GENOMICS AND PHYSIOLOGICAL EVOLUTION OF COLD TOLERANCE IN *DROSOPHILA MELANOGASTER*

by

ALISON RENAE GERKEN

B.S., The University of South Dakota, 2008
M.S., The University of South Dakota, 2010

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

The Division of Biology
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Abstract

Thermal stress impacts animals around the globe and understanding how organisms adapt to changes in temperature is of particular interest under current climate change predictions. My research focuses on the evolutionary genetics involved in cold tolerance and plasticity of cold tolerance using both artificially selected and naturally segregating populations, while tying the genes of interest to their physiological components. First I address cross-tolerance of stress traits following artificial selection to a non-lethal cold tolerance metric, chill-coma recovery. Using these artificial selection populations, we found that stress traits such as desiccation tolerance, starvation tolerance, acclimation, and chronic and acute cold tolerance do not correlate with level of cold tolerance as defined by chill-coma recovery time. We next assessed lifetime fitness of these different cold tolerance lines and found that only at low temperatures did fitness differ among cold tolerance levels. We then analyzed gene expression differences between resistant and susceptible populations at three time points to understand where selection pressures are hypothesized to act on genomic variation. Our gene expression analyses found many differences between resistant and susceptible lines, primarily manifesting themselves in the recovery period following cold exposure. We next utilized a community resource, the *Drosophila melanogaster* reference panel, to identify naturally segregating variation in genes associated with cold acclimation and fitness. We specifically asked if long- and short-term acclimation ability had overlapping genetic regions and if plasticity values from constant rearing environments were associated with demographic parameters in fluctuating environments. We found that long- and short-term acclimation are under unique genetic control and functionally tested several genes for acclimation ability. We also found that acclimation ability in constant environments and fitness in fluctuating environments do not correlate, but that genotypes are constrained in their fitness abilities between a warm and cool environment. Our analyses describe several novel genes associated with cold tolerance selection and long- and short-term acclimation expanding our knowledge of the complex relationship between demographic components and survivorship as well as a unique investigation of the change in gene expression during cold exposure.

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Major Professor
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Chapter 1 - Quantitative Genetics of the Evolution of Cold Tolerance in *Drosophila melanogaster*

The global climate is a variable that many animals must cope with on a daily and seasonal basis in order to maintain reproductive success and fitness. Stressful climatic conditions can significantly lower Darwinian fitness and combatting these stresses through behavioral, physiological, or genetic adaptations is important for population persistence. Recent climate change predictions are conflicted on the role of cold stress in the future (Kodra et al 2011; IPCC 2013). Some models predict increases in duration and intensity of cold stress (Kodra et al 2011; Jentsch et al 2007), while others predict overall decreases in the number and duration of cold days (IPCC 2013). In either circumstance, ectotherms must deal with ever changing patterns of thermal biology. Thus, understanding both their long-term adaptation and short-term acclimation processes on a physiological and genetic scale have been of much interest to biologists. This dissertation work highlights new advances in this field, focusing on artificial selection, fitness, acclimation, and gene expression for cold tolerance in *Drosophila melanogaster*.

Insects are crop pests and disease vectors that can negatively affect many parts of the world. *Drosophila melanogaster* are an example of a cosmopolitan species that has recently expanded to inhabit a wide range of thermal regimes (David et al 2003). *D. melanogaster* also have a large body of literature on many thermal properties, fitness, and survivorship of this insect. Additionally there is a rich community of researchers with genetic tools for quantitative genetic and genomic analyses thus, enabling the mechanistic analysis of natural variation in thermal stress responses. In these organisms, and many others, cold tolerance has been quantified using a variety of techniques and metrics including chill-coma recovery time (David

et al 1988; Gibert et al. 2001; Anderson et al 2005; Rako and Hoffmann 2006; Kristensen et al 2007), acute cold survivorship (Kelty and Lee 2001; Nyamukondiwa et al 2011), chronic cold tolerance (Gibert et al 2001; Sinclair et al. 2007), acclimation (Lee et al 1987; Shintani and Ishikawa 2007; Overgaard and Sorensen 2008; Teets and Denlinger 2013), and knockdown temperatures or critical thermal temperatures (Stillman 2003; Norry et al 2007). These metrics provide an overview of the intricate response to cold stresses in ectothermic organisms.

Ectotherms can be divided into two different classes in association with their cold responses: freeze tolerant and freeze intolerant. Freeze tolerant insects are those often found in Arctic and Antarctic regions where they can endure long durations of cold and can endure ice particle formation within their cells (Danks 2000). These organisms use ice nucleating agents as well as antifreeze proteins to bind water particles and prevent extreme cellular injury during these cold stress situations (Lee 1991; Kelty and Lee 1999; Clark and Worland 2008). Freeze intolerant insects, on the other hand, cannot tolerate ice particle formation within their cells and must physiologically and behaviorally cope with cold temperatures (Bale 1993). These organisms will undergo transformations in membrane fluidity (Hazel 1995; Duman 2001; MacMillan et al 2009), enter chill-coma to slow metabolism and preserve biological function (David et al 1998; Gibert et al 2001; MacMillan and Sinclair 2010), and employ protein components such as heat shock proteins, which help to maintain the integrity of proteins during extreme temperature changes (Kelty and Lee 2001; Feder and Hofmann 1999; Sinclair et al 2003). These organisms will die at temperatures much warmer than their super-cooling point, the point where water in their cells freeze (Ring 1982; Sinclair et al 2003), and thus must change their physiology and behavior in order to survive low temperature.

The role of these physiological changes for cold stress resistance is frequently debated. The maintenance of the cellular membrane from fluid to crystallization stages is critical in maintaining biological functions (Quinn 1985; Denlinger and Lee 2010; Goto and Katagiri 2011), including ionic balance (MacMillan and Sinclair 2010). Regulating cellular injury through appropriate mechanisms like heat shock proteins has also been often studied and reviewed (Goto 2000; Qin et al 2005; Sinclair et al. 2007; Clark and Worland 2008). However, these processes have not proven to be the rule and many exceptions have been found in nature (Kelty and Lee 2001; Chown and Nicholson 2004; Overgaard et al. 2005; Teets et al 2012). Therefore, understanding the unique variation in physiological traits is important for understanding the adaptive and evolutionary nature of the cold stress response.

In turn, we strive to understand the genetic architecture underlying cold tolerance adaptation, as natural genetic variation will drive the overarching adaptive response to perturbations in climate and survivorship under extreme temperature variation. Using artificially selected populations is one avenue through which we can understand genetic changes over time (Goto 2000; Qin et al 2005; Sinclair et al 2007; Ragland et al 2011; Teets and Denlinger 2013). Selecting on fitness and cold response traits has proven successful in *D. melanogaster* and several populations have been studied for their response to selection pressures. Similarly, variation in nature is key to further understanding adaptive potential of these populations and studies examining clinal variation in cold tolerance and acclimation ability provide clear examples of the genetic and adaptive variation in latitude and altitude (Gibert and Huey 2001; Hoffmann et al. 2002; Ayrinhac et al. 2004; Rako et al 2007; Fallis et al. 2011).

Several studies have sought to quantify the genetic architecture influencing cold tolerance and many candidate genes have been found to influence cold tolerance. Some favorite genes of

interest include *Frost* (Sinclair et al 2005, 2007; Bing et al 2012), *Smp30* (Clowers et al. 2010), *Hsp70* (Goto 2001; Anderson et al. 2003; Overgaard et al 2005; Sinclair et al 2007), and *couchpotato* (Schmidt et al 2008). These analyses have focused on quantifying these genes in different populations and focusing on the gene expression of this group of candidate genes. Other work has sought to identify regions of the genome involved in cold tolerance using quantitative trait loci (QTL) mapping (Norry et al 2004; Morgan and Mackay 2006; Svetec et al 2011; Fallis et al *in review*). These studies have often lacked the fine-mapping techniques but have yielded promising genomic regions to identify genes involved in cold tolerance evolution.

In addition to cold tolerance in the form of acute or chronic cold shock, much work has been done focusing on the acclimation affect in insects. This phenomenon is described as a general increase in survivorship following a pretreatment at a non-lethal mild temperature prior to cold shock at a more extreme temperature (Lee et al 1987; Teets and Denlinger 2013). Quantifying this variation in nature is important to understanding phenotypic plasticity of cold tolerance responses and the evolution of populations in response to temperature changes. Plasticity scores, or a quantified ability to respond to pretreatment, can be further defined in cold tolerance as the increase in proportion of survivorship from being pretreated and non-pretreated. This score or value is hypothesized to be a quantitative trait and natural variation in plasticity is hypothesized to shield specific genotypes from selection pressures of predicted temperature changes. From an ecological standpoint, clinal variation can also lead to differences in plasticity ability and quantifying the genetics of this response can also provide a better understanding of the genetic underlying of plasticity and evolvability (Scheiner 1993; Via et al 1995).

Trade-offs in cold tolerance ability among metrics such as plasticity and acute cold tolerance can also be investigated from a population-wide perspective, as many environmental

regimes may not promote maintenance of one particular cold tolerance component over another. For example, in environments with large daily thermal fluctuations, the maintenance of phenotypic plasticity will be favored instead of maintenance of increased survivorship to cold shock. This plasticity ability has been shown in several organisms to share a negative trade-off with basal survivorship (Kellett et al 2005; Nyamukondiwa et al 2011; Foray et al 2013), suggesting that the environmental influence on groups of organisms is critical for understanding the genetic adaptation components as well as the physiological responses to environmental changes and evolution of these traits. As a whole, understanding the genotype-phenotype response to stress of any kind is important for predictability to environmental stressors and evolution of populations (Mackay et al 2009), and this dissertation work improves our understanding of the evolution of cold stress genetics using various metrics of tolerance.

In the second chapter of the dissertation, we describe the response to selection on a non-lethal cold tolerance trait and the cross-tolerances to other stress traits of interest. Understanding these associations can lead to a better understanding of the associations between stress genes and genes associated with distinct selective pressures. It can also help us understand heritability of variation in stress responses and the evolvability of these environmentally influenced traits.

The third chapter discusses gene expression variation in artificially selected lines and allows us to quantify the physiologically critical point at which the environmental influence pushes genotypes to different phenotypic levels. For these analyses we examined our selection lines at three time points (a baseline, exposure to cold stress, and recovery from cold stress) in order to understand the changes in gene expression among differentially selected populations. These analyses will shed light on the three hypotheses describing selection to cold tolerance: Do gene expression changes occur 1) in the baseline expression of the genotype without stress

induction; 2) during cold stress exposure as a maintenance response; or 3) in the recovery phase, as a mechanism to regain physiological and biological functions? Differences in functional gene enrichment among the selection lines will also provide an analysis of what biological and molecular functions are needed to maintain an increased tolerance to cold.

The fourth chapter of the dissertation is an examination of the lifetime fitness and demographic parameters in artificially selected lines. In this chapter we ask whether the more susceptible genotypes also experience a reduction in reproductive success and Darwinian fitness components associated with their decreased ability to cope with cold stress. We test the selection lines across a range of five temperatures in order to better understand the full ability of these genotypes across thermal variation and to determine if differences in reproduction manifest themselves across different environmental pressures.

The fifth chapter uses a community resource of naturally segregating populations called the *Drosophila melanogaster* Genetic Reference Panel (DGRP; Mackay et al 2012), to understand the genetic differences between long-term and short-term acclimation. Through association-mapping analyses we can distinguish differences in the genetic architecture between these acclimation traits and work to understand population variation in these plasticity traits of interest. We can also understand the functional genes associated with these phenotypes and test several candidate genes to confirm their association with acclimation pretreatments.

The sixth chapter uses a subset of the DGRP further to understand variation in fitness components among these genotypes in association with plasticity ability. We use plasticity ability calculated under a constant environmental rearing condition and compare the fitness ability at two daily fluctuating rearing environments. We hypothesize that plasticity should correlate with fluctuating fitness components due to the ability to use pretreatment variation

being linked to fitness ability in a fluctuating environmental context. In other words, fitness in fluctuating environments should reflect plasticity ability in a constant environment, i.e. plasticity in fitness and survivorship should be related across genotypes.

This dissertation attempts to understand the intricate details between multiple stress metrics, fitness, fluctuating environments, and the genetics of naturally varying adaptive thermal traits. Through these experiments we provide new evidence of genetic associations of stress tolerance and new genes involved in these selective traits. We make an effort to link our results from a genetic level with physiological and biological interpretations to understand the whole-organism response and form a predictability frame-work for further research on genotype-phenotype interactions.

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Chapter 2 - Artificial selection on chill-coma recovery time in *Drosophila melanogaster*: Direct and correlated responses to selection

Alison R. Gerken, Trudy F.C. Mackay, and Theodore J. Morgan

Alison R. Gerken

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: aegge@ksu.edu

Trudy F.C. Mackay

Department of Biological Sciences, North Carolina State University Raleigh, NC 27695. email: trudy_mackay@ncsu.edu

Theodore J. Morgan

Corresponding author. Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: tjmorgan@ksu.edu

Abstract

Artificial selection is a process used to create populations with extreme phenotypic responses to environmental stressors. When artificial selection is applied to a single component of a stress response, this selection can have linkages with other stress responses, a phenomenon called cross-tolerance, which is ultimately controlled by genetic correlations among traits. We selected for extreme responses to cold tolerance using chill-coma recovery time in *Drosophila melanogaster*. Chill-coma recovery time is a common metric of low temperature tolerance to a cold, but non-lethal, temperature. Artificial selection was applied to a genetically variable base population for 31 generations, thus resulting in two replicate cold resistant lines, two replicate

cold susceptible lines, and two random bred control lines. To quantify the relationship between selection on chill-coma recovery and other metrics of thermal performance, we measured cross-tolerances to survivorship after acute cold exposure, survivorship after chronic cold exposure, survivorship after cold exposure following a pre-treatment period (rapid cold hardening), desiccation tolerance, starvation tolerance, and heat tolerance. Surprisingly, we found no cross-tolerances between selection on chill-coma recovery time and any of the environmental stress response traits. These results suggest that although artificial selection has dramatically altered chill-coma recovery time the correlated response to selection on other stress response phenotypes has been negligible. The lack of a correlated response suggests that chill-coma recovery time is likely genetically independent from the other environmental stress phenotypes examined here.

INTRODUCTION

Daily and seasonal variation in climate, including temperature, provides potentially stressful environmental variation in which organisms must respond (Koehn and Bayne 1989; Sibly and Calow 1989; Harshamn et al. 1999; Pidwirny et al. 2006). For ectotherms in particular, extremes in temperature directly influence most biochemical and physiological functions of the organism (Overgaard and Sørensen 2008), inducing responses in physiological processes required to maintain an individual's fitness (Hoffmann and Parsons 1991; Brakefield 2003). Low temperature is a particularly damaging stress for an organism as these temperatures will damage cell membranes and decrease overall cellular and physiological function and survivorship (Hazel 1995; Overgaard et al. 2006; Rajamohan and Sinclair 2008).

Improving tolerance to cold stress has been a key component of physiological research both on short-term and long-term time scales (Lee et al 1987; Powell and Bale 2006; Nyamukondiwa et al. 2011). General processes that may increase survivorship after cold stresses include within-generation *acclimation* responses caused via thermal pre-exposures to stress (Lee et al. 1987; Rajamohan and Sinclair 2008) or the across-generation genetic *adaptation* via shifts in allele frequency caused by the differential survivorship of natural genotypes after a stress exposure. These long-term and short-term cold tolerance mechanisms may be closely related physiologically or genetically and selection pressures on a single component of cold tolerance may also select (via genetic correlations) for other fitness or stress survivorship traits (Hoffmann and Parsons 1989; Leips and Mackay 2000; Gibert et al. 2001). Using artificial selection it is possible to change the allele frequencies in genomic regions associated with a particular trait as well as determine the correlations among the trait under selection, cold tolerance, and

physiologically related environmental stress responses (Service 1987; Harshman et al. 1999; Overgaard and Sørensen 2008; MacMillan et al. 2009; Koštál et al. 2012; Mackay et al. 2012).

Cross-tolerance occurs when selection to a given environmental stressor results in a correlated response to tolerance in another environmental stressor (MacMillan et al. 2009; Harshman and Hoffmann 2000). It has been demonstrated in a variety of organisms, and is thought to be especially prevalent in insects, where tolerance to a single extreme environment may correlate with a change in tolerance to other environmental stresses (Ring and Danks 1994; Bayley et al. 2001; Vermeulen and Loeschcke 2007), although these cross tolerances are inconsistent across all insects (Phelan et al 2003; Anderson et al 2005; Bubliy and Loeschcke 2005; Kristensen et al 2007; Norry et al 2007; MacMillan et al. 2009). MacMillan et al. (2009) also hypothesized that selection may have a directionality of correlation and cross-tolerance to other traits. For example, such asymmetric correlations have been observed when an increase cold tolerance is positively correlated to an increase in heat tolerance but tolerance to heat did not correlate to tolerance to cold (Harshman et al 1999; Bubliy and Loeschcke 2005; MacMillan et al. 2009).

Various cross-tolerance studies have examined correlations between stress metrics such as cold tolerance and starvation resistance (Bubliy and Loeschcke 2005; Sinclair et al. 2007a), how cold selection affects heat tolerance capacity (Anderson et al 2005; Kristensen et al 2007), starvation resistance and metabolic mechanisms (Harshman et al 1999), and cold survivorship, desiccation resistance, and body size (Benoit et al 2009; MacMillan et al 2009; Jumbo-Lucioni et al 2010). The goal in quantifying stress related trait correlations is to identify linkages among genetic components associated with many traits and determine the capacity of selection on one trait to constrain or promote phenotypic evolution of another (Brakefield 2003; Bubliy and

Loeschcke 2005; MacMillan et al 2009). As many organisms have shown a wide range of cross-tolerances, several hypotheses have emerged concerning the linkage of genomic regions among various stress responses. A lack of correlations between responses to various environmental stresses may suggest that there is no linkage between tolerances to such stresses—all stress responses are genetically independent of one another (Harshman and Hoffmann 2000; Sinclair et al. 2007a) or that the experiment is too small to detect a weak correlation. Cross-tolerances may also gradually weaken over time as fitness trade-offs occur or spurious associations (i.e., transient LD) are broken down (Phelan et al 2003). However, strong cross-tolerances are likely to occur when the underlying quantitative trait loci controlling each environmental stress trait are shared (Bubliy and Loeshcke 2005; Norry et al 2007).

Previous work on physiological cross-tolerances has used many insect systems including *Drosophila melanogaster* (Hoffmann and Parsons 1989; Chen et al 1991; Bubliy and Loeschcke 2005; Benoit et al 2009). *D. melanogaster* are historically found in tropical climates (David and Capy 1988) and have successfully adapted and evolved to become a cosmopolitan species and inhabit many temperate and sub-tropical regions of the globe (David and Capy 1988; Overgaard and Sørensen 2008). The ability of this particular model organism to be laboratory reared and artificially selected provides a strong base for testing cross-tolerance as well (Hoffman and Parsons 1989; MacMillan et al 2009). Because *Drosophila* are such a widely distributed species, laboratory induced patterns of resistance to stress can be translated to natural populations inhabiting different areas of the globe; for example, *Drosophila* species in the tropics showed a lower resistance to desiccation and cold tolerance as compared to those at higher latitudes (Kellermann et al. 2009).

In this study we specifically examine the cross-tolerances between chill-coma recovery and six other environmental stress responses. Chill-coma recovery is a physiological response to non-lethal cold temperatures (David 1988) that is a genetically variable and adaptive phenotype that exhibiting significant clinal variation on multiple continents (Gibert et al. 2002; Rako and Hoffmann 2005; Kristensen et al 2007) as well as strong habitat associations among species (Gibert et al 2001). Starting with a genetically variable base population, we artificially selected two cold resistant (i.e., fast chill-coma recovery) lines, two cold susceptive (i.e., slow chill-coma recovery) lines, and two random bred control lines for 31 generations. At the end of this selection, six environmental stress responses were measured on this set of lines to examine the cross-tolerance to chill-coma recovery. These stress responses included acute cold exposure (-6° C for one hour), chronic cold exposure (0° C for 16 hours), a rapid cold hardening pre-treatment followed by cold exposure (two hours at 4° C followed by one hour at -6° C), starvation tolerance, desiccation tolerance, and survival after heat stress (38° C for one hour). We predict that selection for increased resistance to cold stress via selection on chill-coma recovery will result in an increased ability to survive this set of environmental stressors while lines selected for a susceptibility to cold tolerance will have decreased survivorship to other environmental stressors.

METHODS

Experimental Flies and The Chill-Coma Recovery Time Assay

Mated *D. melanogaster* females were collected from the Raleigh, NC Farmer's Market using fruit bait and used to establish a population of 60 isofemale lines. This sample of isofemale lines was used to establish a genetically variable base population, by crossing the lines in a round

robin design in separate culture vials, with four males and four females per vial. Three days after the crosses were initiated a single inseminated female from each cross was placed in each of two culture bottles to initiate two replicate base populations. The progeny from these culture bottles were designated generation 0. To begin the first generation upon which selection was applied, approximately 20 virgin individuals per sex per replicate base population were scored for cold tolerance and were used to initiate the three selection regimes (resistant, susceptible, and control) in each base population.

All of the individuals screened in this study were young flies (i.e., 5-7 days old), as previous studies have found that chill-coma recovery time changes as a function of age (David et al 1998). Individuals were maintained at 25^{o} C and 60% humidity and a 12:12h light/dark cycle until they were used in the experimental assay. We scored cold tolerance every generation, rather than every other generation as we have been unable to detect any evidence of 'carry-over' effects, detrimental influence of the cold treatment on future success, (Anderson 2005; Harrison et al 2011) for cold tolerance (data not shown). However, if 'carry over' effects do actually exist within these or other populations the result of these effects will be the underestimation of the genetic variation in the trait rather than the overestimation. Thus our result should be considered either an exact or slight underestimation of the genetic variation influencing chill-coma recovery time and its correlated traits.

To measure chill-coma recovery time, the trait upon which selection was applied, we utilized the assay presented in Morgan and Mackay (2006). Specifically, 50 same sex individuals were transferred without anesthesia into empty vials and placed in chambers containing melting ice (0^{o} C ± 0.5) for 3 hours. After 3 hours, individuals were removed from the cold treatment and returned to room temperature (23^{o} C ± 0.5), and the chill-coma recovery time was measured by

recording the amount of time until an individual was able to stand on its legs.

Artificial Selection Regime

Beginning at generation 1, 50 virgin male and female progeny were collected from each of the six lines every generation. These individuals were assayed for chill-coma recovery time as described above. In the replicate resistant lines the 20 males and females with the fastest recovery time were used as the breeders for the next generation, while in the replicate susceptible lines the 20 males and females with the slowest recovery time were used as the breeders for the next generation, and in the control lines 20 males and females were randomly chosen with respect to chill-coma recovery time and used as the breeders for the next generation. Individuals that did not recover from chill-coma in the 90-minute observation period were given a score of 90 minutes.

Correlated Responses to Selection

Six environmental stress response phenotypes were measured to test for the presence of correlated responses to selection. These include chronic cold exposure, acute cold exposure, acclimated cold exposure, desiccation tolerance, starvation resistance, and survival after heat stress.

Chronic Cold Exposure

Chronic cold exposure was performed by exposing the six selection lines to 0° C for 16 hours in order to simulate a long-term exposure to freezing temperatures (Gibert et al 2001; Sinclair et al. 2007a). Each line was tested separately for males and females, with 10 individuals

per vial (n=3 vials per sex/line). Flies were placed in empty vials during cold exposure to avoid becoming stuck in the food during the treatment; after the 16 hour exposure period, flies were placed in vials containing medium on which they were allowed to recover for 24 hours at 25° C. Survivorship was assessed after 24 hours as the ability to stand or right oneself after a physical knockdown and was recorded as a proportion of dead per vial (Kelty and Lee 2001; Overgaard et al 2005; MacMillan 2009; Colinet and Hoffmann 2010).

Acute Cold Exposure

Acute cold exposure was assayed on the selection lines as a percentage of dead flies after an exposure of flies to -6° C for one hour (Kelty and Lee 2001). Flies were sorted into vials with 10 individuals per vial separated by sex (n=3 vials per sex/line). Before exposure to cold, flies were transferred to empty vials in order to avoid becoming stuck in the food during the treatment and are placed directly into -6° C (Kelty and Lee 2001; Nyamukondiwa et al 2011). After one hour at this temperature, flies are removed and placed back on food and allowed to recover for 24 hours. After 24 hours, survivorship was recorded as the ability to right after physical knockdown (Kelty and Lee 2001; Overgaard et al 2005; Colinet and Hoffmann 2010).

Pre-treatment to Cold Exposure

A rapid-cold hardening response (RCH) has been shown to occur in field studies of insects and is characterized by an increase in survivorship following a pre-treatment exposure before a cold shock (Lee et al. 1987; Leips and Mackay 2000; Overgaard and Sørensen 2008; Nyamukondiwa et al. 2011). A RCH response was assessed via survivorship following a pre-treatment exposure at a mild temperature for minutes to hours two hours followed by an acute

cold shock (Overgaard et al. 2005; MacMillan et al. 2009; Nyamukondiwa et al. 2011). Flies were separated into vials with 10 individuals by sex (n=3 vials per sex/line). Prior to cold exposure, flies are transferred into empty vials as per the previous cold treatments. The flies were then placed in a cold room for exposure to 4° C for two hours. After two hours, flies are placed directly into -6° C for one hour. Following the one hour exposure, flies were transferred to food vials and allowed to recover for 24 hours at 25° C; survivorship was assessed as the ability to right oneself after physical knockdown (Kelty and Lee 2001; Overgaard et al 2005; Colinet and Hoffmann 2010) and will be represented by the percentage of dead flies per vial.

Desiccation Tolerance

Desiccation tolerance was measured in the selection lines by assessing survivorship of flies over time without food or ingestible water. Flies were separated with 10 individuals per sex (n=3 vials per sex/line). At the start of the experiment flies were transferred directly into clean, empty vials and placed at 25° C. Every four hours vials were assessed for mortality, measured as inability to right oneself, until all flies were dead (Gefen et al 2006; MacMillan et al 2009). Each individual received a time until death score in hours without food or moisture.

Starvation Tolerance

Starvation tolerance was measured in the selection lines by utilizing starvation vials (1% agar medium in each vial) with no nutritional value but allowing the flies to obtain ingestible moisture (Harshman et al 1999; Bubliy and Loeschcke 2005; MacMillan et al 2009). Flies were separated by sex and line (n=3 vials per sex/line). At the beginning of the experiment, flies were transferred to vials containing the starvation medium. Flies were kept at 25° C and were counted

every four hours for dead flies until all flies had died (Sinclair et al. 2007a). Each individual received a time until death score in hours without food.

Survival after Heat Stress

Survival after heat stress was measured on all selection lines by exposure to 38° C for one hour. Flies were separated into vials of 10 individuals per sex (n=3 vials per sex/line). Prior to heat exposure, flies were transferred into empty vials in order to avoid becoming stuck in the food during the treatment. Flies were then placed at 38° C for one hour (Overgaard and Sørensen 2008). After one hour, flies were placed on fresh food medium and allowed to recover for 24 hours before survivorship was assessed. Survivorship was recorded as the ability to right oneself and was recorded as a percentage of dead individuals per vial (Kelty and Lee 2001; Overgaard et al 2005; Colinet and Hoffmann 2010).

Analyses

Quantitative Genetic Analysis of the Selection Response

Estimates of the realized heritabilities for chill-coma recovery time were computed for each of the replicate susceptible and resistant selection lines by regression of the cumulative selection response (as a deviation from the control) on the cumulative selection differential based on the data from generation 0 to 31 (Falconer and Mackay 1996).

At generation 31 or the generations when the correlated traits were assayed, we used a nested model analysis of variance (ANOVA) to compare the effect of selection regime on chill-coma recovery time, chronic cold exposure, acute cold exposure, acclimated cold exposure, desiccation tolerance, starvation resistance, and survival after heat stress. Each of these

phenotypes was analyzed using the following mixed model:

$$y = \mu + Sel + Line(Sel) + Sex + Sel \times Sex + Line(Sel) \times Sex + \varepsilon$$

Where y is the phenotype, *Sel* is the fixed effect of selection regime (susceptible, control, or resistant), *Line*(*Sel*) is the random effect of replicate line nested within selection regime (replicate population 1 or 2), *Sex* is the fixed effect of sex, and ε is the residual variance. The primary terms of interest in the analysis are the *Sel*, *Line*(*Sel*), *Sel* x *Sex*, *Line*(*Sel*) x *Sex* as these reflect general or sex-specific genetic differences as a result of selection (significant differences among the selection regimes) or random genetic drift (significant differences among lines within each selection group).

Analysis of Correlated Responses to Selection

For the correlated trait analyses, we used the same model as above on three separate data sets per trait: 1. all three selection regimes; 2. the control vs. the cold resistant selection regime; and 3. the control vs. the cold susceptible selection regime. Each of these analyses was performed to explore the possibility of symmetric and asymmetric correlated responses to selection. That is, symmetric correlated responses can occur in both selection groups relative to the control lines when the direct response to selection is the result of the evolution of *different alleles at the same loci* in both selection regimes. However, an asymmetric correlated response will occur when the direct selection response has occurred because of *responses at different loci in each direct selection regime*. Thus, the correlated responses caused by pleiotropy at different loci under direct selection will result in an asymmetric correlated response to selection.

ANOVAs provide a baseline comparison of treatment and selection interaction effects while correlations between stress tolerance assays were conducted using Pearson's product-

moment correlations. Correlations were performed to compare chill-coma recover to acute cold, chronic cold, desiccation, starvation, heat, and RCH exposures. We also tested correlations with and without the control lines in order to test selection lines independently. If correlations are significant we can conclude on a cross-tolerance effect of selection on chill-coma recovery and the other environmental stress. Directionality of correlations indicate if the cross-tolerance is a trade-off (negative) or a true cross-tolerance effect (positive). All analyses were performed using R statistical software (version 2.13).

RESULTS

Direct Selection Response

The result of 31 generations of replicated selection for cold susceptibility and cold resistance is shown in Figure 2.1A. There were no significant differences in mean chill-coma recovery time among the three selection groups at generation 1 ($F_{2,3}$ = 2.43 P = 0.2356). The selection response was highly asymmetrical in the direction of cold susceptibility. The first consistent significant difference between the susceptible and resistant lines was measured at generation 6 ($F_{2,3}$ = 15.44 P = 0.063) and at generations 30 and 31 there is a highly significant divergence among the all of the selection groups (Table 2.1; Gen30: $F_{2,3}$= 25.34, P = 0.0132; Gen 31: $F_{2,3}$= 22.88, P = 0.0153). At generation 31 the resistant lines recovered on average after 7 minutes 45 seconds (± 48 seconds), the susceptible lines recovered on average after 60 minutes 8 seconds (± 51 seconds), while the random bred controls recovered on average after 15 minutes 8 seconds (± 50 seconds).

Realized heritabilities (h^2) of chill-coma recovery time were computed from the regressions of the cumulative selection response (ΣR) on the cumulative selection differential

(ΣS) for each selection line (Fig. 1B) as well as for the divergence between selection lines within each replicate base population (Falconer and Mackay 1996). Estimate of h^2 (± SE of the regression coefficient) were h^2 = 0.046 (± 0.019; P = 0.0268) and h^2 = 0.103 (± 0.025; P = 0.0003) for replicate 1 and 2 in the resistant lines, respectively. Estimates of h^2 for replicate 1 and 2 of the susceptible lines, respectively, were h^2 = 0.128 (± 0.009; P < 0.0001) and h^2 = 0.066 (± 0.008; P < 0.0001). Heritabilities estimated from the divergence were h^2 = 0.119 (± 0.008; P < 0.0001) and h^2 = 0.070 (± 0.007; P < 0.0001).

Correlated Responses in Environmental Stressors

Variation among lines

Acute cold tolerance did not have a significant difference among selection regimes ($F_{2,3}$= 0.10, P = 0.9064; Figure 2.2A; Table 2.2). The resistant selection regime had an average proportion dead of 0.53 ± 0.42, the susceptible selection had an average of 0.51 ± 0.45, and the control populations had an average of 0.54 ± 0.39. Chronic cold tolerance did not have a significant difference among selection regimes ($F_{2,3}$= 0.76, P = 0.5406; Figure 2.2B). The resistant selection regime had an average proportion dead of 0.52 ± 0.25, the susceptible selection had an average of 0.39 ± 0.24, and the control populations had an average of 0.51 ± 0.27. Starvation time to death had nearly significant differences among selection regimes ($F_{2,3}$= 6.47, P = 0.0816; Figure 2.2C) and showed significant differences in responses for all comparisons (resistant to control, p<0.0001; resistant to susceptible, p<0.0001; susceptible to control, p<0.0001). However, an examination of the means of the selection regimes reveals that both the resistant and susceptible lines have means less than the control lines. The control populations had an average of 68.83 ± 20.49 hours, while the resistant selection regime had an

average time to death of 63.97 ± 17.57 hours, the susceptible selection had an average of 59.13 ± 16.32 hours. Thus, this significant effect of selection is likely caused by inbreeding during the selection process and not a correlated response to selection.

Desiccation had a significant difference among selection regimes in time to death ($F_{2,3}$= 13.96, P = 0.0299; Figure 2.2D). However, similar to starvation, both the resistant lines and the susceptible lines significantly differed from the control lines (P<0.0001, for both), while the resistant and susceptible lines did not significantly differ from one another (P=0.9191). The resistant selection regime had an average time to death of 31.03 ± 11.91 hours, the susceptible selection had an average of 31.46 ± 10.32 hours, and the control populations had an average of 38.05 ± 10.32 hours. Thus, this effect of selection is again likely to be driven by inbreeding. The rapid cold hardening response did not differ among selection regimes ($F_{2,3}$= 0.32, P = 0.7502; Figure 2.2E). The resistant selection regime had an average proportion dead of 0.06 ± 0.10, the susceptible selection had an average of 0.06 ± 0.12, and the control populations had an average of 0.09 ± 0.14. Response to heat stress did not differ among selection regimes ($F_{2,3}$= 0.46, P = 0.6675; Figure 2.2F). The resistant selection regime had an average survival after heat stress of 0.88 ± 0.17, the susceptible selection had an average of 0.77 ± 0.33, and the control populations had an average of 0.72 ± 0.29.

Phenotypic correlations among stress response phenotypes

Pearson's product moment correlations revealed that none of the responses to environmental stressors correlated with chill-coma recovery time, the metric of selection in our experiment (Figure 2.4; Table 2.2). Chill-coma recovery time and chronic cold tolerance had a negative but non-significant correlational trend (r = -0.60, P=0.20; Figure 2.3A). Chill-coma recovery time

and desiccation resistance and starvation resistance individually also both had a negative, non-significant trend (Desiccation: r = -0.22, *P*=0.66; Figure 2.3B; Starvation: r = -0.59, *P*=0.20; Figure 2.3C). Survival after heat stress and chill-coma recovery also had a slightly negative trend which was non-significant (r = 0.12, *P*=0.81; Figure 2.3D). Acute cold tolerance and rapid cold hardening to chill-coma recovery also had non-significant trends where acute cold response was positive (r = 0.20, *P*=0.69; Figure 2.3E) and the rapid cold hardening response was negative but non-significant (r = -0.18, *P*=0.72; Figure 2.3F).

We also tested correlations between chill-coma recovery time and the other stressors by dropping out the lines that were not subjected to selection pressure (i.e. the control lines) to determine if these non-selected lines were causing the correlations to be non-significant; even without the control lines, correlations between chill-coma recovery time and the environmental stressors tested were not significant (Table 2.2). Comparing chill-coma recovery and chronic cold tolerance had a negative but non-significant correlational trend (r = -0.57, *P*=0.42,). Chill-coma recovery time compared to desiccation resistance and starvation resistance individually also both had non-significant trends but desiccation resistance was positive (r = 0.65, *P*=0.34) and starvation resistance was negative (r = -0.58, *P*=0.41). Survival after heat stress and chill-coma recovery had a negative trend, which was non-significant (r = -0.25, *P*=0.74). While acute cold tolerance and the rapid cold hardening response compared to chill-coma recovery time was also non-significant. Acute cold response was positive (r = 0.23, *P*=0.76) and the rapid cold hardening response was negative (r = -0.06; *P*=0.93).

DISCUSSION

Artificial selection is a powerful tool used to shift the allele frequencies in a population. Such selection typically results in populations with trait values that lie on extreme ends of the phenotypic distribution. In our experiment, we were able to successfully select *Drosophila melanogaster* with increased and decreased cold tolerance, as measured by chill-coma recovery (Figure 2.1; Table 2.1). Chill-coma recovery is an ecologically relevant and adaptive trait in *D. melanogaster* as it enables the fly to cope with prolonged periods at non-lethal cold temperatures (David et al 1998; Kelty 2007). The selection procedure developed replicate sets of lines that were both susceptible and resistant to chill-coma recovery time and dramatically different between the selection regimes. The response to selection was more dramatic in the direction of susceptibility than resistance, potentially reflecting an increased number of genotypic possibilities that can make chill-coma recovery time worse (i.e., slow recovery) than will make chill-coma recovery better (i.e. rapid recovery). This is consistent with asymmetric responses to selection on other traits that are closely associated with fitness (Mackay et al. 2005; Edwards et al. 2006). From the selection response we were also able to estimate the realized heritability for chill-coma recovery by regressing the cumulative selection response (ΣR) on the cumulative selection differential (ΣS) for each replicate pair of selection lines (Fig. 2.1B). These heritability estimates were low and consistent with those estimated for most physiological or behavioral traits (Mousseau and Roff 1987; Roff 1997). Our estimates are dramatically lower than those estimated by Hoffmann et al. (2005) and Morgan and Mackay (2006). This is however to be expected because both of these studies estimated heritability for chill-coma recovery time using admixed study populations, thus likely causing an inflation in the level of transient linkage disequilibrium and increasing the estimated heritability.

Using these lines we have the potential to look at costs and benefits of this artificial selection response on an ecologically important trait (Lande 1979; Chown et al. 2009). One way to look at these costs and benefits is to explore cross-tolerance or correlative responses to other environmental stressors. Phenotypic responses to stressful environments are thought to be linked to one another via both physiological and genetic mechanisms (Hoffmann and Parsons 1989; MacMillan et al 2009). A successful response (i.e. increased survivorship) to one trait may mean that the organism or population will be successful in responding to another environmental stressor (e.g. starvation or desiccation). However, in our experiment we found that this was not the case. The environmental stress responses tested—acute and chronic cold exposure, cold exposure following a RCH pretreatment, starvation, desiccation, and heat exposure—did not show any significant correlations with chill-coma recovery, the trait upon which selection was applied (Table 2.2 ; Figure 2.4). In this case it is likely then that the underlying genomic regions responding to these various environmental stressors are different from one another (Brakefield 2003; Bubliy and Loeschcke 2005) and thus, selecting for resistance to cold stress via changes in chill-coma did not result in a correlated response to selection in other survival responses. It is also possible that the lack of a significant correlated response to selection is driven by our limited experimental design (six total lines), thus reducing the power to detect genetic correlations in a small sample of natural genetic variation.

The phenotypes measured in the selection lines in response to different environmental stressors exhibited large variation, but exhibited few consistent trends. However, some patterns do emerge in how *D. melanogaster* act as a whole to different environmental stressors. For acute cold stress, lines behave the most similarly with no significant differences among the selected lines (Figure 2.3). For chronic cold there is some variation among the lines with no patterns

distinguishable for the selection regime of chill-coma recovery time. When comparing starvation and desiccation times until death, desiccation has a shorter overall time to death, demonstrating that provided with a source of water, flies can survive environments with uncertain food resources for longer than if deprived of both food and water. As expected, all selection lines showed an overall increase in survivorship following a RCH pre-treatment before acute cold as compared to acute cold survivorship. This acclimation phenomenon is commonly observed in insects and our experimental lines are no exception (Lee et al 1987). Interestingly, it also appears that the line with the highest mean percentage dead after acute cold exposure (susceptible line 2) experiences the greatest magnitude of decrease in percentage dead after an RCH pretreatment. However, this trend does not follow with the other selection lines and we cannot conclude on any trends in magnitude for the RCH responses compared to acute cold treatment.

Chill-coma is a physiological response to a non-lethal temperature exposure for a moderate amount of time while the other environmental traits we tested were either at lethal temperatures or extended exposures to adverse conditions. As shown by the lack of cross-tolerance among the environmental stressors and chill-coma recovery time, we can further hypothesize that recovery time and survival mechanisms are not linked for this set of environmental stressors. Often traits under selection pressure must compromise other phenotypic responses in order to successfully adapt to a given selection pressure (Brakefield 2003). Although stress overall was tested in this experiment, survivorship and recovery time are two different aspects of an organism's overall fitness and selecting on one metric (chill-coma recovery time) did not lead to any cross-tolerances with our measures of survivorship. By testing these genotypically different lines we are left to conclude that there is limited cross-tolerance among these traits, however it should be noted that these conclusions are based on a limited set

of the total natural genetic variation (Service 1987; Hoffmann and Parsons 1989; Leips and Mackay 2000; Gibert et al. 2001; Jumbo-Lucioni et al. 2010; Mackay et al. 2012).

The underlying genetic make-up of an organism constrains the extent of adaptive evolution (Jumbo-Lucioni et al 2010) and understanding this genetic variation is vital to understanding the evolution of thermal adaptation and plasticity in nature (Falconer and Mackay 1996; Ghalambor et al 2007; Denlinger and Lee 2010). In concert with this underlying genetic variation, the environment drives adaptive evolution of genotypes in response to stress (Harshman et al. 1999). Utilizing artificial selection to genetically differentiate loci affecting one trait, we can begin to associate the underlying genetic mechanisms to relative physiological responses of organisms in nature (Service 1987; Gabriel 2005). By combining such selection pressures with ecologically relevant experiments on cross-tolerance, we can begin to understand the interplay of genetics and physiology of how an organism will respond to environmental stressors (Brakefield 2003). The exposure of our selection lines to chill-coma recovery time demonstrates that for this population of *D. melanogaster* these environmental traits do not exhibit a significant correlation with one another, and thus will likely respond to selection in an independent manner.

