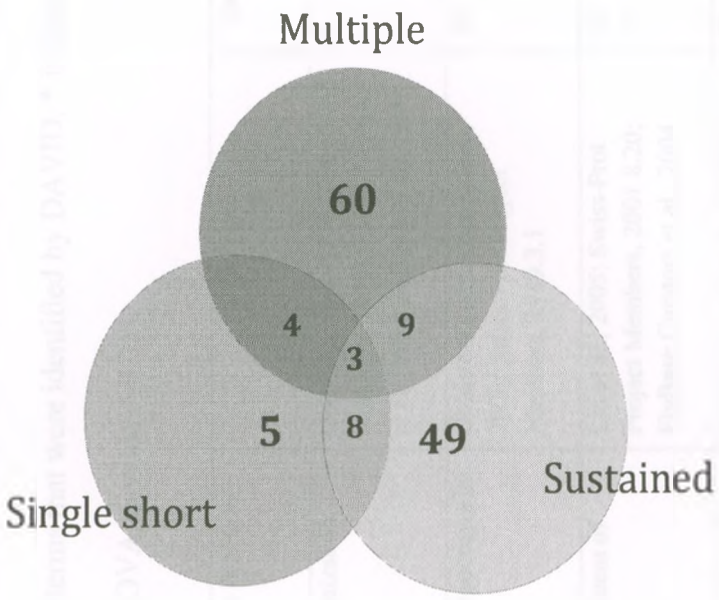


Figure 3.5: Number of candidate genes identified in female *D. melanogaster* after multiple, sustained and single short cold exposures. The candidate genes were selected with fold changes of expression values > 2 compared to the control, and the p-values < 0.05 after a moderated t-test (Table 3.1 - 3.4).

Table 3.1: Genes differentially expressed after multiple cold exposures but not in the flies experienced sustained and single short cold exposures, with a description of associated biological processes or molecular functions, as well as their locations on *D. melanogaster* chromosomes (X, 2L, 2R, 3L, 2R, 4). Genes were classified based on their GO terms that were identified by DAVID. * indicates differentially expressed genes among the three cold treatments after F-test (ANOVA, p-value < 0.05).

Functional category	Gene Name	Fold change	Biological process and molecular function	Reference	Location
Muscle protein/ Actin binding	*Upheld*	2.6 up	Calcium ion binding; sarcomere organization; myofibril assembly	Domingo et al., 1998; Fekete and Szidonya, 1979; Marco-Ferreres et al., 2005; Fyrberg et al., 1990	X
	*Tropomyosin 2**	2.4 up	Actin binding; muscle thin filament tropomyosin	Goldstein and Gunawardena, 2000; Swiss-Prot Project Members, 1989.3.1	3R
	*Paramyosin**	2.4 up	Myofibril assembly; structural constituent of muscle; motor activity	Liu et al., 2005; Swiss-Prot Project Members, 2001.8.20; FlyBase Curators et al., 2004	3L
	Fhos	2.3 up	Actin binding	FlyBase Curators et al., 2004	3L
	Myosin light chain 2	2.1 up	ATPase activity; calcium ion binding; microfilament motor activity	Goldstein and Gunawardena, 2000; FlyBase Curators et al., 2004; FlyBase, 1992	3R
Chorion	*Chorion protein 16**	2.7 up	Structural constituent of chorion; multicellular organismal development	FlyBase, 1992; FlyBase Curators et al., 2004	3L

	*Chorion protein 19**	2.6 up	Structural constituent of chorion; multicellular organismal development	FlyBase, 1992; FlyBase Curators et al., 2004	3L
	*Chorion protein 38**	2.1 up	Eggshell chorion assembly	FlyBase, 1992	X
Metabolic process/ Regulation of biological process	*CG6300**	2.5 up	Catalytic activity; long-chain fatty acid transporter activity	FlyBase Curators et al., 2004; FlyBase, 1992	3R
	*ia2**	2.2 up	Protein tyrosine phosphatase activity; protein amino acid dephosphorylation	Morrison et al., 2000; FlyBase Curators et al., 2004	2L
	Rab GTPase 9E	2.1 up	GTPase activity; protein transport; small GTPase mediated signal transduction	FlyBase Genome Annotators, 2004; FlyBase Curators et al., 2004	X
	CG3635	2.1 up	Triglyceride lipase activity	FlyBase, 1992	2L
	NADH-ubiquinone oxidoreductase chain 4	2.1 up	NADH dehydrogenase (ubiquinone) activity; mitochondrial respiratory chain complex I	FlyBase Curators et al., 2004; FlyBase, 1992	Mitocho-ndrion genome
	CG3894	2.1 up	Intracellular signaling pathway	FlyBase Curators et al., 2004	2R
	Rab GTPase 9Db	2.0 up	GTP binding; protein transport; small GTPase mediated signal transduction	FlyBase Curators et al., 2004	X

	Phosphotidylinositol 3 kinase 59F	2.0 down	Phosphoinositide-mediated signaling; dsRNA transport; cellular response to starvation	FlyBase Curators et al., 2004; Saleh et al., 2006; Juhasz et al., 2008	2R
	*CG32174**	2.1 down	Ubiquinone biosynthetic process	FlyBase, 1992	3L
	*Heat shock protein 22**	2.1 down	Response to heat; determination of adult lifespan; response to oxidative stress; protein refolding	Morrow et al., 2004; Swiss-Prot Project Members, 1986	3L
	*CG8303**	2.1 down	Catalytic activity	FlyBase Curators et al., 2004	2R
	CG6188	2.3 down	Folic acid binding; methionine metabolic process	FlyBase Curators et al., 2004	3R
Metal ion binding	CG13830	2.0 up	Calcium ion binding	FlyBase Curators et al., 2004	3R
	CG14306	2.1 up	Protein binding; zinc ion binding	FlyBase Curators et al., 2004	3R
	CG32850	2.2 up	Zinc ion binding; protein binding	FlyBase Curators et al., 2004	4
	Sirt4	2.1 down	Zinc ion binding; chromatin silencing; NAD binding; protein amino acid deacetylation	FlyBase Curators et al., 2004	X
	*Neu3**	2.1 down	zinc ion binding; proteolysis	FlyBase Curators et al., 2004	3R
Other	*TurandotM**	4.9 up	Encodes a humoral factor	Ekengren and Hultmark, 2001	2L

	CG33669	2.4 up	Unknown		X
	*Histone H3**	2.4 up	DNA binding; chromatin assembly or disassembly; nucleosome assembly	FlyBase Genome Annotators, 2005; Swiss-Prot Project Members, 1986.7.21	2L
	*CG41495**	2.4 up	Unknown		
	*Stellate protein CG33245**	2.4 up	Protein kinase regulator activity; regulation of protein kinase activity	FlyBase Genome Annotators, 2004	X
	CG42287	2.4 up	Unknown		2R
	CG32821	2.3 up	Unknown		X
	CG11585	2.3 up	Unknown		X
	*CG41124**	2.2 up	Unknown		
	CG33223	2.2 up	Unknown		X
	*CG33664**	2.2 up	Unknown		X
	CG32074	2.2 up	Unknown		3L
	*CG1894**	2.2 up	Histone acetyltransferase activity; regulation of transcription	FlyBase, 1992; FlyBase Curators et al., 2004	3R
	Met75C	2.2 up	Unknown		3L
	Z band alternatively spliced PDZ-	2.2 up	Mesoderm development; Z disc	Furlong et al., 2001; Hudson et al., 2008	3L

	*motif protein 66**				
	Lcp65Ag1	2.2 up	Structural constituent of chitin-based cuticle; larval chitin-based cuticle development	Willis, 2007.1.3; Charles et al., 1998	3L
	CG13055	2.2 up	Unknown		3L
	*Sperm-specific dynein intermediate chain 3**	2.1 up	Unknown		X
	*CG12587**	2.1 up	Unknown		3R
	CG13622	2.1 up	Unknown		3R
	CG33667	2.1 up	Unknown		X
	CG18437	2.1 up	Unknown		3L
	Chorion protein a at 7F	2.0 up	Unknown		X
	CG3984	2.0 up	Unknown		3R
	*CG13704**	2.0 down	Unknown		3L
	CG9396	2.0 down	Unknown		3R
	CG4025	2.0 down	Unknown		X

	CG4749	2.1 down	Unknown		2L
	CG5428	2.1 down	Sulfotransferase activity	FlyBase, 1992	2R
	CG14104	2.2 down	Unknown		3L
	CG16711	2.2 down	Unknown		3L
	CG13921	2.3 down	Unknown		3L
	CG14995	2.6 down	Autophagic cell death; salivary gland cell autophagic cell death	Gorski et al., 2003	3L
	*Larval serum protein 1 β**	2.9 down	Larval serum protein complex; nutrient reservoir activity; oxygen transporter activity	Wolfe et al., 1977; Swiss-Prot Project Members, 1989.10.1; FlyBase Curators et al., 2004	2L

Table 3.2: Gene differentially expressed after sustained cold exposure but not in the flies experienced multiple and single short cold exposures, with a description of associated biological processes or molecular functions, as well as their locations on *D. melanogaster* chromosomes (X, 2L, 2R, 3L, 2R, 4). Genes were classified based on their GO terms that were identified by DAVID. * indicates differentially expressed genes among the three cold treatments after F-test (ANOVA, p-value < 0.05).

GO terms	Gene Name	Fold changes	Biological process and molecular function	Reference	Location
Immune response	*Immune induced molecule 23**	2.7 up	Toll signaling pathway; antibacterial humoral response; defense response	Uttenweiler-Joseph et al., 1998	2R
	*Chorion protein b at 7F**	2.5 up	Unknown		X
	*Immune iduced molecule 1**	2.3 up	Defense response	Uttenweiler-Joseph et al., 1998	2R
	*Metchnikowin**	2.3 up	Defense response; antibacterial humoral response; antifungal humoral response	Uttenweiler-Joseph et al., 1998; Levashina et al. 1995, Lemaitre et al. 1997	2R
	*Peptidoglycan-recognition protein SC1a/b**	2.2 up	Peptidoglycan binding; protein binding; defense response; innate immune response	Jiggins and Hurst, 2003; Lemaitre, 2000	2R
Metabolic process	*NADH-ubiquinone oxidoreductase*	2.5 up	NADH dehydrogenase (ubiquinone) activity; mitochondrial electron transport,	FlyBase Curators et al., 2004	Mitocho-ndrion

	chain 3		NADH to ubiquinone		genome
	*CG9747**	2.0 up	acyl-CoA delta11-desaturase activity; lipid metabolic process; oxidation reduction	FlyBase, 1992; FlyBase Curators et al., 2004	3R
	CG4099	2.0 up	Heme binding; oxidation reduction	FlyBase Curators et al., 2004	3R
	*CG13309**	2.2 down	Unknown		3L
	*CG31661**	2.4 down	Unknown		2L
	*Target of brain insulin**	2.4 down	Carbohydrate metabolic process	FlyBase Curators et al., 2004	3R
	CG9466	2.4 down	Zinc ion binding; carbohydrate binding;	FlyBase Curators et al., 2004	2L
	*Larval visceral protein L**	2.9 down	Cation binding; carbohydrate metabolic process	Swiss-Prot Project Members, 1988; FlyBase Curators et al., 2004-	2R
	*Neither inactivation nor afterpotential D**	3.6 down	scavenger receptor activity; phototransduction	Kiefer et al., 2002; Wang and Montell 2005	2L
Reproduction	*Odorant-binding protein 19c**	4.4 up	Odorant binding; sensory perception of chemical stimulus; transport	Hekmat-Scafe et al., 2002; Galindo and Smith, 2001; FlyBase Curators et al., 2004	X
	*Vitelline membrane 32E**	5.1 up	Vitelline membrane formation in chorion-containing eggshell	Lindsley and Zimm, 1992	2L

	*Defective chorion 1**	3.6 up	Chorion-containing eggshell formation	Mauzy-Melitz and Waring, 2003	X
	Lipid storage droplet-1	2.4 up	Lipid storage	Miura, 2001	3R
	*CG13887**	2.4 up	Intracellular protein transport;	FlyBase Curators et al., 2004	3L
	*Chorion protein 15**	2.7 down	Structural constituent of chorion; eggshell chorion assembly	FlyBase, 1992	3L
Transport	*CG14309**	5.9 up	Unknown		3R
	Follicle cell protein 3C	5.7 up	Unknown		X
	*CG10407**	4.4 up	Unknown		3R
Others	*Glutathione S transferase D2**	3.9 up	Glutathione peroxidase activity	Singh et al., 2001	3R
	*Chorion protein b at 7F**	3.9 up	Oxidation reduction	FlyBase Curators et al., 2004	X
	*Heat shock protein 23**	3.3 up	Response to hypoxia; response to heat	Azad et al., 2009; Swiss-Prot Project Members, 1986	3L
	*CG15784**	3.2 up	Unknown		X
	*Vitelline membrane-like**	3.1 up	Dorsal/ventral axis specification	Zhang et al., 2009	X
	*CG15829**	3.0 up	acyl-CoA binding; enzyme inhibitor activity	FlyBase, 1992	3L

	*CG14834**	2.9 up	Unknown		3L
	*Follicle cell protein 26Ac**	2.8 up	Unknown		2L
	*CG13997**	2.8 up	Unknown		2L
	*CG31086**	2.7 up	Unknown		3R
	*Frost**	2.6 up	Response to cold	Goto, 2001	3R
	Cuticular protein 47Eb	2.5 up	Structural constituent of chitin-based cuticle; structural constituent of chitin-based larval cuticle	Willis, 2007; FlyBase, 1992	2R
	*CG13324**	2.5 up	Unknown		2R
	CG13905	2.4 up	Unknown		3L
	*CG15065**	2.3 up	Unknown		2R
	*CG14187**	2.3 up	Unknown		3L
	HMG protein Z	2.2 up	DNA binding	FlyBase Curators et al., 2004	2R
	*CG13114**	2.1 up	Unknown		2L
	CG16775	2.1 up	Unknown		3L
	*CG13323**	2.1 up	Unknown		2R
	Niemann-Pick type C-2e	2.1 up	Proteolysis	FlyBase Curators et al., 2004	3R

	CG10993	2.1 up	Nucleic acid binding; nucleotide binding	FlyBase Curators et al., 2004	X
	*Yellow-g**	2.0 up	Unknown		3L
	*CG12378**	2.0 up	Unknown		3R
	Mucin 12Ea	2.0 down	Unknown		X
	CG10513	2.4 down	Unknown		3R
	*Metallothionein C**	3.1 down	Metal ion binding	FlyBase Curators et al., 2004	3R

Table 3.3: Genes differentially expressed after single short cold exposure, but not in the flies experienced multiple and sustained cold exposures, with a description of associated biological processes or molecular functions, as well as their locations on *D. melanogaster* chromosomes (X, 2L, 2R, 3L, 2R, 4). Genes were classified based on their GO terms that were identified by DAVID. * indicates differentially expressed genes among the three cold treatments after F-test (ANOVA, p-value < 0.05).

Gene Name	Fold changes	Biological process and molecular function	Reference	Location
Raptor	2.1 down	positive regulation of protein amino acid phosphorylation; response to DNA damage stimulus	Sarbassov et al., 2004; Ravi et al., 2009	X
*CG42574**	2.1 down	acid-amino acid ligase activity zinc ion binding; protein modification process	FlyBase Curotors et al., 2004	3R
RhoGEF3	2.2 down	signal transducer activity; regulation of Rho protein signal transduction	Hicks, 2000	3L
*CG11505**	2.5 down	nucleic acid binding	FlyBase Curators et al., 2004	3L
CG34415	2.6 down	regulation of transcription; DNA-dependent	FlyBase Curators et al., 2004	2R

Table 3.4: Genes differentially expressed in female *D. melanogaster* after more than one cold exposure in the three cold treatments (multiple, sustained and single short cold exposures), with a description of associated biological processes or molecular functions, as well as their locations on *D. melanogaster* chromosomes (X, 2L, 2R, 3L, 2R, 4). Genes were classified based on their GO terms that were identified by DAVID.

Name	Fold-change (multiple)	Fold-change (sustained)	Fold-change (single short)	Biological processes and molecular functions	Reference	Location
Differential regulation after three cold treatments						
Turandot A	15.3 up	4.0 up	3.1 up	Encodes a humoral immune factor that from the fat body and accumulates in the body fluids	Ekengren et al., 2001; Agaisse and Perrimon, 2004	3R
CG11374	2.4 up	2.2 up	2.1 up	Ralactoside binding; urate transmembrane transporter activity	FlyBase, 1992	2L
Hephaestus	2.1 up	2.0 up	2.2 up	mRNA binding; notch signaling pathway; imaginal disc-derived wing margin morphogenesis	Lasko, 2000; FlyBase Curators et al., 2004-	2L
Differential regulation after multiple and sustained cold treatments						

Turandot C	9.9 up	2.0 up	1.9 up	Encodes a humoral immune factor	Ekengren and Hultmark, 2001	3R
CG1648	2.8 up	2.1 up	1.0 up	Unknown		2R
CG40116	2.7 up	2.0 up	1.8 up	Unknown		
CG41074	2.1 up	2.1 up	1.4 up	Unknown		
CG15369	2.1 down	2.2 down	1.2 down	Cysteine-type endopeptidase inhibitor activity	FlyBase, 1992	X
Metallothionein D	2.5 down	2.9 down	1.1 down	Metal ion binding	FlyBase Curators et al., 2004-	3R
CG9463	2.6 down	3.3 down	1.6 down	Zinc ion binding; carbohydrate binding	FlyBase Curators et al., 2004-	2L
CG15533	2.6 down	2.6 down	1.1 down	Sphingomyelin phosphodiesterase activity	FlyBase, 1992	3R
Larval serum protein 2	3.5 down	4.5 down	1.2 down	Nutrient reservoir activity; oxygen transporter activity	Swiss-Prot Project Members, 1997; FlyBase Curators et al., 2004-	3L
CG31532	3.2up	2.1 up	5.2 down	Unknown		3R
CG40103	2.0 up	1.7 up	2.1 up	Withdrawn		

Differential regulation after multiple and single short cold treatments						
CG16971	2.0 up	1.4 up	3.1 down	Unknown		3L
CG40275	2.0 up	1.5 up	2.0 down	Withdrawn		
Odorant-binding protein 99b	1.0 up	8.6 up	3.9 up	Olfactory behavior; autophagic cell death	Swiss-Prot Project Members, 2005; Gorski et al., 2003	3R
Differential regulation after sustained and single short cold treatments						
CG10814	1.0 down	4.8 up	2.0 up	Oxidation reduction; gamma-butyrobetaine dioxygenase activity	FlyBase Curators et al., 2004-; FlyBase, 1992	2R
CG15043	1.0 up	4.8 up	2.5 up	Unknown		X
Attacin A	1.1 up	4.6 up	4.4 up	Antibacterial humoral response; defense response to bacteria	Lemaitre et al., 1997; Meister et al., 2000	2R
CG10962	1.0 down	3.4 up	2.1 up	Oxidoreductase activity	FlyBase Curators et al., 2004-	X
Urate oxidase	1.3 down	3.4 up	2.0 up	Urate oxidase activity	FlyBase, 1992; FlyBase Curators et al., 2004-	2L