Although we did not observe any cross-tolerance of chill-coma recovery time to our environmental stress responses, we must note that experiments involving so few lines have limited statistical power and thus decrease our ability to detect correlations among such stress responses. Future experiments should work to expand on the numbers of naturally varying lines to increase power of detection of potential cross-tolerance effects. One such resource would be the *Drosophila melanogaster* Genetic Reference Panel (DGRP), a newly developed community tool for the analysis of phenotypic variation and the underlying genetics of quantitative traits

(Mackay et al. 2012). The DGRP consists of 192 line derived from a single outbred population, each fully sequenced and available for experiments on various stressors (Flint and Mackay 2009). Not only will we be able to increase our power to detect potential cross-tolerance between phenotypes but we will also have access to the complex genetic basis of traits using association mapping techniques (Mackay et al 2012).

In conclusion, our lines selected for increased and decreased tolerance to cold exposure did not show any significant correlations to the other environmental stressors we tested (acute and chronic cold, rapid cold hardening pretreatment, starvation, desiccation, and heat exposure). These results suggest that these phenotypic responses are represented by different regions of the genome and that these stressors in particular do not share particular loci under selection for chill-coma recovery response time. Increasing the numbers of naturally varying lines will increase our power to detect cross-tolerances and thus, future research on these environmental stressors will include a large set of independently segregated lines.

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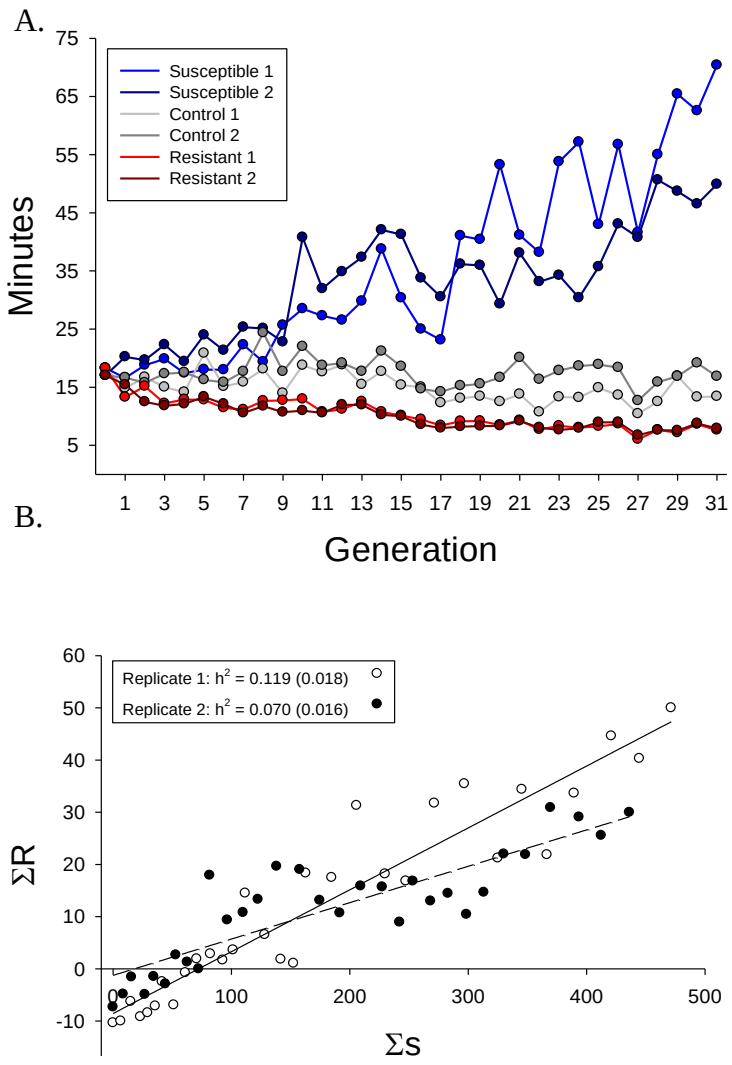

Figure 2.1. Phenotypic Response to Selection for Chill-Coma Recovery Time (A) Mean chill-coma recovery time (minutes) of the selection lines (y-axis) versus generation of selection on the x-axis. The light and dark blue lines are the replicate susceptible lines, light and dark grey lines are the replicate control lines, and light and dark red lines are the replicate resistant lines. (B) Regressions of cumulative response (y-axis) on cumulative selection differential for divergence between high and low selection lines (x-axis). Open circles and solid line are for replicate 1, while closed circles and dashed line are replicate 2.

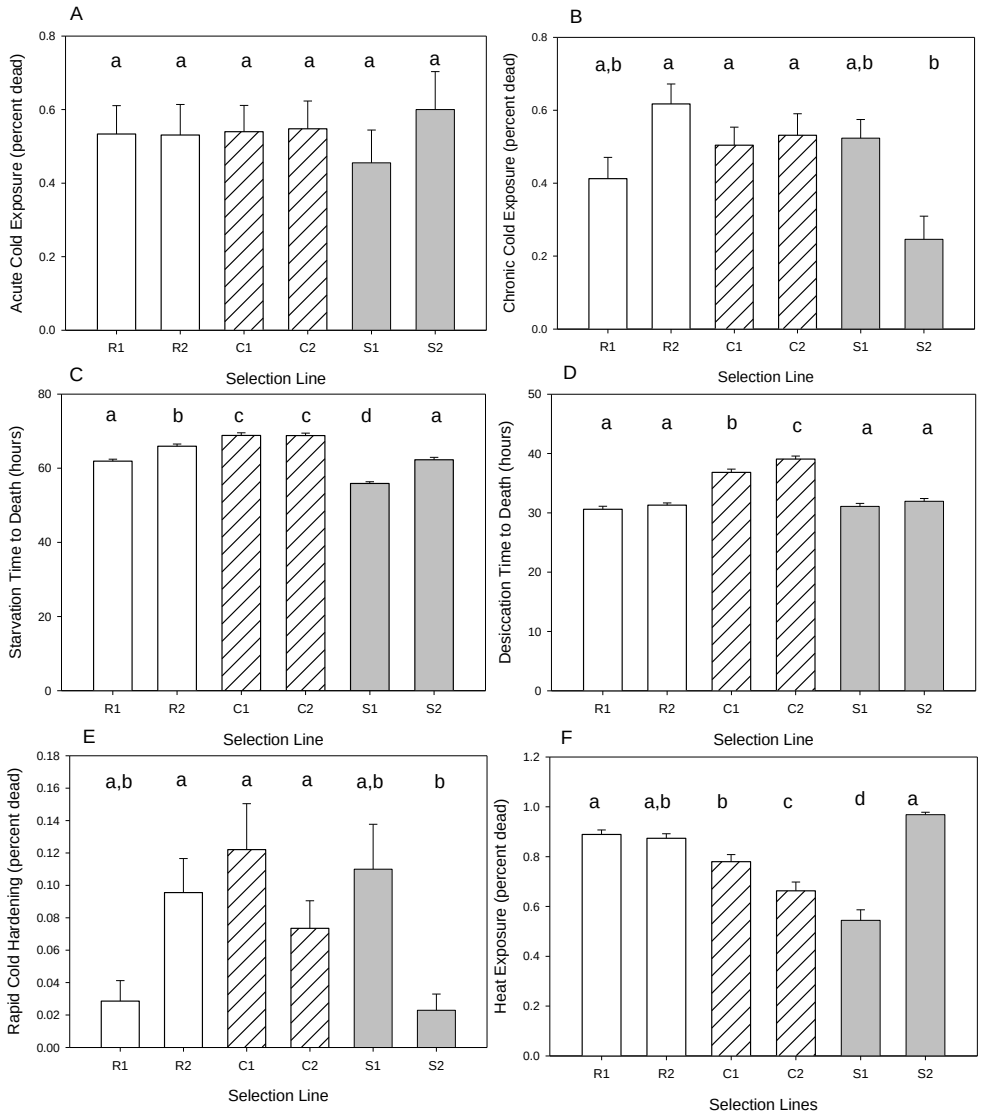

Figure 2.2. Environmental stress responses. Each stress is reported by mean of the selection line on the x-axis and the phenotypic response to stress on the y-axis. Error bars represent standard errors on the mean. Letters above bars represent significantly different means from one another based on $p<0.05$. There are no consistent significant differences among selection regimes for each of the stresses, although for starvation and desiccation differences between susceptible and resistant to control lines is observed. Selection lines are represented by replicates for resistant (R1 and R2), susceptible (S1 and S2), and control (C1 and C2). A) Acute stress survivorship. B) Chronic cold stress survivorship. C) Starvation time to death. D) Desiccation time to death. E) Rapid cold hardening survivorship. E) Heat stress survivorship.

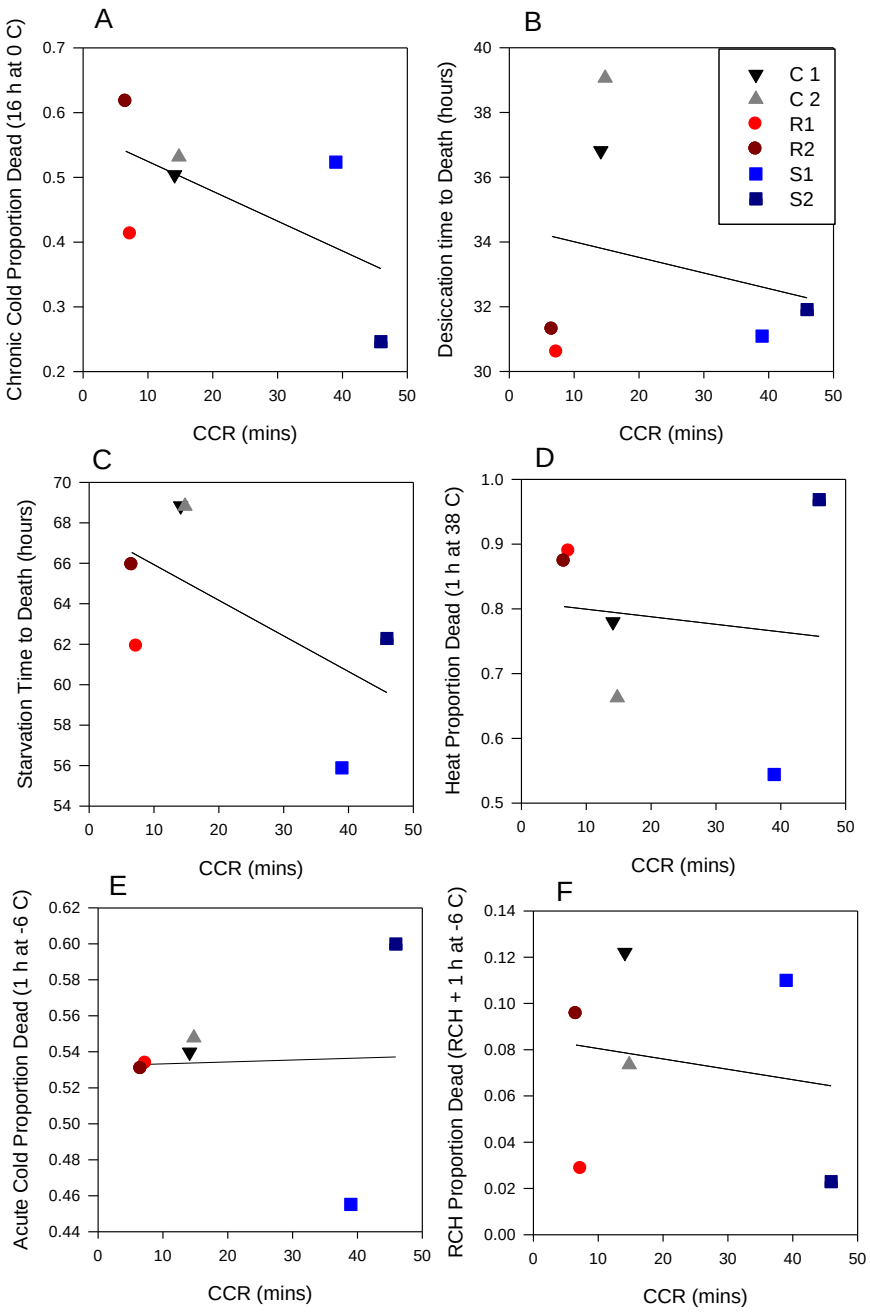

Figure 2.3. Cross-tolerance correlation plots. Chill-coma recovery time is represented on the x-axis in minutes to recovery. Environmental stressors are on the y-axis, either in time until death in hours (starvation, desiccation) or percentage dead (heat, acute cold, chronic cold, RCH). None of the correlations are significant at the $p<0.05$ significance level, although differences between susceptible and resistant to the control lines drives some marginally significant correlations.

Control lines are represented by diamonds (C1 and C2), susceptible lines are represented by squares (S1 and S2), and resistant lines are represented by circles (R1 and R2). A) Chronic cold stress survivorship. B) Time to death desiccation stress. C) Time to death during starvation stress. D) Heat stress survivorship. E) Acute cold stress survivorship. F) Survivorship following rapid cold hardening.

Table 2.1. ANOVA of chill-coma recovery time. Selection regime created a significant influence of selection regime (susceptible or resistant) at both generation 30 and 31. Line nested within selection was significant only at generation 30, as was the effect of sex.

	Generation 30				Generation 31			
Source	***df***	***MS***	***F***	***P***	***df***	***MS***	***F***	***P***
Selection	2	12798	25.34	0.0132	2	159196	22.88	0.0153
Line(Selection)	3	4848.91	62.92	0.0033	3	6961	1.66	0.345
Sex	1	819.62	10.62	0.0468	1	26.78	0.01	0.9414
Selection x Sex	2	45.90	0.60	0.6057	2	9.39	0.00	0.9978
Line(Selection) x Sex	3	77.06	0.43	0.7339	3	4205.39	30.13	<0.0001
Error	615	180.56			596	139.59		

Table 2.2. R values and p-values of correlation between chill-coma recovery time and environmental stressors based on F-test values and Pearson's product moment correlation. Desiccation was significantly associated with chill-coma recovery time but was driven in a non-biological manner. Similarly, starvation was marginally significant but associations were not biologically reasonable. The environmental stressors tested show no significant correlations with lines selected for chill-coma recovery time, with our without control lines present.

			Correlations			
	Selection		With Control Lines		Without Control Lines	
Trait	$F_{2,3}$	*P*	r	*P*	r	*P*
Desiccation	13.96	0.0299	-0.23	0.66	0.66	0.34
Acute Cold	0.10	0.9064	0.04	0.94	0.23	0.76
Starvation	6.47	0.0816	-0.60	0.21	-0.58	0.42
RCH	0.32	0.7502	-0.18	0.72	-0.03	0.93
Chronic Cold	0.76	0.5406	-0.60	0.20	-0.57	0.43
Heat	0.46	0.6675	-0.13	0.81	-0.25	0.74

Chapter 3 - Genetic networks underlying the evolution of cold hardiness

Alison R. Gerken, Tonia S. Schwartz, Caroline Williams, David B. Allison, Xiangqin Cui, Daniel Hahn, and Theodore J. Morgan

Alison R. Gerken

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. E-mail: aegge@ksu.edu

Tonia S. Schwartz

Office of Energetics, University of Alabama at Birmingham, Birmingham, AL, 35233. E-mail: tschwartz@uab.edu

Caroline Williams

Department of Entomology and Nematology, University of Florida, Gainesville, FL, 32611. E-mail: carolinewilliams@ufl.edu

David B. Allison

Office of Energetics, University of Alabama at Birmingham**,** Birmingham, AL, 35233. E-mail: dallison@uab.edu

Xiangqin Cui

Department of Biostatistics, University of Alabama at Birmingham, Birmingham, AL, 35233. E-mail: xcui@uab.edu

Daniel Hahn

Department of Entomology and Nematology, University of Florida, Gainesville, FL, 32611. E-mail:dahahn@ufl.edu

Theodore J. Morgan

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. E-mail: tjmorgan@ksu.edu

Abstract

Thermal limitations can drive species distribution as well as determine variation in fitness and metabolic parameters. Insects are particularly susceptible to thermal stresses, and cold stress significantly influences molecular, physiological, and behavioral phenotypes in nature. The molecular mechanisms underlying variation in the response to cold stress are poorly understood. This variation in cold tolerance may have evolved via three hypothesized mechanisms: 1) constitutive changes in baseline gene expression making them better prepared to deal with a cold stress 2) inducible responses that are activated during cold stress, or 3) inducible responses to recover thermal homeostasis after cold exposure. The goal of this project is to identify the gene expression networks that differ among artificially selected *Drosophila melanogaster* before, during, and after exposure to cold stress. Selection was applied to an outbred population, resulting in two lines with increased cold tolerance (resistant lines) and two lines with decreased cold tolerance hardiness (susceptible lines). Flies were collected from each line at three time points: a baseline expression profile before cold exposure, an exposure time point during cold, and a recovery time point after exposure to cold stress (0^{o} C for 3 hours). Affymetrix microarray analyses provided gene expression analyses. Comparisons between the resistant and susceptible lines identified 80 differentially expressed genes at the exposure time point cold exposure, 2,378 differentially expressed genes for the recovery time point, and no differentially expressed genes at baseline. We conducted network analyses to identify groups of genes and pathways associated with variation in cold tolerance. These analyses identified a highly correlated expression network

of 9-11 odorant binding proteins in resistant lines, and a correlated expression network of 13 immune defense genes in susceptible lines. Additionally, a large network (>500 genes) of mostly unannotated yet significantly correlated up regulated genes was identified in the resistant lines only. The resistant lines also maintained a stably expressed set of genes enriched for mitochondrial function (173 genes), suggesting mitochondrial homeostasis may be important in cold stress. These results provide a novel analysis of the time series response of genes involved in variation in the cold tolerance.

INTRODUCTION

Temperature dictates the species distributions and fitness capacity of many organisms. Insects can be major crop pests and disease vectors and understanding their responses to daily and seasonal thermal fluctuations as well as response to extreme events is important for understanding their economic and health impacts. High and low temperatures can significantly impact the evolution of mechanisms to enhance survivorship and fitness via molecular, behavioral, or physiological changes. Understanding the complex interactions between physiology and genetics will provide a better understanding of how the environment influences the evolution and population dynamics of important ectotherms species.

Physiological and molecular adaptations drive natural population variation in response to cold stress (Gibert & Huey 2001; Gibert et al. 2001). Transcriptomic and metabolomics studies have also associated some molecular processes with the response to cold stress (Teets et al 2012; Nielsen et al. 2006; Kristensen et al. 2006; Sorensen et al. 2007; Pedersen et al. 2008). However, our understanding of the specific genetic networks over a time series in response to cold stress remains unclear. Genetic adaptations to increased cold tolerance and recovery could occur via three overlapping mechanisms. First, cold tolerant individuals may exhibit constitutive gene expression levels that make individuals better prepared to deal with cold stress. Second, cold tolerant individuals may differentially regulate gene expression during cold stress, thus allowing them to modify genetic and physiological mechanisms during cold. Third, cold tolerant individuals may differentially regulate their gene expression during recovery phase, after cold exposure, thus allowing them to recuperate normal biological function and homeostasis more quickly following cold exposure. These patterns of gene expression will provide essential

information on the most critical time point for the regulation of variation of cold tolerance (Sinclair et al 2007).

Previous research has described several physiological mechanisms that are utilized by insects in to maintain biological function and limit the injuries associated with extreme cold stress. These physiological mechanisms include maintaining membrane ionic balances and viscosity (Hazel 1995; Michaud and Denlinger 2006; MacMillian et al 2009; Qin et al 2005), build-up of cryoprotectants and polyol sugars to avoid freezing of hemolymph (Storey 2002; Qin et al. 2005; Sorensen et al. 2007), using antifreeze proteins to avoid ice crystal formation (Kelty and Lee 1999; Sinclair et al 2003; Duman 2001), and preventing cellular injury (Hochachka 1986; Pullin & Bale 1988; Kelty et al. 1996; Kostal et al. 2004 and 2007). Genetic differentiation and network analyses will also allow us to determine which of these physiological responses are most important and will provide insight into both the critical physiological mechanisms and timing responsible for dealing with cold stress and cold recovery.

A comprehensive understanding of the genetic networks associated with cold stress requires the identification of the pathways that are induced when individuals are exposed stress, that are variable among genotype with different cold tolerance phenotypes, and those that are both induced and variable among genotypes. Artificial selection on natural genetic variation is an effective strategy to create genotypes with extreme phenotypes (Service 1987; Brakefield 2003) that can determine the extent to which a population will be able to genetically respond to future environmental change (Brakefield 2003; Tucic 1979; Chen & Walker 1993; Anderson et al. 2005). *Drosophila melanogaster* is an ideal species for artificial selection and thermal biology as they have been very successful in its ability to adapt to novel thermal challenges (David and Capy 1988) and currently occupy a worldwide distribution (David and Capy 1988; Lachaise et

al. 1988). *D. melanogaster* has been used extensively in artificial selection studies, for example, Kristensen et al. (2007) artificially selected *D. melanogaster* for increased tolerance to heat stress, Hoffmann and Parsons (1989) selected lines for desiccation tolerance (also see Sinclair et al 2007), and MacMillan et al (2009) selected for survival following acute cold stress (1 hour at -5° C). In addition, we have previously (Gerken et al. *in prep* a; Chapter 2) created at set of artificially selected lines that are significantly different for increased and decreased cold tolerance, as measured by chill-coma recovery.

Chill-coma recovery is an adaptive quantitative trait that is found in a variety of insects (Hoffmann et al. 2002; Ayrinhac et al. 2004; Collinge et al. 2006; Gibert and Huey 2001; Gibert et al 2001). The trait is expressed as the time to recover as measured by regaining muscle movement and orientation of the fly following the onset of a physiological immobilization caused by exposure to low but non-lethal temperatures (David et al. 1998; Kelty and Lee 2001). The trait also exhibits significant natural genetic variation as has been documented by the response to artificial or natural laboratory selection (Tucic 1979; Anderson et al. 2005; Dierks et al 2012; Bertoli et al 2010; Franke et al 2012) and by the quantification of genetic variation along clines of resistance cold stress (Gibert and Huey 2001; Hoffman et al. 2002; Ayrinhac et al. 2004; Kimura 2004; Fallis et al. 2011; Rako et al 2007). While these studies have documented substantial adaptive genetic variation for chill-coma recovery time in nature, molecular analyses of the chill coma have been limited.

To date, molecular analyses of chill coma have focused on specific candidate genes known to have a role in thermal tolerance, including *Frost*, heat shock proteins, and *Smp30* (or *Dca*) (Overgaard et al 2005; Sinclair et al 2007; Goto 2001; Anderson et al. 2003; Clowers et al. 2010). These studies have provided inconsistent results indicating that some but not all candidate

genes are involved in the induction (Goto 2000, 2001; Daibo et al 2001; Anderson et al 2003; Greenberg et al 2003; Qin et al 2005; Kelty & Lee 2001; Sinclair et al. 2007a; Udaka et al 2013) or recovery (Burton et al 1988; Goto and Kimura 1998; Sejerkilde et al 2003) from cold stress. Other studies have used transcriptional approaches to identify genes induced in response to cold stress or cold acclimation (Qin et al. 2005; Rako & Hoffmann 2006; Udaka et al 2010; Teets et al 2012). These studies found that transcripts associated with recovery from cold shock were associated with heat shock proteins, cytoskeleton organization, and cell shape and signaling genes, as well as genes associated with stress, membrane, and mitochondrial function (Qin et al 2005), but acclimation had little effect on the expression set during the acclimation or recovery period (Teets et al 2012). Other studies have investigated the phenotypic, metabolomic and genetic correlations in response to selection on cold tolerance traits, finding a wide range of levels of correlation among selection processes (Kristensen et al 2007; Colinet and Hoffmann 2012; Colinet et al 2013). Quantitative trait loci mapping of cold tolerance traits have also provided several genomic regions involved in cold tolerance responses, including parts of chromosome II (Morgan and Mackay 2006; Norry et al 2007), chromosome III (Goto 2000, 2001; Daibo et al 2001; Anderson et al 2003; Greenberg et al 2003; Qin et al 2005), and the X chromosome (Svetec et al 2011). These results have identified broad genomic involvement in cold tolerance. However, these experiments lack the fine mapping power to pinpoint specific genes of interest (but see Sevtec et al 2011).

Although multiple specific candidate genes and genomic regions have been associated with responses to cold stress, we do not have a complete understanding of the genetic networks underlying the evolution of natural variation in cold tolerance. We are also lacking an examination of the time course of gene expression levels, before, during, and after cold exposure.

Such information is important to understanding the ecologically relevant molecular mechanisms involved in the response to cold stress. This work provides a first analysis of gene expression in genetically district lines *across* a time course from pre-exposure, induction, and recovery. By assessing differential expression of genes at these three different time points using our artificially selected populations, we can determine the genes that are differentially expressed among the lines and when the genes for cold tolerance are influencing this system.

METHODS

Drosophila melanogaster Populations

Artificially selected lines are described in detail in Gerken et al (*in prep* a; Chapter 2). Briefly, mated *D. melanogaster* females were collected from the Raleigh, NC Farmer's Market to establish a population of 60 isofemale lines. This sample of isofemale lines was used to establish a genetically variable base population, by crossing the lines in a round robin. A single inseminated female from each cross was placed in each of two culture bottles to initiate two replicate base populations. The progeny from these culture bottles were designated generation 0. To begin the first generation upon which selection was applied, approximately 20 virgin individuals per sex per replicate base population were scored for cold tolerance and were used to initiate the three selection regimes (resistant, susceptible, and control) in each base population. Beginning at generation 1, 50 virgin male and female progeny were collected from each of the six lines every generation. These individuals were assayed for chill-coma recovery time. In the resistant lines the 20 males and females with the fastest recovery times were used as the breeders for the next generation, while in the susceptible lines the 20 males and females with the slowest recovery times were used as the breeders for the next generation, and in the control lines 20

males and females were randomly chosen with respect to chill-coma recovery time and used as the breeders for the next generation. Individuals that did not recover from chill coma in the 90-minute observation period were given a score of 90 minutes. Selection was performed in this manner each generation for 31 generations. After 31 generations of selection, resistant lines had a mean chill-coma recovery time of 7 minutes 45 seconds (±48 seconds), while the susceptible lines had a mean chill-coma recovery time of 60 minutes 8 seconds (±51 seconds).

Microarray Samples

Experimental flies were reared at 25° C on a 12 hour/12 hour light/dark cycle prior to collection of samples for microarray analysis. Only female flies were collected for the gene expression analysis and all flies were 5-7 days old at time of collection. Upon eclosion, females were lightly anesthetized using CO_2 to sort by sex and were placed into vials to recover for 48 hours. After 48 hours, all samples were exposed to three different treatments for microarray collection. The three treatments were before, during and after cold exposure. For the before treatment, flies were snap frozen from room temperature to capture gene expression differences before cold exposure. For the during treatment, flies were snap frozen immediately after they were exposed to 0° C for 3 hours to capture gene expression differences during cold exposure. Finally, for the after treatment, flies were snap frozen after 4 minutes at room temperate following an identical exposure to 0° C for 3 hours to capture gene expression difference during cold recovery. Although the three treatments represent a time course of gene expression changes, the snap freezing of the flies occurred during an approximately 10 minute window to minimize differential gene expression between the treatments caused by the circadian clock. The experimental design contained three replicate vials of 25 female flies per line and cold treatment

for a total of 54 experimental samples of female flies (Figure 3.1). Total RNA was extracted independently for each of the samples using the TRIzol reagent (GIBCO/BRL). The samples were treated with DNase and purified on Qiagen (Chatsworth, CA) RNeasy columns. Biotinylated cRNA probes were hybridized to high-density oligonucleotide Affymetrix *Drosophila* GeneChip 2.0 microarrays by the Kansas State University Integrated Genomics Facility.

Gene-by-gene Analysis

All gene expression values were RMA normalized gene expression values prior to all subsquent analyses. Genes differentially expressed between the resistant and susceptible lines were identified within time points (before, during, and after) using the limma function in R (R Statistical Software) using the following model:

$$Expression = m + Selection + e$$

where selection is the difference between the resistant and susceptible populations. *P* values from the selection term were computed via *F*-ratio tests. We used the false discovery rate criterion to determine significance at a FDR level of 0.05 (Storey and Tibshirani 2003; Benjamini and Hochberg 1995). Output from pairwise comparisons were then assessed under high stringency, which generates less functional groups and more tightly correlated gene functions, using DAVID for gene function clustering (Dennis, et al. 2003; Huang da, et al. 2009) with the background list of probes from the Affymetrix gene expression array.

Network Analyses

We used mulitple approaches for network analysis to identify genetic pathways and functions that are associated with cold tolerance. Taking advantage of our time series data we analysed and compared networks both (i) among timepoints, and (ii) between the resistant and susceptible selection lines across the time series. For each analyses, we evaluated the significant subnetwork (e.g. module, group of correlated genes) for annotation enrichment using DAVID (Dennis, et al. 2003; Huang da, et al. 2009) with the background set to the list of probes from the Affymatrix gene expression array specific for each analysis. We used the default parameters for the function clustering, with the exception of including annotation for Panther Pathways and Reactome. We used an enrichment score critical value of 1.3 (Huang da, et al. 2009).

To identify co-expressed genes that are likely to represent functional cellular processes or pathways that are associated with chill-coma recovery at a particular time point (before, during, or after cold stress), we conducted a weighted correlation network analysis using Langfelder and Horvath (2008) WCGNA (R package) using the 5,000 most variable probes for which more than 90% of the individuals had expression data. Using all the samples (n=9) from the respective time point, we built a before network, a during network, and an after network. To identify modules (groups of genes correlated in their expression) we used a soft-threshold power for the network construction, chosen specifically for each network analyses using the scale-free topology index curve; the lowest power for which the index curve flattened out upon reaching a value above 0.8. For building the gene network dendrogram to identify gene modules, we use the following parameters: deepsplit = 2 and merge cut height = 0.25. For the modules that were significantly associated with chill-coma recovery we assessed the correlation between gene significance for the trait and module membership; a strong correlation suggests the module is likely biologically

significant. Functional annotation enrichment was conducted on the significantly associated modules as described above.

We next wanted to identify gene networks and pathways that have diverged in their expression patterns across the time series between the resistant and control lines. For these analyses we wanted to identify gene networks that have evolved under the artificial selection pressure in their coordinated expression patterns (up- or down-regulated transcription) across the time series (referred to as responsive networks) as well as those that evolved to *maintain* their expression consistent across the times series (referred to as homeostatsis networks). We employed two approaches to characterize the responsive networks: (i) partial correlation analysis in GeneNet (Opgen-Rhein and Strimmer 2007a; Opgen-Rhein and Strimmer 2006a, 2007b, 2006b; Schafer, et al. 2006), and (ii) clustering of genes based on the temporal expression patterns using Short Time Series Expression Miner (STEM) that is designed for short time series microarray datasets (Shannon, et al. 2003).

Using GeneNet we analyzed the expression data as a time-series to infer gene association networks with the assignment of putative causal direction to the relationships between genes (nodes) in the networks based on partial correlations. We used the R package longitudinal to code the data matrix as a time series again using the 5,000 most variable probes for which more than 90% of the individuals had expression data. Analyzing the resistant and susceptible lines separately within the R package GeneNet we used the dynamical correlation shrinkage estimator to estimate the correlation matrix for partial correlation (Opgen-Rhein and Strimmer 2006a, b). We focus on the top 150 significant edges to compare across the resistant and susceptible lines.

To understand how the resistant and susceptible lines have diverged the expression patterns across the time-series, we used STEM (Ernst and Bar-Joseph 2006) to cluster genes

based on their temporal expression patterns and to test for significantly enriched patterns in the resistant and susceptible lines separately. For this analysis we started with all the microarray probes and normalized the before time-point to zero. We set the two resistant lines as replicates of "different time period". We focus on genes with a max-min > 0.4 change as the minimum change in the gene's expression value between any two time points; and required a correlation among reps (0.75). Thereby the analyses averaged the three replicates within each line to correlate across the two lines as a method to filter out genes with inconsistent expression patterns across both lines and provide noise estimates. The same approach was used for susceptible lines. Secondly, we compared the resistant and susceptible profiles to test for groups of genes that switched profiles (up- or down-regulated) when comparing two groups (resistant vs. susceptible). To identify gene networks that provide homeostatis under the challenge of cold stress and that differed between the resistant and susceptible lines we filtered for genes that did not change in response to cold exposure using a max-min < 0.2 and > -0.2 change between any two time points and a correlation among reps (0.75).

Overlap gene comparisons

Significant probe lists from pairwise comparisons, GeneNet, and WGCNA analyses were visually compared for overlap in probes for time point and selection line association. STEM results were not compared for overlap since they focus on enrichment of broad gene expression profiles that were not restricted to individually signficant genes.

RESULTS

Pairwise Comparisons

Pairwise comparisons provide a gene-by-gene expression analysis for baseline expression, cold induced expression, and recovery expression, and provides a relative expression contrast between resistant and susceptible lines. We then used DAVID to find functional enrichment of our differentially expressed genes. The pairwise comparisons of the susceptible and resistant lines provide baseline differential expression data for pairwise comparison analyses. Pairwise comparisons provided differentially expressed genes for comparing resistant and susceptible lines at the time points before, during, and after cold stress. There were no differentially expressed genes between resistant and susceptible lines for the before time point.

Comparisons between resistant and susceptible lines during cold stress identified 80 genes that were differentially expressed. Susceptible lines had 61 genes with increased expression relative to the resistant lines, while resistant lines had 19 genes with increased expression relative to the susceptible lines. DAVID clustering did not support any significant clusters, due to small numbers of significant gene expression differences. Genes with higher expression in susceptible lines included biological and functional involvement in cation transmembrane transport, odorant binding, metal ion binding, chitin based cuticle structural components, and DNA binding, as well as 13 unannotated genes. Genes with higher expression in resistant lines were involved in such functions as cytoskeleton actin and organization, immune response, calcium ion binding, olfactory behavior, DNA damage response, phagocytosis, and vesicle mediated transport, as well as 6 unannotated genes.

Comparisons between resistant and susceptible lines after cold stress resulted in 2,378 significantly differentially expressed genes. Susceptible lines had 1,583 genes with increased expression relative to the resistant lines, while the resistant lines had 795 genes with increased expression relative to the susceptible lines. For susceptible lines, there were 184 annotation

clusters in DAVID under high stringency, with the highest enrichment score of 24.49 to a cluster representing mitosis. The top ten clusters were representative of the following functions (enrichment score): cell cycle (24.47); DNA response to stress or stimulus (16.93); nucleotide binding (8.51); replication (7.78); chromosome localization (6.86); meiosis within the cell cycle (5.19); lumen activity (4.61); and eggshell morphogenesis (4.58). For HCH genotypes there were 141 significant annotation clusters, with the highest enrichment score of 7.04 representing a cluster involved with glycosyl hydrolase activity. The top ten clusters for resistant lines were involved in the following functional groups: cytochrome p450 activity, metabolite transportation (6.02); sensory perception (4.43); detection of external stimulus (4.41); detection of light (3.01); CHK kinase-like activity (2.50); tube size of tracheal system (2.43); hormone metabolism, specifically androgen and estrogen (2.43); phototransduction, G-protein coupled receptor activity (2.31); nerve impulse, synaptic transmission, and cell-cell communication (2.28).

Network Analyses

WGCNA network analysis provides us with an examination of clusters of significantly enriched genes differentially expressed before, during, and after cold stress. WGCNA clusters the enriched gene sets by association to chill-coma recovery as a continuous trait. We first wanted to identify co-expressed genes that are likely to represent functional cellular processes or pathways that are associated with chill-coma recovery at a particular time point to build a before network, a during network, and an after network using Langfelder and Horvath's (2008) WGCNA (R package). We identified modules at each of these time-points that are correlated (> [0.7]) with chill-coma recovery (Figure 3.2). At the before time point, the most highly correlated module (yellow: Figure 3.2) was enriched for symporter activity and protease activity. The red module

also highly correlated, had no significant enrichment in DAVID, but included genes involved in sensory perception, specifically vision. A highly significant module was associated with chill-coma recovery at the during timepoint, which also had no significant enrichments, but contained serine proteases and hydrolases. At the after timepoint, the modules were highly significant and were enriched for immune function, chitin cuticle function, and cognition/odorant perception.

GeneNet

GeneNet provides an analysis of significantly enriched clusters between resistant and susceptible lines. Comparing the top 150 most significantly connected nodes from the GeneNet analyses revealed a highly connected network unique to each selection group. The network unique to the resistant lines, the blue network was enriched for odorant binding proteins (Figure 3.3A, blue cluster), whereas the green network in the susceptible lines was enriched for bacterial defense (Figure 3.3B, green cluster). The two selected groups shared two networks (Figure 3.3; red and yellow). Both of these shared networks were more tightly connected in the resistant lines. The red network was enriched for cell fate determination. Interestingly the shared yellow network showed no significant enrichment, as many of those genes not found in DAVID. In most cases the "hub" genes do not have annotation.

STEM

STEM analysis provides a time series analysis of significantly enriched gene sets across time points and provides changes in these gene sets for before, during, and after cold stress. The resistant lines have eight expression profiles that were statistically significantly enriched relative to the number of genes that would have been assigned to the profile by chance (Figure 3.4A).

The total number of genes in theses significant profiles is 1,829. The susceptible lines have four significant profiles (1, 10, 11, and 14) that are significantly enriched (Figure 3.4B) with 1,136 genes. None of these significant profiles overlapped between the resistant and susceptible lines. Notably missing from the susceptible lines are significant profiles that have an increase in expression at the recovery time point (i.e. profiles 6 and 8). We identified genes at the intersection of different profiles between the susceptible and the resistant lines (Table 3.2). Potential homeostatsis genes that are highly correlated between the lines selected in a particular direction. In the resistant lines, the low expression differentiation genes are enriched for mitochondrial function and oxidative phosphorylation, whereas the susceptible lines were enriched for RNA transport; ATP and GTP binding. Between the resistant profile 4 and susceptible profiles 1 and 5 we identified 167 genes, which were enriched for multiple aspects of reproduction.

Overlap of Genes Between Analyses

Between pairwise comparisons, WGCNA, and GeneNet analyses we were able to associate probes with whether they associated with resistant or susceptible lines (Table 3.3). From our analyses 169 probes overlapped between our different analyses; nineteen further overlapped with either selection lines (resistant or sussceptible) or time point (before, during, after) as the common overlap. Between GeneNet and pairwise comparisons we found five probes that overlapped; three overlapped with high lines at the after time point with the resistant line yellow module in the GeneNet analyses (Table 3.3). Of these probes, only two were associated with annotated genes. Probe 1626116_at corresponded to the serine peptidase 212 gene, which acts to cleave peptide bonds in proteins. Probe 1627946_at corresponded to shroud, which is

involved in ecdysone biosynthetic process. In plants this process is important as a protection agent against toxins and p450 is a critical component of this process.

For comparison between WGCNA and pairwise comparisons for time point overlap nine probes overlapped with the WGCNA red module in the after comparison and resistant lines after expression (compared to susceptible line; Table 3.3). Four probes overlapped with the red module in the after comparison and susceptible line after expression. Three probes were unknown to gene associations. Genes from the resistant line after-Red module comparison included functions such as actin cytoskeleton organization, follicle development, ion binding (including calcium ion binding), female courship, growth factors, and p450 elements and oxidation-reduction processes. For susceptible lines after-Red module overlap functions included G-protein coupled receptor activity, adult lifespan, and two genes with unknown molecular and biological functions.

DISCUSSION

Artificial selection experiments can be used to elucidate the mechanistic basis by which populations evolve at both the genetic and physiological levels, in response to environmental perturbations (Tucic 1979; Anderson et al. 2005; Dierks et al 2012; Bertoli et al 2010; Franke et al 2012). Understanding the consequenes of selection on cold tolerance metrics in insects, such as chill-coma recovery, are important for predicting population dynamics in response to climate change in many ecologically, evolutionary and argriculturally important insect species (Jentsch et al 2007; IPCC 2013; Kodra et al 2011). Understanding the time course of a physiological stress where selection has acted is important to understanding constitutive expression versus evironmentally responsive gene expression. By pinpointing specific genomic and genetic

components, as well as functional network involvement that have responded to selection, we can identify how gene regulation contriubutes to the divergent cold tolerance phenotypes. Collectively, our analyses show that there is significant divergence between the resistant and the susceptible lines in gene expression at each time point, but particularly during recovery, time point and significant differences in the genes and networks between the two spectrums of cold tolerance (resistant and susceptible).

Both the pairwise comparisons and the time series network analyses (STEM) emphazise significant gene expression differences between the lines during the recovery phase (i.e., the after time point). These results suggest that there is little constitutive differences responding to selection between the divergent selection lines. We must however, take into account that the cold metric under selection is a recovery trait (Dierks et al 2012; Bertoli et al 2010; Franke et al 2012; David et al. 1998; Kelty and Lee 2001), and therefore, genes responsible for fast or slow recovery time may only demonstrate changes in expression during the recovery time point. However, the whole organism response to cold stress must be considered as a recovery time point is not independent of the before or after gene expression profile or lifetime experience of the flies under selection. Indeed, our network analysis at each time point (WCGNA) identfied modules of genes associated with CCR at the before and during timepoints associated with symporter activity and sensory perception (before) and serine proteases (during).

A unique component of our analyses is that we not only assessed gene-by-gene pairwise comparisions, but we also assess the networks of stress response involved at different time points during cold stress for co-expression analyses. Our analyses are also on a genome-wide basis which provides a global analysis of the gene regulation during cold stress and the identification of the functional networks associated with such changes. Our pairwise analyses point to many

differences between the resistant and susceptible lines for the during and after time points only, again suggesting that evolutionary selection pressures may act primarily on the gene expression at the most significant time point of this chill coma, recovery. This result dovetails Teets et al (2012) study, which found that most of their gene expression differences in cold acclimated flies occurred during the recovery time point as well (also see Clark and Worland 2008; Colinet and Hoffmann 2010). This may indicate that gene expression differences during the recovery phase of stress response may be the most important selection target and may serve as the best indicator of divergent selection and resistance/response to cold stress.