Attacin C	1.3 down	2.5 up	2.0 up	Antibacterial humoral response	Uttenweiler-Joseph el al., 1998	2R
Attacin B	1.0 down	2.3 up	2.0 up	Antibacterial humoral response	Lazzaro and Clark, 2000; Dushay et al., 2000	2R

3.2 *Functional analysis of candidate genes*

Nine functional terms were significantly enriched (p-values lower than 0.05) in the genes differentially expressed after multiple cold exposures, including: "contractile fiber" ($p<0.001$), "sarcomere" ($p<0.001$), "muscle protein"e ($p<0.001$) and "myofibril" ($p<0.001$) (Table 3.5). Eight functional terms were significantly enriched (p-values lower than 0.05) in the genes differentially expressed after sustained cold exposures, including: "response to stress" ($p<0.001$), "vitelline membrane" ($p<0.001$), "secreted" ($p<0.001$) and "immune response" ($p<0.001$) (Table 3.6). No single GO term was enriched after single short cold exposures. A total of 11 functional terms were significantly enriched from the genes differentially expressed after more than one cold treatments with p-values lower than 0.05, including "secreted" ($p<0.001$), and "immune response" ($p<0.001$) (Figure 3.6). Most of the candidate genes after multiple, sustained and single short exposures were associated with the functions of "muscle protein", "ion binding", "immune response", and "reproduction" (Figure 3.7). For the pathway analysis, a total of 16 candidate genes from different cold treatments were associated with cell signaling pathways and metabolic pathways in *D. melanogaster* were identified by KEGG_PANTHER and PANTHER_PANTHER (Table 3.7).

Gene ontology terms	Multiple		Sustained		Single short
Innate immunity	2		3		3
Secreted	2	1	6	1	3
Defense response to bacterium	1		4		4
Oxidoreductase activity		2	3		3
Catalytic activity			3		3
				2	
Metal ion binding		2		2	
Cation binding		2		2	
Organ development	1		2		2
Multicellular organismal process	1		2		2
Protein binding	5	1	6	1	3
Membrane-bounded organelle	2		3		1

Figure 3.6: Enriched GO terms ($p < 0.05$) identified by DAVID of the genes differentially expressed after more than one cold exposure in the three cold treatments (multiple, sustained and single short cold exposures). Light grey boxes indicate number of genes that were down-regulated, while dark grey boxes indicate genes that were up-regulated after multiple, sustained and single short cold exposures in female *D. melanogaster*.

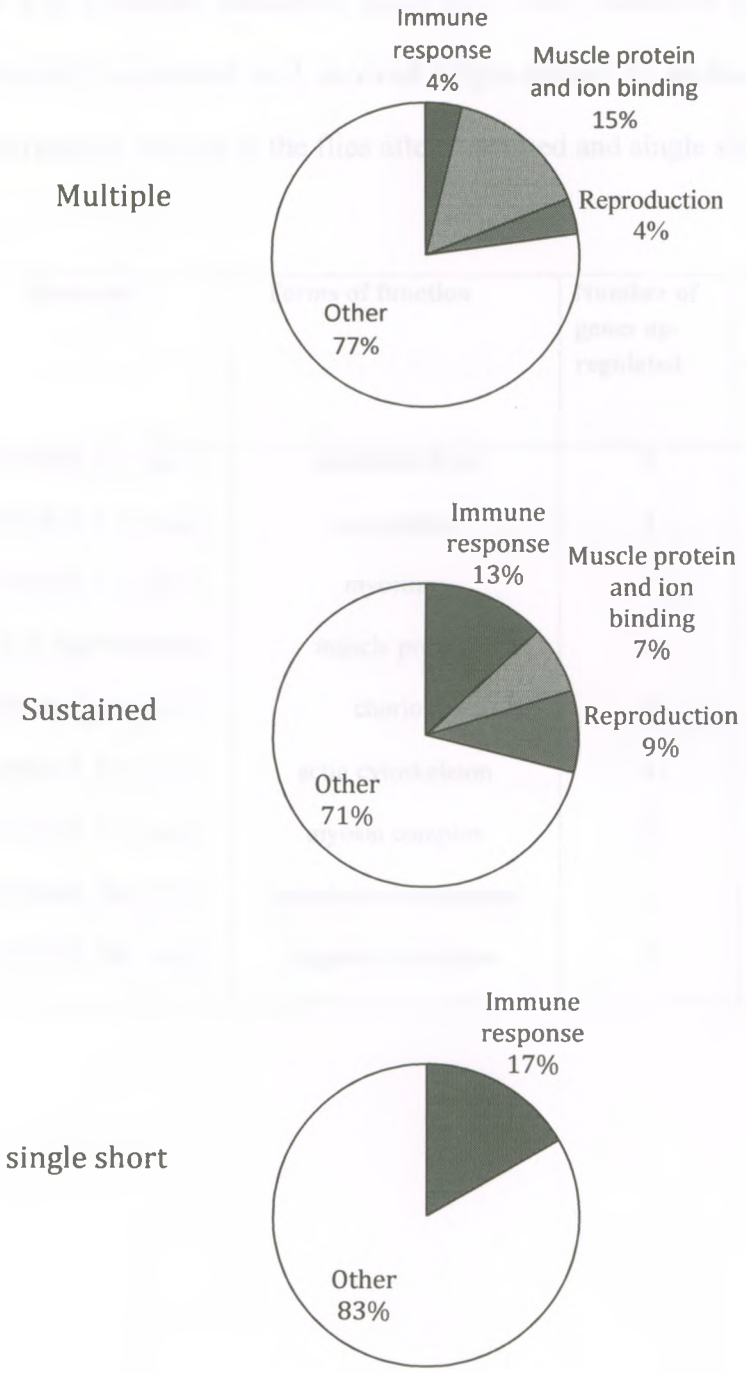


Figure 3.7: Proportion of candidate genes associated with muscle protein and ion binding; immune response; and reproduction in the total of candidate genes after multiple, sustained and single short cold exposures in female *D. melanogaster*.

Table 3.5: Enriched functional terms ($p < 0.05$) identified by DAVID from genes differentially expressed in 5 day-old virgin female *D. melanogaster* after multiple cold exposures, but not in the flies after sustained and single short cold exposures.

Recourse	Terms of function	Number of genes up-regulated	Number of genes down-regulated	% in totally identified genes
GOTERM_CC_ALL	contractile fiber	5	0	8.5
GOTERM_CC_ALL	sarcomere	4	0	6.8
GOTERM_CC_ALL	myofibril	4	0	6.8
SP_PIR_KEYWORDS	muscle protein	4	0	6.8
GOTERM_CC_ALL	chorion	4	0	6.8
GOTERM_CC_ALL	actin cytoskeleton	4	0	6.8
GOTERM_CC_ALL	myosin complex	2	0	3.4
GOTERM_BP_ALL	mesoderm development	3	0	5.1
GOTERM_BP_ALL	eggshell formation	3	0	5.1

Table 3.6: Significantly enriched functional terms ($p < 0.05$) identified by DAVID from genes differentially expressed in 5 day-old virgin female *D. melanogaster* after sustained cold exposure, but not in the flies after multiple and single short cold exposures.

Recourse	Terms of function	Number of genes up-regulated	Number of genes down-regulated	% in totally identified genes
GOTERM_CC_ALL	response to stress	7	1	16.3
SP_PIR_KEYWORDS	innate immunity	4	0	8.2
GOTERM_CC_ALL	defense response	5	0	10.2
GOTERM_CC_ALL	chorion	3	0	6.8
GOTERM_CC_ALL	carbohydrate metabolic process	1	4	10.2
GOTERM_CC_ALL	eggshell formation	3	0	6.1
SP_PIR_KEYWORDS	glycosidase	0	3	6.1

Table 3.7: Identified pathways by KEGG_PANTHER and PANTHER_PANTHER from genes differentially expressed after multiple, sustained, and single short cold exposures.

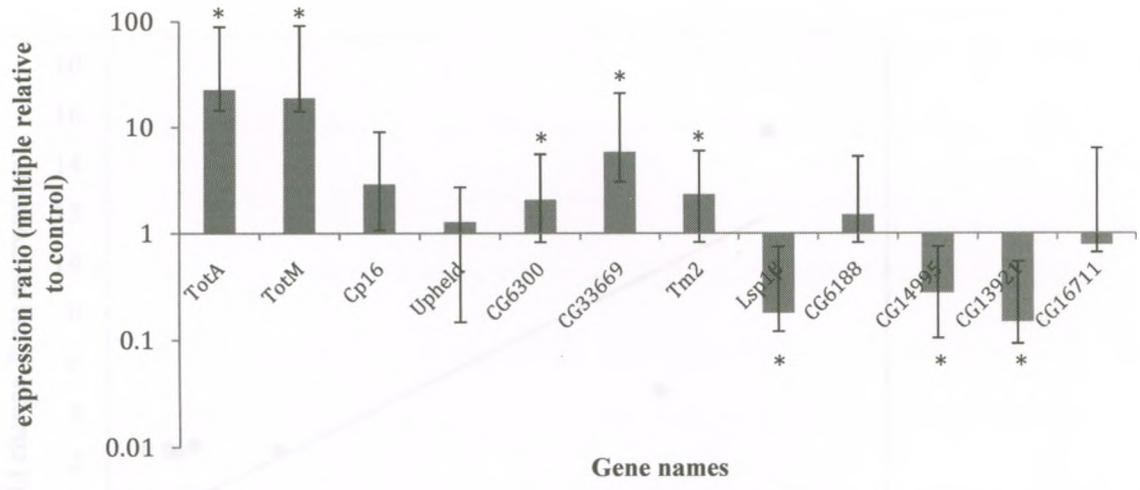
Database	Pathway terms	Multiple	Sustained	Single short
KEGG	Lysine degradation		*CG10814*	*CG10814*
	Lysosome	*CG9463*	*CG9463* *CG9466*	
	Purine metabolism		*Urate oxidase*	*Urate oxidase*
	Glycine	*CG6188*		
	Oxidative phosphorylation	*NADH-ubiquinone oxidoreductase chain 4*	*NADH-ubiquinone oxidoreductase chain 3*	
	p53 pathway	*CG3187* *Phosphotidylinositol 3 kinase 59F*		
	Ras pathway	*Phosphotidylinositol 3 kinase 59F*		*Raptor*
	Galactose metabolism		*CG11909* *Larval visceral protein L*	
	Biosynthesis of unsaturated fatty acids		*CG9747*	

	Glutathione metabolism		*Glutathione S transferase D2*	
PANTHER	Notch signaling pathway	*CG3894*		
	Angiogenesis	*Phosphotidylinositol 3 kinase 59F*	*Heat shock protein 23*	
	T cell activation	*Phosphotidylinositol 3 kinase 59F*		
	Wnt signaling pathway	*Stellate protein CG33245*		

3.3 *Quantitative real-time PCR validation*

To validate the changes of expression in candidate genes from the microarray, I measured the transription of seven up-regulated genes and five down-regulated genes in female *D. melanogaster* after multiple cold exposures. *Actin79B* did not change its expression after three treatments and so was considered a good reference gene for target gene normalization. Five genes (*TotA*, *TotM*, *CG6300*, *CG33669*, *Tm2*) showed significant up-regulation and three genes (*lsp1β*, *CG14995*, *CG13921*) showed significant down-regulation after multiple cold exposures (Figure 3.8a-b). In addition, the expression levels of *TotA,* which was significantly up-regulated after mutiple, sustained and single short cold exposures was screened. *TotA* was also up-regulated after multiple cold exposures with real time PCR validation. Microarray and qRT-PCR data were also significantly correlated ($r = 0.86$, $df = 9$, $p < 0.001$), indicating the reliability of the microarray data (Figure 3.9).

a)



b)

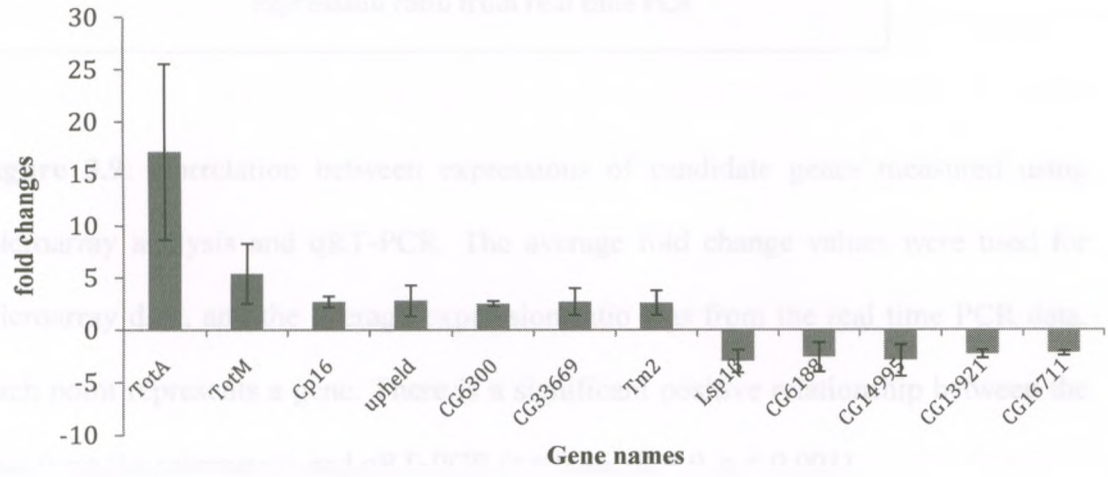


Figure 3.8: a) Expression ratio of 11 candidate genes after multiple cold exposures vs. control measured by qRT-PCR (normalized by *Actin79B*). Columns labeled with * indicate genes significantly up- or down-regulated ($p < 0.05$). Error bars represent the standard error from three biological replicates. b) Fold changes in 11 candidate genes significantly up- or down- regulated after multiple cold exposures vs. control that as indicated from microarray. Error bars represent the standard error from three biological replicates.

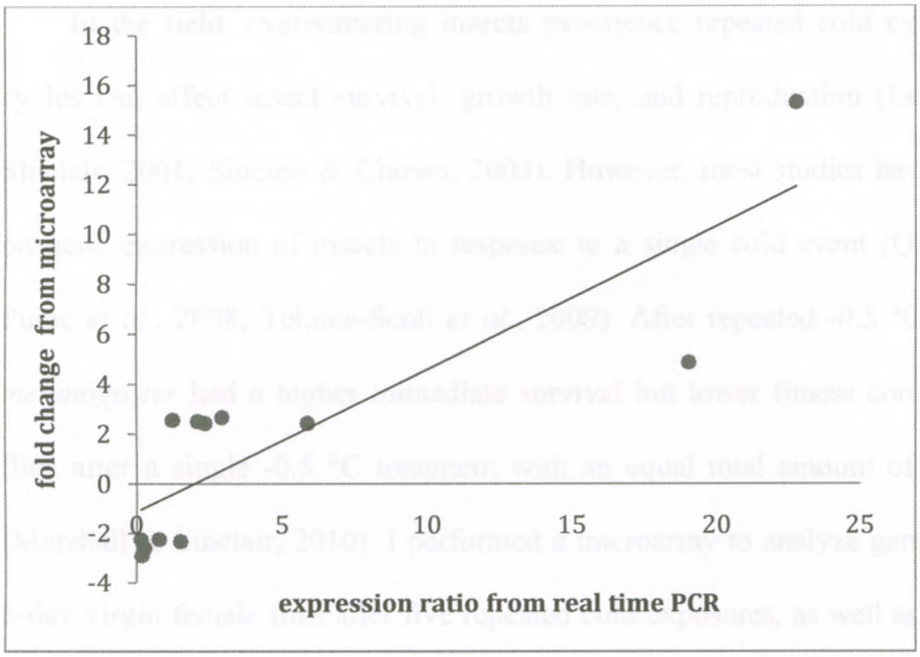


Figure 3.9: Correlation between expressions of candidate genes measured using microarray analysis and qRT-PCR. The average fold change values were used for microarray data, and the average expression ratio was from the real time PCR data. Each point represents a gene. There is a significant positive relationship between the data from the microarray and qRT-PCR ($r = 0.86$, $df = 9$, $p < 0.001$).

CHAPER 4: DISCUSSION

In the field, overwintering insects experience repeated cold cycles, and these cycles can affect insect survival, growth rate, and reproduction (Lee *et al.*, 1987; Sinclair, 2001; Sinclair & Chown, 2003). However, most studies have only focused on gene expression of insects in response to a single cold event (Qin *et al.*, 2005; Purac *et al.*, 2008; Telonis-Scott *et al.*, 2009). After repeated -0.5 °C exposures, *D. melanogaster* had a higher immediate survival but lower fitness compared with the flies after a single -0.5 °C treatment with an equal total amount of exposure time (Marshall & Sinclair, 2010). I performed a microarray to analyze gene expression in 5-day virgin female flies after five repeated cold exposures, as well as flies subjected to a single cold exposure (including prolonged and short cold exposures), to select candidate genes that were differentially expressed in response to repeated cold exposures. To my knowledge, my project is the first to study gene expression in any ectotherm in response to repeated cold exposures. I identified 60 genes, which had altered transcription only in response to multiple cold exposures. These genes were generally associated with muscle and actin binding. I also found that increased mRNA abundance of genes associated with secreted proteins, for example, the Turandot families, which might contribute to removing cold damaged protein or tissue during the recovery periods.

4.1 *Gene expression differs between single and multiple cold exposures*

Drosophila melanogaster may respond differently in terms of gene expression in response to multiple cold exposures, compared to single cold exposures. Molecular

investigations of low temperature responses are limited in *D. melanogaster* (Qin *et al.*, 2005; Purac *et al.*, 2008; Telonis-Scott *et al.*, 2009). Qin et al. (2005) identified 37 genes that changed their mRNA accumulation in female *D. melanogaster* after a similar cold treatment as the single short cold exposure in this study: a cold shock of 0 °C for 2 h + recovered at 25 °C for 30 min (Qin *et al.*, 2005). In the study described here, a single brief cold exposure also did not alter the transcription of a large number of genes (Figure 3.4): there were only 20 candidate genes differentially expressed after a single exposure to -0.5 °C for 2 h. However, 79 genes were differentially expressed after repeated cold exposures, and 60 of those did not change their transcription in response to one time prolonged and short cold exposures. There were 69 genes differentially regulated after a single prolonged cold exposure as well. This work suggests that with increasing times of repeated cold exposures or length of cold exposure time, more genes in *D. melanogaster* are differentially regulated.