Our gene-by-gene analyses between resistant and susceptible lines within time points suggest that differences in functional response in gene expression may be driving the differences in recovery time between these two populations. For example, the low cold tolerance lines have differential, up-regulation in genes primarily involves with general cell maintenance responses such as mitosis, meiosis and some DNA stress response. Alternatively, the resistant lines have a response in regulating metabolic responses and perception and response to stimulus, indicating their increased ability to detect and successfully manage these cold stress situations (Portner et al 2000; Scholander et al 1950). The resistant lines are also significantly enriched in response to glycosyl hydrolase activity which has been shown to be a component of cell wall activity and could contribute to maintenance of cell wall (Lee et al 2006).

Similarly the GeneNet analyses indicated several differences between the resistant and susceptible lines in their response to cold stress on a network scale. A large network of immune defense genes was found in the susceptible lines, while a network of odorant binding genes was significantly enriched in the resistant lines. In this case, resistant lines are again detecting and responding to cold stress via perception response in these networks (Vieira and Rozas 2011),

while susceptible lines may be allocating resources inappropriately, which possibly contributes to the slow recovery from cold stress. In addition, there was also a large network of unannotated genes within the resistant lines that could indicate a large, novel list of genes for increased cold stress response and should be further analysed for functional associations to cold.

Understanding whole network changes between time points and selection lines allows us to investigate the overall changes following cold stress. Our analyses expands on this and provides a detailed examination into the genetic changes at the three specific time points. Tracking responsive genes over the three time points allows us to highlight groups of genes enriched for responses over time. In the resistant lines there are more significantly enriched profiles suggesting a more coordinated response among the filtered genes relative to the filtered genes in the susceptible lines. These analyses also highlight a number of differences between the resistant and susceptible lines in their progress from the during to after cold exposure, particularly in the differences in genes that return to expression levels in the before condition in the susceptible lines that remain altered in the resistant lines. For example, genes involved in female reproduction are down-regulated in both resistant and susceptible lines during cold exposure, but they remain down-regulated in resistant lines while returning to before levels in the susceptible lines (Table 3.1). This pattern is of particular interest as allocating resources to reproduction is a costly endeavor (Harshman and Zera 2007; Calow 1979), and thus possibly creates a slowed recovery from cold as the susceptible lines reallocate resources to metabolism (Calow 1979).

In addition, genes that do not change across the time series are indicative of "homeostasis" or maintenance genes and provide an indication of the ability of these selection lines to maintain critical functions throughout cold exposure (Table 3.2). For resistant lines, a set

of genes involved in mitochondrial activity and oxidization phosphorylation (Gnaiger et al 2000; Johnston and Dunn 1987) remain enriched throughout the time series analysis, suggesting that resistant lines have evolved mechanisms that enable them to maintain metabolic and energetic activity throughout cold stress environments. The susceptible lines on the other hand maintain RNA transport components as well as ATP and GTP binding abilities, again maintaining basic cellular components (Johnston and Dunn 1987) but not immediately responding efficiently to regain metabolic function to handle the cold stress.

Our comparison of the network, time series, and pairwise comparisons indicated several shared genes of interest. These genes could be powerful candidate genes for further inspection as they were present in several analyses. Among them was again a gene for cytochrome p450 and oxidation reduction processes (*Cyp12a4*). This gene was present in the after time point and the resistant lines, indicating that they may be more prepared to maintain metabolic processes during and after cold shock. In addition, several ion binding genes including *Neuroglian* (*Nrg*) and *Cyp12a4* were present in the resistant lines at the after time point. These again may indicate the resistant lines are able to maintain membrane activity throughout cold stress. Maintaining these two processes is of great importance importnace as they will help the organism avoid cold injury and regain metabolic and biological functions following cold stress.

Throughout these analyses it is clear that certain components of gene expression and network differentiation affect recovery from chill coma. The network time series comparisons as well as the pairwise comparisons indicate that most differences in gene expression lie in the after time point. The resistant lines are more responsive in terms of perception of the cold stress and maintenance of cellular components in the face of stress; in addition, in the after time point, significant modules are associated with response to stress and perception (odorant/cognition) as

well as components of cuticular response, a mechanism hypothesized to play an important role in cold tolerance (Chen et al 1991; Denlinger and Lee 2010; Michaud and Denlinger 2006). In addition, our time series analyses comparing resistant and susceptible lines indicate similar properties to our pairwise comparisons, indicating benefits to recovery time manifesting in mitochondrial and oxidative phosphorylation homeostasis. The susceptible lines again, maintains basic cellular components such as ATP/GTP binding and RNA transport as indicated by time series analyses and meiosis and mitosis maintenance as indicated by the pairwise comparisons.

In conclusion, our results describe a time series response to gene expression differences in *Drosophila* lines artifically seleced for high and low cold hardiness. From these anlayses we pinpoint that much of the evolutionary change occurs in gene expression during recovery from cold stress, similar to that of Teets et al (2012; see also Clark and Worland 2008; Colinet and Hoffmann 2010). In addition, we examined the impact of selection on the baseline gene expression patterns and find that there are no constitutive changes in gene expression. This could indicate that responses in gene expression to cold stress are too costly to maintain consistently and changes only following stress exposure provide the most efficient mechanism of evolutionary change in these organisms. Our analyses also combine gene-by-gene comparisons alongside network comparisons which provide a more comprehensive view of gene expression responses to cold tolerance evolution. Convergence of similar functional enrichments and underlying gene expression influences over time points and selection lines provide confidence in our functional interpretations. These results also provide an examination of one component of selection: gene expression. Further comparisons with metabolomics, genome evolution, fitness (Gerken et al. *in prep* b, Chapter 4), and correlated responses (Gerken et al. *in prep* a, Chapter 2) can provide a complete examination of the effects of selection to cold stress (Malmendal et al

2013). Finally, our results provide a novel look at a time series of cold tolerance response in gene expression; overlapping these analyses with artificially selected lines pushes these comparisons and understanding of evolutionary changes involved in gene expression of cold tolerance to a new level.

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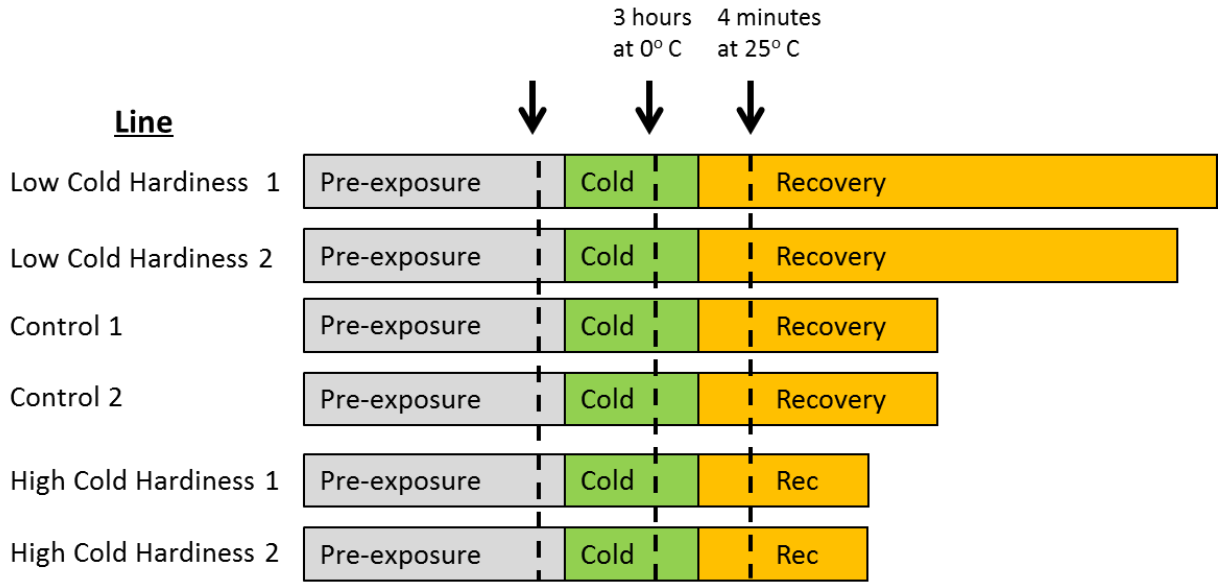
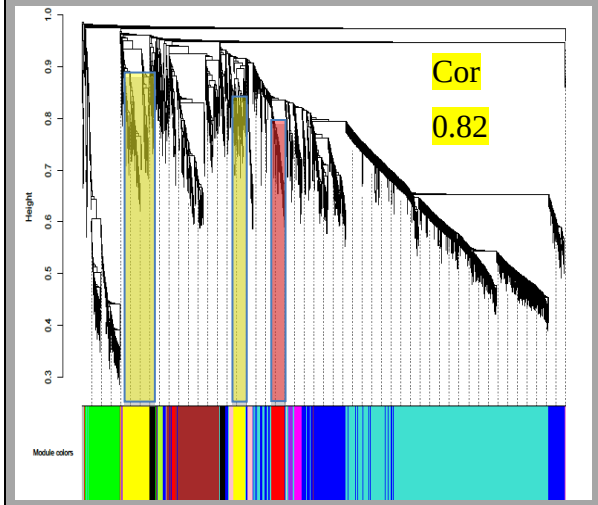
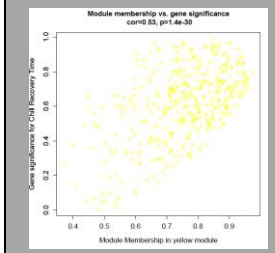


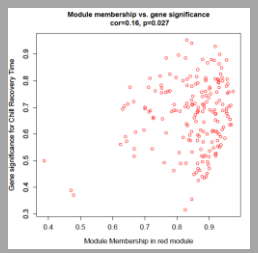
Figure 3.1. Experimental design. At each time point, as indicated by the arrow, three replicate samples of 50 female flies were snap-frozen for gene expression analysis

Figure 3.2. WCGNA networks and gene modules that are significantly associated with chill coma recovery at each time point: Before, During and After Cold Exposure. The colors are completely arbitrary across time points. A. The networks constructed using the microarray gene expression data at each time-point. The modules that are significantly associated with CCR with at least a correlation coefficient of 0.70 are highlighted. B. Biological relevance of each model based on correlations of each gene's membership in the module and its significance for Chill Coma Recovery, based on membership in the module and C. The annotation enrichment of the highlighted modules based on DAVID.

BEFORE







Enrichment

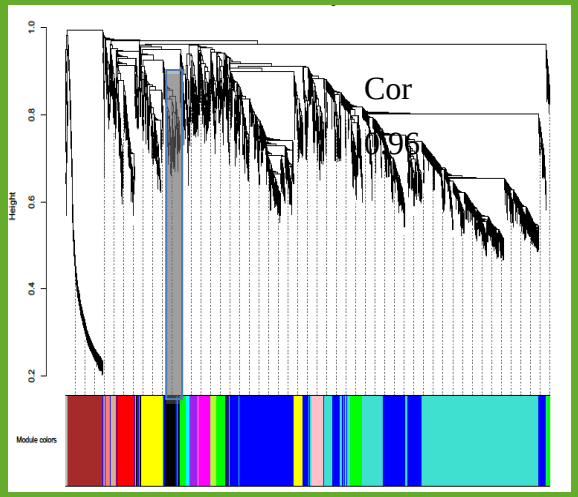
Yellow Module:

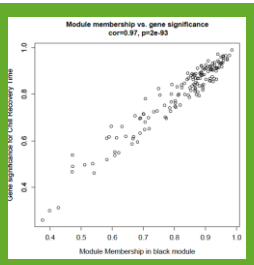
Symporter activity ES 2.71
Proteolysis ES 2.41
Phospholysis ES 2.22

Red Module:

Sensory Perception, Vision ES 1.01

DURING



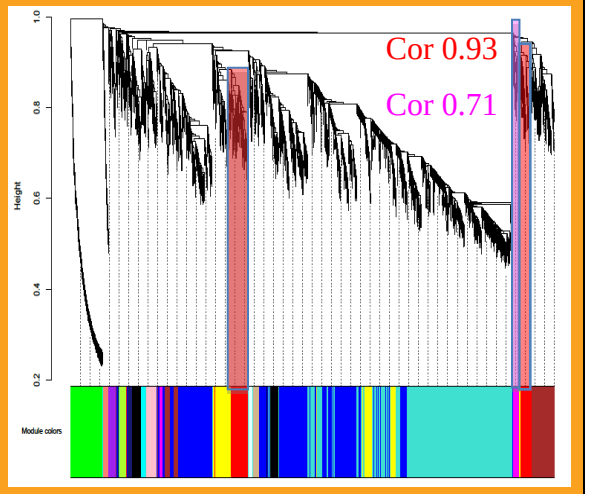


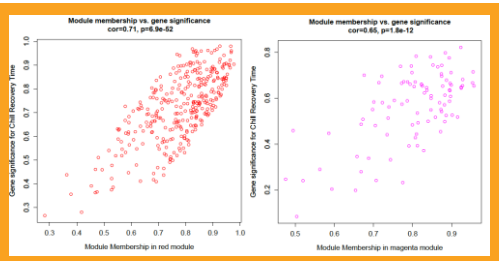
Enrichment

Black Module:

Serine Proteases, hydrolases ES 1.17

AFTER





Enrichment

Red Modules:

Immune response, humoral ES 1.68

Biotinidase, hydrolase activity ES 1.42

Alkaline phosphatase ES 1.4

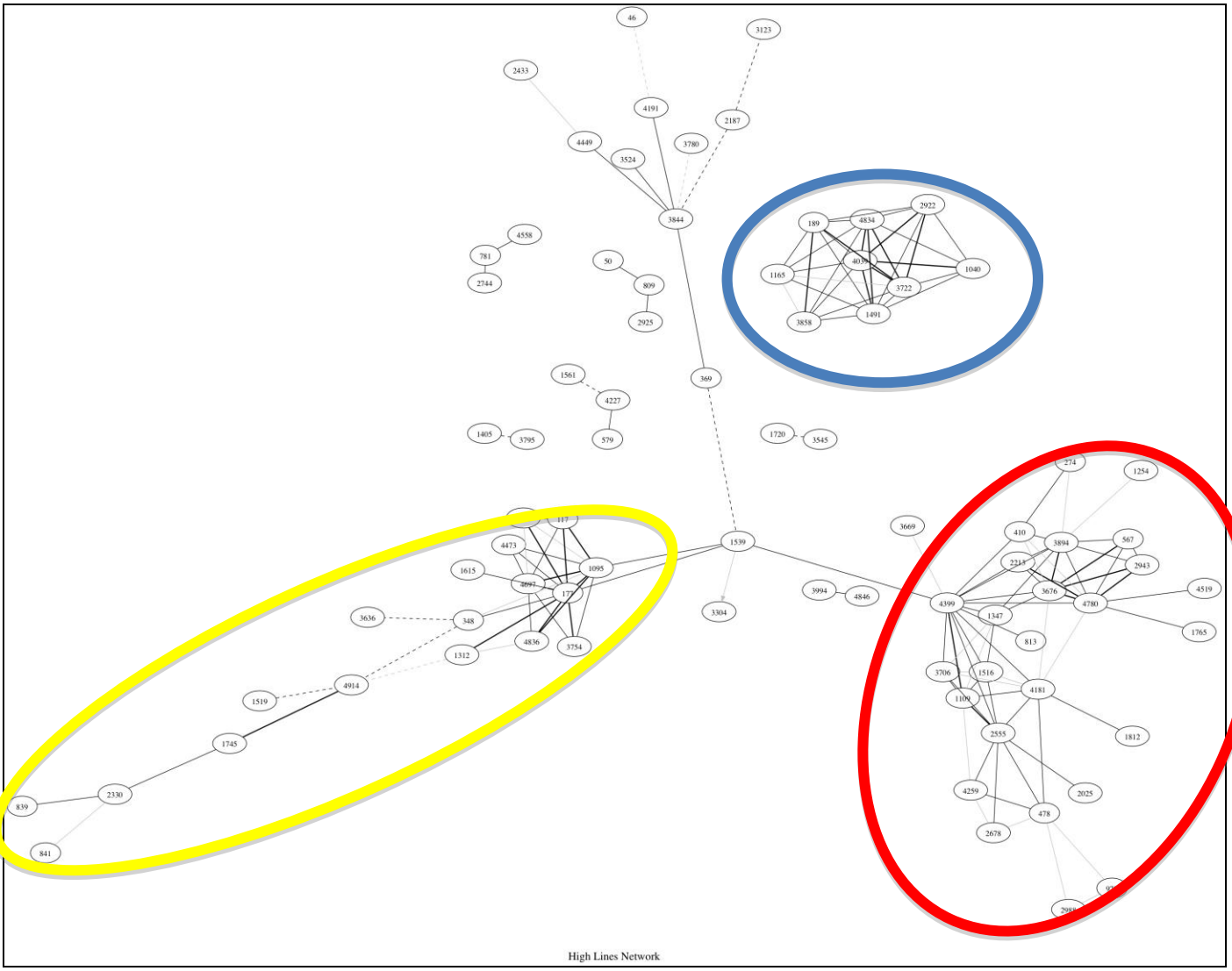
Cognition/perception/olfaction ES 1.18

Magenta Module:

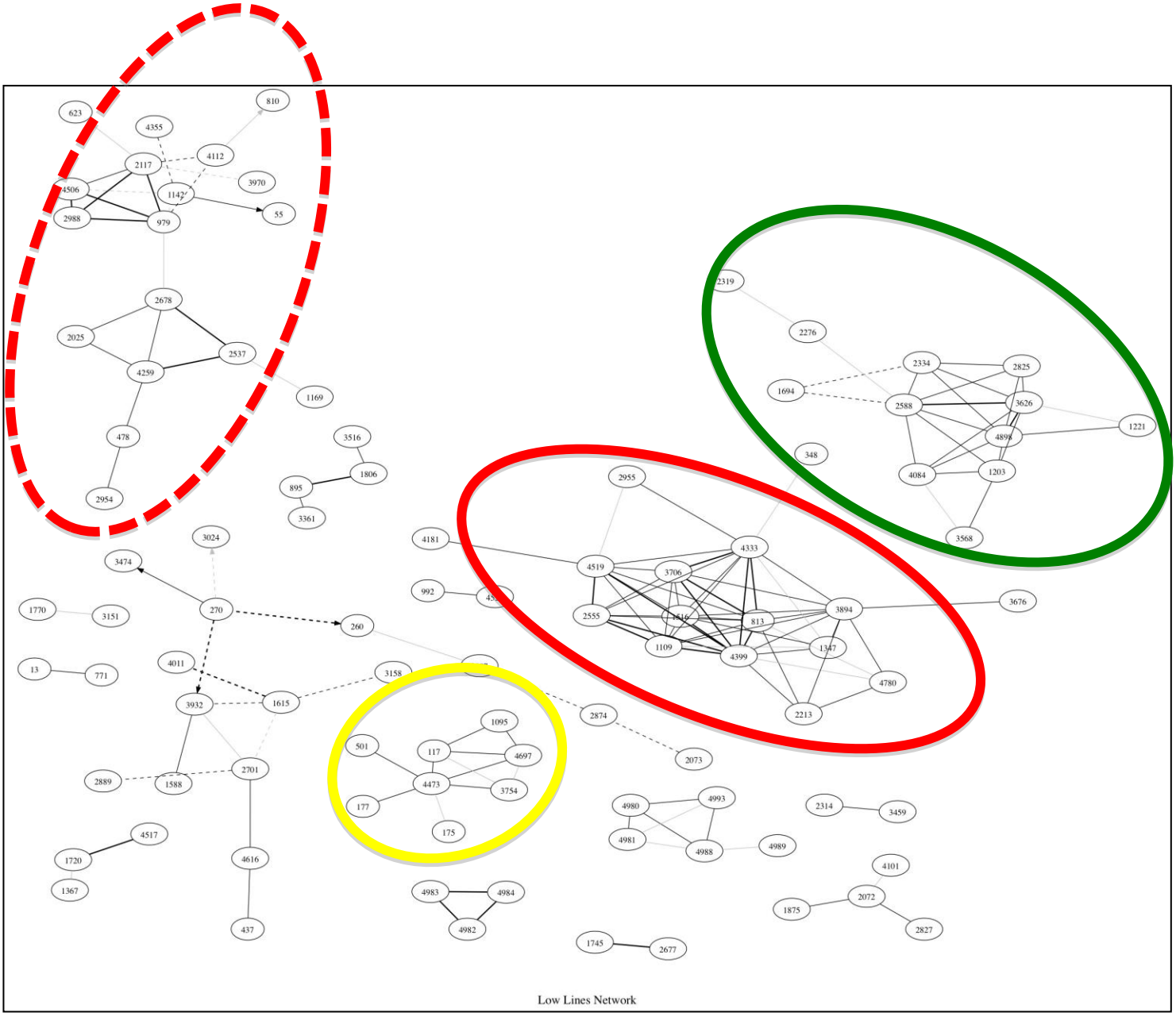
Chitin, cuticle ES 10.1

Figure 3.3. The top 150 significant edges of the correlation networks for (A) resistant and (B) susceptible lines. Solid lines indicate positive correlation coefficients; dotted lines indicate negative correlation coefficients; line intensity indicates strength of correlations. Blue circle contains a resistant network enriched for odorant binding proteins. The green circle contains a susceptible network enriched for immune defense genes. The red circles represent a network shared among the resistant and the susceptible lines that is involved in cell fate determination; likewise the yellow network is shared and mostly contains unannotated genes.

A. Resistant Cold Tolerance Network



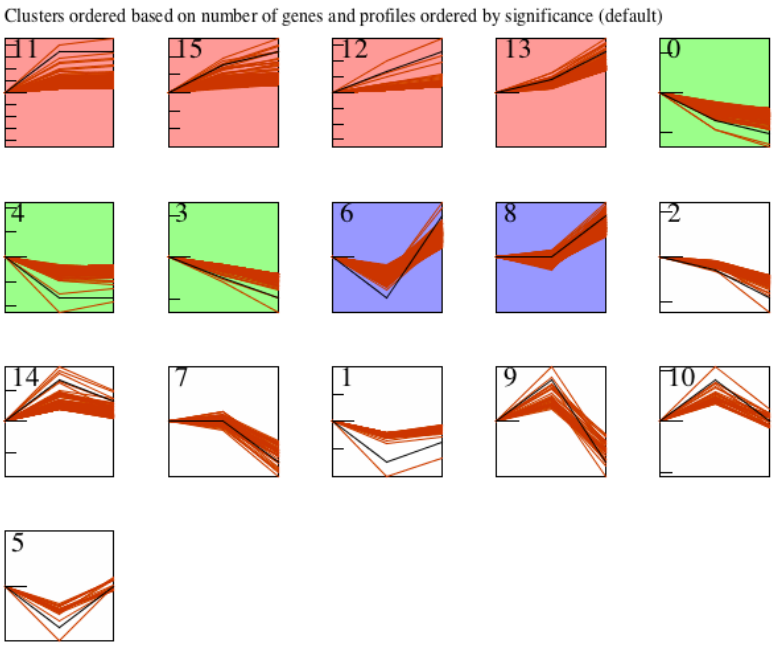
B. Susceptible Cold Tolerance Network



Low Lines Network

Figure 3.4. The 16 profiles of gene expression patterns across the three time points for resistant lines (A), and susceptible lines (B). The expression values are normalized to the before time point. Within each profile, the black line defines the expression patter across the time points, and each red line represents the expression patter of a gene. The colored profiles are significantly enriched for more than expected number of genes. Profiles of the same color are clusters having similar broad patterns of expression. None of the significantly enriched profiles overlap between the resistant and susceptible lines.

A.



B.

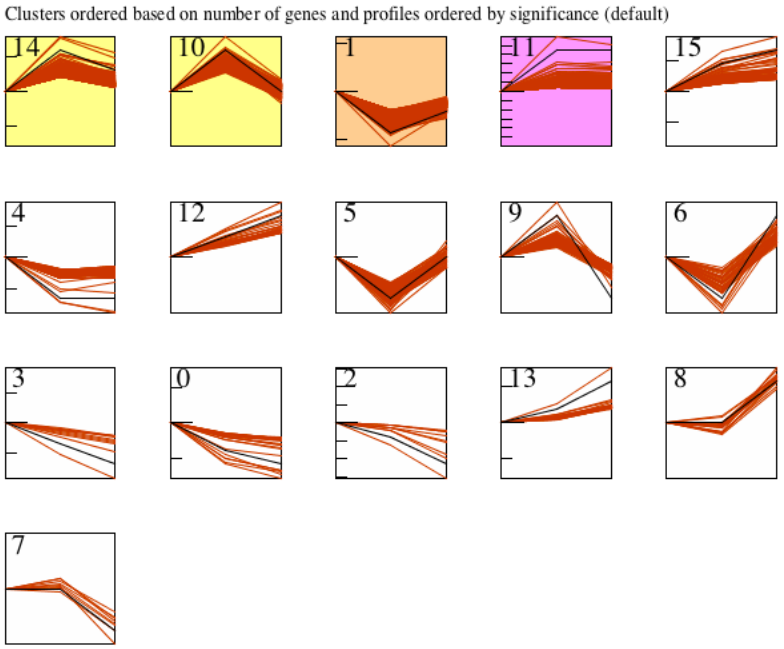


Table 3.1. Summary of STEM analysis of the responsive genes. These genes are at the intersection of profiles that differed between the resistant and susceptible lines. Bolded annotations are involved in growth/reproduction at cell and organismal levels

Resistant	Susceptible	No. of Genes	Annotation and Enrichment (n=number of genes; ES = Enrichment Significance)
		152	1. Neg Regulation Macromolecule biosynthesis (n=21, ES=5.67) **2. Chromosome/chromatin organization (n=20, ES=5.5)** 3. DNA binding (n=33, ES=5.19) **4. Chromosome, chromatin (n=19, ES=4.7)** **5. DNA metabolic process/replication (n=15, ES=3.9)** **6. Female gamete generation/reproduction/oogenesis (n=20, ES=3.9)** **7. Chromatin/heterochromatin (n=10, ES=3.4)** 8. Zinc-finger, metal ion / cation binding (n=24, ES=3.28)
		15	**9. Regulation of mitotic cell cycle (n=12, ES=3.26)** 10. Neuron development & differentiation (n=13, ES=2.1) 11. Positive regulation Macromolecule biosynthesis (n=9; ES =2.05)

Table 3.2. Summary of STEM analysis for nonresponsive "homeostasis" or maintenance genes. Genes enriched for mitochrondria or energy production are enriched in the resistant lines only, while general transcription is enriched in the susceptible lines.

	No. of Genes	**Annotation enrichment (n = No. of genes; ES = Enrichment Significance)**
Resistant	4417	1**. Mitochondria; oxidative phosphorylation ; ETC (n=173; ES=5.12**)
		2. Intracellular transport; protein localization (n=97, ES=3.86)
		3. vesicle docking; cell-cell signaling; neuortramsimter secretion; SNAPE interactions (n=17, ES=3.18)
		4. **Mitochondrion; transit peptide (n=59; ES=2.93)**
		5. Casein kinase II (involved in cell cycle, DNA repair, circadian rhythm); regulation of kinase activity; regulation of phosphate metabolic processes (n=20, ES=2.93).
		6. Vesicle targeting; membrane docking (n=17; ES =2.17
		7. Vesicle-mediated transport; phagocytosis (n=101, ES=2.12)
		8. Mitochondrial ribosome (n=30; ES=2.01)
		9. Protein complex biogenesis (n=48, ES=1.83)
Susceptible	4551	1. Protein localization and transport (n=115, ES=5.5)
		2. protein biosynthesis; translation initiation (n=44, ES=3.83)
		3. nucleotide binding, ATP binding (n=298, ES=3.24)
		4. vesicle-mediated trasnport; endocytosis; phagocytosis (n=98; ES=2.82)
		protein folding; chaperone (n=36; ES=2.81)
		5. GTP binding; RAS GTPASE (n=56; ES=2.72)
		6. RNA transport; mRNA export; (n=27, ES=2.57)
		7. ncRNA and tRNA metabolic process; aminoacyl-tRNA synthease (n=42, ES=2.44)
		8. cytoskeleton organization; cell cycle process (n=117, ES=2.13)
		9. Secretion; neurotramsmiter; synaptic transmission; cell-cell signaling (n=40; Es=2.08)
		10. coenzyme metabolic process (n=28, ES=1.97)

Table 3.3. Overlap of genes from WGCNA, pairwise comparisons and GeneNet. Molecular and biological functions are briefly summarized. If probe did not correspond to a gene "None" is present in the Gene Name column. Only significant probes from each analyses were compared.

Probe	Gene Name	WGCNA Module	Pairwise Comparison	GeneNet	Function
1623636_at	None		HCH After	HCH Blue	
1626116_at	Serine peptidase 212		HCH After	HCH Yellow	
1627946_at	shroud		HCH After	HCH Yellow	Ecdysone-steroidal pheromone; molting; protection agent (toxins); insect development
1628730_at	None		HCH After	HCH Yellow	
1629334_at	None		LCH After	LCH Green	
1624807_at	Ppcs	After Red	HCH After		Actin cytoskeleton organization; chaeta development; ovarion follicle cell migration
1628793_at	CG6106	Before Red		Hub	Cobalt ion binding; zinc ion binding; allontoinase activity
1630964_at	CG15211	After Red	HCH After		Gene product from CG15211
1633386_s_at	mthl8	After Red	HCH After		G-protein coupled receptor activity; adult lifespan
1635443_at	CG18539	After Red	LCH After		No known functions
1636146_at	Nrg	After Red	HCH After		Calcium ion binding; axon ensheathment, genesis, and extension; female courship behavior; imaginal disc morphogenesis; nerve maturation

1637161_s_at	myo	After Red	HCH After		Growth factor activity; mushroom body development and growth
1637253_s_at	CG17570	After Red	LCH After		No known functions
1637622_s_at	Cyp12a4	After Red	HCH After		Cytochrome p450 family; electron carrier activity; heme binding; iron ion binding; oxidation-reduction process
1638899_s_at	CG15312	After Red	HCH After		Imaginal disc-derived wing morphogenesis
1641189_a_at	puffyeye	After Red	HCH After		Positive regulation of cell growth; regulation of protein stability; wing disc development
1635802_at	None	After Red	HCH After		
1638331_s_at	None	After Red	LCH After		
1641442_a_at	None	After Red	HCH After		

Chapter 4- Demographic parameters reflect cold susceptibility only at cool rearing temperatures in an artificially selected population of *Drosophila melanogaster*

Alison R. Gerken, Christopher Berger, Rebecca Sullivan, and Theodore J. Morgan

Alison R. Gerken

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: aegge@ksu.edu

Christopher Berger

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: cberger@ksu.edu

Rebecca Sullivan

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506.

Theodore J. Morgan

Corresponding author. Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: tjmorgan@ksu.edu

Abstract

Artificial selection can create genotypic differences among naturally occurring populations with differing levels of phenotypic values. Selection for chill-coma recovery can create divergent populations of susceptible (increased time to recovery) and resistant (decreased time to recovery) genotypes. Associations with many other stress or fitness traits may also be under influence by these selection pressures. In turn, susceptible genotypes may be poor at many different life

history and stress components compared to the resistant genotypes. We set out to test this hypothesis using artificially selected lines in *Drosophila melanogaster*, two replicate cold resistant, two replicate cold susceptible, and two control lines. We examined lifetime fitness across five temperatures ranging from 14° C to 33° C to estimate thermal performance curves for each selection or control line. We calculated net reproductive rate (R_0), generation time, and intrinsic rate of population increase (λ) and compared confidence intervals for these parameters across our five temperatures. We found at only at cool temperatures (14° and 18° C) were susceptible and resistant lines significantly different in their demographic parameters from one another. At cool temperatures, resistant lines had significantly faster generation times and overall higher λ values. These data demonstrate that there is canalization at warmer temperatures only and that selection differences are only manifested within a cooler, more stressful environment.

INTRODUCTION

Environmental stress can force organisms to respond either long-term or short-term in order to maintain Darwinian fitness to promote population stability (Sibly and Calow 1989). Genotypic differences within a population drive the primary response to stresses and quantifying these genotype-by-environment interactions is critical to predicting species distributions and responses to climatic changes (Lande 1979; Baer and Travis 2000; Williams et al 2007). Shifts in environmental temperatures have not only influenced migratory and breeding patterns (Gibert et al 2001; Koehn and Bayne 1989) but also force organisms to respond either through phenotypic plasticity, a short-term genotype specific response to environmental variation, or through adaptation, a long-term population-wide response to environmental changes (Dierks et al 2012; David et al 2003; Angilletta et al 2003). Performance curves allow researchers to quantify variation in phenotypic plasticity within a genotype, allowing a visual inspection of fitness responses over a range of environmental pressures, including temperature (Angilletta 2006; Foray et al 2011; Lande 1979).

Construction of performance curves relies on the investigation of life-history data including fecundity and longevity (Hoffmann and Parsons 1989; Gunay et al 2010; Huey and Berrigan 2001; Caswell 2010). Many studies have projected performance curves by assessing traits associated with reproduction such as development time and larval or pupal mass (Franke et al 2012; Sambuccetti et al 2006). Others have assessed reproductive output on a short-term basis and have used this short-term egg laying and longevity as projections for lifetime fitness (Chevin et al 2010). However, Kingsolver and Huey (2008) discuss the variation in reporting various demographic values such as r, the intrinsic rate of increase, and R_0, the net reproductive rate, and how each value is context dependent (see also Gilchrist and Huey 2001). Collecting data on

entire lifespan egg production and survival rates may provide the most accurate and complete picture of performance curves and plasticity responses across different environments (Norry and Loeschcke 2002; Gunay et al 2010).

Temperature variation can drive organisms to adjust their survival, mating behaviors as well as affect their fitness in appreciable ways. Natural clinal variation studies have indicated that thermal variation is found throughout ectotherms at various altitudes and latitudes (Sambucetti et al 2006; Folguera et al 2008; Hoffmann et al 2002). Temperate insects generally have an increased resistance to cold stress compared to tropical insects, driven by environmental selection pressures (Angilletta 2009). *Drosophila* populations at various altitudes also show varying degrees of plasticity, with plasticity increasing as altitude increases (Folguera et al 2008; Fallis et al 2011). However, Schnebel and Grossfield (1984, 1986) also demonstrated in laboratory experiments that various temperatures did not have any effect on mating behavior in *Drosophila* including the number of eggs laid over a short-term period. Conversely Franke et al (2012) found that butterflies reared or acclimated at two different temperatures showed substantial differences in developmental time, larval and pupal sizes, and larval growth.

Many theoretical and empirical studies have also shown that temperature can have profound impacts on size and developmental time, often suggesting conflicting views of thermal optima (Angilletta 2009; Cooper et al 2010; Gilchrist and Huey 2001). Studies suggest that for ectotherms, hotter temperature is better since generation time is decreased and thus eggs laid at warmer temperatures develop and hatch quickly, re-populating the environment quickly (Gilchrist and Huey 2001; Gunay et al 2010). In contrast, colder temperatures increase development time but also produce larger individuals, suggesting that cooler temperatures are best for population persistence (Gilchrist and Huey 2001; Angilletta 2009). Each thermal

environment has trade-offs including increased metabolism and decreasing lifespan at warmer temperatures and increasing developmental time at colder temperatures increasing susceptibility to competitive pressures among larvae and death from extreme temperatures (Kostal et al 2012; Franke et al 2012; Dillon et al 2009). Thus, calculating entire lifespan fecundity and longevity performance curves over a broad range of temperatures can provide a complete picture of thermal optima for ectotherms and identify trade-offs in fecundity and fitness over this range (Barton and Partridge 2000).

Artificial selection experiments allow substantial trait manipulations on a short-term environmental scale (Bertoli et al 2010; Norry and Loeschcke 2002). By imposing pressures of temperature selection, we not only observe the response to the trait of interest but can also explore cross-tolerance and trade-offs to other traits that may be correlated to traits such as cold tolerance (Brakefield 2003; Bubliy and Loeschcke 2005). These populations of artificially selected individuals will mimic ecologically relevant environmental clines with varying degrees of tolerance and will provide essential data to investigate the impact of cold tolerance on population-wide success at various environmental temperatures. Few studies have looked at how selection regimes impact lifetime fitness (Chevin et al 2010) and this experiment aims to answer questions on the effects of selection on demographic life history characteristics for populations with varying levels of cold tolerance (Gunay et al 2010; Caswell 2010).

Selection for cold tolerance was applied to lines of *Drosophila melanogaster* from Raleigh, NC via chill-coma recovery time (Kelty 2007; Bertoli et al 2010; Franke et al 2012; Gibert et al 2001). Chill-coma recovery time is one of several ecologically relevant adaptive measures of cold tolerance (Gibert et al 2001; Gibert and Huey 2001; Anderson et al 2005; Hoffmann et al 2003). Selection pressures followed by several generations of inbreeding create

homozygous lines from the original population with allelic variation in response to cold tolerance (Gerken et al. *in prep*, Chapter 2; Service 1987). Chill-coma recovery time also provides a unique opportunity for assessment of cold tolerance in divergent directions, with selection for increased susceptibility as well as resistance (Brakefield 2003; Gibert et al 2001). The *Drosophila melanogaster* selection lines previously described in Gerken et al. (*in prep*, Chapter 2) and consist of two resistant, two susceptible, and two control lines which also provides an experimental assessment of variation in artificial selection pressures within a single population under a single environmental pressure. Determining the magnitude of variation in fitness within and among the replicates of each selection regime will provide an important assessment of the variation in selection pressures within a natural population.

Using the four selection lines and two control lines our experiment aims to evaluate underlying genetic variation produced through artificial selection to assess genotypic response of varying levels of cold tolerance to different environmental temperatures. We will measure eggs laid for females of each line of *Drosophila melanogaster* as well as life time survivorship at five stable temperature regimes. We predict that life history demographic measures will vary substantially across this variation in temperature. We also hypothesize that selection regime will show significant differences in overall fitness, with cold susceptible lines showing a decrease in overall fitness in cooler temperatures and resistant lines showing decreased overall fitness at warmer temperatures. Variation in overall fitness by selection regime will indicate that selection for cold tolerance based on chill coma recovery is tied either genetically or physiologically to overall fitness, specifically longevity and fecundity. Direction of response in fitness to selection regime will also indicate whether this association is a trade-off due to selection or if selection pressures for chill coma recovery provide a mechanism of cross-tolerance to fitness components.

METHODS

Selection Lines

Drosophila melanogaster were selected for chill coma recovery time as in Gerken et al. (*in prep*, Chapter 2). Briefly, fifty male and 50 female flies were exposed to a mild cold stress of 0 C for three hours and then allowed to recover at room temperature (approximately 25° C). The top and bottom 20 males and females were then selected as founders for populations with a resistance to cold (quick recovery time) or a susceptibility to cold (slow recovery time). Selection was imposed at 40% to limit inbreeding depression. After 31 generations of selection, four lines, two with resistance to cold and two with susceptibility to cold, were inbred. Two control lines were also maintained via inbreeding unselected flies. Population trait means of chill-coma recovery responded to selection pressure and selection provided two extreme responses (Gerken et al *in prep*, Chapter 2). Selection followed by inbreeding has established six varying genotypes, as determined by chill-coma recovery metric.

Thermal Treatments

We reared the four selection lines and two control lines from egg to adult at 25° C under 12:12 light:dark cycles (David et al 1983). Stock populations were kept in 5 mL vials on cornmeal, yeast, agar, and molasses substrate. For each selection and control line, 1-5 day old females were selected at random, using light CO_2 anesthesia, from the stock populations. The selected females were placed individually into 10 mL vials containing ~1 mL of substrate with blue food coloring added to aid in egg identification later (Tatar et al. 1996; David et al. 2003; Rauser et al. 2005; Jumbo-Lucioni et al. 2010). Vials were lightly sprinkled with active yeast to

stimulate oviposition (Chapman and Partridge 1996; Leips and Mackay 2000). In addition to the single females, 2 males of the same line were transferred to vials, to ensure mating. Twenty vials per line, containing single females and two males (Tatar et al 1996), were randomly assigned to each of the five thermal treatments: low (14° C), high (33° C), and intermediate (18°, 25°, and 30° C), with 12:12 light:dark cycles (Gilchrist and Huey 2001; Chown et al. 2009). Flies were kept for their lifetime at these experimental temperatures.

Measuring fecundity and longevity

Flies were transferred, without anesthesia, into fresh vials every day at 25°, 30°, and 33° C, every two days at 18° C and every three days at 14° C (Chapman and Partridge 1996; Pitnick 1991; Rauser et al. 2005), due to faster egg hatching at warmer temperatures and slower hatching at cooler temperatures. Vacated vials were observed under a dissecting microscope and egg counts were recorded (Rauser et al 2005). If a male died during the lifetime of a female, a new one of the same selection line was transferred to the vial with anesthesia the same day, maintaining a minimum of two males per female at all times (Leips et al. 2006). Egg counts were recorded immediately after vials were vacated or kept up to four days at 4° C, to inhibit hatching, before egg counts were recorded. Flies were kept at the experimental temperatures for the duration of their lifetime and survivorship was recorded for individuals (Bertoli et al 2010; Gunay et al 2010). Data was averaged on a six day time step in order to align egg counting data.

Statistical Analysis

Analysis of variance (ANOVA) was calculated for overall age averages and egg counts per line to assess if selection regime or selection line was different for longevity and fecundity

(Norry and Loeschcke 2002; Franke et al 2012). Lines were also pooled to assess overall differences in temperature regime. Life history measures were analyzed using R statistical programming (R version 2.13.0). Averages and bootstrap confidence intervals were calculated for λ, R_0, and generation time (Caswell 2010; Huey and Berrigan 2001). R_0 is the net reproductive rate and is calculated as $R0= \Sigma(l_x m_x)$ where l_x is proportion surviving at each life stage and m_x is the average fecundity at the life stage. Generation time (T) is the average time of maturity to laying an egg and is calculated as $T= \Sigma(l_x m_x)/R_0$. Lambda (λ) is defined as the rate of growth from one interval to the next and is defined as $\lambda=e^r$ where r is the intrinsic rate of increase, and is calculated as $r=\ln R_0/T$ (Begon et al 1996). Bootstrapping analyses provided 95% confidence intervals to assess overlapping life history values for each selection line and temperature interaction. Lambda assesses both generation time as well as fecundity and longevity values and provides a complete assessment of selection line and temperature differences. We also plotted egg counts and survivorship over time in order to assess where differences in life history metrics were found (Foray et al 2011).