Functional analysis of candidate genes also implied that the responses of *D. melanogaster* after repeated and single cold exposures were different at the gene expression level. In general, 15 % of genes differentially expressed after multiple cold exposures were associated with muscle proteins and ion binding, compared with 7 % of genes from flies that experienced a single prolonged cold exposure, and none from the single short cold exposure. Interestingly, 13 % of and 17 % of gene differentially expressed after the single prolonged or short cold exposures respectively were involved in the immune response, in contrast to only 4 % of genes from the multiple cold treatment (Figure 3.6). The differences in candidate gene functions indicate that, in response to repeated cold exposures, *D. melanogaster* mainly regulate genes to

recover muscle activity, while they activate the immune system in response to single prolonged or single short cold exposures.

The differences in gene expression patterns among flies after multiple, sustained and single short cold exposures can be related to the differences in chronic and acute chilling injury. Prolonged cold exposure or cold acclimation may cause the mismatching of metabolic pathways and loss of ion homeostasis (Koštál *et al.*, 2004; Koštál *et al.*, 2006; Colinet *et al.*, 2007), while chilling injury from RCH is thought to be associated with changes of membrane phase (Drobnis *et al.*, 1993). Chen & Denlinger (1992) found that, in *S. crassipalpis*, recovery at high temperature reduced the acute chilling injury after cold shock, but not chronic injury after 10 days of cold. In this study, the expression pattern of genes after multiple cold exposures was more similar to the pattern after sustained cold exposure, rather than single short cold exposure (Figure 3.1). I hypothesize that repeated cold exposure resulted in accumulated chilling injuries that were close to chronic but not to acute chilling injury.

The duration of recovery time after cold treatments may also be important to the change in gene expression in *D. melanogaster*. Although the single short cold exposure designed in my work was equal to the cold treatment used by (Qin *et al.*, 2005), there were no candidate genes identified that were shared with their study. The reason for the lack of overlapping genes may be that flies in my work were allowed 6 h recovery time from low temperature, compared to only 30 min cold recovery time in (Qin *et al.*, 2005). Sinclair et al. (2007) found that the mRNA abundance of genes *Frost* and *Hsp70* was decreased with increasing cold recovery time in *D.*

melanogaster. I suggest that while the expression of genes responsible for short-term cold recovery was fading, genes identified from microarray in my work were responsible for long-term cold recovery. For example, I found four genes (*Raptor, RhoGEF3, CG11505,* and *CG34415*) were up-regulated after a single short cold exposure that were associated with the regulation of transcription (Table 3.3). These genes did not change their expression during short period of cold recovery (Qin *et al.*, 2005), but might regulate the response to cold in the long-term.

4.2 *Muscle-related genes were up-regulated after multiple cold exposures*

Drosophila melanogaster are chill-susceptible: they suffer chilling injuries after cold exposure (Bale & Hayward, 2010). Since more female adult *D. melanogaster* survived after multiple cold exposures than flies after sustained cold exposure with an equal total amount of exposure time, it has been suggested that repairing chilling injuries might be activated during recovery from cold (Marshall & Sinclair, 2010). The genes *Tm2*, *Paramyosin*, *upheld*, *Fhos*, and *Myosin light chain 2* associated with muscle construction, actin binding or motor activity were significantly up-regulated after multiple cold exposures (Table 3.1). The data presented here suggests that repairing in *D. melanogaster* after multiple cold exposures occurs in the muscle. For example, the muscle protein gene *Tm2* is important for actin binding and the organization of muscle thin filament (Lin & Storti, 1997; Gunning, 2008), and the gene *Paramyosin* is involved in motor activity and muscle thick filaments (Levine *et al.*, 1976). Similarly, the gene *upheld* is involved in muscle thin filament and myofibril assembly (Nongthomba *et al.*, 2007).

Rapid cold-hardening protected *S. crassipalpis* from cold shock-induced damage of neuromuscular transmission and reduction of the mean resting membrane potential of tergotrochanteral muscle (Kelty *et al.*, 1996). Rapid cold-hardening also decreased apoptotic cell death in flight muscles of *D. melanogaster* after -7 °C (Yi *et al.*, 2007). However, genes encoding muscle proteins were not up-regulated after sustained and single short cold exposures. I hypothesize that *D. melanogaster* up-regulate muscle protein genes to repair accumulated chilling injuries in their muscles only after multiple recoveries from low temperatures.

Previous work suggested that the concentration of sodium ions was decreased but potassium ion increased in the coxal muscle of the tropical cockroach *Nauphoeta cinerea* after the cockroaches suffered chilling injuries. But the changing rate of ion concentration slowed down in cold-acclimated cockroaches, also there was no chilling injuries in the cockroaches after cold acclimation (Koštál *et al.*, 2006). Another work found that the changes of ion concentration in the bug *Pyrrhocoris apterus* and the beetle *Alphitobius diaperinus* during fluctuating thermal regimes were smaller than bugs during constant low temperature treatments (Koštál *et al.*, 2007). No genes associated with Na^+ and K^+ ion binding were differentially regulated after either multiple or sustained cold exposures, nor were there genes directly coding ATPase subunits found. Only one ATP-binding gene *CG32318* was down-regulated after single short cold exposure. In contrast, Qin et al. (2005) identified three genes related to ATP binding and Na^+/ H^+ regulation in *D. melanogaster* after cold shock. Hence their data suggest that transcriptional changes in ATP-binding or ion transport

genes could happen quickly during cold recovery and might have returned to basal levels after 6 hours cold exposure, when the samples in this study were collected.

4.3 *Metabolic and signaling pathways were associated with multiple cold exposures*

Repeated cold exposures may change the regulation of metabolism in insects. Colinet et al. (2007) analyzed the protein expression profiles in the parasitic wasp *Aphidius colemani* after fluctuating thermal regimes (FTR) that interrupted a 4 °C cold exposure with 2h at 20 °C, and found up-regulation of energy metabolism in glycolysis, the TCA cycle and ATP synthesis. In a separate series of experiments in *D. melanogaster* after multiple cold exposures had lower glycogen stores than flies after 10 h and 2 h cold exposures (Marshall & Sinclair, 2010), and oxidative stress in house flies after prolonged cold exposure may cause chilling injuries since higher oxygen consumption caused higher mortality in houseflies after seven days at low temperatures (Rojas & Leopold, 1996). The gene expression analysis in this study suggests oxidative stress responses may be related to multiple and prolonged cold exposure responses in *D. melanogaster*. The genes *NADH-ubiquinone oxidoreductase chain 3* and *NADH-ubiquinone oxidoreductase chain 4* were up-regulated in flies with multiple and sustained cold exposures respectively. These two genes are located in the *D. melanogaster* mitochondrial genome and encode proteins associated with the first enzyme in the electron transfer chain (complex I), which transfers electrons from NADH to ubiquinone during oxidative phosphorylation.

Previous work has found that, in insects, cold stress activates cellular signaling pathways as well. For example, the p38 MAPK pathway was activated in *S.*

crassipalpis within 10 min exposure to 0 °C (Fujiwara & Denlinger, 2007). KEGG and PANTHER pathway analysis in the study presented here identified five signaling pathways that were associated with repeated cold exposures in *D. melanogaster*, and two of them are associated with apoptosis pathways (Table 3.7). The up-regulated gene *Phosphotidylinositol 3 kinase 59F* and the down-regulated gene *CG3187* involve the p53-signaling pathway (Sherr & McCormick, 2002). This signaling pathway is associated with repairing DNA damage and regulates apoptosis (Brodsky *et al.*, 2004). Yi et al. (2007) found that rapid cold-hardening may block cold-induced cell death to protect *D. melanogaster* at low temperatures (Yi *et al.*, 2007). Taken together, this data suggests that the p53 pathway is activated to repair cold-induced DNA damage, especially in response to multiple cold exposures. Another apoptosis related pathway that was identified in the current study was the Notch signaling pathway (Table 3.7). The Notch pathway in *Drosophila* regulates the cell surface proteins, which control the signal transmission of cell fate and cell communication (Artavanis-Tsakonas *et al.*, 1999). *Hephaestus*, a gene that encodes a polypyrimidine tract-binding protein, and regulates the Notch signaling of wing development (Dansereau *et al.*, 2002), was significantly up-regulated after all three cold treatments. *Hephaestus* also has the molecular function of mRNA binding and alternative splicing in signal transduction (Lasko, 2003). Thus, the apoptosis-related Notch signaling pathway may be important to *D. melanogaster* in its response to cold stress.

4.4 *Stress related genes were up-regulated in response to multiple, single prolonged and single short cold exposures*

Many environmental stresses induce different Hsps, and their chaperone function may act during the recovery from cellular stresses. For example, *Hsp70* and *Hsp23* have been up-regulated during the recovery from heat shock and desiccation stresses in *S. crassipalpis* (Hayward *et al.*, 2004). In *D. melanogaster*, *Hsp70* has been up-regulated during the recovery from cold shock (Sinclair *et al.*, 2007). Another stress-related gene *Frost* has been up-regulated during the recovery from cold and desiccation exposure, but not from heat exposure (Sinclair *et al.*, 2007; Goto, 2001). In my work, genes encoding two heat shock proteins have been identified as candidate genes. *Hsp23* and *Frost* have been up-regulated after single prolonged cold exposure.

Three genes from the Turandot family (*TotA*, *TotC* and *TotM*) were significantly up-regulated in response to multiple cold exposures. In particular, *TotA* was up-regulated in response to all three cold stresses, with a 15-fold up-regulation after multiple cold exposures. *TotM* was only up-regulated in response to multiple cold exposures. Another gene in the Turandot family, *TotC*, was up-regulated after both multiple and single chronic cold exposures. The proteins encoded by the genes in Turandot family are stress proteins. Previous work has found that the expression of genes in this family can be induced by many environmental stresses including heat shock, cold shock, septic injury or bacterial infection (Ekengren & Hultmark, 2001). Recently, a cDNA microarray experiment found that *TotA* was up-regulated in cold-adapted *D. subobscura* (Laayouni *et al.*, 2007). The regulation of *TotA* may involve

different pathways under different environmental stresses. It has been shown that this gene was activated by Mekk1-p38 pathway after septic injury, and the JAK-STAT or Imd pathway after bacterial infection (Ekengren & Hultmark, 2001) (Agaisse *et al.*, 2003). Based on the protein sequences of the Turandot family, it was predicted that these genes encode 11-14kDa highly-charged proteins, with N-terminal signal peptides that would direct the protein out of the cell, e.g. the product of *TotA* is be secreted into hemolymph from the fat body (Ekengren *et al.*, 2001).

In response to damage from different environmental stresses, the heat shock proteins concentrate in nucleus and at cell membranes. Heat shock proteins can bind to the determinants and then refold or degrade aberrant proteins (Parsell & Lindquist, 1993). Nevertheless, the function of stress proteins during cold recovery remains unknown. Based on this work I hypothesize that the up-regulation of *TotA*, *TotC*, and *TotM* in *D. melanogaster* may be triggered by tissue or protein damage after low temperature exposure, and their functions might be similar to the heat shock proteins (Ekengren & Hultmark, 2001). However, unlike heat shock proteins, which work on the intracellular level, the Turandot proteins may operate extracellularly; they somehow are involved in signal transductions, and activate downstream proteins to protect cell damage. In addition, the regulation of Turandot proteins in response to cold might be slow – I found increased expression of *TotA* in 6 h cold recovery, yet previous work only measured gene expression changes in within 3 hours cold recovery period (Qin *et al.*, 2005; Sinclair *et al.*, 2007).

4.5 *Immune related genes were induced after a single cold exposure*

Surprisingly, 13 % of genes after sustained cold exposure and 17 % candidates of genes upregulated after a single short cold exposure were immune-induced genes that have been shown to contribute to bacteria or fungal defenses (Figure 7). The candidate genes from sustained and single short cold treatments were compared with other microarray data from *D. melanogaster* after septic injury and natural infection (De Gregorio *et al.*, 2002). The genes *Immune induced molecule 1*, *Immune induced molecule 23, Attacins*, *Metchnikowin*, *PGRP-SC1b*, and *yellow* were involved in both cold and infection responses. In particular, the up-regulation of five candidates (*Immune induced molecule 1*, *Immune induced molecule 23, AttacinA*, *AttacinB*, *AttacinC* and *Metchnikowin*) may be involved in producing antimicrobial peptides in *D. melanogaster*. In response to infection, antimicrobial peptides target invading cell membranes, bind peptides to the membranes that increase the membrane permeability, thus kill the invading organisms (Zasloff, 2002). The Toll and Imd pathways are the major signaling pathways that regulate the expression of antimicrobial peptides in *D. melanogaster* (Zasloff, 2002). One candidate gene, *PGRP-SC1*, which was up-regulated after sustained cold exposure, may regulate the activation of immune pathways (Bischoff *et al.*, 2006). A potential interpretation of this finding is that cold stress activates immune pathways in *D. melanogaster* and this may be because flies have evolved similar responses to both cold and infection stresses, or the flies are sensitive to pathogens at low temperatures and so have adapted by inducing their immunes response after cold exposures.

4.6 *Genes associated with reproduction after cold exposures*

After multiple and sustained cold exposures, 4 % and 9 % of candidate genes (respectively) were associated with reproduction (Figure 3.7). The Chorion protein families (*Cp16*, *Cp19*, and *Cp38*) were up-regulated in the flies after multiple cold exposures, while the *defective chorion 1*, *Chorion protein b at 7F*, *Chorion protein c at 7F* were up-regulated and the *Chorion protein 15* were down-regulated by flies in the sustained group. Other genes (*Vitelline membrane 32E*, *Follicle cell protein 26Ac* and *Vitelline membrane-like*) genes increased their expression in the flies after prolonged cold exposure. The Chorion proteins in the vitelline membrane layers play important roles in the assembly and stabilization of the mature eggshell (Noguerón *et al.*, 2000). Since Vitelline membrane proteins are usually synthesized during the early stages of eggshell development, while Chorion proteins are synthesized in later stages (Pascucci *et al.*, 1996), this process may be involved in multiple stages of egg development.

Marshall and Sinclair (2010) found that the total number of offspring produced by 3-day old adult female *D. melanogaster* after multiple cold exposures was higher than by the flies after sustained cold exposure, although there was no difference between the groups at five and seven days of age. More egg development-related genes were up-regulated in flies after sustained cold exposure rather than after multiple cold exposures, indicating that prolonged cold exposure was more stressful to flies in immediate reproduction. However, after multiple cold exposures *D. melanogaster* had a significantly male-biased sex ratio, leading to a lower intrinsic rate of population increase than the flies after sustained and single short cold

exposures (Marshall & Sinclair, 2010). It is possible that 6 h recovery was not long enough to observe the gene expression changes underlying this survival-reproduction trade-off in *D. melanogaster* after repeated cold exposures.

4.7 *Project limitations*

The microarray analysis and experimental design of this project have certain limitations and the conclusions from such studies must be treated with caution. First of all, this experiment only examined gene expression profiles after 6 hours cold recovery in *D. melanogaster*. However, as disscussed above, different recovery periods may regulate different genes in response to cold stress (Qin *et al.*, 2005; Sinclair *et al.*, 2007) and it is entirely possible that longer term studies are needed to identify trade-offs at the different life history stages of the flies. Secondly, post-translational modification plays an important role in protein translation, therefore changes in gene expression identified from this work may not be equate to protein expression changes in response to different types of cold stresses. Moreover, although I performed biological replicates on the microarrays, and used qRT-PCR to validate candidate genes, I did not hybridize the same samples on different arrays as technical replicates. Of the 11 candidate genes measured using qRT-PCR, four of them (*cp16*, *upheld*, *CG6188* and *CG16711*) did not show consistant expression with the microarray data. This difference might be due to the biological variation from different samples, or the difficulty of detecting transcription variants from qRT-PCR. For example, in the microarray data, the transcription variant A of gene *upheld* was up-regulated but its transcription variant B was down-regulated after multiple cold exposures. My primers amplified both transcription variant A and transcription

variant B in the PCR reaction. Even given these limitations, this work remains a valuable analysis for insects in response to repeated cold exposures. The data generated in this project have provided candidate genes involved in the response to different types of cold stresses with statistical confidence, and hypothesized a potential mechanisms uderlying recovery from different cold stresses.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

In response to multiple cold exposures, female *D. melanogaster* increased short-term survival rate but decreased long-term reproduction. In this study, a microarray analysis of *D. melanogaster* after multiple, single but prolonged, and single short cold exposures suggested that different types of cold exposures changed gene regulation in female flies, and provided many clues for future research in the area of insect cold responses. After repeated cold exposures, the up-regulation of genes *TotA*, *TotC* and *TotM* might result in the accumulation of stress proteins and thus improve the survival of female *D. melanogaster* during the repeated cold recovery periods. In particular, many candidate genes differentially expressed in response to multiple cold exposures were associated with muscle activities, suggesting the flies may prioritize repairing chilling injury in muscle tissue after repeated cold exposures. Since this work used whole body tissue of flies as samples for the microarray analysis, future work should focus on analyzing regulation of muscle protein in specific muscle tissue of *D. melanogaster*.

This work provides, for the first time, gene expression evidence to suggest that the *D. melanogaster* immune response is up-regulated in response to acute cold stress. This mechanism may be induced by chilling injury and involve expressing antimicrobial peptides that remove or repair damaged proteins after cold exposures. Thus, future work should also focus on testing cross-tolerance to cold and infection stresses on chill-susceptible species.

The microarray data indicates candidate genes differentially expressed in response to cold stress, but the function of many candidate genes at 6 h cold recovery

remains unknown. RNA interference (RNAi) experiments can be used to analyze these "unknown" gene functions after cold stress. In the future, it would also be useful to analyze the function of genes overlapping after both multiple and single cold exposures (*TotA*, *CG11374*, and *Hephaestus*). Moreover, microarray analysis suggested that the metabolic regulation in *D. melanogaster* after cold exposures was changed. However, the changes may be more complex than suggested on the gene expression level, and may require the application of additional methods, such as biochemistry and proteomics to provide a finer detail and more holistic analysis of the cellular processes affected.

CHAPTER 6: REFERENCES

Agaisse, H., Petersen, U., Boutros, M., Mathey-Prevot, B. & Perrimon, N. (2003) Signaling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury. *Developmental Cell,* **5**, 441-450.

Agaisse, H. & Perrimon, N. (2004) The roles of JAK/STAT signaling in *Drosophila* immune responses. *Immunological Reviews,* **198**, 72-82.

Anderson, N.L. & Anderson, N.G. (1998) Proteome and proteomics: New technologies, new concepts, and new words. *Electrophoresis,* **19**, 1853-1861.