Analysis of survivorship curves for each temperature treatment was conducted using the package survival in R (version 2.13.0). Kaplan-Meier estimates of survivorship curves were used to estimate 95% upper and lower confidence intervals for the survivorship curves. The function survdiff was then used to calculate Chi-squared differences among the selection and control lines, using an un-weighted analysis (rho=0) and a weighted analysis for the later portion of the survivorship curve (rho=1.5). Post-hoc one-way ANOVAs were also performed on survivorship curves to assess Tukey pairwise contrasts among the selection and control lines.

RESULTS

As previously described in Gerken et al. (*in prep*, Chapter 2), selection lines for both susceptibility and resistance to cold stress significantly differed from control lines. Susceptible lines (S1 and S2) had significantly longer times to recover from chill coma recovery while resistance lines (R1 and R2) had significantly shorter times to recover.

Longevity

Longevity varied substantially across temperatures and individual lines of selection regime. ANOVA of a full mixed model had significant effects of the interaction with selection with line nested within selection and temperature ($p=0.033$) as well as a highly significant effect of temperature ($p<0.0001$) and selection regime ($p=0.0086$; Table 4.1). Increasing temperatures decreased longevity, with longest living flies living at 14° C (Figure 4.1). Selection regime does not indicate a clear trend over the temperature gradient, although it is a significant factor of longevity (Table 4.1; Figure 4.2F). For example, control lines have the longest longevity at 14° C but comparable longevity at 25° and 30° C. Similarly, susceptible lines show shortest lifespan at 33° but similar lifespans at 18°, 25°, and 30° C.

Survivorship curves were significantly influenced by temperature, with a quick time to death at 30° and 33° C (Figure 4.2D-E) and extended curves at both 14° and 18° C (Figure 4.2A-B). Pairwise comparisons (Table 4.2) indicate significant differences between C1 and R1, R2, S1, and S2 ($p=0.001$, $p<0.0001$, $p=0.0059$, $p=0.0080$, respectively). There are also significant differences between C2 and R1 and R2 ($p=0.015$, $p=0.0075$, respectively). These differences do not associate strictly with selection regime, as the resistant lines are not significantly different than susceptible lines. Therefore, significant ANOVA main effect term of selection is most likely due to differences in control lines and not selection lines necessarily.

Survivorship curves at 14° C showed significant overlap in the upper and lower confidence intervals for resistant and susceptible lines (Table 4.3). Overall chi-squared analysis had significant differences among the survivorship curves (X^2=30.5, df=5, p=1.16x10^{-5}). Weighted analysis on the later portion of the curve also indicated significant differences in survivorship among the lines (X^2=16, df=5, p=0.0068). Post-hoc Tukey pairwise comparisons indicated only significant differences between resistant 1 and control 1 (p=0.012; Table 4.4). Survivorship curves at 18° C had significant overlap among selection lines for confidence intervals (Table 4.3) with no differentiation by selection regime present. Chi-squared analysis showed significant overall differences among the selection lines for survivorship curves (X^2= 22.3, df=5, p=0.00045) as well as for weighted analysis on the later portion of the curve (X^2=17.5, df=5, p=0.00368). Post-hoc Tukey pairwise comparisons indicated only significant differences between resistant 2 and control 1 (p=0.029) and susceptible 1 and control 1 (p=0.010; Table 4.4).

Survivorship curve analysis for 25° C had significant overlap in confidence intervals among selection regimes (Table 4.3). Overall chi-squared analysis indicated significant differences among selection lines (X^2=29.5, df=5, p=1.84x10^{-5}) and weighted analysis on the later portion of the curve was also significant (X^2=19.2, df=5, p=0.0017). Post-hoc Tukey pairwise comparisons indicated significant differences between resistant 2 and control 1 (p=0.029) and susceptible 1 and resistant 2 (p=0.029; Table 4.4). Survivorship curve analysis for 30 C had significant overalp in confidence intervals among selection regimes (Table 4.3). Overall chi-square analysis indicated significant differences among selection lines (X^2=87.3, df=5, p=0) and weighted analysis was also significant (X^2=66.1, df=5, p=6.53x10^{-13}). Post-hoc Tukey pairwise comparison indicated significant differences between resistant 1 and control 1

(p=0.0089), susceptible 2 and control 1 (p<0.001), resistant 1 and control 2 (p=0.0079), resistant 2 and control 2 (p<0.001), resistant 1 and susceptible 2 (p=0.0030), resistant 2 and susceptible 2 (p=0.0080), and susceptible 1 and susceptible 2 (p<0.001; Table 4.4).

Survivorship curves at 33° C have significant overlap among selection regimes in confidence intervals (Table 4.3). Overall chi-squared analysis indicated significant differences among the selection lines (X^2=48.8, df=5, p=2.49x10^{-9}) and weighted chi-squared analysis was also significant (X^2=28, df=5, p=3.6x10^{-5}). Post-hoc pairwise comparisons were significantly different between resistant 2 and control 1 (p=0.012) and susceptible 2 and control 2 (p<0.001; Table 4.4).

Fecundity

A significant interaction effect was found when assessing fecundity (number of eggs laid) for line nested within selection, temperature, and line nested within selection by temperature interaction (Table 4.5). General trends indicate that fecundity was highest at mid-range temperatures (25° C) with lowest fecundity at 14° C (Figure 4.3). The main effect of temperature was significant for all lines pooled together (p<0.0001; Table 4.5). Highest fecundity for all lines pooled is found at 25° C with 18° C following. Lowest fecundity is at 14° C (Figure 4.3). Individual selection lines showed substantial variation across temperatures with no clear trends emerging for selection regime and fecundity (Figure 4.4; Table 4.6). Differences between the individual lines drove much of the interaction effect significance and fecundity did not adhere to selection regime. Pairwise comparisons indicate significant differences in fecundity between C2 and every other line, S1 and all other lines and R2 and S1 (Table 4.6). These pairwise comparisons again do not correspond strictly with selection regime but instead are associated in

various ways based solely on line. For each temperature, peak egg laying differed for each line and temperature but for 14°, 18° and 25° C lines showed a peak egg laying from 18 to 40 days on average. Lines reared at 14° C had a low, steady rate of egg production (Figure 4.4A), while shifting to 18° C increased egg production and even created a peak point in production around day 25 and again at day 50 (Figure 4.4B). Flies reared at 25°, 30°, and 33° C shared similar patterns of egg peaks with lots of eggs early and a quick decline (Figure 4C-E).

Life History Demographics

Performance curves for R_0, net reproductive rate, were plotted and 95% confidence intervals indicated variation across temperatures as well across selection lines (Figure 4.5). R_0 ranged from 2.40 average offspring per female to 149.54 at 14° C, 100.93 to 519.27 at 18° C, 188.82 to 803.03 at 25° C, 49.27 to 294.79 at 30° C, and 19.84 to 214.60 at 33° C. Across temperatures, 14° or 33° C had the lowest R_0 confidence intervals for each line when considered independently (Table 4.7). R_0 had the highest confidence intervals at 25° or 18° C for each line. Confidence interval rankings for each line at each temperature show some significant overlap at each temperature regardless of selection regime (Table 4.7). For example, resistant 1 and susceptible 1 show overlap in their confidence intervals at 14° and 33° C. Susceptible 2 showed the lowest confidence intervals at all temperatures and only overlapped with resistant 1 at 30° C. Overall, net reproductive rate indicates that females of each line at each temperature are producing a large number of average offspring during their lifetime.

Generation time showed a wide range of variation based on temperature and selection regime and selection line (Figure 4.6). Mean generation time ranged from 5.27 to 7.16 days at 14° C, 3.08 to 3.97 at 18°, 2.50 to 2.82 at 25° C, 2.18 to 2.35 at 30° C, and 2.02 to 2.25 at 33° C.

Across temperatures generation time consistently increased as temperature decreased, showing a negative correlation in all selection lines (Table 4.8). At low temperatures selection lines significantly differed from each other with resistant lines showing quicker generation times than susceptible lines (Table 4.8). Control lines overlapped with both susceptible and resistant lines at these low temperatures as well. At 25° and 30° C, selection lines overlapped in their confidence intervals but at 30° C selection lines were significantly different from one another, although not in a consistent higher or lower pattern. Overall, generation times are relatively short for all lines and temperature treatments, decreasing at warmer temperatures for all lines and selection regimes.

The finite rate of increase, λ, showed significant non-overlapping variation in some temperature and selection line combinations. Lambda ranged from 1.14 to 2.96 at 14° C, 3.67 to 7.23 at 18° C, 8.07 to 11.98 at 25° C, 5.53 to 11.19 at 30° C, and 4.36 to 11.15 at 33° C (Figure 4.7). For all lines, λ showed a peak at 25° C and was lowest at 14° C. Confidence intervals showed significant differences between selection regimes at 14° and 18° C (Table 4.9), with susceptible lines having lower λ than the resistant lines. Control lines overlapped with the resistant lines in their generation time confidence intervals at both of these temperatures.

DISCUSSION

Ectotherm survival and reproduction is highly susceptible to stressful conditions including changes in temperature. In our experiment we exposed artificially selected *Drosophila melanogaster* lines to five different environmentally realistic temperatures. The range of temperatures highlighted in this study allows a complete picture of a thermal performance curve for this highly adaptable species. A complete lifespan analysis of fecundity also allows for exact

calculations of life history demographics for each cold tolerance genotype (Barton and Partridge 2000). Few studies have use life time fecundity analysis and such a range of temperatures to develop a complete performance curve for such genetically distinct phenotypes of *D. melanogaster* or any other ectotherm (but see Franke et al 2012; Gunay et al 2010). The complete analyses presented here allows a look into the adaptability and evolvability of life history traits as impacted by a range of temperatures (Leips and Mackay 2006) and can be extrapolated to other organisms that must deal with shifting temperature regimes due to climate change (Williams et al 2007, 2008).

We found that temperature greatly affects both longevity and fecundity with a peak in fecundity at mid-range temperatures (25° C) and a declining of longevity as temperature increases (Figure 4.1). Because high temperatures can be particularly stressful, organisms will shorten generation time because of increased mortality pressures (Sibly and Calow 1989; Bubliy and Loeschcke 2005) and need to quickly produce offspring. Thus, peaks in egg laying for high temperature (30° and 33° C) came within the first six days for females, while peaks in egg production occurred around days 18 to 40 for long-lived, cooler temperature flies (14°, 28, and 25° C). A decreased metabolic rate at lower temperatures allows these populations to live substantially longer than at other temperatures; however, the lengthened lifespan could not serve as a rescue mechanism for consistently low numbers of eggs laid per female. At 14° C especially low fecundity leads to a decreased λ values for each of the lines such that these populations may struggle to persist at these cool temperatures. However, at every other temperature all the lines had λ values high enough for a population size increase. These high λ values are common in insects as they have short generation times and can produce many offspring per generation (Gibert and Huey 2001; Angilletta 2009).

Similar to λ values, generation time also showed trends highly dependent on temperature, with shortest generation time at warm temperatures (30° and 33° C) and longest generation time at 14° C for all selection lines. Among selection lines, only 14° C had non-overlapping confidence intervals consistent with selection regime. Susceptible lines had the longest generation times which contributed greatly to identifying differences between selection regime in life history demographics such as λ. Accounting for generation time provides a more accurate estimate of population success compared to measures of R_0, which is dependent on age specific mortality rates and not generation time (Caswell 2010; Gunay et al 2010) and did not show significant differentiation of selection regime in confidence intervals.

Similarly, analysis of survivorship curves did not indicate any significant differences between selection regimes at any temperature. Survivorship curves at 30° C showed the most significant Tukey pairwise comparisons as well as highly significant chi-squared analysis for overall differences in survivorship (Table 4.3 and 4.4). However, like the other temperatures tested, the significant differences between individual selection lines did not align in a biologically relevant manner (i.e. by selection pressure). Upper and lower confidence intervals for survivorship curves had significant overlap among selection lines and selection treatments; survivorship curves for individual selection lines also differ among temperatures. For example, susceptible line 1 has the lowest confidence interval value at 14° C and 18° C, also with the largest range, but susceptible line 2 had the lowest confidence interval value at 25° and 30° C, with susceptible 1 having the highest lower limit (Table 4.3). In other words, survivorship curves for each selection line are behaving inconsistently as compared to the other selection lines at each temperature.

During artificial selection as in natural selection, traits associated with the trait under selection may either experience trade-off or cross-tolerance effects from the selection process (Hoffmann et al 2003; Anderson et al 2005; Kellerman et al 2009; Harshman et al 1999). Cross-tolerance associations are indicated by positive responses of secondary traits in association with the trait of interest while trade-offs are negative responses of the secondary traits to the trait of interest (Stillman 2003; Portner et al 2006; Angilletta et al 2003). These possible correlative relationships may be apparent when populations are selected for varying levels of cold tolerance as a cost may underlie the ability to be cold tolerant (Norry and Loeschcke 2002; Watson and Hoffmann 1996; Parsons 1992). In our analyses, we did not find any apparent trade-offs in life history associated with resistance to cold as selected by chill coma recovery time. Resistance lines compared to control lines often showed overlapping confidence intervals in demographic parameters such as λ, r, and generation time indicating that the pressure of cold tolerance selection does not impact the life history traits when compared to lines that have not undergone selection pressures.

Susceptible lines however showed significantly lowered demographic parameters, λ in particular, and an increased generation time as compared to both resistant and control lines at cooler temperatures. As temperatures increased across the range tested, confidence intervals of the selection lines often overlapped suggesting that the stressful conditions of 14° or 18° C force differential life history traits in these selection lines. This pattern of temperature driven demographic differentiation and genetic variability was suggested by Koehn and Bayne (1989) since demographic differences of genetically different lines should be more apparent under potentially stressful conditions as compared to more optimal conditions. This suggests that in our selection lines, the cold susceptible lines are not only susceptible to increased chill coma

recovery times but are also susceptible to cooler environmental temperatures and thus populations of these susceptible genotypes may not compete well with resistant or control lines in a natural population.

Differences in the susceptible lines are also often driven by what we have called susceptible line 2. This line has dramatic decreases in λ and longest generation time across all temperatures. Between the two susceptible lines there we also find more variation in chill coma recovery response (Gerken et al *in prep*, Chapter 2; Bertoli et al 2010). The variability of selection in susceptibility may be driven by the natural genetic variation found within the original population of *D. melanogaster*. For natural populations, long-term temperature pressures may have led to fixation on resistant alleles and although we can select for susceptibility to chill coma recovery, alleles responsible for this physiological response may be rare and thus selection in this direction is highly variable and somewhat random (Bertoli et al 2010). The difference in susceptibility lines, however, did not affect the larger trends we observed among the selection lines.

Fecundity and longevity are highly variable and dependent on temperature. Although we cannot see trends based on our selection genotypes in longevity and fecundity alone, calculating demographic history parameters combining generation time, longevity, and egg production provide a clear differentiation of selection lines at cooler temperatures. Observations of egg production and longevity suggest that susceptible lines may have alternative components of fitness that lead to the decrease in intrinsic rate of increase. For example, although susceptible line 2 had longevity similar to the other selection lines at 14° C, females from this line laid very few eggs at 14° C over their lifespan which lowers their λ values. In contrast, susceptible 1 had shorter longevity than susceptible 2 but laid eggs in similar numbers to the other lines, thus

upping their λ values (Figure 4.2 and 4.4). A complete lifespan fecundity and longevity analysis may seem complex, but demographic parameters provide a clear analysis of how genetically adaptable lines behave at variable temperatures.

Thermal performance curves can be complex for genetically different selection lines within a given population. This variation reflects the underlying natural variation of cold tolerance and the association that cold tolerance has with fitness capacities (Watson and Hoffmann 1996; Caswell 2010; Franke et al 2012). Our analyses of artificially selected lines for chill coma recovery time suggest that there is no overall trade-off in fitness to selection regime as they become increasingly cold tolerant. Differences in demographic parameters indicate that cold susceptible lines will do poorly at cool temperatures (14° and 18° C), reflecting the impacts of cold susceptibility selection at stressful environmental temperatures. Traditionally optimal temperature of 25° C (Cohet and David 1978; Gilchrist and Huey 2001) reflects the highest most consistent λ values for all selection lines in our study, indicating that laboratory rearing temperatures of 25° C continue to be most appropriate for genetically variable lines. Although egg counts provide a proximate measure of population success, future studies should also include offspring viability measures to estimate total success.

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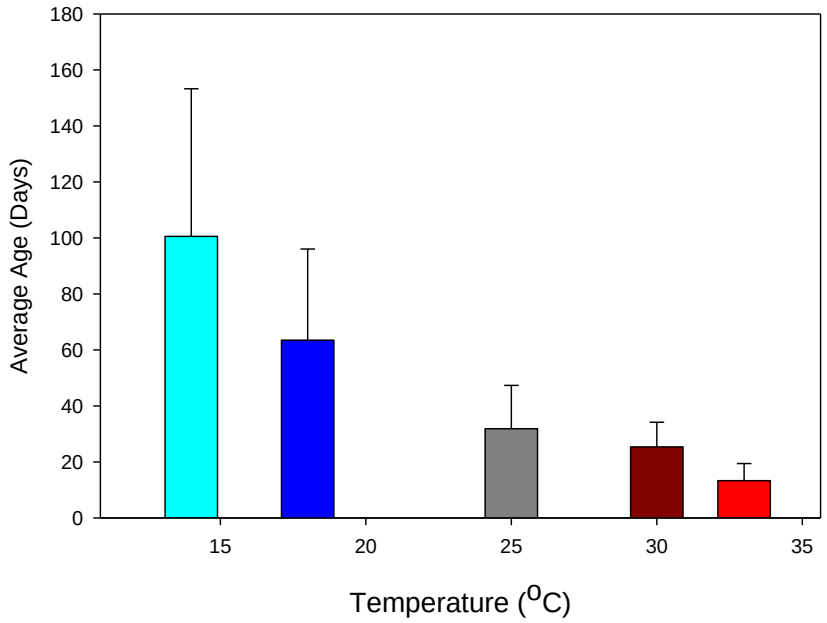

Figure 4.1. Longevity by temperature effect. Means ± standard deviation. There was a significant effect of temperature on longevity ($p<0.0001$) as well as selection ($p=0.0086$). Longevity decreases as temperature increases.

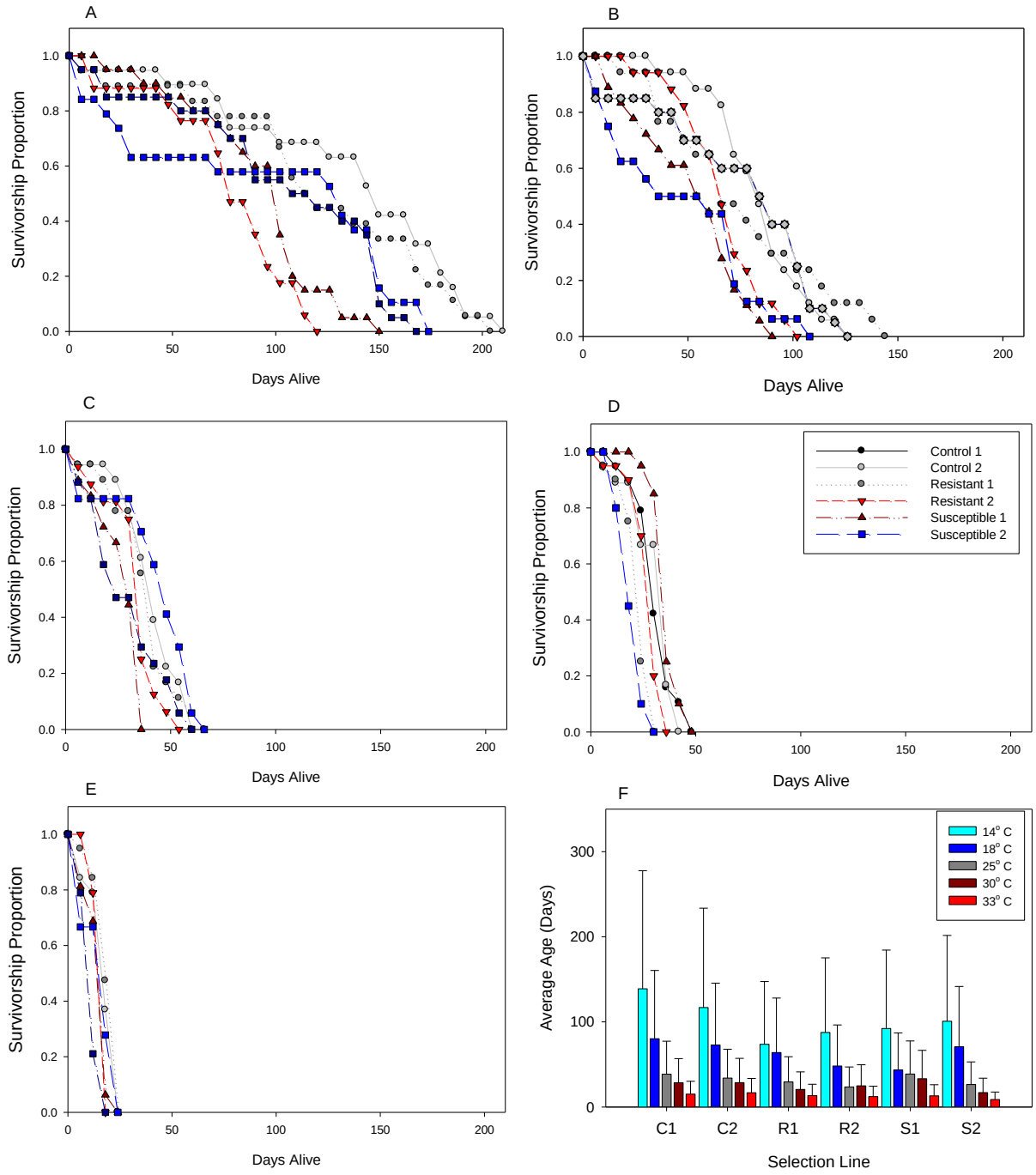

Figure 4.2. Longevity by selection line and temperature. Survivorship curves for flies reared at A) 14° C B) 18° C, C) 25° C, D) 30° C, E) 33° C. Red shades indicate resistant lines, blue represent susceptible lines, and gray represent control lines. F) Average age (means ± standard deviation) for line across all temperatures. Survivorship curves are significantly different from one another but differences are not consistent between selection pressures.

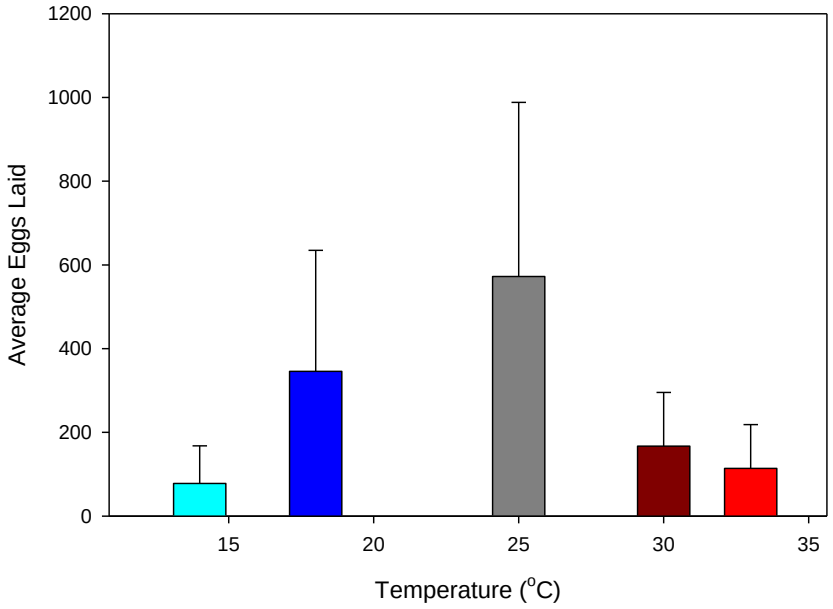

Figure 4.3. Average eggs laid across temperatures. Temperature had a significant effect on the numbers of eggs laid across the populations ($p<0.001$). There is a peak in average numbers of eggs at 25° C, with the lowest numbers at the extreme ends of the temperatures tested.

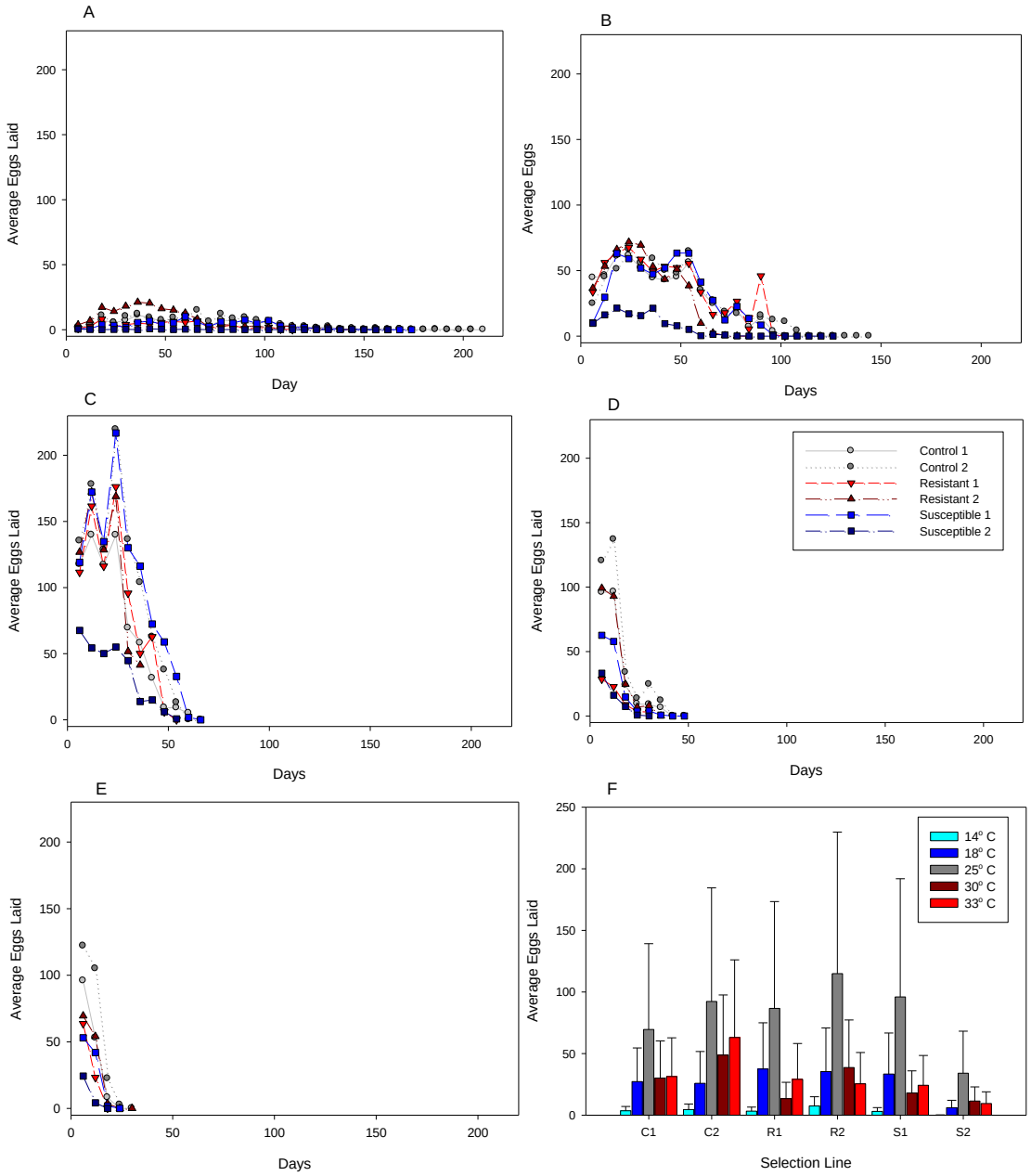

Figure 4.4. Mean eggs laid for each temperature per line. A) 14° C, where fecundity is relatively stable over time. B) 18° C, where fecundity shows a peak for most lines around day 25 and 50. C) 25° C, with most lines showing a peak at day 25. D) 30° C, with a quick peak in production early on and a sharp decline. E) 33° C, with a quick peak in production and a sharp decline in production. Red shades indicate resistant lines, blue represent susceptible lines, and gray represent control lines. F) Average eggs laid (means ± standard deviation) for line across all temperatures.

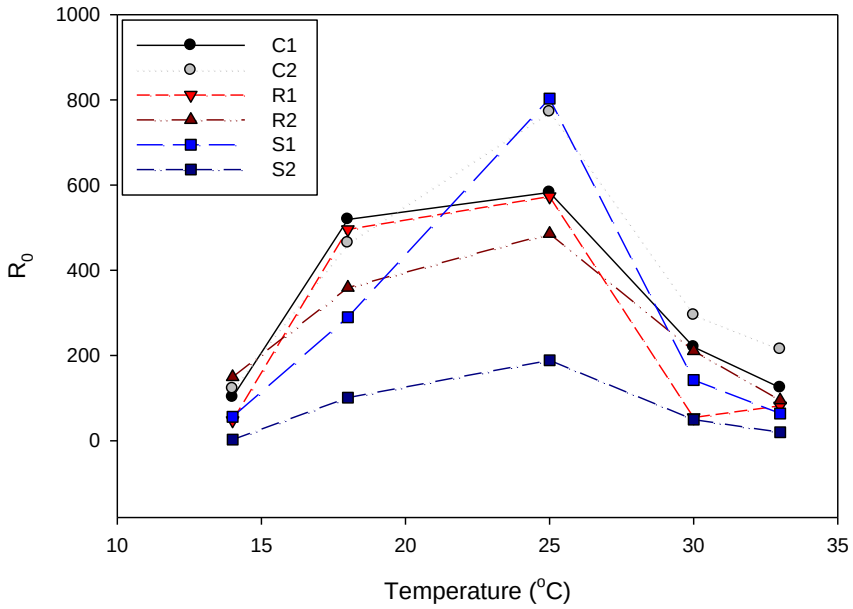

Figure 4.5. Net reproductive rate, R_0, for each temperature and line. Blue lines represent susceptible lines, red represent resistant lines, and grays are control lines. Significant differences by selection regime are not present in R_0, as several confidence intervals overlap across selection lines.

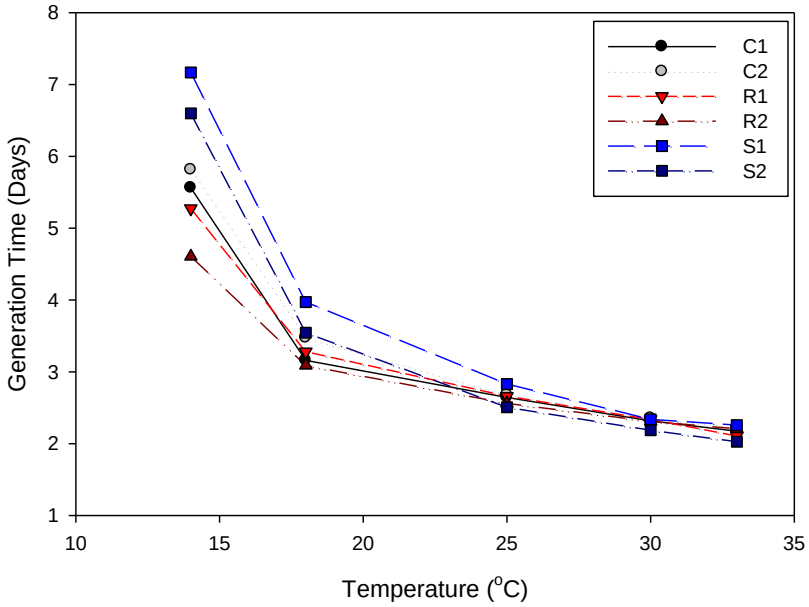

Figure 4.6. Generation time or average age at which female gives birth to her offspring. Blue lines represent susceptible lines, red represent resistant lines, and gays are control lines. Significant differences by selection regime are only present at 14° and 18° C, with resistant lines having quicker generation times than susceptible lines; at warmer temperatures generation times overlap across selection lines and regimes.

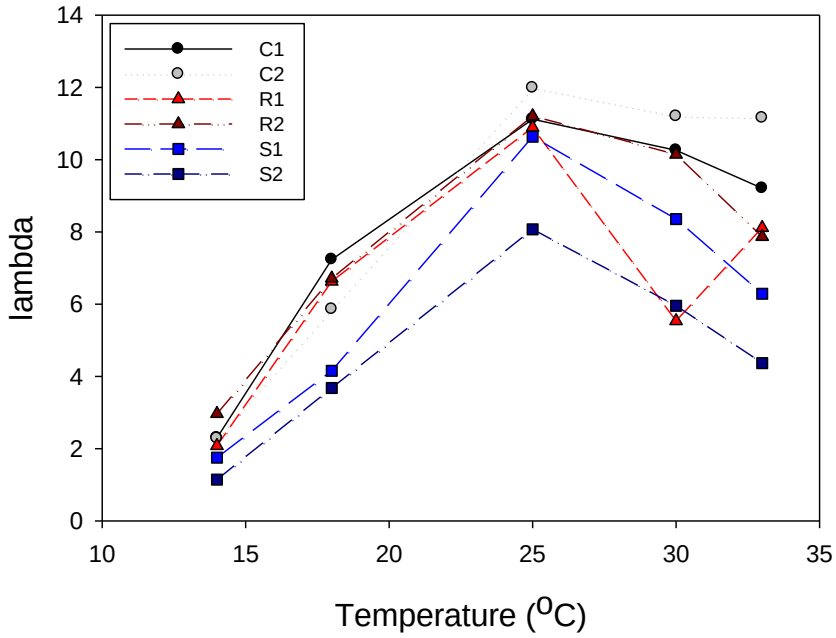

Figure 4.7. Intrinsic rate of increase or λ. Blue lines represent susceptible lines, red represent resistant lines, and gays are control lines. Significant differences by selection regime are only present at low temperatures, with resistant lines having higher λ values than susceptible lines at these temperatures.

Table 4.1. Full ANOVA for longevity. Selection represents selection pressure (resistant, susceptible, control), Line(selection) represents line effects nested within selection regime. Temperature represents the five test temperatures. Selection*Temperature is the interaction effect of these two terms. Line*Temperature(Selection) is the interaction of Selection nested within line and temeperature.

Source	**df**	**MS**	***F***	***P***
Selection	2	12763	34.19	0.0086
Line(selection)	3	373.30	0.26	0.85
Temperature	4	137383	95.85	<0.0001
Selection x Temperature	8	2671.79	1.86	0.15
Line(Selection) x Temperature	12	1433.80	1.89	0.033
Error	519	759.91		

Table 4.2. Pairwise comparison p-values of average longevity. Calculated by least squares means and adjusted using Tukey HSD. Comparisons suggest that selection differences in the main effects ANOVA are driven by differences in control lines and not by differences between susceptibility and resistance to cold.

Line	C1	C2	R1	R2	S1	S2
C1		0.8407	0.0001	<0.0001	0.0059	0.0080
C2			0.015	0.0075	0.18	0.22
R1				1.00	0.93	0.88
R2					0.87	0.79
L1						1.00
L2						

Table 4.3. Survivorship curve analysis. Median and 95% confidence intervals for each temperature show overlap among selection regimes. Selection lines do not show consistent patterns across temperatures, as upper and lower confidence intervals vary overall for each line at each temperature.

Temperature	Selection Line	Median	Lower Limit	Upper Limit
14° C	Control 1	144	123	180
	Control 2	111	99	168
	Resistant 1	75	69	99
	Resistant 2	97.5	78	102
	Susceptible 1	129	24	147
	Susceptible 2	102	84	147
18° C	Control 1	80	70	96
	Control 2	64	48	110
	Resistant 1	64	58	82
	Resistant 2	55	34	70
	Susceptible 1	42	12	74
	Susceptible 2	84	58	102
25° C	Control 1	38	34	53
	Control 2	36	30	43
	Resistant 1	31	30	41
	Resistant 2	26	22	31
	Susceptible 1	43	36	55
	Susceptible 2	20	17	46
30° C	Control 1	28	27	35
	Control 2	32	23	35
	Resistant 1	22.5	19	24
	Resistant 2	25	24	29
	Susceptible 1	34	32	37
	Susceptible 2	17	15	21
33° C	Control 1	18	17	19
	Control 2	18	17	21

	Resistant 1	14	12	16
	Resistant 2	15	8	17
	Susceptible 1	17	5	20
	Susceptible 2	10	9	14

Table 4.4. Post-hoc Tukey Pairwise Comparisons for survivorship curve analysis. P-values for each pairwise comparison for each temperature indicate few significant differences between susceptible and resistant lines. There are no consistent patterns of differentiation between selection regime.

14° C	Control 1	Control 2	Resistant 1	Resistant 2	Susceptible 1	Susceptible 2
Control 1		0.95	0.012	0.088	0.17	0.43
Control 2			0.12	0.48	0.67	0.92
Resistant 1				0.96	0.88	0.58
Resistant 2					0.99	0.96
Susceptible 1						0.99
Susceptible 2						
18° C	Control 1	Control 2	Resistant 1	Resistant 2	Susceptible 1	Susceptible 2
Control 1		0.97	0.63	0.029	0.010	0.93
Control 2			0.95	0.17	0.074	1.00
Resistant 1				0.64	0.39	0.98
Resistant 2					0.99	0.21
Susceptible 1						0.091
Susceptible 2						
25° C	Control 1	Control 2	Resistant 1	Resistant 2	Susceptible 1	Susceptible 2
Control 1		0.92	0.45	0.029	1.00	0.15
Control 2			0.95	0.27	0.91	0.66
Resistant 1				0.84	0.44	0.99
Resistant 2					0.029	0.99
Susceptible 1						0.14
Susceptible 2						
30° C	Control 1	Control 2	Resistant 1	Resistant 2	Susceptible 1	Susceptible 2
Control 1		1.00	0.0089	0.58	0.27	<0.001
Control 2			0.0079	0.54	0.33	<0.001
Resistant 1				0.41	<0.001	0.57
Resistant 2					0.0030	0.0080
Susceptible 1						<0.001

Susceptible 2						
33° C	Control 1	Control 2	Resistant 1	Resistant 2	Susceptible 1	Susceptible 2
Control 1		0.94	0.93	0.66	0.89	0.012
Control 2			0.43	0.17	0.36	<0.001
Resistant 1				0.99	1.00	0.14
Resistant 2					0.99	0.51
Susceptible 1						0.21
Susceptible 2						

Table 4.5. ANOVAs for average eggs laid. Differences among lines drive the highly significant interaction term. Temperature is a highly significant predictor of differences in longevity. Differences in Line also drives the Line nested within selection term, since selection is not significantly different in longevity. Selection represents selection pressure (resistant, susceptible, control), Line(selection) represents line effects nested within selection regime. Temperature represents the five test temperatures. Selection*Temperature is the interaction effect of these two terms. Line*Temperature(Selection) is the interaction of Selection nested within line and temeperature.

Source	**df**	**MS**	***F***	***P***
Selection	2	1292.94	2.78	0.2077
Line(selection)	3	465.72	3.79	0.0401
Temperature	4	4100.83	33.34	<0.0001
Selection x Temperature	8	153.36	1.25	0.35
Line(Selection) x Temperature	12	123.06	4.75	<0.0001
Error	519	25.92		

Table 4.6. Pairwise comparison p-values of average eggs laid. Comparisons indicate C2 significantly differs from all other lines, as does S2. R2 and S1 also are significantly different from one another. Calculated by least squares means and adjusted using Tukey HSD.

Line	**C1**	**C2**	**R1**	**R2**	**S1**	**S2**
C1		<0.0001	0.9967	0.4005	0.4294	<0.0001
C2			<0.0001	0.0464	<0.0001	<0.0001
R1				0.1692	0.7506	<0.0001
R2					0.0027	<0.0001
L1						<0.0001
L2						

Table 4.7. Means and 95% confidence intervals for R_0 for each selection line. Confidence intervals were calculated with bootstrap permutations in R Statistical Software and provide a look at significant overlap between selection lines. Superscripts represent differences of lines within a given temperature and alphabetical order represent increasing values.

		Resistant		Control		Susceptible	
Temperature		1	2	1	2	1	2
14° C	Mean	47.52	149.54	102.47	122.21	56.01	2.40
	Confidence Interval^{Group}	38.48-54.92^{B}	125.85-162.96^{E}	58.92-116.97^{C}	93.73-114.75^{C,D}	35.13-73.29^{B,C}	1.94-2.77^{A}
18° C	Mean	495.74	359.42	519.27	464.34	289.77	100.93
	Confidence Interval^{Group}	442.46-545.32^{D}	276.10-423.18^{B,C}	482.02-544.76^{D}	397.63-527.99^{C,D}	190.29-399.57^{B}	76.56-118.41^{A}
25° C	Mean	572.90	485.62	582.64	771.95	803.03	188.82
	Confidence Interval^{Group}	465.98-652.70^{B,C}	392.90-569.32^{B}	507.87-639.34^{B,C}	655.32-875.00^{D}	589.09-939.03^{C,D}	140.49-240.27^{A}
30° C	Mean	54.37	210.52	220.03	294.79	142.49	49.27
	Confidence Interval^{Group}	45.72-61.35^{A}	177.29-255.92^{C}	198.53-233.64^{C}	249.79-324.40^{D}	135.14-151.52^{B}	45.02-54.76^{A}
33° C	Mean	81.93	94.82	124.59	214.60	63.81	19.84
	Confidence Interval^{Group}	77.45-85.75^{B,C}	64.98-116.0^{B,C,D}	100.49-143.03^{C,D}	187.25-236.93^{E}	42.06-85.57^{B}	14.49-24.68^{A}

Table 4.8. Means and 95% confidence intervals for generation time for each selection line. Confidence intervals were calculated with bootstrap permutations in R Statistical Software and provide a look at significant overlap between selection lines. Superscripts represent differences of lines within a given temperature and alphabetical order represent increasing values.