Artavanis-Tsakonas, S., Rand, M.D. & Lake, R.J. (1999) Notch signaling: Cell fate control and signal integration in development. *Science,* **284**, 770-776.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M. & Sherlock, G. (2000) Gene ontology: Tool for the unification of biology. *Nature Genetics,* **25**, 25-29.

Bale, J.S. (2002) Insects and low temperatures: From molecular biology to distributions and abundance. *Philosophical Transactions of the Royal Society of London.Series B: Biological Sciences,* **357**, 849-862.

Bale, J.S. & Hayward, S.A.L. (2010) Insect overwintering in a changing climate. *Journal of Experimental Biology,* **213**, 980-994.

Bale, J.S., Worland, M.R. & Block, W. (2001) Effects of summer frost exposures on the cold tolerance strategy of a sub-antarctic beetle. *Journal of Insect Physiology,* **47**, 1161-1167.

Benjamini, Y. & Hochberg, Y. (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society.Series B (Methodological),* **57**, 289-300.

Bischoff, V., Vignal, C., Duvic, B., Boneca, I.G., Hoffmann, J.A. & Royet, J. (2006) Downregulation of the *Drosophila* immune response by peptidoglycan-recognition proteins SC1 and SC2. *PLoS Pathology,* **2**, e14.

Bolstad, B.M., Irizarry, R.A., Astrand, M. & Speed, T.P. (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics,* **19**, 185-193.

Brodsky, M.H., Weinert, B.T., Tsang, G., Rong, Y.S., McGinnis, N.M., Golic, K.G., Rio, D.C. & Rubin, G.M. (2004) *Drosophila melanogaster* MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. *Molecular and Cellular Biology,* **24**, 1219-1231.

Brown, P.O. & Botstein, D. (1999) Exploring the new world of the genome with DNA microarrays. *Nature Genetics,* **21**, 33-27.

Burton, V., Mitchell, H.K., Young, P. & Petersen, N.S. (1988) Heat shock protection against cold stress of *Drosophila melanogaster*. *Molecular and Cellular Biology,* **8**, 3550-3552.

Butte, A. (2002) The use and analysis of microarray data. *Nature Reviews Drug Discovery,* **1**, 951-960.

Chen, C. & Denlinger, D.L. (1992) Reduction of cold injury in flies using an intermittent pulse of high temperature. *Cryobiology,* **29**, 138-143.

Chown, S.L. & Nicolson, S.W. (2004) *Insect Physiological Ecology: Mechanisms and Patterns,* Oxford University Press.

Clark, M.S. & Worland, M.R. (2008) How insects survive the cold: Molecular mechanisms: A review. *Comparative Biochemistry and Physiology B,* **178**, 917-933.

Colinet, H., Nguyen, T., Cloutier, C., Michaud, D. & Hance, T. (2007) Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. *Insect Biochemistry and Molecular Biololgy,* **37**, 1177-1188.

Colinet, H., Fai Lee, S. & Hoffmann, A. (2010) Functional characterization of the *frost* gene in *Drosophila melanogaster*: Importance for recovery from chill coma. *PLoS ONE,* **5**, e10925.

Colinet, H., Nguyen, T.T.A., Cloutier, C., Michaud, D. & Hance, T. (2007) Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. *Insect Biochemistry and Molecular Biology,* **37**, 1177-1188.

Czajka, M. & Lee, R. (1990) A rapid cold-hardening response protecting against cold shock injury in *Drosophila melanogaster*. *Journal of Experimental Biology,* **148**, 245-254.

Dansereau, D.A., Lunke, M.D., Finkielsztein, A., Russell, M.A. & Brook, W.J. (2002) Hephaestus encodes a polypyrimidine tract binding protein that regulates notch signalling during wing development in *Drosophila melanogaster*. *Development,* **129**, 5553-5566.

De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M. & Lemaitre, B. (2002) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila. The EMBO Journal,* **21**, 2568-2579.

Denlinger, A.L. & Lee, R.E.J. (2010) *Low Temperature Biology of Insects,* Cambridge, Cambridge University Press.

Doniger, S., Salomonis, N., Dahlquist, K., Vranizan, K., Lawlor, S. & Conklin, B. (2003) MAPPFinder: Using gene ontology and GenMAPP to create a global gene-expression profile from microarray data. *Genome Biology,* **4**, R7.

Drobnis, E.Z., Crowe, L.M., Berger, T., Anchordoguy, T.J., Overstreet, J.W. & Crowe, J.H. (1993) Cold shock damage is due to lipid phase transitions in cell membranes: A demonstration using sperm as a model. *Journal of Experimental Zoology,* **265**, 432-437.

Ekengren, S. & Hultmark, D. (2001) A family of turandot-related genes in the humoral stress response of *Drosophila. Biochemical and Biophysical Research Communications,* **284**, 998-1003.

Ekengren, S., Tryselius, Y., Dushay, M.S., Liu, G., Steiner, H. & Hultmark, D. (2001) A humoral stress response in *Drosophila. Current Biology,* **11**, 714-718.

Fujiwara, Y. & Denlinger, D.L. (2007) p38 MAPK is a likely component of the signal transduction pathway triggering rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Journal of Experimental Biology,* **210**, 3295-3300.

Goto, S.G. (2001) A novel gene that is up-regulated during recovery from cold shock in *Drosophila melanogaster*. *Gene,* **270**, 259-264.

Gunning, P. (2008) *Tropomyosin*, New York, Springer.

Gygi, S.P., Rochon, Y., Franza, B.R. & Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. *Molecular and Cellular Biology,* **19**, 1720-1730.

Hartigan, J.A. & Wong, M.A. (1979) Algorithm AS 136: A k-means clustering algorithm. *Journal of the Royal Statistical Society.Series C (Applied Statistics),* **28**, 100-108.

Hayward, S., Rinehart, J.P. & Denlinger, D.L. (2004) Desiccation and rehydration elicit distinct heat shock protein transcript response in flesh fly pupae. *Journal of Experimental Biology,* **207**, 963-971.

Hoffmann, J.A. (2003) The immune response of *Drosophila. Nature,* **426**, 33-38.

Hoffmann, J.A. & Reichhart, J. (2002) *Drosophila* innate immunity: An evolutionary perspective. *Nature Immunology,* **3**, 121-126.

Hosler, J.S., Burns, J.E. & Esch, H.E. (2000) Flight muscle resting potential and species-specific differences in chill-coma. *Journal of Insect Physiology,* **46**, 621-627.

Huang, D.W., Sherman, B.T. & Lempicki, R.A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols,* **4**, 44-57.

Huey, P. (2001) Chill-coma temperature in *Drosophila*: Effects of developmental temperature, latitude, and phylogeny. *Physiological and Biochemical Zoology,* **74**, 429-434.

Ioannidis, J.P.A., Allison, D.B., Ball, C.A., Coulibaly, I., Cui, X., Culhane, A.C., Falchi, M., Furlanello, C., Game, L., Jurman, G., Mangion, J., Mehta, T., Nitzberg, M., Page, G.P., Petretto, E. & Van Noort, V. (2009) Repeatability of published microarray gene expression analyses. *Nature Genetics,* **41**, 149-155.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U. & Speed, T.P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics,* **4**, 249-264.

Jinwook, S. & Shneiderman, B. (2002) Interactively exploring hierarchical clustering results. *IEEE Computer,* **35**, 80-86.

Joanisse, D.R. & Storey, K.B. (1998) Oxidative stress and antioxidants in stress and recovery of cold-hardy insects. *Insect Biochemistry and Molecular Biology,* **28**, 23-30.

Joplin, K.H., Yocum, G.D. & Denlinger, D.L. (1990) Cold shock elicits expression of heat shock proteins in the flesh fly, sarcophaga crassipalpis. *Journal of Insect Physiology,* **36**, 825-834.

Kelty, J.D., Killian, K.A. & Lee, R.E. (1996) Cold shock and rapid cold-hardening of pharate adult flesh flies (*Sarcophaga crassipalpis*): Effects on behaviour and neuromuscular function following eclosion. *Physiological Entomology,* **21**, 283-288.

Kelty, J.D. & Lee, J.,Richard E. (1999) Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster. Journal of Insect Physiology,* **45**, 719-726.

Košťál, V., Vambera, J. & Bastl, J. (2004) On the nature of pre-freeze mortality in insects: Water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus. Journal of Experimental Biology,* **207**, 1509-1521.

Koštál, V., Yanagimoto, M. & Bastl, J. (2006) Chilling-injury and disturbance of ion homeostasis in the coxal muscle of the tropical cockroach (*Nauphoeta cinerea*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **143**, 171-179.

Koštál, V., Renault, D., Mehrabianová, A. & Bastl, J. (2007) Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes: Role of ion homeostasis. *Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology,* **147**, 231-238.

Koštál, V. & Tollarová-Borovanská, M. (2009) The 70 kDa heat shock protein assists during the repair of chilling injury in the insect, *Pyrrhocoris apterus*. *PLoS ONE,* **4**, e4546.

Laayouni, H., Garcia-Franco, F., Chavez-Sandoval, B., Trotta, V., Beltran, S., Corominas, M. & Santos, M. (2007) Thermal evolution of gene expression profiles in *Drosophila subobscura*.

Lamberty, M., Zachary, D., Lanot, R., Bordereau, C., Robert, A., Hoffmann, J.A. & Bulet, P. (2001) Insect immunity. *Journal of Biological Chemistry,* **276**, 4085-4092.