		Resistant		Control		Susceptible	
Temperature		1	2	1	2	1	2
14° C	Mean	5.27	4.60	5.56	5.81	7.16	6.59
	Confidence Interval[Group]	5.05-5.52[B]	4.45-4.71[A]	5.35-5.77[B,C]	5.62-5.97[C]	6.72-7.63[D]	6.13-6.97[D]
18° C	Mean	3.27	3.08	3.15	3.47	3.97	3.54
	Confidence Interval[Group]	3.20-3.34[C]	2.96-3.17[A]	3.11-3.19[B]	3.37-3.54[D]	3.75-4.14[E]	3.42-3.67[D]
25° C	Mean	2.65	2.55	2.64	2.67	2.82	2.50
	Confidence Interval[Group]	2.56-2.71[A]	2.49-2.60[A]	2.59-2.67[B]	2.61-2.72[B]	2.79-2.87[C]	2.39-2.59[A]
30° C	Mean	2.33	2.30	2.31	2.35	2.33	2.18
	Confidence Interval[Group]	2.28-2.38[B]	2.29-2.32[B]	2.28-2.34[B]	2.31-2.37[B]	2.31-2.36[B]	2.13-2.22[A]
33° C	Mean	2.10	2.20	2.17	2.22	2.25	2.02
	Confidence Interval[Group]	2.07-2.12[B]	2.15-2.23[C]	2.14-2.19[C]	2.19-2.25[D]	2.23-2.28[D]	2.00-2.04[A]

Table 4.9. Means and 95% confidence intervals for λ for each selection line. Confidence intervals were calculated with bootstrap permutations in R Statistical Software and provide a look at significant overlap between selection lines. Superscripts represent differences of lines within a given temperature and alphabetical order represent increasing values.

		Resistant		Control		Susceptible	
Temperature		1	2	1	2	1	2
14° C	Mean	2.07	2.96	2.29	2.28	1.75	1.14
	Confidence Interval^{Group}	1.98-2.15^{C}	2.87-3.04^{E}	2.18-2.38^{D}	2.16-2.36^{D}	1.66-1.82^{B}	1.10-1.17^{A}
18° C	Mean	6.63	6.71	7.23	5.85	4.15	3.67
	Confidence Interval^{Group}	6.49-6.75^{C}	6.54-6.84^{C}	7.07-7.39^{D}	5.73-5.96^{B}	3.85-4.48^{A}	3.30-3.96^{A}
25° C	Mean	10.88	11.20	11.11	11.98	1.63	8.07
	Confidence Interval^{Group}	10.21-11.31^{B}	10.39-11.87^{B}	10.44-11.54^{B}	11.21-12.38^{B}	9.36-11.48^{B}	7.27-8.59^{A}
30° C	Mean	5.53	10.14	8.35	5.95	10.25	11.19
	Confidence Interval^{Group}	5.06-5.89^{A}	9.33-10.48^{C}	10.03-10.53^{C}	10.42-11.65^{C}	8.05-8.68^{B}	5.75-6.21^{A}
33° C	Mean	8.11	7.86	9.20	11.15	6.28	4.36
	Confidence Interval^{Group}	7.99-8.24^{B}	6.63-8.61^{B,C}	8.41-9.82^{C}	10.59-11.52^{D}	5.13-7.31^{B}	3.75-4.89^{A}

Chapter 5- Genetics of long-term and short-term cold acclimation: Different genes for seasonal and daily plasticity

Alison R. Gerken, Olivia C. Eller, Daniel Hahn, and Theodore J. Morgan

Alison R. Gerken

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: aegge@ksu.edu

Olivia C. Eller

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: oceller@ksu.edu

Daniel Hahn

Department of Entomology and Nematology, University of Florida, Gainesville, FL, 32611. E-mail:dahahn@ufl.edu

Theodore J. Morgan

Corresponding author. Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: tjmorgan@ksu.edu

Abstract

Organisms must deal with thermal variation on both a daily and seasonal basis. Prepratory drops in temperature can shield organisms from the extreme stresses. Rapid cold hardening (RCH) is a pretreatment of minutes to hours at non-lethal temperatures prior to extreme cold shock and has been shown to increase survivorship. Developmental acclimation (DACC) is a pretreatment of egg-to-adult development at a cooler temperature and has also been shown to increase survivorship. Comparison of these two acclimation pretreatments has shown both similarities and

differences in their physiology. However, the genetic mechanisms underlying these two phenomena have yet to be identified. We used the *Drosophila melanogaster* Genetic Reference Panel (DGRP) to assess natural variation in these two acclimation pretreatments. Our analyses show significant variation among this population of *D. melanogaster* as well as no significant overlap in the association mapping analyses of these traits. This suggests that these acclimation pretreatments are under different genetic control. Functional genetic analyses found several significant genes involved in RCH, DACC, and DACC+RCH pretreatments, including Atg7, Syt12, Eip74EF, and VGlut. These genes point to a role for autophagy regulation influencing successful acclimation ability. Our analyses also suggest that there are significant negative trade-offs between basal tolerance (acute and chronic cold tolerance) and plasticity scores (acclimation). These trade-offs suggest that different environmental pressures can lead to maintenance of one cold tolerance metric in lieu of another. These results show a first look at the differences in the genetic profiles between long-term and short-term acclimation, and suggest that these two processes are under different genetic control.

INTRODUCTION

Organisms must respond and adapt to environmental variation, whether it be in the form of behavioral or physiological adaptations. These responses and adaptations can also be invoked on a short-term or long-term scale, creating either a permanent change to the physiology of the organism or a transient, reversible response (Service 1987; Bowler 2005; Overgaard et al. 2005). When environmental change causes decreases in fitness and survival, we can classify the environment as a stress (Koehn and Bayne 1989; Sibly and Calow 1989; Harshman et al. 1999). A potent environmental stress, especially for ectotherms, is temperature variation, affecting most biochemical and physiological functions (Overgaard and Sørensen 2008) and impacting outward phenotypic traits such as growth, fecundity, and survival (David et al. 1983; Hoffmann and Parsons 1991; Gilchrist and Huey 2001).

As the climate changes, organisms must adapt not only to changes in mean temperature but also changes in extreme fluctuations (Irwin and Lee 2000; Sinclair et al. 2003), including extreme minimum temperature, which for ectotherms are extremely detrimental (Jentsch et al. 2007). Physiological research on insects indicate that some species die at temperatures that are much above their super cooling point, the temperature at which all liquid in the organism is frozen (Czajka and Lee 1990; Bale 1993; Sinclair et al. 2003; Nilson et al. 2006; Clark and Worland 2008; Koštál et al. 2011). Ectotherms employ mechanisms of behavioral, morphological, and physiological adaptations in order to prevent chilling injury, both on the long-term and short-term scales (Quinn 1985; Drobnis et al. 1993; Koštál et al. 2004; Kelty et al. 1996). The ability to mitigate these injuries is thus an integral part of an insect's physiological response to low temperature stress (Lee et al. 1987; Sinclair and Chown 2006; Nyamukondiwa et al. 2010, 2011).

An acclimation event, such as an exposure to a mild temperature prior to an extreme low temperature, has been shown to increase survivorship when compared to survivorship with no pre-treatment, whether it be a short-term exposure of minutes to hours (Lee et al. 1987; Bowler 2005; Overgaard and Sørensen 2008; Nyamukondiwa et al. 2011) or a long-term exposure of days to weeks (Powell and Bale 2005; Shintani and Ishikawa 2007; Ju et al. 2011) and is a form of phenotypic plasticity (West-Eberhard 2003; Ghalambor et al. 2007). Coping with low temperature extremes can benefit from two types of acclimation processes: rapid cold hardening (RCH) for short-term acclimation and developmental acclimation (DACC) for long-term acclimation (Lee et al. 1987; Goto 2000; Shintani and Ishikawa 2007; Bale and Hayward 2010; Colinet and Hoffmann 2012). These two types of acclimation responses have been classified as ecologically advantageous mechanisms for many insects (Bale and Hayward 2010; see review Teets and Denlinger 2013a). Rapid cold-hardening (RCH) has been shown to occur in variety of organisms and is especially important for ectotherms, as it conveys temperature stress protection in as little as a few minutes and (Lee et al. 1987; Coulson and Bale 1990), is phylogenetically widespread (Denlinger and Lee 2010) and thought to be a ubiquitous component for animals to adapt to cyclic environments (Sinclair and Chown 2006).

The ecology of the RCH response may provide the initial resistance of an organismal response to daily fluctuations (Shreve et al. 2004), environmental temperature extremes (Nyamukondiwa et al. 2010) and has been shown to occur in laboratory as well as field studies (Overgaard and Sørensen 2008; Shintani and Ishikawa 2007; Sinclair and Chown 2006). RCH can be especially beneficial during environmental transitions in autumn or spring when a long-term acclimation is incomplete or reversing, but temperatures are still fluctuating (Lee et al.

1987; Coulson and Bale 1990; Kelty and Lee 1999; Chown and Nicholson 2004; Powell and Bale 2005).

The long-term acclimation response is hypothesized to represent a seasonal preparation to dramatic changes in seasonal thermal variation (Bowler 2005). Acclimation is typically triggered at temperatures within the viable range of temperatures for the organism (Shintani and Ishikawa 2007) and follows induction by longer cues (Lee 1989). Whereas RCH is valuable for sudden extreme drops in temperature (Sinclair and Chown 2006), acclimation alters physiological mechanisms for temperature shifts of longer duration (Lee 1989; Bowler 2005). Organisms that have undergone long-term acclimation typically also have the capacity to act as if they had undergone RCH but not vice versa, as if the responses are linked by similar mechanisms of protection (Loeschcke and Sørensen 2005; but see Teets and Denlinger 2013a). Both RCH and acclimation are shown to have little cost in the induction of the response (Tollrian and Harvell 1999; Kingsolver et al. 2002; Gabriel 2005) in both metabolism (Basson et al. 2011) and fecundity (Shreve et al. 2004; Overgaard et al. 2007; but see Coulson and Bale 1992) and have even been shown to increase the length of reproductive life and longevity in the aphid *Sitobion avenae* (Powell and Bale 2005).

As such, it has been proposed that long-term acclimation and RCH responses are most effective in different environmental conditions and different time-scales, as DACC is best for long-term exposure to mid-range temperatures and RCH is best for protection from extreme cold at shorter durations (Powell and Bale 2006; Shintani and Ishikawa 2007; Ju et al. 2011). This continuum may indicate that RCH is the beginning in a flow of events that can turn into long-term acclimation response over time (Sinclair and Roberts 2005) and may even act in tandem with one another (Powell and Bale 2005; Ju et al. 2011). However, temperatures at which a RCH

pre-treatment is applied would become lethal if experienced as a long-term acclimation pre-treatment (Goto 2000) demonstrating the distinct effective temperature ranges, as well as differential response to cold tolerance measures (Goto 2000; Ransberry et al. 2011; Colinet and Hoffmann 2012) and injuries (Shintani and Ishikawa 2007; Ju et al. 2011).

Much work has been done on the overall responses of organisms to both long- and short-term acclimation as well as acute and chronic cold stress; however, the underlying mechanisms responsible for each of the acclimation responses are unclear and overlap between these two acclimation phenomenons are under much debate (Teets and Denlinger 2013a). Cold stress seriously affects membrane capabilities by increasing membrane ion pump injuries and causing loss of osmotic functionality (Koštál et al. 2004; Overgaard et al. 2005; Rajamohan and Sinclair 2008). Acute stress injury occurs via membrane phase transitions that cause damage (Lee 1991; Hazel 1995) and ion leakage (Quinn 1985; Drobnis et al. 1993; Sinclair et al. 2007); similarly, chronic stress can lead to lethality by attempts at ion equilibration across membranes (Koštál et al. 2004). To combat temperature stresses, a suite of mechanisms have been proposed such as maintenance of metabolic activity via protein function (Hawkins et al. 1987) and membrane organization and fluidity (Hazel 1995; Overgaard et al. 2005; MacMillan et al. 2009). Organisms may also have lower metabolic rates to conserve resources and minimize exposure (Service 1987; Hoffmann and Parsons 1989; Portner et al 2000; Scholander et al 1950), accumulate metabolic energy reserves, protect proteins through the use of heat shock proteins (HSPs) (Bubliy and Loeschcke 2005; Rinehart et al 2006; Sinclair et al 2007), utilize cryoprotectants such as glycerol, sorbitol, trehalose (Storey et al. 1991) or proline (Koštál et al. 2011), ice nucleators or antifreeze proteins (Lee 1991; Sinclair and Roberts 2005; Clark and Worland

2008), and adjust water content (Overgaard et al. 2005; Clark and Worland 2008) as universal mechanisms of cold protection.

Much debate has taken place on whether the mechanisms underlying RCH and DACC are shared or if a different suite of physiological or genetic changes underlies each acclimation response (Precht 1973; Sinclair and Roberts 2005; Rako and Hoffmann 2006; Colinet and Hoffmann 2012; Teets and Denlinger 2013a). Long-term acclimation has been shown to include an increase in cryoprotectants (Lee 1991; Rako and Hoffmann 2006) but the accumulation of cryoprotectants in RCH has been inconclusive as Sinclair and Chown (2006) and Michaud and Denlinger (2006) indicate their presence (see also Chown and Nicolson 2004; Overgaard et al. 2007), but Kelty and Lee (2001) did not. Proteins such as ice nucleators, which regulate the temperature at which ice forms in the insect body, and antifreeze proteins have been detected to increase in long-term acclimation processes (Lee 1991; Clark and Worland 2008); the RCH response instead has shown evidence that ice nucleators and antifreeze proteins are not used during the process, but expression of heat-shock proteins is increased in some (Goto 2000; Qin et al 2005; Sinclair et al. 2007; Clark and Worland 2008) but not all cases (Kelty and Lee 2001; Chown and Nicolson 2004; Overgaard et al. 2005; Teets et al 2012).

One of the primary physiological benefits of RCH and long-term acclimation is membrane restructuring via lipid configuration (Hazel 1995) in order to maintain membrane fluidity (Overgaard et al. 2005, 2008; Michaud and Denlinger 2006; MacMillan et al. 2009). This maintenance has been shown to occur in a variety of ways and differ for long-term and RCH responses (Russell 1997; Lee et al. 2006). Overgaard et al. (2005) found an overall decrease in fatty acid saturation following a RCH pre-treatment, which lowers the temperature of liquid-crystal to gel phase transition of the membranes (Russell 1997; Lee et al. 2006). Fatty acid

organization was also under construction for long term acclimation as well, and included a proportional increase in unsaturated fatty acids to saturated fatty acids in some studies (Michaud and Denlinger 2006; Kayukawa et al. 2007) but little change or a lowering of unsaturation in others (Overgaard et al. 2008; Goto and Katagiri 2011). Differentiation between long-term acclimation and rapid cold hardening relies on inconsistent information in the similarities and differences in physiological membrane restructuring and accumulation of proteins (Hazel 1995; Kelty and Lee 1999; Overgaard et al. 2005; Sinclair and Roberts 2005; Teets and Denlinger 2013a).

Although there is a wealth of information on rapid cold hardening and acclimation treatments as separate entities, an examination comparing both cold-acclimation responses has been lacking (but see Shintani and Ishikawa 2007; Ju et al. 2011; Colinet and Hoffmann 2012; Teets and Denlinger 2013a for review). Prior studies focused solely on the phenotypic outcome of the treatments, i.e. survivorship, and concluded that differences between long-term acclimation and RCH are evident through differences in survivorship following each pre-treatment compared to survivorship with no pre-treatment. Physiological evidence on membrane restructuring, including protein and cryoprotectant accumulation, is also contradictory and experiments have thus far been only conducted on single genotypes or small sample sizes (Hazel 1995; Overgaard et al. 2005; Michaud and Denlinger 2006; Franke et al 2012). Genomics research has been limited, although Teets et al (2012) conducted a microarray analysis on the flesh fly *Sarcophaga bullata* and concluded that RCH had very little impact on gene expression during and after cold shock. Thus natural, genetic variation and evaluation of associations between traits and genotypes has yet to be sufficiently explored (Loeschcke and Sørensen 2005) and is necessary as phenotypic variation can be found among the many genotypes within a single population

(Mackay et al. 2012). This population-wide genetic variation reflects the underlying constraints in adaptive responses: an organisms' phenotype only mirrors the ability of its genotype to alter physiological, metabolic, and behavioral changes following pre-treatment exposures (Goto 2000; Kelty et al. 1996; Sinclair et al. 2007; Kellermann et al. 2009; Marshall and Sinclair 2009).

Targeted gene expression studies have focused primarily on genes such as *Frost*, Smp-30 (the *Drosophila* cold acclimation gene), and a variety of heat shock proteins and their up- or down-regulation to cold hardening (Goto 2000, 2001; Qin et al. 2005; Overgaard et al. 2005; Sinclair et al. 2007; Ellers et al. 2008). Low temperature tolerance responds to selection pressures demonstrating an underlying genetic component to temperature adaptations (Bubliy and Loeschcke 2005; Sinclair et al. 2007). Investigating this temperature tolerance through population-wide genomic assays will allow us to identify cold tolerance or adaption gene regions and the regulatory mechanisms behind these adaptive responses (Loeschcke and Sørensen 2005). These analyses will also allow us to compare known molecular and biological functions of genes of interest to understand the impacts of possible convergent evolution on these acclimation pre-treatments. Although differing long-term and short-term environmental pressures drive different acclimation processes, similar functions (i.e. physiology) with alternate genetic regions could drive the overall increase in survivorship, suggesting a convergent evolution for long-term and short-term cold acclimation (Thurber et al 2013; Arendt and Reznick 2007).

Basal tolerance levels must also be considered when analyzing the independence and constraints on cold tolerance traits. Comparisons of basal thermal tolerance and phenotypic plasticity ability (acclimation responses) have focused primarily on interspecific comparisons, typically across an ecological gradient such as latitude (Stillman 2003; Kellett et al 2005; Calosi et al 2008; Chown et al 2010) or a phylogeny (Nyamukondiwa et al 2011). These studies have

found both evidence for and against an association between basal thermotolerance and phenotypic plasticity capacity. When comparing basal thermotolerance and plasticity capacity, three hypotheses are possible. First, the two traits may show no association with one another; the traits are independent of one another within an organism or population. Second, the two traits may show a positive correlation. In this association, basal tolerance and plasticity are positively related, where organisms fall on continuum from cold tolerant in both basal and plasticity capacities to cold intolerant for both basal and plasticity. Finally, the basal thermotolerance and plasticity capacity may be negatively correlated with one another, indicating a trade-off in these two cold tolerance traits.

Evidence for all three hypotheses has been gathered in multiple species. Kellett et al (2005) found that eight species of *Drosophila* had a positive correlation between basal thermotolerance and plasticity; however, this relationship was driven by one species' extreme positive relationship. Calosi et al (2008) also found a positive relationship with heat tolerance and acclimation abilities in European diving beetles. This relationship predicts that those species with lowest basal thermotolerance levels are most at risk for climate change pressures, as they not only have poor survival abilities at high heat but also poor acclimation abilities. However, Stillman (2003), found a trade-off between basal thermotolerance and acclimation ability in Porcelain crabs but only when using critical thermal maximum (CT_{max}) as an index; critical thermal minimum (CT_{min}) did not show a significant correlation (but see Nyamukondiwa et al 2011; Foray et al 2013). These results suggest that crabs with poor survival abilities may be trading off the ability to survive acute heat stress with the positive ability to acclimate to heat stress. With current climate change conditions, including increases in temperature variation (Jentsch et al 2007; IPCC 2013), an increase in performance curve breadth, i.e. the range of

temperatures at which an organism can perform, is predicted to lead to a decrease in phenotypic plasticity (Angilletta 2009), as predictability of climate extremes declines and maintaining phenotypic plasticity capacity could be costly (Chown et al 2010). In other words, organisms will have to maintain capacity to respond to a larger range of temperatures and in consequence may lose their ability to acclimate in the face of extreme temperature changes. Expanding sample sizes and within species variation of the basal thermotolerance and plasticity capacity association should provide insight into trade-offs within populations that could constrain evolution of cold tolerance abilities necessary for adapting to extreme thermal fluctuations predicted under current climate change scenarios (IPCC 2013).

The *Drosophila melanogaster* Genetic Reference Panel (DGRP) provides us with an easy to use, fully genotyped population to explore natural, population level variation and the underlying genetic variation driving adaptation responses (Flint and Mackay 2009; Mackay et al. 2012). *Drosophila melanogaster* are a cosmopolitan species that has evolved thermal adaptations in order to expand its range to many temperate and sub-tropical regions (David et al. 1983; Umina et al. 2005; Overgaard and Sørensen 2008). *D. melanogaster* die at temperatures well above their super-cooling point (Czajka and Lee 1990), demonstrate a high degree of plasticity, and must adapt to thermal variation through physiological mechanisms such as the RCH response and long-term acclimation (Leips and Mackay 2000; Gibert et al.2001; Overgaard and Sørensen 2008; Chown et al. 2009; Koštál et al. 2012) and thus, are interesting for cold tolerance. Although *D. melanogaster* are a highly used model organism for physiology and genetics, the association of these two concepts lacks an understanding and identifying genomic regions involved in thermal adaptation in *D. melanogaster* will give us insight as to how genetic adaptations drive the phenotypic responses to cold stress and acclimation.

We used the DGRP resource to explore population-wide genetic variation in adaptation responses for a first look at the underlying genetic components of acclimation, allowing us to pair physiological and molecular differences between rapid cold hardening and long-term acclimation. First, set out to determine if there is underlying variation within a population by testing 182 iso-female lines of the DGRP with pre-treatments of developmental acclimation (DACC), rapid cold hardening (RCH), and DACC combined with RCH (DACC+RCH). Using two different rearing temperatures allows us to identify not only genotype-by-environment interactions but also potential genotype-by-environment-by-environment interactions (Hoffmann and Parsons 1989; Harshman et al. 1999; Bubliy and Loeschcke 2005). We conducted association mapping of these genotypes (see Mackay et al. 2012) to determine genetic variation between the adaptation responses of the population, comparing survivorship between pre-treated and non-pretreated cold shock treatments. The association mapping provides us with a list of potential candidate genes involved in the DAC, RCH, and DACC+RCH responses (Brakefield 2003; Mackay et al. 2012). A portion of these potential candidate genes were then be screened for functional roles in survivorship traits in DACC and RCH responses via knockout mutations. We also exposed the genotypes to a chronic cold stress to analyze the basal tolerance levels within the naturally segregating population. Our hypotheses are: 1) the physiological responses of DACC, RCH, and DACC+RCH will differ from one another; 2) the genetic components associated with each acclimation treatment will differ from one another; 3) basal tolerance and acclimation response will be negatively associated in a trade-off response in the DGRP in physiological response; and 4) the underlying genetics of basal tolerance and acclimation responses will overlap. These correlation analyses of genetic architecture of acclimation responses and correlation of basal tolerance and acclimation are useful in understanding how

cold tolerance and acclimation abilities evolve, whether they evolve independently or if they are constrained by another factor of cold tolerance (Franke et al 2012; Chown et al 2010).

METHODS

Drosophila melanogaster Genetic Reference Panel

All 192 lines were obtained from the Bloomington Stock Center (Bloomington, IN, USA). Stocks were maintained at 25°C on a 12 hour light, 12 hour dark cycle (Basson et al. 2011). Flies were maintained in variable numbers in *Drosophila* stock vials with approximately (20 mL) agar, molasses, and cornmeal standard fly medium. When flies were ready to be used in experimental treatments, five males and five females from each individual line were anesthetized via CO_2 (Nilson et al. 2006) and placed in a new vial for egg laying; flies reared at 25° C were removed from the vials every three days in order to control larval densities. Flies reared at 18° C were removed every five days. Offspring were then allowed to develop at their given rearing temperature, eclose from pupal state, and experiments were conducted on 5 to 7 day old flies to avoid developmental differentiation effects (Czajka and Lee 1990; Marshall and Sinclair 2009; Basson et al. 2011). All acute and acclimated cold tolerance treatments are summarized in Figure 5.1.

Chronic Stress Treatment

A chronic cold treatment was performed on flies reared at 25° C for the entirety of their life span in order to examine the population-wide differences of chronic cold exposure. Once flies had eclosed, they were anesthetized using CO_2 and separated into males and females in individual fly stock vials with 10 mL standard food medium in each vial. Each vial contained 10-

20 flies from individual lines of separate sex. Flies were then allowed to age to 5-7 days old, after which they were placed in empty vials for the treatment in order to avoid influence of the food on the freezing temperature of the flies. Vials were placed at 0° C for 16 hours to simulate a chronic cold exposure. After 16 hours, flies were removed from the cold, placed in new vials containing food medium and allowed to recover for 24 hours at 25° C (Kelty and Lee 2001; Overgaard et al. 2005; Colinet and Hoffmann 2010). After 24 hours of recovery, survivorship was assessed. Flies were considered alive if they showed coordinated movement (Powell and Bale 2005). Three replicates of each line were performed at this treatment (Leips et al. 2006; Shintani and Ishikawa 2007).

Acute Stress and Acclimation Treatments

Rearing flies at two different temperatures results in two diverse groups of flies, suitable for identifying an effect of developmental acclimation. As stated above, flies from each DGRP line were allowed to lay eggs at either 18° C or 25° C; these eggs were then allowed to fully develop and eclose into adults at these two different temperatures. Even though flies reared at 18° C and 25° C experience differences in developmental rate, all flies were 5-7 days old when used in cold stress treatments. Upon eclosion, flies were removed from their vials and anesthetized with CO_2, separated into males and females, and placed into new vials containing food. There were 10-20 flies per vial per sex (Rajamohan and Sinclair 2008; Benoit et al. 2009; Jumbo-Lucioni et al. 2010), and flies were allowed to age until 5-7 days old with no further exposure to CO_2.

Upon reaching the required age for experiments, flies reared at both 18° and 25° C were experimentally tested. First, flies were placed into empty vials in order to prevent cold

temperature effects to be confounded by food. The flies were then placed in one of several cold stress treatments. Extreme cold stress and discriminating temperatures were previously determined (Gerken et al *in prep*, Chapter 2; Powell and Bale 2005; Sinclair and Chown 2006). Flies reared at 25° C were exposed to one of two different cold treatments. The first consisted of a pre-treatment of 2 hours at 4° C followed by cold exposure at -6° C for 1 hour (i.e. RCH). Flies in the second treatment were placed directly from room temperature to -6° C for 1 hour (i.e. no RCH; David et al. 2003; Overgaard and Sørensen 2008; Marshall and Sinclair 2009). Flies reared at 18° C were subjected to one of three different treatments: flies were placed in a cold incubator at -6° C or -8° C directly from room temperature and stressed for 1 hour (i.e. no RCH) or they were pre-treated with an exposure to 4° C for 2 hour followed by -8° C for 1 hour (i.e. DACC+RCH). After all cold shock treatments, whether pre-treated or not, flies were allowed to recover for 24 hours on standard fly medium and survivorship was assessed. Survivorship was scored as the ability of the fly to perform coordinated movements. We performed three replicates on each line by sex treatment for each rearing temperature.

Statistical Analysis and Comparisons

Survivorship was recorded as the proportion of adult *D. melanogaster* surviving each cold stress treatment (Rajamohan and Sinclair 2008; Benoit et al. 2009; Jumbo-Lucioni et al. 2010) after 24 hours recovery. Males and females were analyzed separately, but were highly correlated (Pearson Product Moment, Supplemental Table 5.S1) so survivorship for sexes is pooled and data reported for acclimation scores, survivorship, and association mapping results is from pooled sexes only. Replicates were pooled for survivorship proportions. Means and standard deviations were calculated using R statistical software; means represent the phenotypic

trait value for each line within the population (Chown et al. 2009). Variation in survivorship proportions were compared among the 182 viable lines of the DGRP using R statistical software (R Statistical Software Version 2.13.0). Prior to conducting association mapping, acclimation scores were calculated using the survivorship means of each individual line in order to determine the magnitude and direction of the RCH, DACC, or DACC+RCH response for each line using the formula

$$Plasticity(\text{acclimation})score = \%Survival_{Hardened} - \%Survival_{NonHardened}$$

These phenotypic values represent the ability of each individual line to either successfully or unsuccessfully use DACC or RCH as an adaptive process to increase survival as well as the intensity of the response. RCH values for flies reared at 18° C (DACC+RCH) were calculated by subtracting the survivorship proportion of flies exposed directly to -8° C for 1 hour from those exposed to a pre-treatment of 4° C at 2 hours then exposed to 1 hour at -8° C. RCH values for flies reared at 25° C were calculated by subtracting the survivorship proportions of flies exposed directly to -6° C for 1 hour from those exposed to a pre-treatment of 4° C for 2 hours followed by exposure of 1 hour at -6° C. DACC values were calculated by subtracting survivorship proportions of flies reared at 25° C tested directly with -6° C for 1 hour from flies reared at 18° C tested directly with -6° C for 1 hour (Figure 5.1).

Phenotypic values for each acclimation score were assessed via Pearson's Product Moment Correlation to assess physiological associations between short-term and long-term acclimation and the combination of the two. Survivorship proportions for cold survival without any pretreatment will be compared to acclimation scores for each line to assess basal thermotolerance and plasticity (acclimation) capacity. Chronic survivorship for each line is compared to each acclimation score to assess basal tolerance and plasticity correlations.

Pearson's Product Moment Correlation analysis in R statistical software was used to assess strength of correlations and significance of correlative variables to one another.

Genome-wide Association Mapping

Genome-wide association mapping for 182 lines of the DGRP tested was performed for survivorship proportions for flies reared at 25° C and tested for chronic cold stress survival, flies reared at 18° C and tested at -8° C, flies reared at 25° C and tested at -6° C, RCH scores, DACC scores, and DACC+RCH scores. Publicly available single nucleotide polymorphism (SNP) data from Mackay et al 2012 (http://dgrp.gnets.ncsu.edu; DGRP Freeze 1.0) were used to perform genome-wide association analysis using 2,425,403 publicly available SNPs. Associations are computed for SNPs with minor polymorphism in a minimum of 4 lines with mean coverage of 2-30 (Mackay et al 2012). Phenotypes were analyzed for pooled sexes (see above) and the ANOVA used was phenotype= mean + M, where M is the SNP marker. A significance level of $p<10^{-5}$ is used as a cut-off for significant SNPs. MMC clustering is performed to analyze LD among significant SNPs in order to assess long-distance LD that could be found because of the small number of lines tested. Output provided genes and chromosome positions for single nucleotide polymorphisms at a cut-off value of $p<10^{-5}$ (Mackay et al. 2012). Overlap for significant SNPs for each acute cold stress survivorship association mapping will be compared to the appropriate SNPs associated with acclimation ability to assess overlap in genetic architecture of the traits at the $p<10^{-5}$ significance level. Minor allele frequencies were calculated as the frequency of the less common allele compared to total alleles and effect sizes were calculated as half the difference in mean phenotype for major and minor alleles (Mackay et al 2012).

Mutant Analysis

Gene lists from the association mapping output provided potential candidate genes that were highly significant among the naturally segregating populations of the DGRP. These gene lists were then compared among the survivorship proportions, DACC+RCH, RCH, and DACC phenotypes, noting any trends in gene representation among the phenotypes. Candidate genes were then selected based on three categories: highly significant p-values ($p<10^{-5}$); those of physiological interest; and those in missense regions of the chromosomes. Twelve mutant lines and two background lines were obtained from the Bloomington Stock Center (Bloomington, IN, USA). Chromosome, position, and genetic mutant are identified in (Table 5.1).

Functional mutant tests, using knockout mutants, were conducted using the same parameters of rearing temperature to assess RCH, DACC+RCH, and DACC via acute stress as described above (Brakefield 2003; Jumbo-Lucioni 2010; Mackay et al. 2012). Backgrounds of the knockout mutants were used as a control for RCH, DACC+RCH, and DACC ability. ANOVAs were calculated for each mutant compared to each background independently using the formula

$$Survival = m + Mut + Treat + Mut \times Treat + e$$

where survival is the proportion of survivorship, mutant is either the background or the knockout mutant, and treatment is either without acclimation or with an acclimation pretreatment. Significant interaction effects of Mutant*Treatment indicate a difference in survival between mutant and background when comparing with- and without-acclimation treatments and indicate the genes involvement in the appropriate acclimation response.

RESULTS

Chronic Cold Stress

Chronic cold stress survival showed a normal distribution for the DGRP lines with an average survivorship of 52.62 ± 26.10 % (mean ± standard deviation) for all lines (Figure 5.2). Males had lower survivorship than females, with 42.99 ± 29.04% and 62.06 ± 29.34% survivorship respectively. For further analyses, sexes are pooled as their survivorship proportions are highly correlated ($r=0.59$, $p<0.0001$).

Acute Cold Stress and Rapid Cold Hardening Survivorship

Acute cold stress for flies reared at 25° C resulted in a normal distribution for lines of the DGRP (Figure 5.3A). The average survivorship for all lines combined is 53.38 ± 21.68% (mean ± standard deviation) with males and females similar in survivorship with 54.21 ± 25.74% and 52.63 ± 26.27% survivorship, respectively. A rapid cold hardening pre-treatment of 2 hours at 4° C, significantly increased survivorship for flies reared at 25° C and then tested at -6° C for 1 hour, and skewed the distribution to the right (Figure 5.3B). Overall survivorships between hardened and non-hardened flies were significantly different ($p<0.001$; Figure 5.4). Average survivorship for all lines was 71.77 ± 20.34%, with female survivorship at 68.34 ± 24.71% and male survivorship at 75.17 ± 21.41%.

Acute cold stress for flies reared at 18° C showed a strong skew for cold stress at -6° C for 1 hour and a normal distribution for cold stress at -8° C for 1hour (Figure 5.3 C and D). Average survivorship for all lines for cold stress of -6° C for 1 hour for flies reared at 18° C is 97.03 ± 3.55%, with 96.01±0.06% for females and 97.97 ± 6.13% for males. For cold stress of 1 hour at -8° C for all lines reared at 18° C, the average survivorship is 42.17 ± 18.12%. For females the average survivorship is 38.81 ±19.96% and 44.86 ± 21.81% for males at -8° C for 1

hour. Survivorship of all lines after an RCH pre-treatment of 2 hours at 4° C followed by 1 hour at -8° C is highly skewed right for DGRP lines and is on average 89.03 ± 12.41% (Figure 5.3E). For females, average survivorship of all lines with RCH pretreatment is 87.94 ± 15.05% and for males it is 90.45 ± 13.18%. Comparisons of means of lines tested with a pretreatment at -8° C for 1 hour and with no pretreatment at -8° C for 1 hour indicate that pretreatment of 2 hours at 4° C significantly increases survivorship for all lines combined ($p<0.001$, DACC+RCH; Figure 5.4). Paired t-tests of survivorship of all lines reared at 25° C and tested for 1 hour at -6° C compared to survivorship of lines reared at 18° C and tested for 1 hour at -6° C indicate that development at 18° C significantly increases survivorship at -6° C ($p<0.001$; Figure 5.4, DACC).

For all acute cold stresses, sexes were pooled as they were significantly correlated. Thus, acclimation scores reflect those calculated with sexes pooled. For flies reared at 25° C and tested for 1 hour at -6° C, males and females had $r=0.40$; flies reared at 25° C and tested for 2 hours at 4° C and then for 1 hour at -6° C had $r=0.55$ for males and females ($p<0.0001$ for both correlations; Supplemental Table 5.S1). For flies reared at 18° C and tested at -6° C and -8° C, males and females had an $r=0.37$ and 0.49, respectively; for flies reared at 18° C and tested for 2 hours at 4° C and then 1 hour at -8° C, $r=0.49$ ($p<0.0001$ for all correlations; Supplemental Table 5.S1).

Acclimation Effects

Acclimation scores (plasticity) were calculated and showed a high amount of variability among the lines of the DGRP. Analysis of all of the DGRP lines allows us to compare and observe the range of phenotypic and genotypic variation in a naturally segregating population. Short-term acclimation, rapid cold-hardening (RCH) for flies reared at 25° C, showed an overall

increase in survivorship following a pre-treatment at 4° C for 2 hours and was highly variable among the lines for acclimation score. A total of 34 of the 182 DGRP lines tested for RCH showed negative RCH scores (Figure 5.5), meaning that survivorship in these lines was lower following a pre-treatment of 4° C for 2 hours. The average RCH score for all DGRP lines is 0.18 ± 0.20 (mean ± standard deviation). The RCH score here represents an 18% increase in survivorship following pretreatment. All subsequent acclimation scores can be interpreted in such a way as well.

For long-term, developmental acclimation (DACC) compared survival of flies reared at both 18° C and 25° C and tested at -6° C for 1 hour. Two lines of the 182 tested had slightly negative DACC scores and the average DACC score for all DGRP lines is 0.43 ± 0.21 (Figure 5.5). The combination treatments of long-term and short term acclimation (DACC+RCH) did not have any lines that showed a negative acclimation score and the average DACC+RCH score is 0.47 ± 0.18 (Figure 5.5).

Assessment of physiological overlap among the acclimation scores is assessed through Pearson's product moment correlations. Each combination of acclimation scores were compared to one another individually (Figure 5.6). DACC+RCH score had no significant correlation to RCH score ($r=0.06$, $p=0.28$). Similarly, DACC+RCH score had no significant correlation with DACC score ($r=0.12$, $p=0.09$). RCH score and DACC have a significant correlation of acclimation scores ($r=0.55$, $p<0.001$).

Basal tolerance versus Plasticity Correlations

Basal tolerance (acute or chronic survivorship) and acclimation (plasticity) correlations were compared using survivorship and plasticity scores for all three acclimation treatments

(Figure 5.7). All comparisons showed a significant, negative correlation. Survival of flies reared at 25° C and tested for 1 hour at -6° C showed a significant, negative correlation to RCH score for all lines ($r= -0.56$, $p<0.001$). Survival of flies reared at 18° C and tested for 1 hour at -8° C showed a negative, significant correlation with DACC+RCH score ($r= -0.15$, $p<0.001$) and survival for flies reared at 25° C and tested for 1 hour at -6° C had a strong, significantly negative correlation with DACC score ($r= -0.46$, $p<0.001$).

Chronic cold tolerance was also used as a measure of basal tolerance for the DGRP genotypes and was compared to acclimation scores. Chronic cold tolerance and RCH score did not have a significant correlation (Figure 5.8; $p=0.87$, $r=0.011$). DACC score and chronic cold tolerance had a significant negative correlation ($p<0.0001$, $r= -0.98$) as did DACC+RCH score and chronic cold tolerance ($p=0.04$, $r= -0.76$).

Association Mapping Analysis

Association mapping analyses revealed a significant number of genes associated with chronic cold stress exposure for flies reared at 25° C and tested for 16 hours at 0° C, with little linkage disequlibrium represented (Supplemental Figure 5.S1). Twenty-two SNPs were significant at a $p<10^{-5}$ level with 14 unique genes represented in the association mapping list (Figure 5.9; Table 5.2). Genes associated with significant SNPs for chronic cold stress had biological functions related to synaptic transmission, programmed cell death, biological regulation, and carbohydrate metabolic processes and molecular functions related to protein binding, calcium ion binding, and cytoskeleton structure and binding.

Association mapping analysis revealed a significant number of genes associated with each acclimation treatment. A total of 34 SNPs were associated with RCH score for the 182 lines

of the DGRP tested ($p<10^{-5}$; Figure 5.10; Table 5.3), with 25 unique genes housing these SNPs. There was also little linkage disequlibrium for this phenotype (Supplemental Figure 5.S2). Multiple genes associated with RCH had molecular functions associated with nucleic acid binding, protein binding, and imaginal disc-derived wing morphogenesis and biological functions related to chitin processes, autophagy, adult lifespan, and synaptic transmission (FlyBase.org). For DACC score, 34 SNPs were significantly associated with DACC score in the DGRP lines at $p<10^{-5}$ (Figure 5.11; Table 5.4), representing 22 unique genes. There was some mid-level linkage disequlibirum in this phenotype but was not extreme (Supplemental Figure 5.S3). Genes associated with DACC had molecular functions associated with transmembrane signaling, protein binding, calcium phospholipid binding, and voltage gated potassium channels and biological functions associated with phagocytosis, programmed cell death, and proteolysis.

The combination acclimation treatment identified 38 SNPs significantly associated with DACC+RCH score in the DGRP at $p<10^{-5}$ (Figure 5.12; Table 5.5) with 18 unique genes containing the SNPs. There was little linkage disequlibrium in this phenotype (Supplemental Figure 5.S4). Genes were associated with molecular functions such as protein binding, extracellular channel activity, transmembrane transporters, calcium ion binding, sodium ion transport, and RNA polymerase II and biological functions associated with proteolysis, sodium ion transport, autophagy, and synaptic transmission. For all phenotypes tested, there was little long range LD (Supplementary Figures 5.S2-4) shown by low r^2 (blue) in associated heat maps with significant SNPs for each phenotype.

Out of the 128 SNPs that were significant at the $p<10^{-5}$ significance level for chronic cold survivorship, DACC score, RCH score, and DACC+RCH score, only one SNP associated with RCH and DACC overlapped, while no SNPs overlapped between DACC and DACC+RCH or

RCH and DACC+RCH. The only SNP to overlap between DACC and RCH is in the gene Cda4 on the X chromosome, which is associated with broad functions such as chitin binding, hydrolase activity, and neurogenesis. Chronic cold tolerance and plasticity scores did not overlap any of their significant SNPs at $p<10^{-5}$.

Basal tolerance (acute survivorship) and plasticity (acclimation) scores had significant SNP overlap in two of the three comparisons. When comparing DACC and survivorship after 1 hour at -6° C for flies reared at 25° C, the association mapping indicated 13 SNPs precisely overlapped out of a total of 40 total unique SNPs for both treatments combined. For RCH and survivorship after 1 hour at -6° C for flies reared at 25° C, association mapping results indicated that out of 47 unique SNP locations, two overlapped at $p<10^{-5}$ significance level, 10 overlapped at $p<10^{-3}$, and 7 overlapped at $p<10^{-2}$. For DACC+RCH and survivorship after 1 hour at -8° C for flies reared at 18° C, only 1 SNP out of 66 unique SNPs from the association mapping for both traits significantly overlapped at $p<10^{-2}$ significance level.

Functional Gene Analysis

Functional gene analysis of candidate genes using knockout mutants identified via association mapping provided evidence of one gene negatively functionally associated with DACC ability, two genes functionally associated with RCH ability, and two genes functionally associated with DACC+RCH ability. Genes were compared to the survivorship of their genetic background (w^{1118}) and were only compared to phenotypic behavior in which the gene was significant in the original association mapping (Table 5.1). A total of three genes were selected for DACC; two of the three knockout mutants (Pros26.4 and Prosap) failed to produce viable offspring at 18° C, so they could not be successfully tested for DACC ability. Syt12 tested

significantly different from control (w^{1118}) for DACC ability as evidenced by a significant mutant*treatment interaction (p=0.016; Figure 5.13) and had a lower DACC score (0.55) than background (0.74).

Five genes were selected and tested for RCH ability. We used two knockout mutants to test the function of one of these genes, CG33275. Both Atg7 and CG32111 had significant mutant x treatment interaction effects (p= 0.017 and p=0.0002, respectively; Figure 5.14). Atg7 had a decreased RCH score (0.24) as compared to the background control (w^{1118}; 0.48), while CG32111 had an increased RCH score (0.71). The other three genes (four mutants tested) did not have significant mutant x treatment interactions (CG33275, p=0.14 and p=0.86; Fili, p=0.51; px, p=0.18). Four genes were selected and tested for DACC+RCH ability. Two of the knockout genes showed significant functional association compared to background response. Eip74EF and VGlut both had a significant mutant x treatment interaction (p=0.005 and p=0.0007, respectively; Figure 5.15). Both genes had a reduced DACC+RCH score (Eip74EF=0.71; VGlut=0.66) as compared to the background control (w^{1118}; 0.91). CG30101 (p=0.41) and CG30069 (p=0.19) did not have significant mutant x treatment interactions.

Overlapping SNP function

Although specific SNPs did not overlap among acclimation treatments, investigation into molecular and biological function of the genes represented by the SNPs from the association mapping indicate significant overlap in functions represented by the genes (Flybase.org; Table 5.6 and 5.7). For example, SNPs within the genes lola and Eip74EF (DACC+RCH), Atg7 (RCH), and L (DACC) have biological function overlap of autophagy or programmed cell death. Similarly Sytalpha (DACC+RCH) has molecular overlap with 18w, Ptp99A, and Syt12 (DACC).

DISCUSSION

Understanding whole organism responses to thermal stress is crucial for understanding population-wide evolutionary responses to global climate change. Genetic and physiological interactions between cold tolerance mechanisms will drive the overall success of specific genotypes within given environmental conditions (Brakefield 2003). Our main question was to assess variation in acclimation ability in naturally segregating populations of *Drosophila melanogaster*. Our association mapping analyses indicate no SNP overlap between DACC and RCH, suggesting that specific genes involved in these two acclimation processes are not the same. Functional inspection of the genes of interest for DACC, RCH, and DACC+RCH does suggest that similar mechanisms may be playing a role in RCH and DACC, including common biological and molecular functions such as sodium and calcium pumps, autophagy processes, proteolysis, and transmembrane properties (Table 5.6 and 5.7). Physiological experiments have recently suggested that calcium may be an important player in RCH ability (Teets et al 2012; Teets and Denlinger 2013b) and maintenance of membranes is known to be critical to organism survival at cold temperatures (Hazel 1995; Michaud and Denlinger 2006). Reduction in autophagy have also been proposed as mechanisms involved in various stress responses including longevity (Juhasz et al 2007) and dehydration (Teets et al 2012). Autophagy genes are present in our association mapping analyses (RCH and DACC), and Atg7 tested significantly functional in RCH response, with knockout mutants having lowered RCH ability compared to the background control. Autophagy may be important for clearing injured cells due to cold shock and preventing this ability may lower acclimation ability if injured cells cannot be cleared at a

reasonable rate and build up in the organism, creating a toxic and death inducing environment (Fujita 1999; Shelley et al 2009; Teets and Denlinger 2013b).

Functional mutant analyses of candidate genes indicated two genes functionally associated with RCH response. CG32111 has unknown molecular and biological functions, while Atg7 has suggested functions of autophagy in the fly midgut, macroautophagy, and regulation of adult lifespan (Flybase.org; Hou et al 2008; Barth et al 2011; Juhasz et al 2007). Regulation of autophagy has been suggested as a mechanism of acclimation, as controlling the impact of cold stress on cellular death can successfully increase the lifespan of an organism (Juhasz et al 2007; Fujita 1999). For DACC, the candidate gene, Syt12, we were able to successfully test showed a significant functional component to developmental acclimation and Syt12 is associated with calcium-dependent phospholipid binding (Flybase 1992). Calcium binding has been shown to be involved in short-term acclimation responses and maintenance of ion transport across membranes is critical for maintaining osmotic balances and subsequent survival of an organism (Monroy et al 1993; Teets et al 2008; MacMillan and Sinclair 2010). In addition, the two knockout mutants for Prosap and Pros26.4 did not produce viable offspring at 18 C which suggests that these two gene knockouts may have a role in thermal sensitivity. Although, we do not have any quantification of these results, we can suggest that these two knockouts are critical for growth at various temperature regimes. For DACC+RCH, two of the four genes tested were significantly associated with this combination acclimation treatment. Eip74EF has biological and molecular functions associated with autophagy as well as functions in transcription (Lee and Baehrecke 2001; Flybase 1992). Again, autophagy could be an important component of regulating cell death via injury to cold stress, and mitigating these injured cells could alleviate this injury and prevent death. VGlut is involved in transmembrane

transport, synaptic transmission and glutamatergic activities, critical for glutamate transmission responsible for excitatory processes in the central nervous system (Daniels et al 2004; Daniels et al 2006; Flybase Curators et al 2004). Maintaining these processes is critical for cold tolerance, as transmembrane transport is important for flow of ions and response of the nervous system helps perceive and cope with cold stress as it occurs.

As with the comparison between RCH and DACC, DACC+RCH did not share any SNPs in common with the other acclimation treatments but did share common biological and molecular functions such as ion pumps, autophagy, proteolysis, and membrane maintenance. Overlap of functions but not of specific SNPs may suggest that a form of convergent evolution may be occurring among the acclimation processes, resulting in similar overall increases in survivorship following DACC, RCH, and DACC+RCH but alternative pathways at which this result is achieved (Thurber et al 2013; Arendt and Reznick 2007). These results suggest that while SNP variation may be uncoupled among the acclimation pre-treatments similar evolutionary pressures may cause similarities in the physiology of each response to the preparatory, mild temperature. Further analyses of these processes contributing to overlap will help us understand the extent of this convergent evolution and overlap of molecular and biological function.

In addition, we have shown here, using the DGRP, that two cold tolerance mechanisms, chronic cold survivorship (basal tolerance) and long-term acclimation ability (plasticity) have negative correlations indicating a basal tolerance-plasticity trade-off (Figures 5.7 and 5.8; Nyamukondiwa et al 2011). Physiologically these mechanisms appear to be constrained by one another and environmental conditions will dictate which cold tolerance capability will be selected for and evolve in the population. Interestingly, RCH and chronic cold survivorship were

not significantly correlated. Rapid cold hardening has been proposed to be an energetically low-cost response and a lack of correlation suggests that not only are RCH and chronic cold stress independent evolutionarily from one another, but that this may be due to the low cost of RCH. DACC and DACC+RCH show negative correlations with chronic cold tolerance which indicates a trade-off between a long-term acclimation treatment and the ability to survive a long-term cold exposure. Evolutionarily, these cold tolerance mechanisms are constrained by one another and environmental pressures will dictate which mechanism is favored in natural selection. For example, for organisms in tropical and temperate environments, temperature range of an organism's lifespan may inhibit or promote evolution of better chronic tolerance (tropics) or greater plasticity (temperate).

The DGRP showed significant variation in acclimation ability among all the lines tested. Overall, acclimation significantly increased survivorship for the whole population but the natural variation maintained in the DGRP allows us to examine the range of phenotypes a population may experience. Interestingly, several genotypes reared at 25° C showed a negative acclimation response indicating that acclimation or plasticity is capable of a "maladaptive" phenotype in natural populations (Angilletta 2009). However, as mentioned above, environmental pressures dictate selective components of thermal tolerance and selection may drive higher tolerance for extreme temperature drops rather than acclimation ability; therefore, genotypes with negative acclimation scores are not maladaptive to their environment at all.

DACC had a greater effect on the increase in survivorship on average than RCH, while the combination treatment of DACC+RCH had only a slightly higher increase in survivorship than DACC alone. Rako and Hoffmann (2005) suggested that ectotherms that have experienced DACC will no longer maintain the ability to be impacted by RCH pre-treatments; the only slight

increase in survivorship found here may support this hypothesis (Overgaard et al 2008). Physiologically, a longer pre-treatment time may allow changes to occur more completely and therefore, protect the organism to a greater extent. DACC and RCH did show a significant correlation among the DGRP lines suggesting that some physiological mechanisms overlap between these two acclimation treatments. The insignificant increase in survivorship following DACC+RCH acclimation, may also suggest that similar mechanisms are working in both RCH and DACC (Overgaard et al 2008; Rako and Hoffmann 2005). However, because of the extreme increase in survivorship after DACC and tested at -6° C for 1 hour, we tested flies reared at 18° C at -8° C for one hour in order to evaluate the effect of an RCH pre-treatment. Rank ordering DACC, RCH, and DACC+RCH as to which is the most beneficial of the three treatments is tricky since test temperature was not consistent among the three acclimation treatments.

Physiological work on acclimation treatments have previously suggested that short-term and long-term acclimation perform on a continuum in time and temperature (Shintani and Ishikawa 2007; Colinet and Hoffmann 2012; see review Teets and Denlinger 2013a). In a similar vein, basal tolerance and plasticity ability may also lie on an environmental continuum and selection for either cold tolerance mechanism is dictated by the natural environmental fluctuations. A benefit of using the DGRP is that natural genetic variation is maintained and we are able to see all potential phenotypes reflecting a range of natural genotypes. The survivorship--acclimation trade-offs show a snap shot of how these genotypes respond to environmental stresses and how their genotypes have maintained their natural abilities to survive in the face of direct cold extremes and a more variable, acclimation treatment. Lines in the DGRP tend to maximize at their statistical limit when calculating acclimation from survivorship scores, while rarely falling into a poor performing range at both cold tolerance mechanisms. Genotypes do not

perform poorly at both acute survivorship and after acclimation treatments; this suggests that natural selection has successfully weeded out genotypes that could not survive at either extreme cold stresses or using acclimation ability. Genotypes with poor survivorship under acute and acclimation conditions would not survive in our naturally fluctuating environments, as organisms rarely experience completely static environments in which there is no need to adapt (Terblanche et al 2011).

In conclusion, both long- and short-term acclimation have been shown to significantly increase survivorship after an extreme cold stress. We have shown here that there is substantial variation in the natural phenotypic distribution of these acclimation scores as well as survivorship in general and that acclimation and survivorship are significantly negatively correlated in a trait trade-off association. We also identified a number of significant candidate SNPs associated with DACC, RCH, and DACC+RCH as well as survivorship for flies reared at both 25° and 18° C. Functional gene analysis found significant functional association of five genes for the acclimation treatments, with similarities in molecular and biological functions such as autophagy, ion gradients, and nervous system synapses. There was no overlap in significant SNPs associated with the three acclimation treatments, suggesting that although similar mechanisms may be used in long- and short-term acclimation, specific genetic regions underlying these acclimation treatments are not the same. Selection via natural climatic variation may drive this genetic variation in long- and short-term acclimation responses as daily and seasonal fluctuations are highly variable around the globe. Our analyses find that acclimation responses can evolve independently but chronic tolerance and long-term acclimation abilities are tightly linked via trade-off mechanisms, both physiologically and genetically. These associations may have large impacts as our climate changes and we experience more extreme fluctuations

with less acclimation time (Franke et al 2012; Chown et al 2010). Maintenance of both survivorship and acclimation ability may therefore decrease survivorship abilities overall; further large scale empirical experiments are needed in order to determine if these patterns hold for a large range of stressful temperatures.

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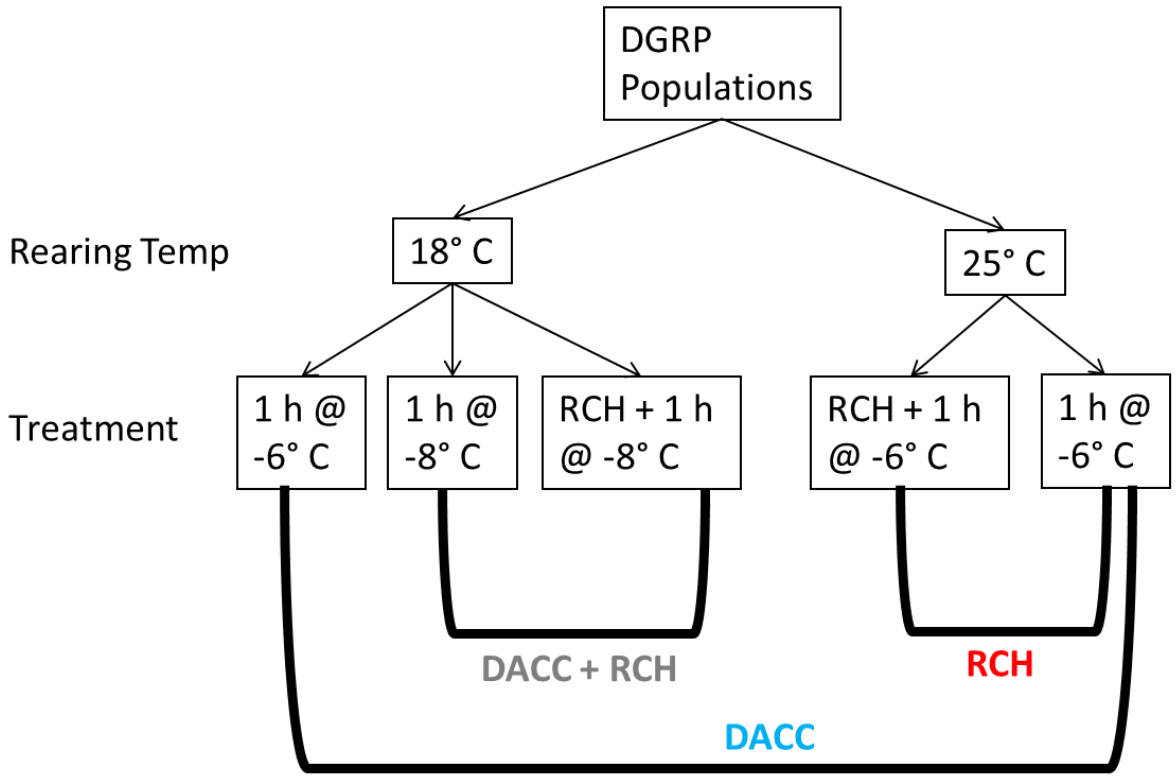

Figure 5.1. Diagram of acute and acclimated cold tolerance treatments. Survivorship following each treatment was calculated as a proportion of the three replicates for each genotype after 24 hours at room temperature. Scores calculated ($Survivorship_{Hardened} - Survivorship_{NonHardened}$) are represented by the loops at the bottom of the diagram. Rapid cold hardening (RCH) score is calculated for flies reared at 25° C and subjected to either -6° C directly for one hour or following a pretreatment of 2 hours at 4° C. Developmental acclimation+RCH (DACC+RCH is RCH score for flies reared at 18° C, with the additional component of being reared at a cooler temperature. DACC is the differences in survivorship after 1 hour at -6° C between the two rearing environments.

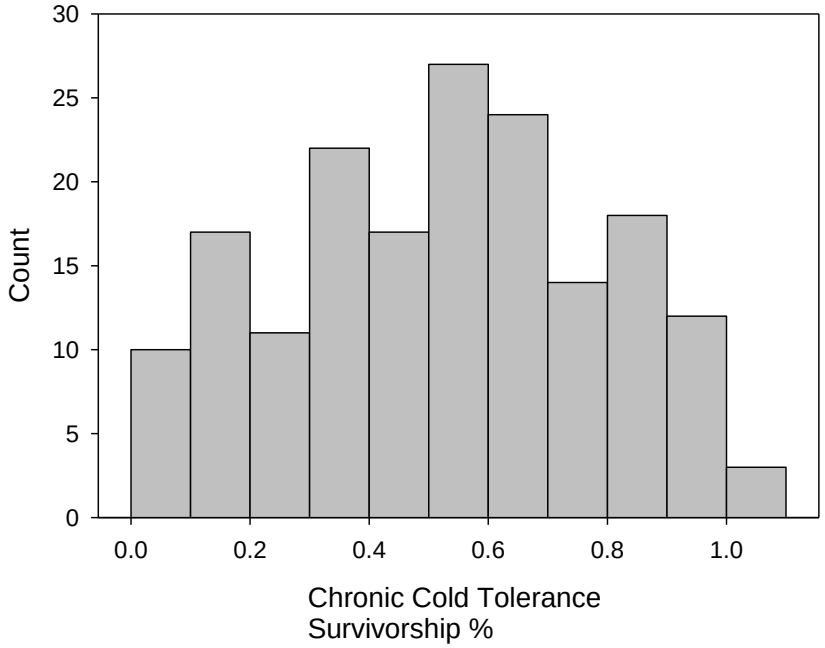

Figure 5.2. Overall distribution of chronic survivorship in the DGRP population as measured by survival after 16 hours at 0° C. The distribution shows high variation in this trait and a somewhat normal distribution of phenotypes across the population.

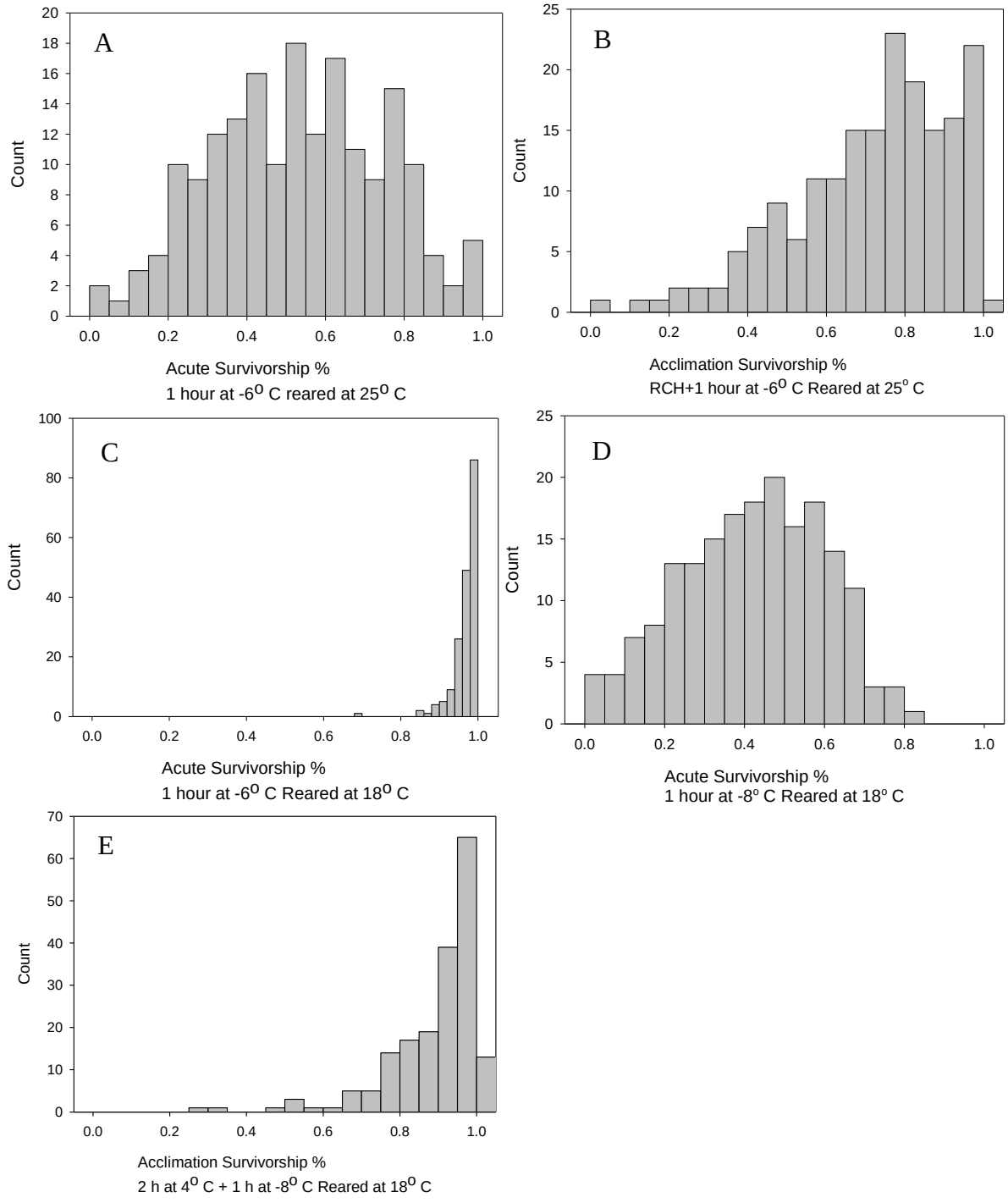

Figure 5.3. Histograms of survivorship distribution of the genotypes of the DGRP. A) Survivorship following acute cold stress for 1 hour at -6° C for flies reared at 25° C. B) Survivorship following pretreatment of 2 hours at 4° C followed by 1 hour at -6° C. C) Survivorship following 1 hour at -6° C for flies reared at 18° C. D) Survivorship following 1

hour at -8° C from flies reared at 18° C. E) Survivorship following pretreatment of 2 h at 4° C followed by 1 hour at -8° C for flies reared at 18° C.

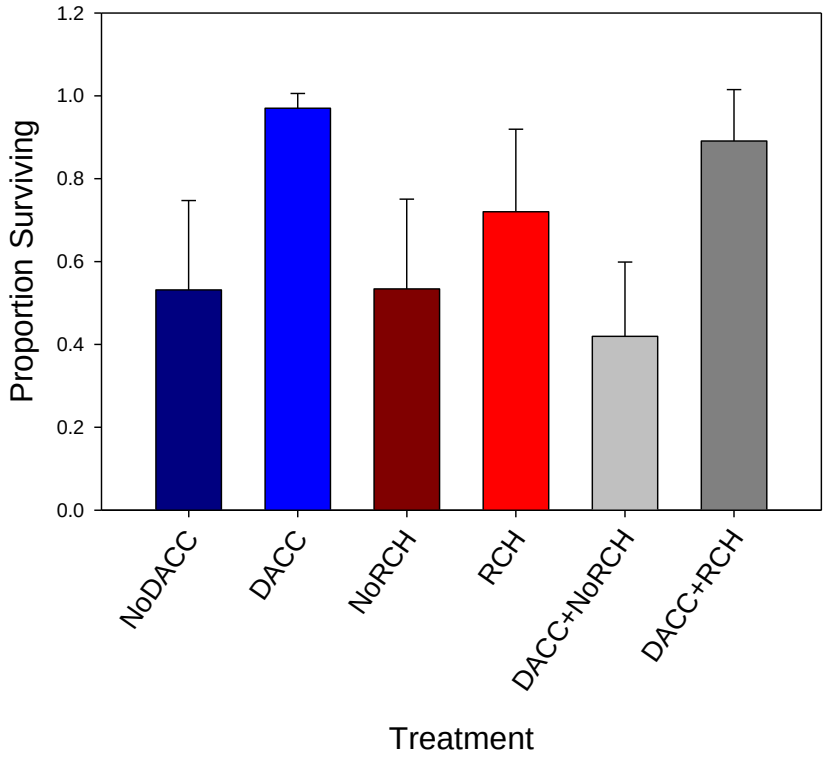

Figure 5.4.Means ± standard deviation for survivorship for acute and acclimated flies. Each No acclimation vs acclimation paired t-test is significantly different ($p<0.001$).

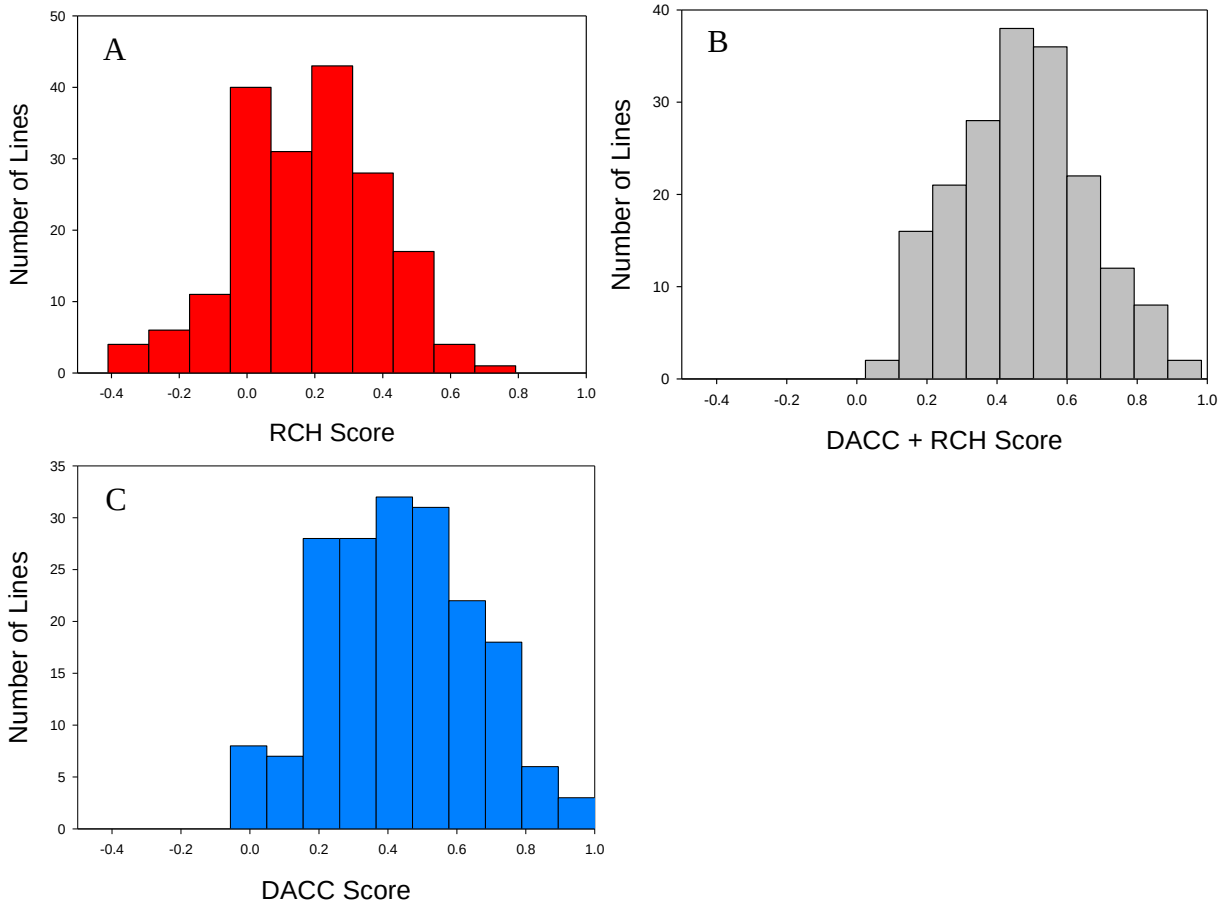

Figure 5.5. Histograms for acclimation scores for the DGRP (n=182). A) Rapid cold hardening scores for flies reared at 25° C. B) DACC +RCH scores for flies reared at 18° C. C) Long-term acclimation scores for flies reared at 18° C and 25° C and tested for 1 hour at -6° C.

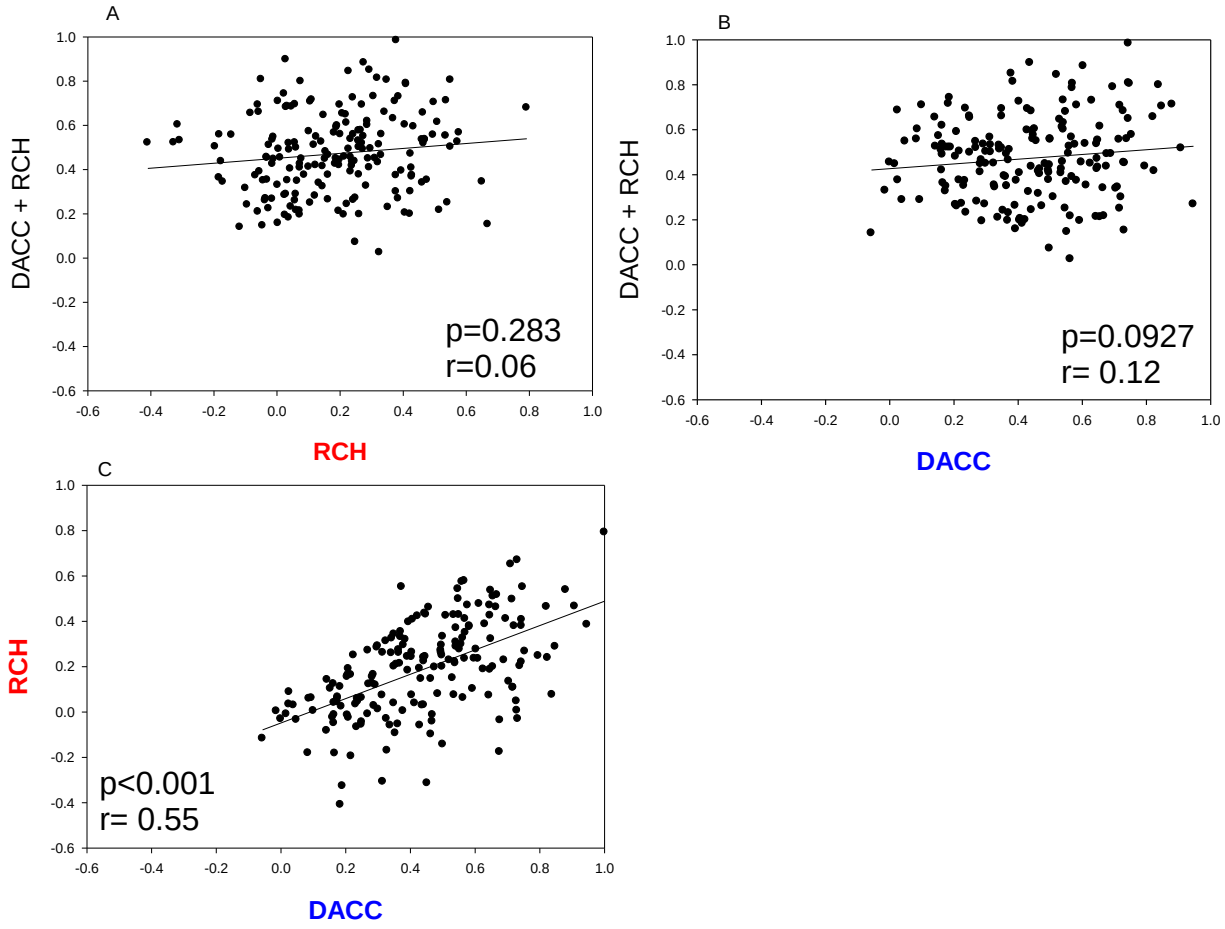

Figure 5.6. Testing for physiological correlations between acclimation scores. Pearson's Product moment correlations among lines of the DGRP for each acclimation score pair shows significant correlations between RCH and DACC but not between DACC+RCH and either DACC or RCH.

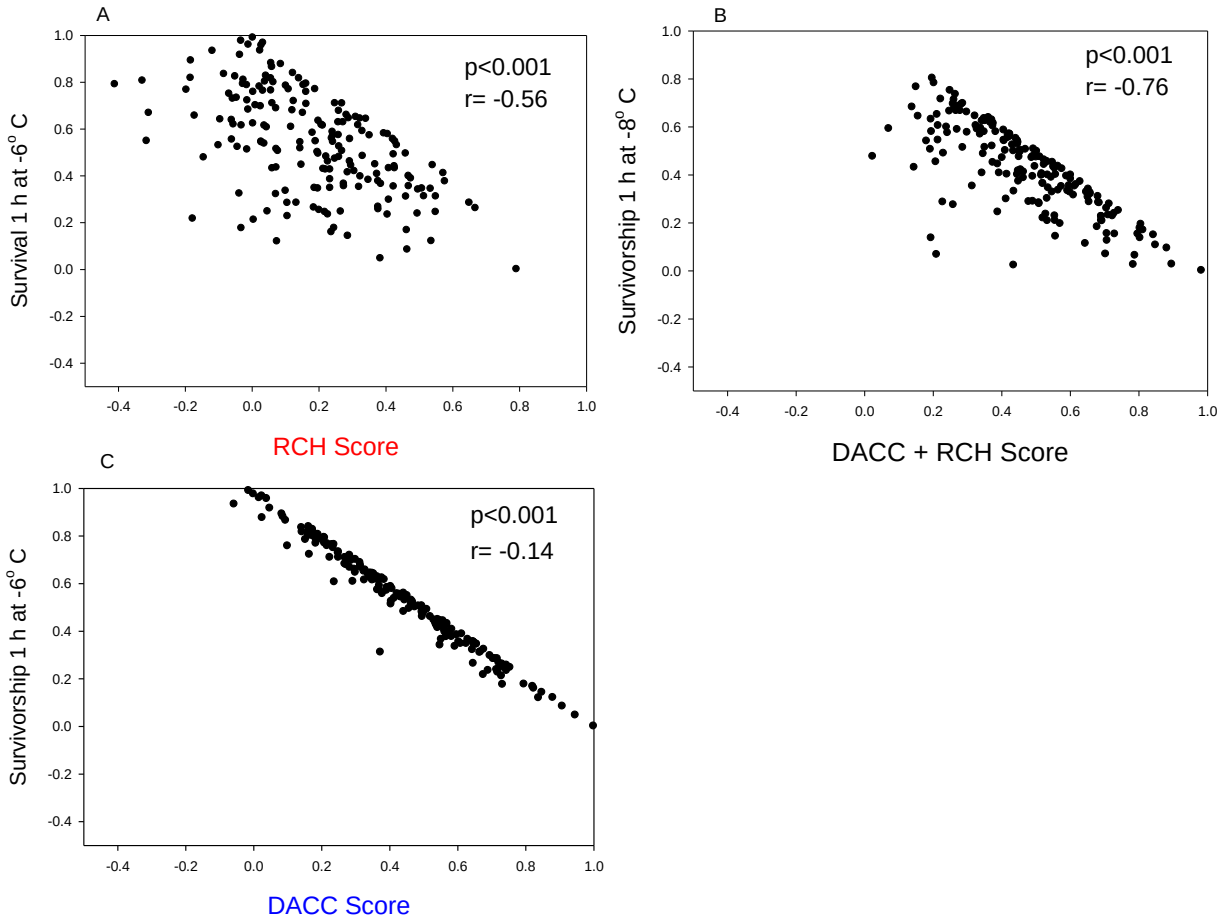

Figure 5.7. Basal tolerance as measured by acute survivorship correlations to acclimation scores. A) Survivorship at 1 hour at -6° C for flies reared at 25° C were highly correlated with RCH score for flies reared at 25° C. B) Survivorship at 1 hour at -8° C for flies reared at 18° C were highly correlated with RCH score for flies reared at 18° C. C) Survivorship at 1 hour at -6° C for flies reared at 25° C were highly correlated with DACC score for flies reared at 25° C compared to flies reared at 18° C.

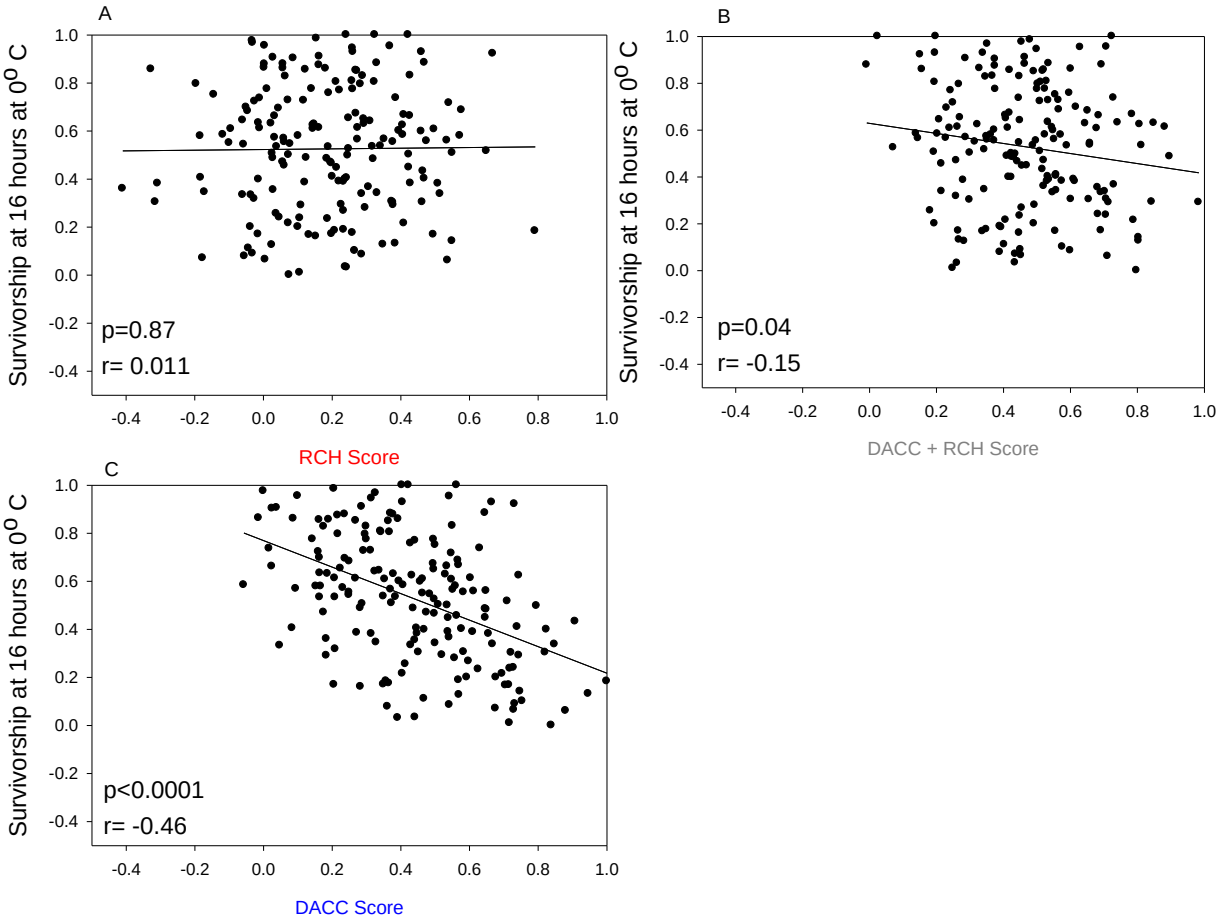

Figure 5.8. Correlations between chronic cold survivorship and acclimation scores. A) Survivorship at 16 hours at 0° C for flies reared at 25° C were highly correlated with RCH score for flies reared at 25° C. B) Survivorship at 16 hours at 0° C for flies reared at 25° C were highly correlated with DACC score for flies reared at 25° C compared to flies reared at 18° C. C) Survivorship at 16 hours at 0° C for flies reared at 25° C were highly correlated with RCH score for flies reared at 18° C.

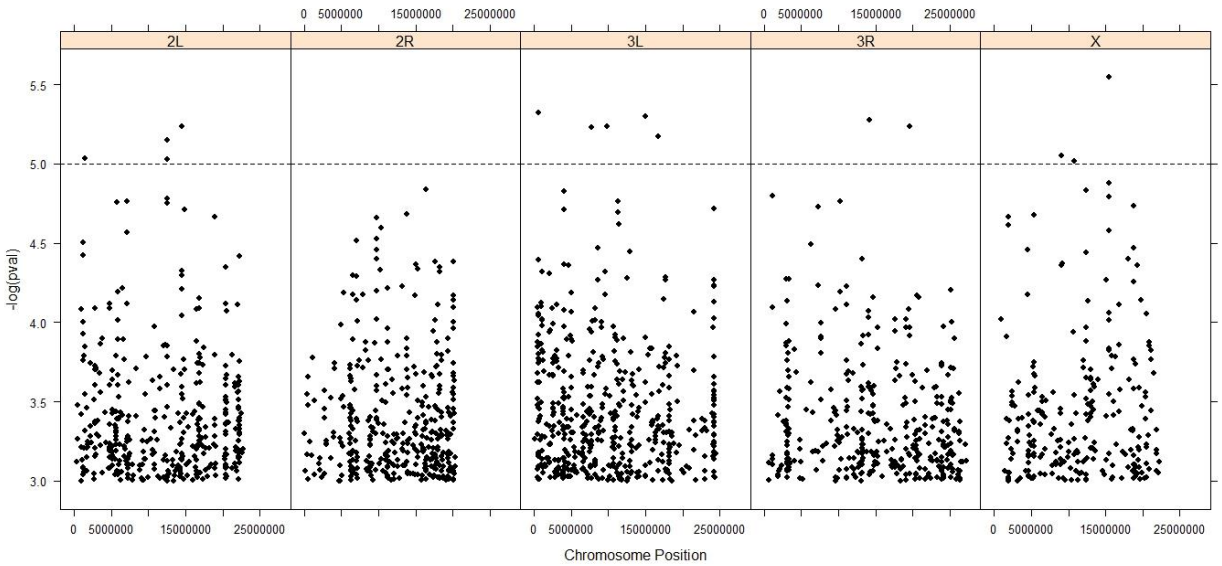

Figure 5.9. Chronic cold stress significant SNPs for DGRP lines association mapping. The dotted line signifies a threshold of –log(pval)=5. Minimum –log(pval) represented is 3. SNPs above this line are highly significant. Each panel represents a chromosome of the *Drosophila melanogaster* genome.