Lasko, P. (2003) Gene regulation at the RNA layer: RNA binding proteins in intercellular signaling networks. *Science Signaling,* **2003**, re6.

Le Bourg, E., Massou, I. & Gobert, V. (2009) Cold stress increases resistance to fungal infection throughout life in *Drosophila melanogaster*. *Biogerontology,* **10**, 613-625.

Lee, R.E.J., Damodaran, K., Yi, S. & Lorigan, G.A. (2006) Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. *Cryobiology,* **52**, 459-463.

Lee, R.E.J., Chen, C. & Denlinger, D.L. (1987) A rapid cold-hardening process in insects. *Science,* **238**, 1415-1417.

Lee, M.T., Kuo, F.C., Whitmore, G.A. & Sklar, J. (2000) Importance of replication in microarray gene expression studies: Statistical methods and evidence from repetitive cDNA hybridizations. *Proceedings of the National Academy of Sciences of the United States of America,* **97**, 9834-9839.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J., Caroff, M., Lee, W., Mengin-Lecreulx, D. & Lemaitre, B. (2003) The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. *Nature Immunology,* **4**, 478-484.

Levine, R.J., Elfvin, M., Dewey, M.M. & Walcott, B. (1976) Paramyosin in invertebrate muscles. II. content in relation to structure and function. *The Journal of Cell Biology,* **71**, 273-279.

Li, C. & Wong, W.H. (2001) Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection. *Proceedings of the National Academy of Sciences of the United States of America,* **98**, 31-36.

Lin, S. & Storti, R.V. (1997) Developmental regulation of the *Drosophila tropomyosin I* (*TmI*) gene is controlled by a muscle activator enhancer region that contains multiple cis-elements and binding sites for multiple proteins. *Developmental Genetics,* **20**, 297-306.

Linder, J.E., Owers, K.A. & Promislow, D.E.L. (2008) The effects of temperature on host–pathogen interactions in *D. melanogaster*: Who benefits? *Journal of Insect Physiology,* **54**, 297-308.

Lockhart, D.J., Dong, H.L., Byrne, M.C., Follettie, M.T., Gallo, M.V., Chee, M.S., Mittmann, M., Wang, C.W., Kobayashi, M., Horton, H. & Brown, E.L. (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology,* **14**, 1675-1680.

Marshall, K.E. & Sinclair, B.J. (2010) Repeated stress exposure results in a survival–reproduction trade-off in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences,* **277**, 963-969.

McLachlan, G.J., Do, K. & Ambroise, C. (2004) *Analyzing Microarray Gene Expression Data,* Wiley interscience.

McMullen, D.C. & Storey, K.B. (2008) Mitochondria of cold hardy insects: Responses to cold and hypoxia assessed at enzymatic, mRNA and DNA levels. *Insect Biochemistry and Molecular Biology,* **38**, 367-373.

Misra, S., Crosby, M.A., Mungall, C.J., Matthews, B.B., Campbell, K.S., Hradecky, P., Huang, Y., Kaminker, J.S., Millburn, G.H., Prochnik, S.E., Smith, C.D., Tupy, J.L., Whitfield, E.J., Bayraktaroglu, L., Berman, B.P., Bettencourt, B.R., Celniker, S.E., de Grey, A.,D.N.J., Drysdale, R.A., Harris, N.L., Richter, J., Russo, S., Schroeder, A.J., Shu, S., Stapleton, M., Yamada, C., Ashburner, M., Gelbart, W.M., Rubin, G.M. & Lewis, S.E. (2002) Annotation of the *Drosophila melanogaster* euchromatic genome: A systematic review. *Genome Biology,* **3**, 12.

Moseley, P. (2000) Stress proteins and the immune response. *Immunopharmacology,* **48**, 299-302.

Mutch, D., Berger, A., Mansourian, R., Rytz, A. & Roberts, M. (2002) The limit fold change model: A practical approach for selecting differentially expressed genes from microarray data. *BMC Bioinformatics,* **3**, 17.

Nielsen, M., Overgaard, J., Sørensen, J.G., Holmstrup, M., Justesen, J. & Loeschcke, V. (2005) Role of HSF activation for resistance to heat, cold and high-temperature knock-down. *Journal of Insect Physiology,* **51**, 1320-1329.

Noguerón, M.I., Mauzy-Melitz, D. & Waring, G.L. (2000) *Drosophila* dec-1 eggshell proteins are differentially distributed via a multistep extracellular processing and localization pathway. *Developmental Biology,* **225**, 459-470.

Nongthomba, U., Ansari, M., Thimmaiya, D., Stark, M. & Sparrow, J. (2007) Aberrant splicing of an alternative exon in the *Drosophila Troponin-T* gene affects flight muscle development. *Genetics,* **177**, 295-306.

Ohtsu, T., Kimura, M.T. & Katagiri, C. (1998) How *Drosophila* species acquire cold tolerance. *European Journal of Biochemistry,* **252**, 608-611.

Overgaard, J., Malmendal, A., Sørensen, J.G., Bundy, J.G., Loeschcke, V., Nielsen, N.C. & Holmstrup, M. (2007) Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster. Journal of Insect Physiology,* **53**, 1218-1232.

Padgett, D.A. & Glaser, R. (2003) How stress influences the immune response. *Trends in Immunology,* **24**, 444-448.

Parsell, D.A. & Lindquist, S. (1993) The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. *Annual Review of Genetics,* **27**, 437-496.

Pascucci, T., Perrino, J., Mahowald, A.P. & Waring, G.L. (1996) Eggshell assembly in *Drosophila*: Processing and localization of vitelline membrane and chorion proteins. *Developmental Biology,* **177**, 590-598.

Piper, M.D.W., Daran-Lapujade, P., Bro, C., Regenberg, B., Knudsen, S., Nielsen, J. & Pronk, J.T. (2002) Reproducibility of oligonucleotide microarray transcriptome analyses. *Journal of Biological Chemistry,* **277**, 37001-37008.

Pockley, A.G. (2003) Heat shock proteins as regulators of the immune response. *Lancet,* **362**, 469-476.

Purac, J., Burns, B., Thorne, M., Grubor-Lajsic, G., Worland, M.R. & Clark, M.S. (2008) Cold hardening processes in the antarctic springtail, *Cryptopygus antarcticus*: Clues from a microarray. *Journal of Insect Physiology,* **54**, 1356-1362.

Qin, W., Neal, S.J., Robertson, R.M., Westwood, J.T. & Walker, V.K. (2005) Cold hardening and transcriptional change in *Drosophila melanogaster*. *Insect Molecular Biology,* **14**, 607-613.

Rinehart, J.P., Li, A., Yocum, G.D., Robich, R.M., Hayward, S.A.L. & Denlinger, D.L. (2007) Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proceedings of the National Academy of Sciences,* **104**, 11130-11137.

Rojas, R.R. & Leopold, R.A. (1996) Chilling injury in the housefly: Evidence for the role of oxidative stress between pupariation and emergence. *Cryobiology,* **33**, 447-458.

Sherr, C.J. & McCormick, F. (2002) The RB and p53 pathways in cancer. *Cancer Cell,* **2**, 103-112.

Leather, S.R., Walters, K.F.A. & Bale, J.S. (1993) *The Ecology of Insect Overwintering,* Cambridge, Cambridge University Press.

Sinclair, B.J., Gibbs, A.G. & Roberts, S.P. (2007) Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. *Insect Molecular Biology,* **16**, 435-443.

Sinclair, B.J. (2001) Field ecology of freeze tolerance: Interannual variation in cooling rates, freeze-thaw and thermal stress in the microhabitat of the alpine cockroach *Celatoblatta quinquemaculata*. *Oikos,* **93**, 286-293.

Sinclair, B.J. & Roberts, S.P. (2005) Acclimation, shock and hardening in the cold. *Journal of Thermal Biology,* **30**, 557-562.

Sinclair, B.J. & Chown, S.L. (2005) Deleterious effects of repeated cold exposure in a freeze-tolerant sub-antarctic caterpillar. *Journal of Experimental Biology,* **208**, 869-879.

Sinclair, B.J. & Chown, S.L. (2003) Rapid responses to high temperature and desiccation but not to low temperature in the freeze tolerant sub-antarctic caterpillar *Pringleophaga marioni* (lepidoptera, tineidae). *Journal of Insect Physiology,* **49**, 45-52.

Sohail, M. & Southern, E.M. (2002) Oligonucleotide scanning arrays: Application to high-throughput screening for effective antisense reagents and the study of nucleic acid interactions. *Advances in Biochemical Engineering Biotechnology.Chip Technology,***77**, 43-56.

Storey, K.B. & Storey, J.M. (1988) Freeze tolerance in animals. *Physiological Reviews,* **68**, 27-84.

Telonis-Scott, M., Hallas, R., McKechnie, S.W., Wee, C.W. & Hoffmann, A.A. (2009) Selection for cold resistance alters gene transcript levels in *Drosophila melanogaster*. *Journal of Insect Physiology,* **55**, 549-555.

Thomas, M.B. & Blanford, S. (2003) Thermal biology in insect-parasite interactions. *Trends in Ecology & Evolution,* **18**, 344-350.

Wilson, K., Cotter, S.C., Reeson, A.F. & Pell, J.K. (2001) Melanism and disease resistance in insects. *Ecology Letters,* **4**, 637-649.

Yi, S., Moore, C.W. & Lee, R.E. (2007) Rapid cold-hardening protects *Drosophila melanogaster* from cold-induced apoptosis. *Apoptosis,* **12**.

Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature,* **415**, 389-395.