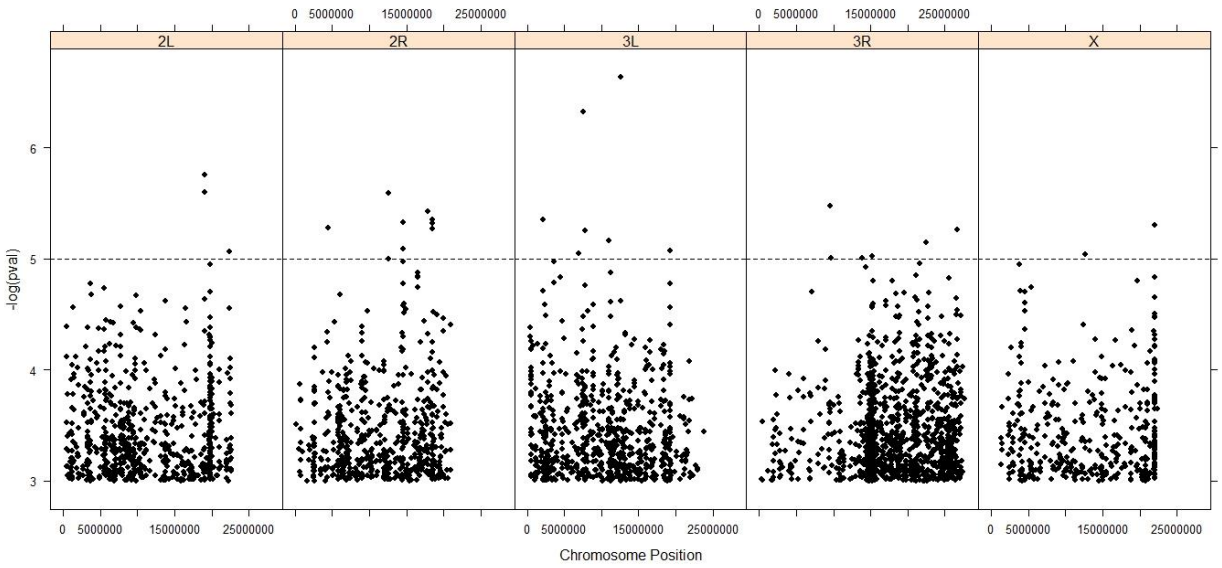

Figure 5.10. RCH score significant SNPs for DGRP lines association mapping. The dotted line signifies a threshold of –log(pval)=5. Minimum –log(pval) represented is 3. SNPs above this line are highly significant. Each panel represents a chromosome of the *Drosophila melanogaster* genome.

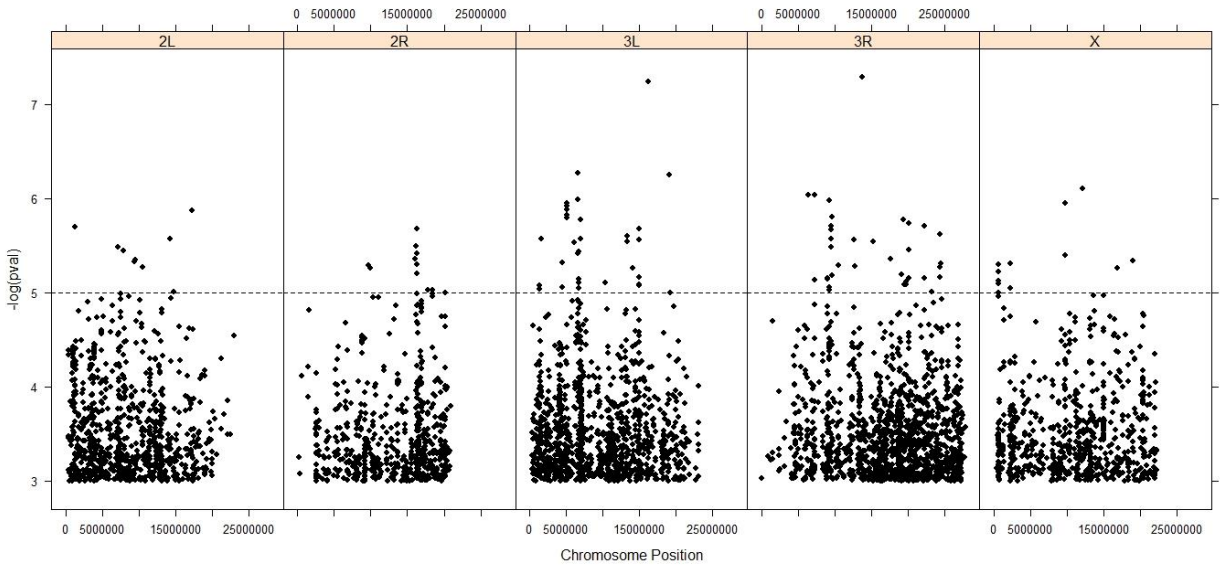

Figure 5.11. DACC score significant SNPs for DGRP lines association mapping. The dotted line signifies a threshold of –log(pval)=5. Minimum –log(pval) represented is 3. SNPs above this line are highly significant. Each panel represents a chromosome of the *Drosophila melanogaster* genome.

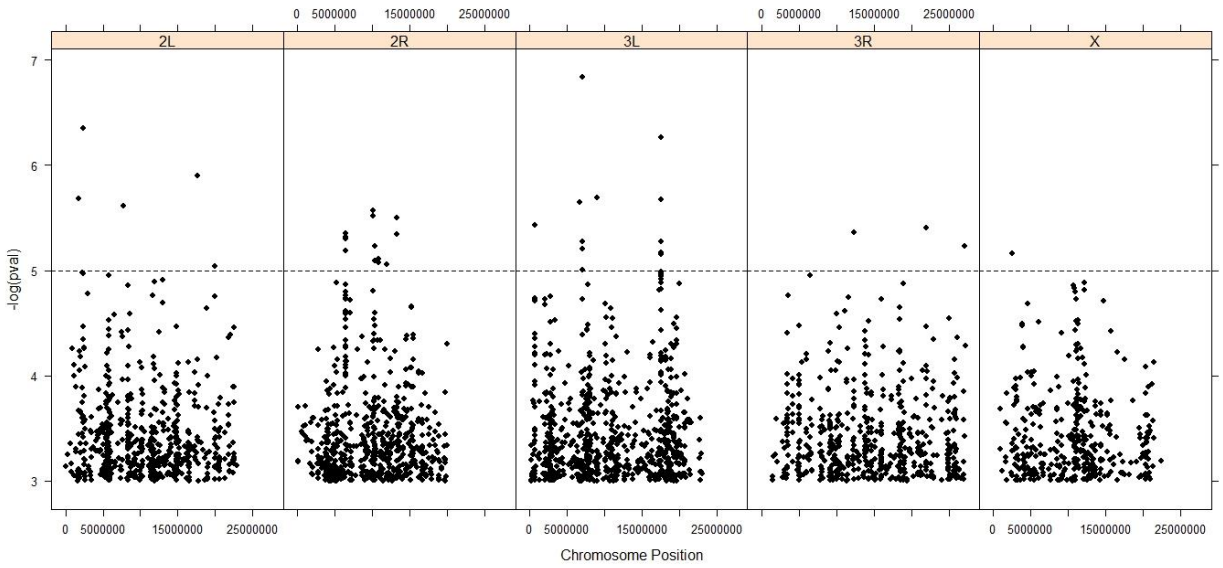

Figure 5.12. DACC+RCH score significant SNPs for DGRP lines association mapping. The dotted line signifies a threshold of –log(pval)=5. Minimum –log(pval) represented is 3. SNPs above this line are highly significant. Each panel represents a chromosome of the *Drosophila melanogaster* genome.

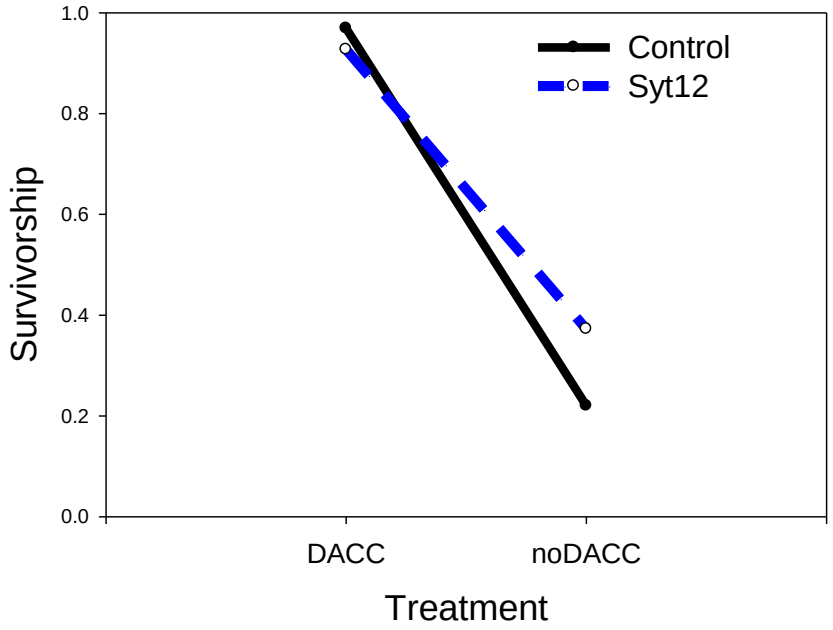

Figure 5.13. DACC functional mutant analysis. Knockout mutant for Syt12 tested significant for mutant x treatment effect (p=0.016) with a lower DACC score than the background control.

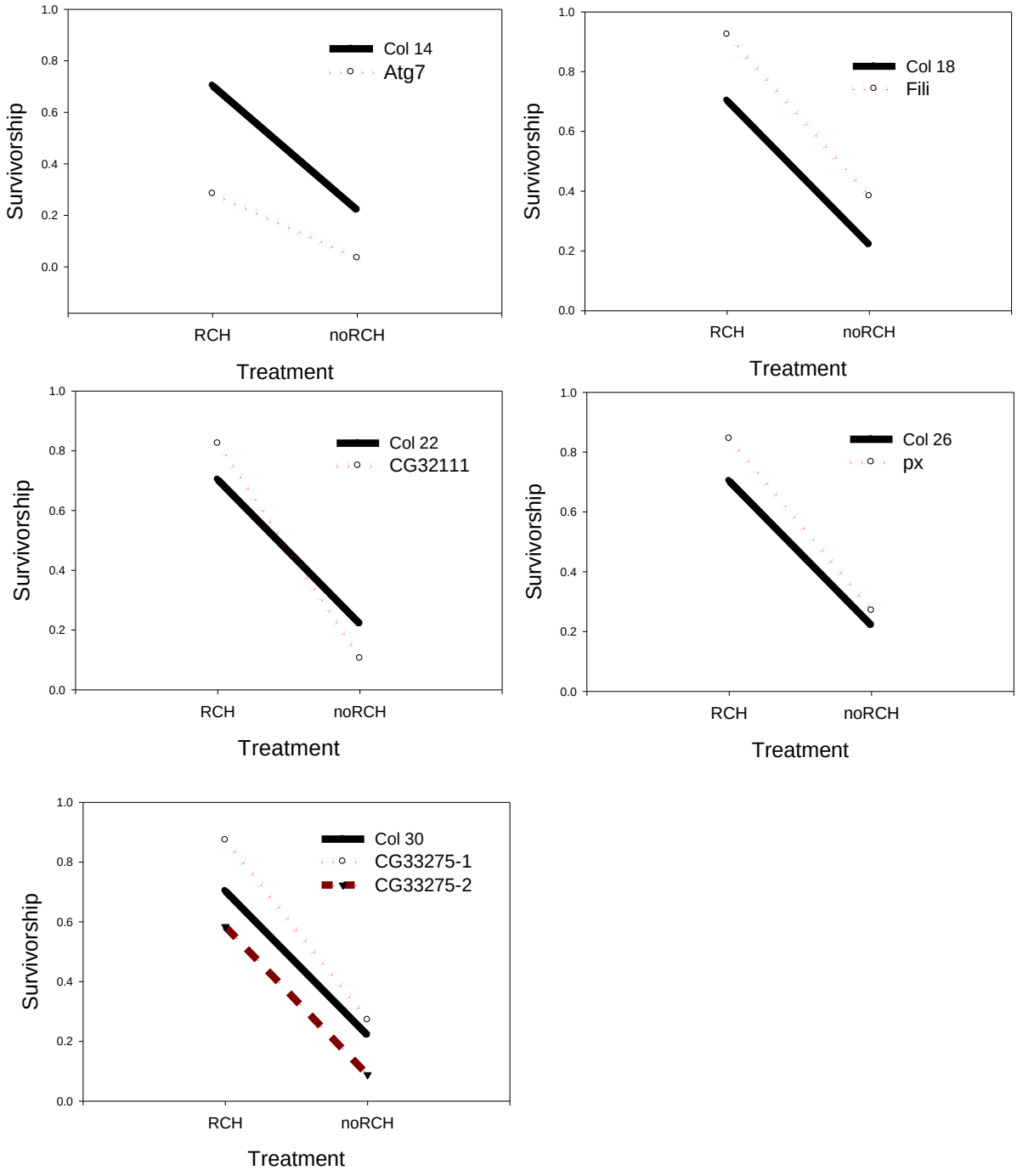

Figure 5.14. RCH score functional mutant analysis. Atg7 (p=0.017) and CG32111 (p=0.0002) were both significantly associated with mutant x treatment and RCH score. Atg7 showed a decrease in in RCH ability while CG32111 had increase RCH score. None of the other knockout mutants tested had a significant mutant x treatment interaction. CG32275, p=0.14 and p=0.86. Fili, p=0.51. px, p=0.18.

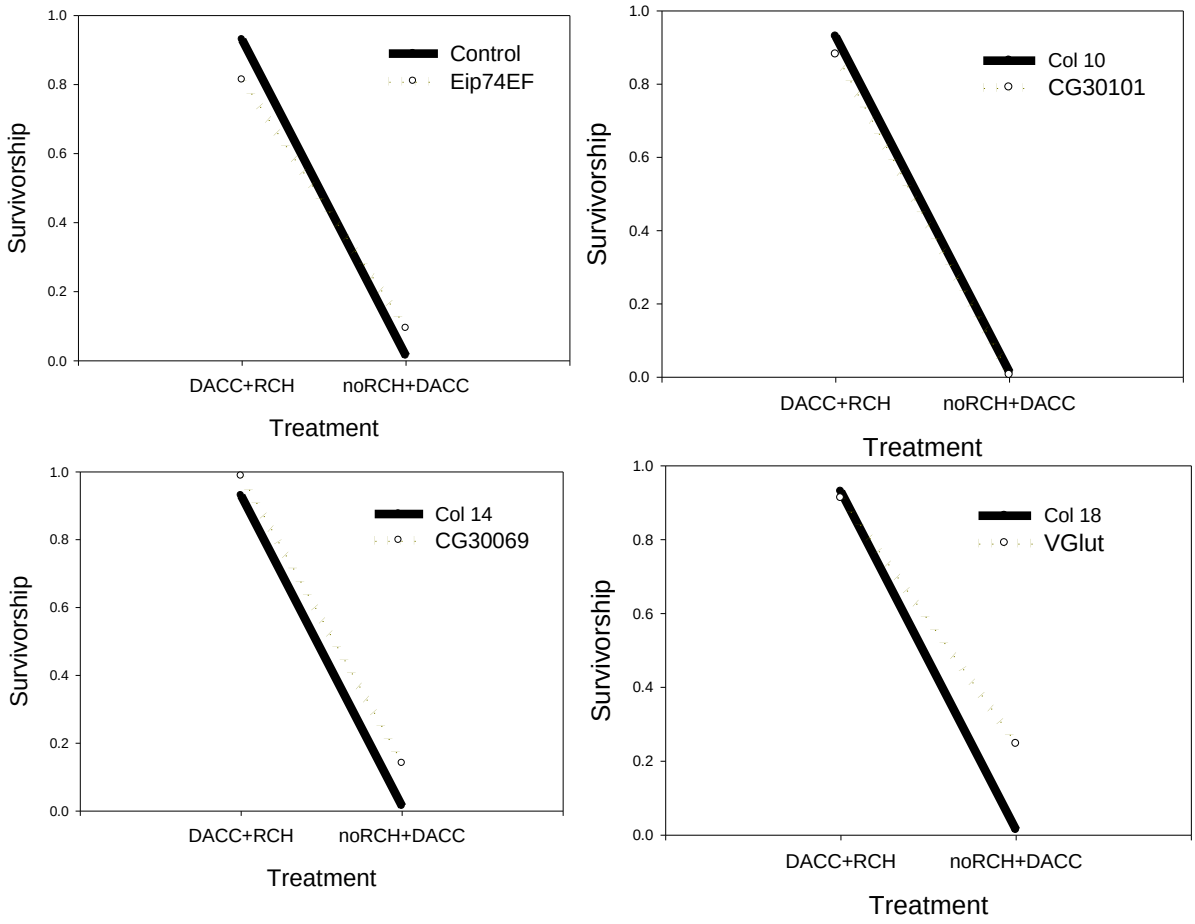

Figure 5.15. DACC+RCH functional mutant analyses. Eip74EF (p=0.005) and VGlut (p=0.0007) are significantly different from background in the mutant x treatment interaction term and both have lowered DACC+RCH ability than the background. Other mutants tested were not significant at mutant x treatment interaction term. CG30101, p=0.41. CG30069, p=0.19.

Table 5.1. Functional mutant details including chromosome, location, mutant knockout stock used, acclimation treatment analyzed, and molecular and biological function (Flybase.org). Mutants were only analyzed for functional association with the indicated phenotype. Significant interactions are reported in the results.

Chromosome	Position	Gene Name/ Site Class	Phenotype Tested	Mutant Stock	Gene Function Molec= Molecular Function, Biol= Biological Function
2R	18436363	px / intron	RCH	24021	Molec: Unknown. Biol: Inter-male aggressive behavior; imaginal disc-derived wing vein morphogenesis
2R	17808016	Fili / intron	RCH	23968	Molec: Unknown. Biol: Regulation of apoptotic process; imgainla disc-derived wing morphogenesis
3L	7607714	CG33275 / intron	RCH	11292, 18470	Molec: Guanyl-nucleotide exchange factor activity. Biol: imaginal disc-derived leg morphogenesis
3L	12632223	CG32111 / intron	RCH	24287	Molec: Unknown. Biol: Unknown
2R	14507546	Atg7 / downstream	RCH	19257	Molec: catalytic activity; nucleotide binding. Biol: determination of adult lifespan; macroautophagy; regulation of defense response to virus; regulation of autophagy; larval midgut cell programmed cell death
2R	13297069	CG30101 / synonymous coding	DACC+RCH	18887	Molec: Unknown. Biol: Unknown
3L	17595338	Eip74EF / intron	DACC+RCH	12619	Molec: RNA polymerase II core promoter proximal region sequence-spefic DNA binding transcprtion factor activity involved in positive regulation of transcription. Biol: positive regulation of transcription from RNA polymerase II promoter; cellular process; autopahgy; salivary gland autophagic cell death
2R	10073554	CG30069 / synonymous coding	DACC+RCH	24260	Molec: Unknown. Biol: Unknown

2L	2396535	VGlut / upstream	DACC+RCH	26160	Molec: nuerotransmitter transporter activity. Biol: synaptic transmission, glutamatergic.
X	13249383	Syt12 / upstream	DACC	18369	Molec: calcium-dependent phospholipid binding. Biol: neurotransmitter secretion
3R	19756448	Pros26.4 / synonymous coding	DACC	16094	Molec: endopeptidase activity. Biol: mitotic spindle elongation; cell proliferation; cellular process; response to DNA damage stimulus; mitotic spindle prganization; proteolysis
2R	9974040	Prosap / intron	DACC	17047	Molec: protein binding. Biol: Postsynaptic density assembly
Background	w^{1118}			6326	

Table 5.2. Significant SNPs for chronic cold survival association mapping results at $p<10^{-6}$. A total of 22 SNPs representing 14 unique genes were significant. Chromosome position, gene symbol (Flybase.org), site class, minor allele frequency, p-value, and effect size are given.

Chromosome	Position	Gene Symbol	Site Class	Minor Allele Frequency	P-value	Effect Size
2L	1449882		SNP is more than 500 bp away from known gene	0.2167	$9.27x10^{-6}$	0.1128
2L	12478252	bun	Intron	0.2014	$7.09x10^{-6}$	0.1214
2L	12478256	bun	Intron	0.2189	$9.32x10^{-6}$	0.1170
2L	14532569	tRNA:G3:35	Upstream	0.2553	$5.79x10^{-6}$	-0.1129
3L	534006	klar	Intron	0.2533	$4.78x10^{-6}$	0.1084
3L	7710287	Ank2	Intron	0.4800	$5.88x10^{-6}$	-0.09336
3L	7710287	GC32373	Intron	0.4800	$5.88x10^{-6}$	-0.09336
3L	7710297	Ank2	Intron	0.4800	$5.88x10^{-6}$	-0.09336
3L	7710297	CG32373	Intron	0.4800	$5.88x10^{-6}$	-0.09336
3L	7710299	Ank2	Intron	0.4800	$5.88x10^{-6}$	-0.09336
3L	7710299	CG32373	Intron	0.4800	$5.88x10^{-6}$	-0.09336
3L	9788154	CG14168	Intron	0.2142	$5.84x10^{-6}$	0.2253
3L	15001009	Reck	Intron	0.4929	$5.06x10^{-6}$	-0.09586
3L	16725379	CG9701	Upstream	0.2635	$6.75x10^{-6}$	-0.1052
3L	16725379	Dbp73D	Upstream	0.2635	$6.75x10^{-6}$	-0.1052
3R	14184132	CG15803	Intron	0.1006	$5.30x10^{-6}$	-0.1597
3R	14184148	CG15803	Intron	0.1006	$5.30x10^{-6}$	-0.1597
3R	19621583	mbc	Intron	0.1944	$5.83x10^{-6}$	0.1193
X	9094708	su(r)	Intron	0.2333	$8.95x10^{-6}$	-0.1087
X	10762911	CG1637	Downstream	0.3355	$9.58x10^{-6}$	-0.09612
X	10762911	CG2157	Upstream	0.3355	$9.58x10^{-6}$	-0.09612
X	15423393		SNP is more than 500 bp	0.1830	$2.85x10^{-6}$	0.1269

			from known gene			

Table 5.3. Significant SNPs for RCH score association mapping results at $p<10^{-6}$. A total of 34 SNPs representing 25 unique genes were significant. Chromosome position, gene symbol (Flybase.org), site class, minor allele frequency, p-value, and effect size are given.

Chromosome	Position	Gene Symbol	Site Class	Minor Allele Frequency	P-value	Effect Size
2L	19078223	CG10700	Non-Synonymous Coding	0.3636	$2.19x10^{-6}$	0.09232
2L	19078238	CG10700	Non-Synonymous Coding	0.3611	$1.52x10^{-6}$	0.09403
2L	19078239	CG10700	Non-Synonymous Coding	0.4097	$1.52x10^{-6}$	0.09403
2L	22389948		SNP is more than 500 bp from known gene	0.1363	$8.60x10^{-6}$	0.1665
2R	4470515	CG42326	Intron	0.472973	$5.21x10^{-6}$	-0.08174
2R	12602120	CG30463	Intron	0.091549	$9.92x10^{-6}$	0.132377
2R	12602131	CG30463	Intron	0.07483	$2.56x10^{-6}$	0.151626
2R	14505980	CG5493	Intron	0.062112	$4.73x10^{-6}$	0.162133
2R	14507546	Atg7	Downstream	0.031056	$8.12x10^{-6}$	0.221622
2R	14507546	CG5493	Upstream	0.031056	$8.12x10^{-6}$	0.221622
2R	17808016	Fili	Intron	0.245161	$3.72x10^{-6}$	0.093497
2R	18436363	px	Intron	0.04908	$4.80x10^{-6}$	0.180537
2R	18436599	px	Intron	0.049689	$4.43x10^{-6}$	0.180607
2R	18436912	px	Intron	0.049689	$5.35x10^{-6}$	0.179996
3L	2174513	CG15820	Upstream	0.051282	$4.43x10^{-6}$	0.18388
3L	2174513	CR15821	Upstream	0.051282	$4.43x10^{-6}$	0.18388
3L	6894035		SNP is more	0.047619	$8.92x10^{-6}$	0.192237

			than 500 bp from known gene			
3L	7607714	CG33275	Intron	0.086957	$4.76x10^{-7}$	0.152481
3L	7800732	CG32369	Intron	0.33125	$5.54x10^{-6}$	0.083114
3L	11053305	CG6321	Non-Synonymous Coding	0.490566	$6.84x10^{-6}$	0.078318
3L	12632223	CG32111	Intron	0.089431	$2.32x10^{-7}$	0.173148
3L	19282005	CG9619	Upstream	0.33758	$8.50x10^{-6}$	0.083446
3R	9520384	CG14367	Intron	0.067901	$3.36x10^{-6}$	-0.15786
3R	9639769	tal-1A	UTR 3’	0.025974	$9.85x10^{-6}$	0.233324
3R	9639769	tal-2A	UTR 3’	0.025974	$9.85x10^{-6}$	0.233324
3R	9639769	tal-3A	UTR 3’	0.025974	$9.85x10^{-6}$	0.233324
3R	9639769	tal-AA	UTR 3’	0.025974	$9.85x10^{-6}$	0.233324
3R	13822328		SNP is more than 500 bp from known gene	0.030864	$9.89x10^{-6}$	0.218058
3R	15184920	CG43203	Downstream	0.043478	$9.53x10^{-6}$	0.187118
3R	22434099	scrib	Downstream	0.027397	$7.18x10^{-6}$	0.250357
3R	26652668	cindr	UTR 3’	0.254777	$5.45x10^{-6}$	-0.09137
X	12667459	CG32647	Downstream	0.142857	$9.06x10^{-6}$	-0.11897
X	12667459	CG3812	Downstream	0.142857	$9.06x10^{-6}$	-0.11897
X	22030597	Cda4	Intron	0.287671	$4.93x10^{-6}$	0.086468

Table 5.4. Significant SNPs for DACC score association mapping results at $p<10\text{-}^{6}$. A total of 34 SNPs representing 22 unique genes were significant. Chromosome position, gene symbol (Flybase.org), site class, minor allele frequency, p-value, and effect size are given.

Chromosome	Position	Gene Symbol	Site Class	Minor Allele Frequency	P-value	Effect Size
2L	9412787	Shawl	Intron	0.3581	$3.58x10^{-6}$	-0.08291
2R	9974040	Prosap	Intron	0.2666	$5.71x10^{-6}$	0.08978
2R	10367967	L	Upstream	0.162791	$1.52x10^{-6}$	0.1213
2R	16004645	18w	Downstream	0.4285	$3.34x10^{-6}$	0.08117
2R	16414768	CG13422	Downstream	0.4575	$1.16x10^{-6}$	-0.08324
2R	16414768	CG30151	Downstream	0.4575	$1.16x10^{-6}$	-0.08324
2R	18257477	Liprin-gam	Intron	0.4407	$9.33x10^{-6}$	0.07778
3L	6073449	CG6619	Synonymous Coding	0.3333	$1.81x10^{-6}$	-0.1675
3L	6073458	CG6619	Synonymous Coding	0.3125	$7.16x10^{-6}$	-0.1586
3R	15376788	Dys	Intron	0.07142	$7.46x10^{-6}$	0.1473
3R	15376973	Dys	Intron	0.07051	$9.26x10^{-6}$	0.1468
3R	17795762		SNP is more than 500 bp from known gene	0.183007	$5.14x10^{-6}$	-0.1021
3R	17802135		SNP is more than 500 bp from known gene	0.1920	$8.75x10^{-6}$	-0.09742
3R	18852029	lmd	Non-synonymous Coding	0.4933	$4.49x10^{-6}$	0.07900
3R	19068657	CG4467	Intron	0.4866	$3.50x10^{-6}$	0.07984
3R	19127380	pnt	Intron	0.2774	$7.02x10^{-6}$	0.08551

3R	19756448	Pros26.4	Synonymous Coding	0.2302	$1.07x10^{-6}$	0.09955
3R	19756514	Pros26.4	Synonymous Coding	0.2317	$1.18x10^{-6}$	0.09960
3R	19833492	KrT95D	Intron	0.406897	$7.08x10^{-6}$	0.07903
3R	20118368	CG6364	UTR 3’	0.2281	$4.36x10^{-6}$	-0.09616
3R	20127470	crb	Intron	0.3904	$9.31x10^{-6}$	-0.08005
3R	22200341		SNP is more than 500 bp from known gene	0.1103	$4.48x10^{-6}$	-0.1284
3R	22722782	T48	Intron	0.1409	$1.84x10^{-6}$	-0.1210
3R	25276666	Ptp99A	Intron	0.1612	$4.20x10^{-6}$	-0.1074
3R	25366182		SNP is more than 500 bp from known gene	0.2272	$8.42x10^{-6}$	-0.09223
3R	25529707		SNP is more than 500 bp from known gene	0.2580	$8.43x10^{-6}$	-0.08695
3R	25897494	sima	Intron	0.1082	$6.17x10^{-6}$	0.1231
3R	26833865	5-HT7	Intron	0.3851	$6.14x10^{-6}$	-0.08153
3R	26834116	5-HT7	Intron	0.4054	$2.58x10^{-6}$	-0.08385
3R	26844737	5-HT7	Upstream	0.2733	$2.05x10^{-6}$	-0.09198
X	13249382	Syt12	Upstream	0.3670	$1.19x10^{-6}$	-0.08448
X	13249383	Syt12	Upstream	0.3653	$8.13x10^{-7}$	-0.08658
X	22029557	Cda4	Intron	0.2406	$6.57x10^{-6}$	0.09619
X	22030724	Cda4	Intron	0.230216	$5.12x10^{-6}$	0.09564

Table 5.5. Significant SNPs for DACC+RCH score association mapping results at $p<10\text{-}^{6}$. A total of 38 SNPs representing 18 unique genes were significant. Chromosome position, gene symbol (Flybase.org), site class, minor allele frequency, p-value, and effect size are given.

Chromosome	Position	Gene Symbol	Site Class	Minor Allele Frequency	P-value	Effect Size
2L	1776930		SNP is more than 500 bp from known gene	0.1397	$2.10\text{x}10^{-6}$	-0.12
2L	2396535	VGlut	Upstream	0.427536	$4.44\text{x}10^{-7}$	0.07916
2L	7691643	CG7025	Synonymous Coding	0.025	$2.46\text{x}10^{-6}$	0.2143
2L	17602435	Sytalpha	Synonymous Coding	0.2156	$1.27\text{x}10^{-6}$	0.08576
2L	19939669		SNP is more than 500 bp from known gene	0.04458	$9.18\text{x}10^{-6}$	0.1527
2R	6415796	lola	Intron	0.0875	$4.99\text{x}10^{-6}$	-0.1154
2R	6415813	lola	Intron	0.08805	$4.80\text{x}10^{-6}$	-0.1140
2R	6417925	lola	Intron	0.08695	$4.48\text{x}10^{-6}$	-0.1157
2R	6421858	lola	UTR 5’	0.1572	$6.56\text{x}10^{-6}$	-0.08901
2R	10073554	CG30069	Synonymous Coding	0.08974	$2.67\text{x}10^{-6}$	0.1182
2R	10073555	CG30069	Non-Synonymous Coding	0.09032	$3.02\text{x}10^{-6}$	0.1179
2R	10287490		SNP is more than 500 bp from known	0.3178	$8.03\text{x}10^{-6}$	0.06904

			gene			
2R	10328122	Oaz	Intron	0.1125	$5.90x10^{-6}$	-0.1025
2R	10795846	CG10249	Intron	0.2767	$8.39x10^{-6}$	-0.08643
2R	10795858	CG10249	Intron	0.3412	$7.83x10^{-6}$	-0.07845
2R	11924347	SP2353	Intron	0.2101	$8.76x10^{-6}$	-0.0792
2R	13297069	CG30101	Synonymous Coding	0.3207	$3.18x10^{-6}$	-0.07164
2R	13297127	CG30101	Intron	0.3291	$4.58x10^{-6}$	-0.07027
3L	772422	tRNA: CR324	Downstream	0.07947	$3.72x10^{-6}$	0.1274
3L	6665147	Gr65a	Upstream	0.325	$2.25x10^{-6}$	0.1274
3L	7057306	CG8641	Intron	0.3522	$6.24x10^{-6}$	0.07204
3L	7057324	CG8641	Intron	0.475	$9.82x10^{-6}$	-0.06299
3L	7057335	CG8641	Intron	0.3552	$5.33x10^{-6}$	0.06917
3L	7058000	CG8641	Intron	0.4832	$1.47x10^{-7}$	0.07763
3L	9013735	Doc2	Upstream	0.1304	$2.03x10^{-6}$	0.1000
3L	17587415	Eip74EF	Intron	0.3381	$5.36x10^{-6}$	0.07353
3L	17588102	Eip74EF	Intron	0.04968	$6.74x10^{-6}$	-0.7454
3L	17588284	Eip74EF	Intron	0.04968	$6.74x10^{-6}$	-0.7454
3L	17588285	Eip74EF	Intron	0.04968	$6.74x10^{-6}$	-0.7454
3L	17588286	Eip74EF	Intron	0.04968	$6.74x10^{-6}$	-0.7454
3L	17588291	Eip74EF	Intron	0.05	$6.77x10^{-6}$	0.1477
3L	17589824	Eip74EF	Intron	0.05063	$7.05x10^{-6}$	0.1473
3L	17595331	Eip74EF	Intron	0.4052	$2.10x10^{-6}$	0.06980
3L	17595338	Eip74EF	Intron	0.4294	$5.51x10^{-7}$	0.07264
3R	12416239	CG14892	Upstream	0.1194	$4.32x10^{-6}$	0.1010
3R	22015013	CG34130	Non-Synonymous Coding	0.2866	$3.91x10^{-6}$	-0.07281
3R	27097113	CG15555	Downstream	0.02649	$5.87x10^{-6}$	0.2071
X	2463724	Pdfr	Intron	0.175	$6.85x10^{-6}$	0.08469

Table 5.6. Molecular Function of significant genes of interest for acclimation scores. Genes may appear in more than one molecular function and are placed according to Flybase.org assignments. Columns represent acclimation treatments and describe the overlap in molecular function, even though there are different genetic regions of acclimation. Gene symbol from Flybase.org is represented.

Molecular Function	DACC+RCH Represented Gene	RCH Represented Gene	DACC Represented Gene
Transporter	VGlut		
Ion Binding	Oaz	CG32369	
Nucleotide Binding	Oaz	Atg7	
Catalytic Activity	CG14892 CG34130 CG7025	Cda4 Atg7 CG6321 CG5493 CG3812 CG32369 CG30463 CG10700	Cda4 CG4467 CG6364 Pros26.4 Ptp99A
Receptor Activity	Pdfr		5-HT7 sima
GTP Activity	CG8641		Shawl
Transcriptional Binding	Doc2 Eip74EF		lmd pnt
Chitin Binding		Cda4	Cda4
Protein Binding	lola	cindr scrib CG9619	crb Dys pnt Prosap sima
Transmembrane Activity	Sytalpha		18w Ptp99A Syt12

Table 5.7. Biological Function of significant genes of interest for acclimation scores. Genes may appear in more than one biological function and are placed according to Flybase.org assignments. Columns represent acclimation treatments and describe the overlap in biological function, even though there are different genetic regions of acclimation. Gene symbol from Flybase.org is represented.

Biological Function	DACC+RCH Represented Gene	RCH Represented Gene	DACC Represented Gene
Proteolysis	CG14892 CG7025	CG32369	CG4467 Pros26.4
Biogenesis	lola	scrib	pnt crb
Immune/Virus/Bacterial Response	lola	Atg7	18w CG13422
Neurogenesis		Cda4	Cda4 pnt
RNA transcription	Doc3 Eipt74EF		sima lmd
Tracheal Morphogenesis	Oaz		crb
Synaptic Transmission	VGlut		Dys
Sensory Organ Development	lola	scrib	crb pnt
Reproduction	CG34130		crb
Organ Morphogenesis		scrib	pnt
Wing Morphogenesis		Fili px tal-1A scrib	Dys
Cell Junction Organization		scrib	crb
Cell Differentiation	Doc2 lola	scrib	
Biological Regulation	lola	scrib	crb pnt
Autophagy/Cell Death	lola Eip74EF	Atg7	L
Developmental Process	lola	scrib	pnt crb
Localization		scrib	pnt crb
Polarity		scrib	pnt Dys crb

Eye Development	lola	cindr	L crb
Regulation of Neurotransmitters/ Secretion	Sytalpha		Dys Syt12
K/Na ion transport	CG15555		Shawl
Cellular Process	Eip74EF		Pros26.4
Cell Proliferation		scrib	crb pnt Pros26.4

Chapter 6- Fitness constraints in two fluctuating environments among naturally segregating populations

Alison R. Gerken, Olivia C. Eller, and Theodore J. Morgan

Alison R. Gerken

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: aegge@ksu.edu

Olivia C. Eller

Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: oceller@ksu.edu

Theodore J. Morgan

Corresponding author. Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506. e-mail: tjmorgan@ksu.edu

Abstract

Phenotypic plasticity is described as the ability of a genotype to have alternative phenotypes in different environments. Rapid cold hardening (RCH) is a metric of plasticity in the face of a pretreatment at a mild temperature prior to a cold shock treatment. The ability to acclimate can be measured at different rearing environments and compared to other fitness and survivorship components to provide a predictive thermal profile for a genotype. The *Drosophila melanogaster* Genetic Reference Panel (DGRP) consists of a naturally segregating population of allelic variation and shows large variation in the rapid cold hardening response for flies reared in a constant environment. Fitness metrics for flies reared in fluctuating environments should correlate with levels of acclimation ability, if this metric of plasticity corresponds with a

genotype's ability to act plastically in fluctuating environments. We examined this interaction by rearing forty genotypes of the DGRP at two daily fluctuating environments, a warm environment with a mean of 23.8° C, and a cool environment with a mean of 16.8° C. We measured lifetime egg production and survival rate on female flies of these genotypes. Analyses to compare fitness and demographic metrics with RCH score from constant environments showed no correlation with fitness in fluctuating environments and plasticity ability from constant environments. Demographic parameters showed significant correlations between the two rearing environments, demonstrating a genetic constraint of fitness, in concordance with current environmental rearing conditions. These results suggest that no association with RCH from constant environments and fitness in fluctuating environments may indicate that different genetic and physiological mechanisms are under differential selection within environments. Correlations of demographic parameters between environments suggest that although fitness components are shifted by environmental conditions, genotypes are constrained in overall numbers of eggs laid and survivorship parameters.

INTRODUCTION

Thermal biology is studied via various metrics such as survival, reproductive output, movement responses to temperature, as well as upper and lower thermal limits. The majority of this research is conducted within a laboratory under constant thermal conditions. For many species, studies of optimal temperature have indicated that populations are successful at a given temperature, but they have variation in the breadth of performance curves that are significantly influenced by temperature and thermal fluctuations (Angilletta et al 2002). Such optimal conditions result in increased reproduction, survival, and population growth rates, however they are not truly representative of what an organism will experience in nature during its lifetime (Kelty and Lee 2001; Terblanche et al 2011). Seasonal and daily thermal fluctuations can drive alterations in physiology and metabolism throughout an organism's lifespan (Martin and Huey 2008; Williams et al 2012), theoretically affecting both survival and reproductive output.

Alternatively, an organisms response to one metric of thermal tolerance may not reflect their overall thermal tolerance, i.e. differing metrics of cold tolerance may not correlate (Gerken et al *in prep* a, Chapter 2; MacMillan et al. 2009; Harshman and Hoffmann 2000; Kristensen et al 2007; Anderson et al 2005; Bubliy and Loeschcke 2005; Phelan et al 2003; Norry et al 2007). For example, individuals that may survive well long-term at cool temperatures may not survive well short-term at extreme temperatures (Gerken et al *in prep* a, Chapter 2). Ectotherms are especially susceptible to environmental influence as the rearing temperature can also significantly alter survival capabilities of individual genotypes (Colinet and Hoffmann 2012; Cooper et al 2010). Faced with a rapidly changing climate, organisms must adapt to new mean temperatures as well as new variations of temperature (Jentsch et al 2007; IPCC 2013; Hoffmann et al 2013). The changes in standard deviation around the mean temperature have been

hypothesized to have as much influence as shifts in mean temperature (Garcia-Carreras and Reuman 2013). Thus, understanding what effect thermal regimes have on fitness and survival following cold stress are key to predicting population-wide impacts (Andrew et al 2013).

The rapid cold hardening (RCH) response is prevalent within many groups of ectotherms, especially insects (Lee et al 1987; Coulson and Bale 1990; Kelty et al. 1996). It is characterized as an increase in survivorship following extreme cold stress with the addition of a pretreatment at a mild temperature (Lee et al 1987). It has been shown that this pretreatment often triggers several physiological responses to prepare an organism for cold stress, including increases in cryoprotectants (Quinn 1985; Lee et al. 1987; Lee 1989), activation of heat shock proteins (Burton et al. 1988; Bubliy and Loeschcke 2005; Sinclair and Chown 2006), and stabilization of the membrane structures (Hazel 1995; Overgaard et al 2005). The RCH score, which is the difference in survivorship of pretreated and non-pretreated organisms, can be used as a measure of the plasticity capacity, or the degree to which an organism can respond to different environmental pressures (Scheiner 1993; Via 1995; Gerken et al *in prep* b, Chapter 5).

Previous work in the *Drosophila melanogaster* Genetic Reference Panel (DGRP; Mackay et al 2012) has shown that the RCH response has significant genetic variation within a naturally segregating population, including genotypes that show a negative response to the acclimation pretreatment, i.e. greater survivorship without pretreatment (Gerken et al *in prep* b, Chapter 5). From these data, we hypothesized that the RCH response could be a good proxy for fitness and demographics in daily fluctuating environments.

Analyses of the association between fitness and RCH response may provide important insights into the thermal profile of a specific genotype; we also hypothesized that there will be high genetic correlation in fitness parameters between similar but alternative fluctuating

environments (Bell 1992). To assess the genetic correlations in fitness, we tested fitness in two fluctuating environments, 23.8±6° C and 16.8±6° C, which represent a warm and cool environment, respectively (Figure 6.1; Niehaus et al 2012). We also examine the correlations between the stress responses in fluctuating environments to acute, chronic, and acclimation cold stress. These responses allow us to compare RCH responses in fluctuating and constant environments (Gerken et al *in prep* b, Chapter 5; Paaijmans et al 2013; Cooper et al 2010).

Understanding the degree to which organisms' fitness and survival differ between environments can provide us with important empirical data on the complex dynamics of naturally occurring genotypes in a changing climate. Assessing associations between fitness and survivorship will also provide an analysis of how the organismal response at different levels is either correlated or independent of one another. Individual assessments of cold tolerance metrics are important but understanding the complex interactions of reproduction and survivorship provide a more complete look into how populations will change over time in response to environmental stress (Chown and Hoffmann 2013).

METHODS

Drosophila melanogaster Populations

Forty genotypes from the *Drosophila melanogaster* Genetic Reference Panel (DGRP; Mackay et al 2012) were chosen based rapid cold hardening (RCH) plasticity scores previously calculated when flies were reared at a constant 25° C (Gerken et al *in prep* b, Chapter 5). These genotypes were chosen based on their RCH score, the difference in a line's ability to survive cold stress at -6° C for one hour with a pretreatment of 2 hours at 4° C and without a pretreatment. Twenty genotypes were chosen on both extreme ends of the DGRP distribution, representing

high and low acclimation abilities. These genotypes were chosen to test the relationship between genotype-by-environment interactions in fluctuating and constant environments and to determine how fitness components relate to acclimation responses.

Stock populations of these 40 genotypes were maintained at 25° C until ready for use in our experiments. Parental generations were placed with five males and five females in a vial with standard corn mean-molasses-agar *Drosophila* food and were removed after three days to control for larval density. As offspring emerged, they were lightly anesthetized with CO_2, separated by sex, and allowed to recover and age until they were 5-7 days old.

Lifetime Reproduction and Survival

Each of the forty genotypes selected based on RCH scores at constant 25° C were tested for lifetime reproduction and survival at 16.8±6° C and 23.8±6° C (Figure 6.1; Ayrinhac et al 2004; Schnebel and Grossfield 1984). Twenty females, aged 5-7 days old and including both virgins and non-virgins, were placed in individual *Drosophila* vials with approximately 5 mL of standard *Drosophila* food, colored blue with food coloring for egg counting purposes. Females were provided with two males of the same genotype at all times in order to ensure mating. Vials were also lightly sprinkled with active yeast to stimulate egg laying.

Flies within each vial were transferred daily, before the peak temperature (Figure 6.1), to a new vial and empty vials were retained to count number of eggs laid. Eggs were counted by placing the vials under a dissection microscope and visually counting eggs. Vials were counted at maximum two days after they were vacated, and vials were stored at 4° C if they were not counted on the day they were vacated. Storing vials at 4° C slowed development time so eggs

would not hatch before they were counted. Egg counts were recorded daily until the female had died. Survivorship of females was also recorded and is reported in days until death.

Cold Tolerance and Plasticity Phenotypes

All genotypes were exposed to several cold stress treatments in order to understand how variability in environment corresponds with constant environmental rearing as well as how cold tolerance plasticity scores correspond to demographic and fitness parameters. Flies were again reared from egg to adult at two different temperatures: 16.8±6° C and 23.8±6° C. At emergence, flies were lightly anesthetized with CO_2, sorted by sex, and allowed to age to 5-7 days old. At this age, they were exposed to cold stress treatments (see below); survivorship was recorded 24 hours after the treatment was completed (citations). All treatments were conducted before daily peak temperature (Kelty and Lee 2001; Figure 6.1). Only female survivorship was used in analyses, as it is directly comparable to fitness metrics.

Flies reared at 23.8±6° C were exposed to three different cold stress treatments. These treatments are slight modifications of the cold stress treatments used in Gerken et al (*in prep* b; Chapter 5), because the changes in the rearing conditions between constant and fluctuating environments significantly influenced the overall mean cold tolerances. First, to measure acute stress tolerance they were exposed to -5.5° C for one hour. To measure plasticity, flies were exposed to a pretreatment of 4° C for 2 hours before exposure for one hour at -5.5° C. For chronic cold tolerance flies were exposed to 0° C for 16 hours. Flies reared at 16.8±6 C were exposed to four different cold stress treatments. To measure acute stress tolerance, and provide a comparison to flies reared at 23.8±6° C, they were exposed for one hour to -5.5° C. To assess differential cold tolerance flies were exposed to -7° C for one hour. To test for plasticity ability,

flies were exposed to a pretreatment of 4° C for 2 hours before exposure to -7° C for one hour. Finally, to test for chronic cold tolerance, flies were exposed to 0° C for 16 hours.

Data Analyses

Daily egg counts and survivorship for both 16.8±6° C and 23.8±6° C were compiled and mean egg counts for each genotype and mean age until death was assessed for each genotype. Time step data from each day was also organized into a demographic fitness matrix for analysis with custom R code (R Statistical Software Version 2.13.0; Gerken et al *in prep* c, Chapter 4). Daily egg counts and survivorship probabilities provide m(x) and l(x) values in order to assess lifetime fitness demographic parameters such as generation time, net reproductive rate (R_0), and population growth rates (lambda or λ). R_0 is the net reproductive rate and is calculated as $R0= \Sigma(l_x m_x)$ where l_x is proportion surviving at each life stage and m_x is the average fecundity at the life stage. Generation time (T) is the average time of maturity to laying an egg and is calculated as $T= \Sigma(l_x m_x)/R_0$. Lambda (λ) is defined as the rate of growth from one interval to the next and is defined as $\lambda=e^r$ where r is the intrinsic rate of increase, and is calculated as $r=\ln R_0/T$ (Begon et al 1996). Analysis in R also provided 95% confidence intervals for each of these three demographic parameters.

Survivorships following cold stress treatments were calculated for females of each genotype also in R. Plasticity scores were calculated for each rearing environment by subtracting survivorship without pretreatment from survivorship with a pretreatment. These scores provide a measure of the ability of a genotype to successfully (positive) or unsuccessfully (negative) enhance survivorship following a pretreatment. Previously calculated RCH scores for these forty

lines reared at a constant 25° C were taken directly from previous work (Gerken et al *in prep* b, Chapter 5).

Pearson's product moment correlations were conducted for all appropriate comparisons to reflect our hypotheses. First, we asked if fitness in fluctuating environments was correlated with RCH scores from constant environments. We also can test these correlations in both the warm and cool fluctuating environments and compare RCH scores from constant environments to average eggs laid, average age, generation time, λ, and R_0. In these analyses we will only be comparing data for flies reared in one environment and not across environment comparisons. We next ask if demographic parameters are constrained by genotype. For these analyses we will compare average eggs laid, average age, generation time, λ, and R0 for all genotypes between warm and cool fluctuating environments. Third, we wanted to know if RCH scores in fluctuating environments correlate with previous RCH scores from a constant environment. For these analyses we will compare RCH scores for flies reared in the warm and cool fluctuating environments to previous RCH scores from constant environments. Finally, we asked if cold tolerance survival metrics are constrained by genotype. For these analyses we compare acute, chronic, plasticity survivorships, and plasticity scores between warm and cool fluctuating environments. In addition, with these comparisons we can ask if there are trade-offs in basal tolerance and plasticity score to further understand genotypic constraints. One-way Analysis of Variance to compare plasticity levels (high and low) to overall fitness, cold tolerance survival, and RCH scores to assess how dependent these metrics in fluctuating environments were to those calculated in constant environments.

Heritability estimates for average eggs laid (fecundity) and average age (longevity) were also analyzed by calculating variance components of line (or genotype) and replicate nested

within line using the lmer function in R statistical software. Association mapping was also performed on average age, average eggs laid, lambda (λ), R0, and generation time for both experimental temperatures, using the ANOVA formula phenotype= mean + M, where M is the SNP marker (Mackay et al 2012). Analyses were run on February 12, 2014 using the intermediate web version between DGRP1 and DGRP2.

RESULTS

Lifetime Fitness

Averages for all genotypes are given in Table 6.1; averages were calculated by using all twenty females tested. Females reared at 23.8±6° C had an average age of 46.44 ± 8.84 days (mean ± standard deviation), with a minimum lifespan of 31.47 days and a maximum lifespan of 65.85 days. Females reared at 16.8 ± 6° C had an average age of 55.65 ± 15.23 days, with a minimum of 24.68 days and a maximum of 98.35 days (Figure 6.2). Females reared at 23.8 ± 6° C laid on average 13.73 ± 3.96 eggs per day, with a maximum of average of 22.65 for one genotype and a minimum of 5.58 eggs per day for another genotype. Females reared at 16.8 ± 6° C laid on average 4.57 ± 2.46 eggs per day, with a maximum of 11.70 on average for one genotype and a minimum of 1.10 eggs laid per day for another genotype (Figure 6.3). Rearing at warmer temperatures led to a peak in egg production at approximately days 6 through 13 (Figure 6.4A) with egg production tapering quickly following. Survivorship curves at warm temperatures show fast declines over time (Figure 6.4B) compared with survivorship at cooler temperatures (Figure 6.4D). Average number of eggs laid for flies reared at cooler temperatures peaked around days 9 and 10 (Figure 6.4C) but stayed at relatively the same rate for a longer time than flies

reared at the warmer temperatures. Overall there was much variation in survivorship curves and average number of eggs laid across the genotypes through time (Figure 6.4).

Flies reared at 23.8 ± 6° C had an average generation time of 5.74 ± 0.64 days, with a minimum of 4.77 days and a maximum of 7.55 days (Figure 6.5C). They also had a net reproductive output (R_0) of 633.39 ± 2.32 eggs, with a minimum of 263.09 and a maximum of 1113.60 (Figure 6.5B). These females had an average lambda value of 3.10 ± 0.39, with a minimum of 2.43 and a maximum of 3.95 (Figure 6.5C). Flies reared at 16.8 ± 6° C had an average generation time of 11.51 2.32 days, with a minimum time of 6.39 days and a maximum of 18.10 days (Figure 6.5F). These females also had an average R_0 of 253.80 ± 156.60, with a minimum of 51.76 and a maximum of 156.60 (Figure 6.5D). They also had an average λ value of 1.63 ± 0.19, with a minimum of 1.33 and a maximum of 2.41 (Figure 6.5E).

Cold Stress Survival

Rapid cold hardening scores previously calculated in a constant rearing environment of 25° C are given in table 6.2, as are average survivorships for each genotype for acute cold stress (1 hour at -5.5° C), chronic cold stress (16 hours at 0° C), and acclimation survivorship (2 hours at 4° C + 1 hour at -5.5° C) for flies reared at 23.8±6° C fluctuating. RCH scores are also calculated by subtracting acute cold stress survivorship from acclimation survivorship (Table 6.2). Mean survivorship for flies reared at 23.8±6° C after acute cold stress was 0.57±0.24 (mean standard deviation), with a maximum of 1.0 and a minimum of 0.022 for individual genotypes. Mean survivorship after chronic cold stress was 0.58±0.24, with a maximum of 1.0 and a minimum of 0.025. Mean survivorship after an acclimation pretreatment followed by 1 hour at -

5.5° C was 0.76±0.23, with a maximum survivorship of 1.0 and a minimum of 0.050. The mean RCH score for these females was 0.19±0.17, with a minimum of -0.10 and a maximum of 0.72.

For flies reared at 16.8±6° C, individual genotype survival and RCH score averages are reported in Table 6.3. Mean survivorship following acute stress of 1 hour at -5.5° C was 0.94±0.07, with a maximum of 1.0 and a minimum of 0.59. For acute stress at -7° C for 1 hour, mean survivorship was 0.39±0.21, with a maximum of 0.82 and a minimum of 0.047. For chronic survivorship, the mean was 0.97±0.03, with a minimum of 0.86 surviving and a maximum of 1.0. Acclimation pretreatment followed by 1 hour at -7° C had a mean survivorship of 0.91±0.14 with a maximum of 1.0 and a minimum of 0.27 survivorship. RCH score was an average of 0.48±0.26, with a maximum of 0.86 and a minimum of -0.40.

Fitness versus RCH

Pearson's product moment correlations did not indicate any significant correlations with our original RCH score for flies reared at constant 25° C and any fitness or demographic parameters (Table 6.4). RCH score for fluctuating 16.8 ± 6° C was significantly correlated with generation time at 16.8±6° C but was not significantly correlated with any other fitness parameter (Table 6.5; figure 6.6). RCH score for females reared at 23.8±6° C was not significantly correlated with any fitness parameter (Table 6.6). RCH score for flies reared at constant 25° C also did not significantly correlated to RCH scores at either 16.8±6° C ($r=0.27$, $p=0.10$; Table 6.5) or RCH score for flies reared at 23.8±6° C ($r=0.22$, $p=0.18$; Table 6.6).

We also tested for significant grouping of both RCH scores and survivorship probabilities to our original RCH score for flies reared at constant 25° C by performing one-way ANOVAs. RCH score for flies reared at 16.8±6° C fluctuating was significantly different between the high

and low RCH scores for flies reared at constant 25° C, as was acclimation survivorship for flies reared at 23.8±6° C fluctuating (Table 6.7; Figure 6.7). No other survivorship proportion or RCH score was significantly different between the high and low RCH groupings.

Fitness versus Cold Tolerance in Fluctuating Environments

Generation time for flies reared at 16.8±6° C was significantly correlated with acute survival after 1 hour at -5.5° C (r=0.32, p=0.05; Figure 6.8A), acute survival after 1 hour at -7° C (r=0.50, p=0.0015; Figure 6.8B), and chronic survival for 16 hours at 0° C (r=0.44, p=0.0065; Figure 6.8C). R_0 was significantly correlated with RCH score for flies reared at 16.8±6° C (r=0.32, p=0.052; Table 6.8), but was not significantly correlated with any other cold stress metric. λ was not correlated with any stress metric for flies reared at 16.8±6° C. Average age of flies reared at 16.8±6° C was marginally significant to acute cold stress for 1 hour at -7° C (r=0.32, p=0.053), as well as RCH score (r=-0.33, p=0.043). Average eggs laid was not significantly correlated with any cold stress metric for flies reared at 16.8±6° C (Table 6.8).

For flies reared at 23.8±6° C, generation time, λ, R_0, and average age were all not significantly correlated with any metric of cold stress (Table 6.9). Average eggs laid was significantly correlated with acute stress at -5.5° C for 1 hour (r=0.35, p=0.034).

Correlations among of Demographic Parameters

Pearson's product moment correlation tests revealed significant correlations between demographic and fitness parameters between females reared at 16.8±6° C and 23.8±6° C (Table 6.10). Generation time was marginally significant between the two rearing environments (r=0.29, p=0.083; Figure 6.9A). Lambda values for the two environments showed significant correlation

(r=0.32, p=0.056; Figure 6.9B). Lambda at 23.8±6° C was also significantly correlated with R_0 at 16.8±6° C (r=0.34, p=0.042). R_0 values were significantly correlated between rearing environments (r=0.59, p=0.0001; Figure 6.9C). Average age for the two rearing environments was also significantly correlated (r=0.33, p=0.048; Figure 6.10A). Average number of eggs laid was also strongly correlated between the two rearing environments (r=0.59, p=0.0001; Figure 6.10B).

Correlations among Cold Stress Tolerance

Cold stress tolerances also had some significant correlations between traits, females reared at 16.8±6° C and 23.8±6° C (Table 6.11). Tolerance for 1 hour at -5.5° C was significantly correlated between both rearing environments (r=0.59, p=0.0001; Figure 6.11A). Survivorship for 1 hour at -5.5° C for flies reared at 23.8±6° C was also significantly correlated with survivorship after acclimation and stress for 1 hour at -7° C for flies reared at 16.8±6° C (r=0.58, p=0.0002). Chronic cold tolerance for flies reared at 23.8±6° C was significantly correlated with survivorship after 1 hour at -7° C for flies reared at 16.8±6° C (r=0.33, p=0.049).

Survivorship following acclimation was highly correlated between the two rearing environments (r=0.64, p<0.0001; Figure 6.11B). Acclimation survivorship was also significantly correlated with survivorship after 1 hour at -5.5° C (r=0.63, p<0.0001), chronic cold survival (r=0.32, p=0.050), and RCH score (r=0.39, p=0.016) all for flies reared at 16.8±6° C. RCH score between the two rearing environments was also highly significantly correlated (r=0.39, p=0.016; Figure 6.11C). RCH score for flies reared at 23.8±6° C was also correlated with survival for 1 hour at -7° C (r=-0.37, p=0.0067) for flies reared at 16.8±6° C. RCH score for flies reared at

constant 25° C did not significantly correlate with any cold survival metrics or RCH scores for fluctuating environments.

We also tested for trade-offs between basal tolerance plasticity using Pearson's product moment correlations. RCH score at 16.8±6° C is significantly negatively correlated with acute tolerance at 1 hour at -7° C at 16.8±6° C ($r=-0.77$, $p<0.0001$; Figure 6.12A) and RCH score at 23.8±6° C is significantly negatively correlated with acute tolerance for 1 hour at -5.5° C at 23.8±6° C ($r=-0.44$, $p=0.0067$; Figure 6.12B). No other acute or chronic tolerances were significantly correlated with RCH scores within rearing environment (Table 6.12).

Heritability Estimates and Association Mapping

Heritabilities were calculated using the lmer function in R and revealed relatively low heritability for average egg laying and average age among our subset of our naturally segregating population. For flies reared at 16.8±6° C, heritability was 0.28 for average eggs laid and 0.18 for average age. For flies reared at 23.8±6° C, heritability was 0.34 for average eggs laid and 0.24 for average age.

DISCUSSION

When developing a comprehensive understanding of cold tolerance, we must integrate several phenotypes in order to determine the impact of environmental change and extreme stresses have on a population's survival and persistence. Our initial hypothesis testing whether rapid cold hardening (RCH) response is a good proxy for fitness in fluctuating environments was not supported by the fitness and demographic parameters in either one of our daily fluctuating environments (Table 6.4). These results indicate that although both survivorship and ability to

acclimate are good indicators of success of an organism in a stressful environment, under these fluctuating environments the responses to cold stress do not correspond to fitness. This lack of correspondence between RCH and fitness in a variable environment was surprising and is could be caused by a lack of stress in our rearing environments (David et al 2003; Gilchrist and Huey 2001; Chown et al. 2009). Therefore, the lack of correlation of RCH response and fitness could be due to differences in physiological processes based on the severity of stress for which the flies are exposed.

Our analyses comparing phenotypic values of "low" and "high" plasticity (RCH) scores at constant 25° C also exhibited some significant interactions with survivorship at our fluctuating environments (Table 6.7; Figure 6.2). For example, RCH score at cooler fluctuating temperatures significantly correlated with high and low plasticity values, but RCH score at warmer fluctuating temperatures did not (Figure 6.7). This may indicate more similarity in RCH properties with cooler temperatures with constant rearing environments than warmer temperatures with constant environments. Additionally, this pattern may be the result of cooler fluctuating temperatures mimicking a constant environment more accurately in terms of metabolic influence and overall metabolic alterations during fluctuations (Martin and Huey 2008; Williams et al 2012). Constant environments do not require such metabolic adjustments during periods of fluctuations and cooler environments may require less severe adjustments that may be reflected in their RCH abilities. However, no other cold tolerance metric for flies reared at cooler fluctuating temperatures was significantly correlated with previously measure RCH scores in a constant environment, thus we cannot conclusively say that this is the case.

The above two analyses and lack of correlations and associations to RCH scores from constant environments and lifetime fitness parameters from fluctuating environments likely

indicate that these two comparisons are on differential levels of the stress response (Shintani and Ishikawa 2007). For instance, analyses of fitness were performed in seasonally variable environments with a daily fluctuation component but no extreme cold stress during the flies' lifetime. Within a benign environment, selection pressures on survival in extreme environments may not present themselves within a fecundity phenotype; evolutionary parameters dictating everyday fitness may not be subject to selection and thus, may not differentiate themselves as dictated by survival after extreme environmental stresses. In a similar vein Teets and Denlinger (2013) discussed the overlap between long-term and short-term acclimation and the long-term fitness analyses may not correspond to short-term cold shock survivorship, just as long-term and short-term acclimation are hypothesized to be under different genetic and physiological control (Gerken et al *in prep* b, Chapter 5). Providing stressful environmental influences during the lifetime of a reproductively active fly may alter the fitness components in a way that would reflect the overall survivorship components, as measured by such cold tolerance metrics (e.g., RCH score, survivorship after chronic and acute stress) and then we may observe expected correlations as well as the thermal profiles of genotypes within these naturally segregating populations. Further experimentation is needed to address this hypothesis.

Testing fitness within fluctuating environments did provide us with evidence that there is high variation in demographic parameters within a sub-set of a larger naturally segregating population of *Drosophila melanogaster* (Figures 6.2-6.4). Heritability of fitness metrics is consistently low in natural populations at about 0.1-0.2 (Visscher et al 2008; Mousseau and Roff 1987), and our analysis of heritability in egg laying and survivorship follow these trends ranging from 0.18 for longevity at cooler temperatures and 0.34 for eggs laid at warmer temperatures. In both environments, we found that heritability in average eggs laid was higher than average

survivorship, suggesting to more genetic influence in egg laying constraints than in lifetime survival. In addition, we did not find any trade-offs between reproduction and longevity for either temperature, unlike Nunney and Cheung (1997), who found that there was an evolutionary trade-off in these two.

An important aspect of these analyses is demonstrating that there is significant variation among genotypes of a naturally segregating population (Figure 6.4). These survivorship and egg laying curves show that there are different rates among the genotypes of this population but that the peaks and slopes of these curves are greatly influenced by environment. However, environmental influence does not trump genotypic influence as we see that both demographic parameters and fitness components (eggs laid and average age) are correlated between the two rearing environments. Earlier peaks in egg laying at warmer temperatures is common in *Drosophila* as is the pattern of more consistent egg laying over time at cooler temperatures (Gerken et al *in prep* c, Chapter 4; Angilletta 2009; Angilletta et al 2003). These patterns are also accompanied by a longer lifespan in the cooler flies; however, overall patterns of total eggs laid between these two environments are correlated but not similar (Figure 6.5B & E) making environmental influence a crucial component of overall lifespan and egg laying timing, and ultimately total number of eggs laid. Thus, over time if temperature changes too greatly, those genotypes with low λ and R_0 may experience great competition in reproductive fitness and alleles associated with these lowered levels will be selected from the population.

Comparisons of demographic values and RCH scores within fluctuating environments provided an examination of the interactions of fitness parameters with survivorship. RCH score was significantly correlated with both generation time and average age for flies reared at the cooler temperature (Table 6.5; Figure 6.6); however, for flies reared at warmer fluctuating

temperatures, these interactions do not occur. The interaction at cooler temperatures suggests a trade-off in timing of first offspring laid and the ability to act plastically in response to cold stress. Flies at this cooler temperature could be devoting more resources to survivorship early on in their lifespan and delaying reproduction early on, in an attempt to bet-hedge against stressful conditions early in life (Angilletta 2009). At warmer temperatures, this bet-hedging may not be occurring because sensing of warmer temperatures may indicate a less likely occurrence of cooler temperatures and devoting resources to cold tolerance may be detrimental to reproduction efforts (Gilchrist and Huey 2001). This is again evident with comparisons to survivorship for cooler fluctuating temperatures for acute, chronic, and acclimation treatments and generation time comparisons (Table 6.8; Figure 6.8).

Our analyses for testing genetic correlations between of demographic parameters between environments indicated significant genetic correlations for fitness components. Generation time was marginally significant (Figure 6.9A), but both λ and R0 were significantly correlated between environments (Table 6.10; Figures 6.9B-C). These associations suggest that although environment has a strong influence on the reproductive output and timing within *Drosophila melanogaster*, the genetic components underlying these fitness traits are directly impacting a genotype's reproductive output and survivorship. In each fitness parameter case, the degree to which a genotype shifted with an environmental shift was limited and thus, fitness components may be able to be tracked with environmental changes. In addition, average age and average number of eggs laid between the two environments were also strongly constrained (Figure 6.10), indicating a specific capacity for aging and egg laying highly influenced by environment but tightly constrained by genotype. This indicates that as climate changes, genotypes that do well in a given environment will continue to do well relative to other genotypes within the same species

or population, while genotypes with low reproductive output will continue to suffer from low fitness components. Thus, certain genotype will continue to have advantage over others and depending on severity of climate stress, may outcompete and out-reproduce other genotypes (Andrew et al 2013; Garcia-Carreras and Reuman 2013).

Similarly, survivorship at cold extremes (chronic and acute) as well as following acclimation pretreatments, are strongly correlated by genotype suggesting a strong influence of genotype on survivorship, regardless of the environment. Again, the degree of survivorship changes significantly depending on rearing environment, but the overall survivorship patterns are tightly linked to genotypic components (Service 1987). Interestingly, the patterns are not consistent when comparing chronic and acute survivorship or acute survivorship with acclimation survivorship. Within acute and chronic metrics, survivorship is strongly genetically constrained but when metrics become increasingly different (i.e. timing of stress, severity of stress) comparisons across environments is not consistent. This demonstrates the intricacies of cold tolerance and highlights the complexity within relying on single metrics to assign cold tolerance levels to a given genotype or population (Denglinger and Lee 2010).

RCH scores for fluctuating environments also show correlations across warm and cool fluctuating environments (Figure 6.11C). This interaction suggests that even plasticity values as calculated as RCH scores are strongly genetically correlated; this may indicate that plasticity values are under heritable control and may be an adaptive form of cold tolerance in this population (Via et al 1995; Scheiner 1993). RCH scores from constant 25° C, however, are not significantly correlated with either fluctuating environment RCH score. This could be due to the differences in cold stress level used between previously conducted work (-6° C for one hour) and our current study (-5.5° C for one hour for warmer fluctuating environment and -7° C for cooler

fluctuating environment) but the fact that our two fluctuating environments' RCH scores significantly correlate suggests that the extreme stress environment should be translatable when assessing plasticity ability. The lack of correlation with constant reared environment RCH scores is more likely due to lifetime adjustments made in a fluctuating environment, that do not occur within a constant environment, that influence plasticity ability as reflected in RCH score (Paaijmans et al 2013; Cooper et al 2010).

Trade-offs within plasticity and levels of basal tolerance, as described as survivorship following acute or chronic stress, can play an important role in defining what type of environment a specific genotype may be best suited for, in terms of severity and duration of cold stresses (Nyamukondiwa et al 2011; Shintani and Ishikawa 2007; Gerken et al *in prep* b, Chapter 5). In our experiment we found trade-offs at both warmer and cooler fluctuating environments with acute tolerance and RCH score (Figure 6.12). This suggests that genotypes show genotypic components associated with specific environmental influences. For example, genotypes with high plasticity scores are best suited for environments with a high degree of fluctuation and preparation for cold stress, while being less equipped for environments with sudden, intense cold stress.

In conclusion, fitness in thermal environments is highly variable for naturally segregating populations and shows consistent genetic constraint for fitness and demographic parameters. However, fitness in fluctuating environments shows little correlation to plasticity (RCH) response for flies reared in constant environments. This suggests that although plasticity responses are highly genetically correlated across environments, plasticity in constant environments and fitness in variable environments may be under different genetic control and may be subject to independent on a selection level. However, we understand that these two

components may not be reflecting a consistent thermal profile under stressful conditions, as fitness was measured in benign environments. Measuring fitness under stressful conditions may provide a more complete understanding of the correlations between fitness and cold stress survival as a whole organism thermal profile. Understanding linkages of highly variable phenotypic responses to thermal changes is crucial in understanding the complete picture of how current climate change scenarios may affect many components of physiological and genetic thermal biology and our research begins at understanding the complexities of stressful and benign conditions on thermal profiles.

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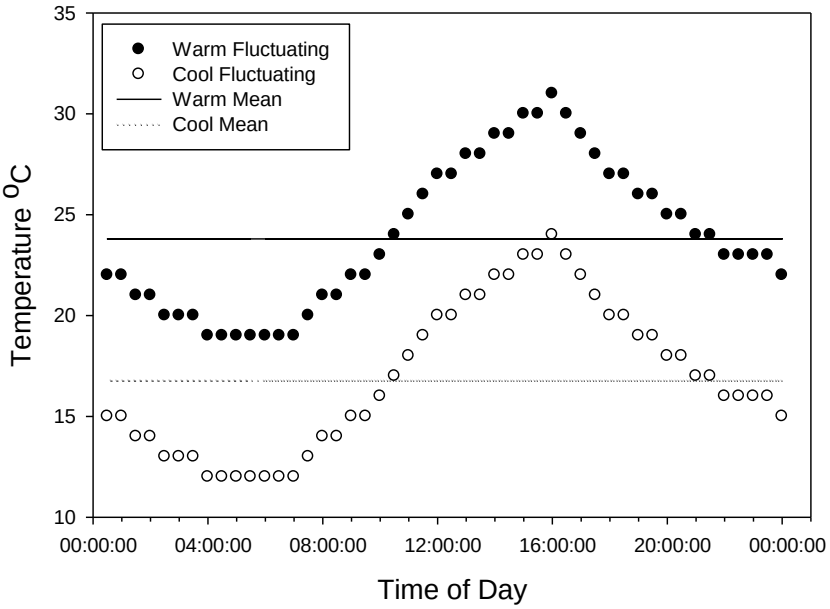

Figure 6.1. Thermal rearing environments. Forty genotypes of the DGRP were reared at two different fluctuating environments. Data was collected for survivorship at the up-swing of temperature (Kelty and Lee 2001) around 11 am to noon. The light cycle was 7:00 AM to 8:00 PM. Means of the fluctuating environments are represented by the straight lines. Open circles are for the cool environment; closed circles are for the warm environment.

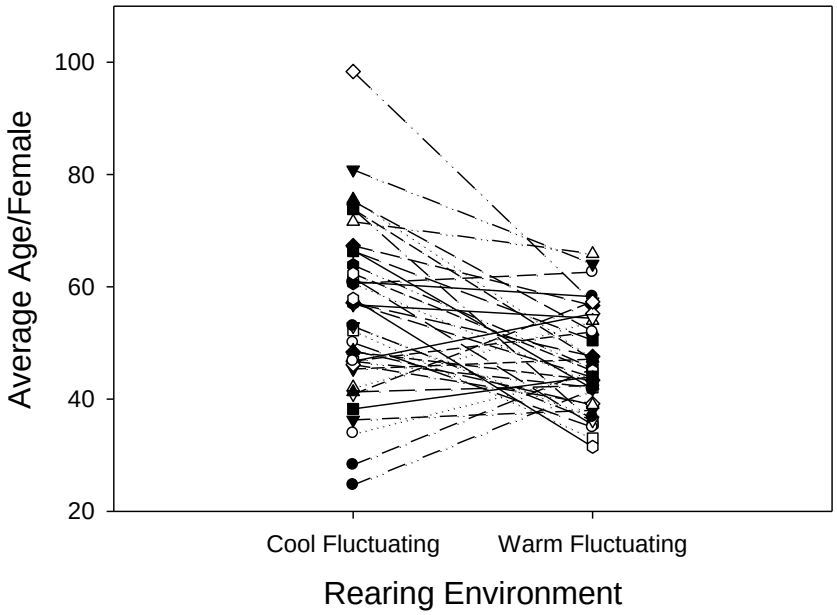

Figure 6.2. Average ages at two rearing temperatures, 16.8±6° C and 23.8±6° C. There is significant variation in the performance curves between these two environments. The cool fluctuating environment had a larger range of averages ages than the warm environment.

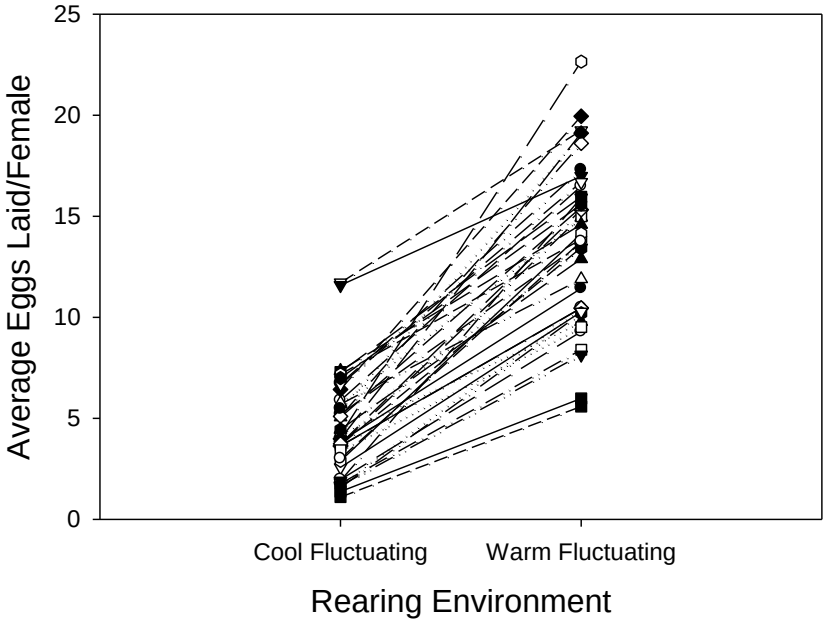

Figure 6.3. Average eggs laid across two rearing environments, 16.8±6° C and 23.8±6° C, for all genotypes. There is significant variation in performance curves among the genotypes in these two environments. The warm fluctuating environment has more variation in average number of eggs laid than the cool environment.

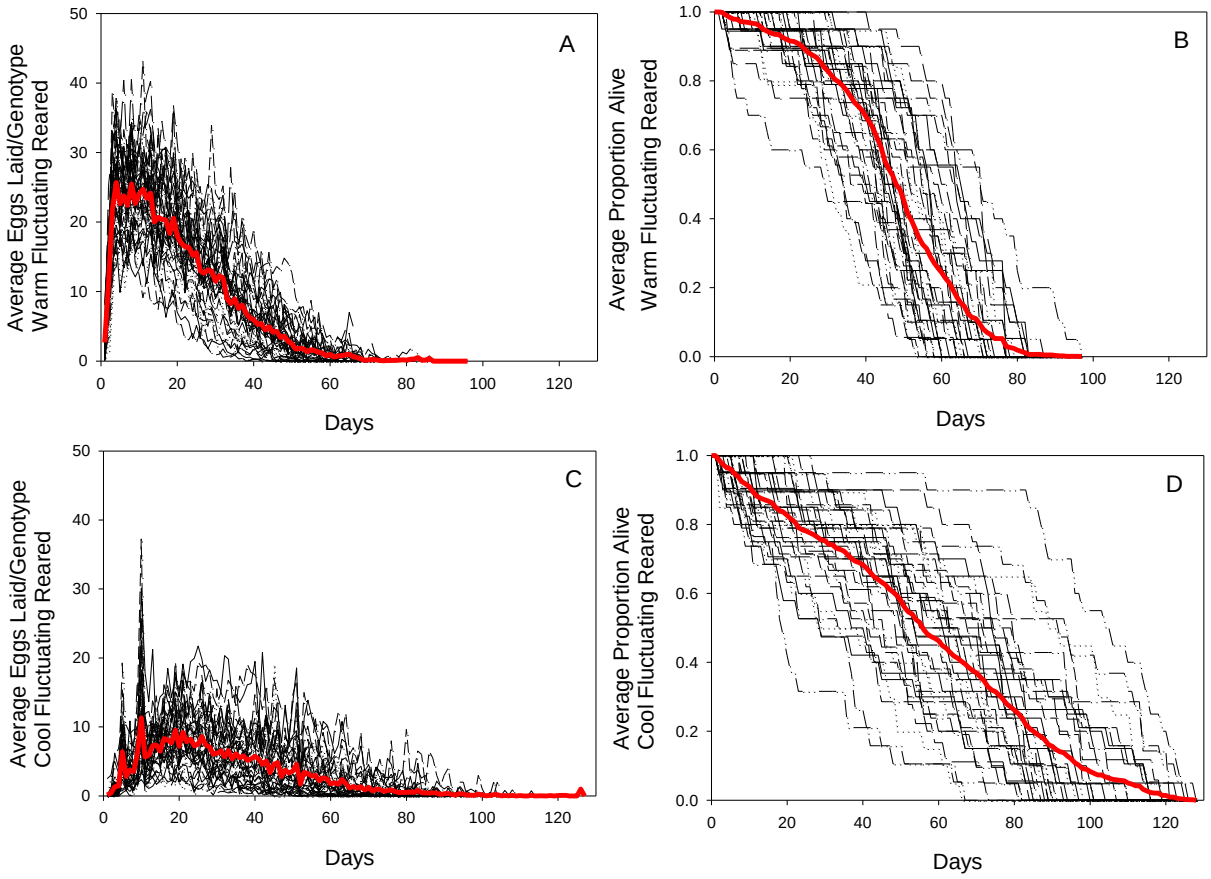

Figure 6.4. Survivorship and longevity curves for the forty genotypes examined. Red line indicates overall average curve for all genotypes. A) Average number of eggs laid per genotype over time at warm fluctuating environments. B) Survivorship curve for genotypes reared in warm fluctuating environments. C) Average number of eggs laid per genotype over time at cool temperature. D) Survivorship curve for genotypes reared in cool fluctuating environments.

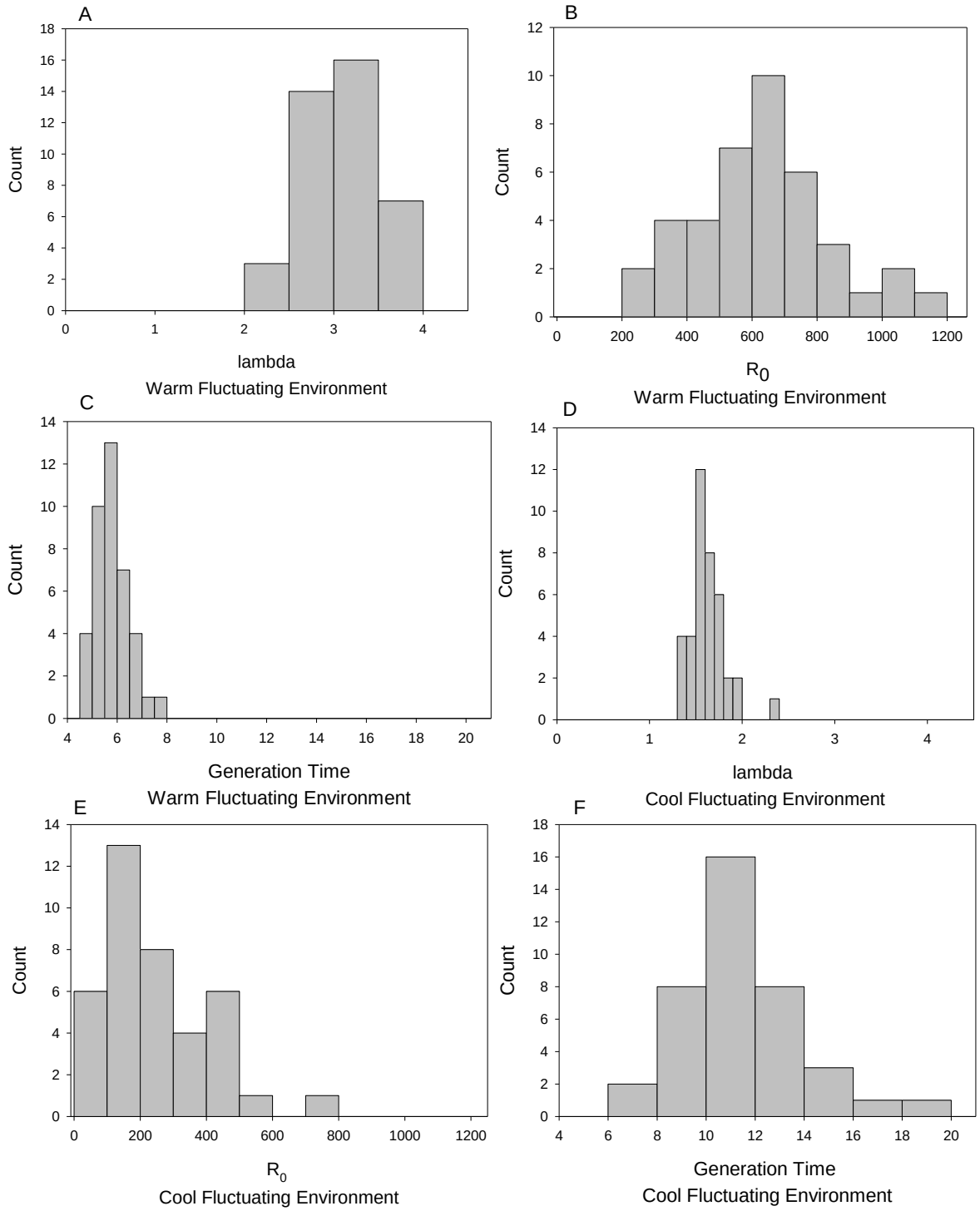

Figure 6.5. Histograms of demographic parameter distributions for warm and cool fluctuating environments. A-C are for warm fluctuating environment. A) λ B) R0 C) Generation time. D-F are for cool fluctuating environments. D) λ E) R_0 F) Generation time.

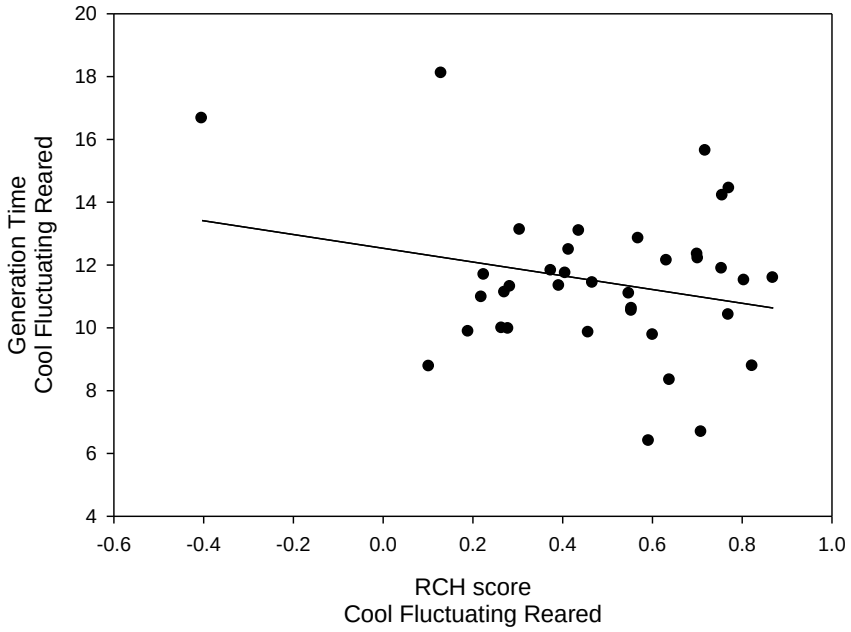

Figure 6.6. Correlation between generation time for flies reared at 16.8±6° C and RCH score for the same rearing conditions. The correlation is significant at p=0.047.

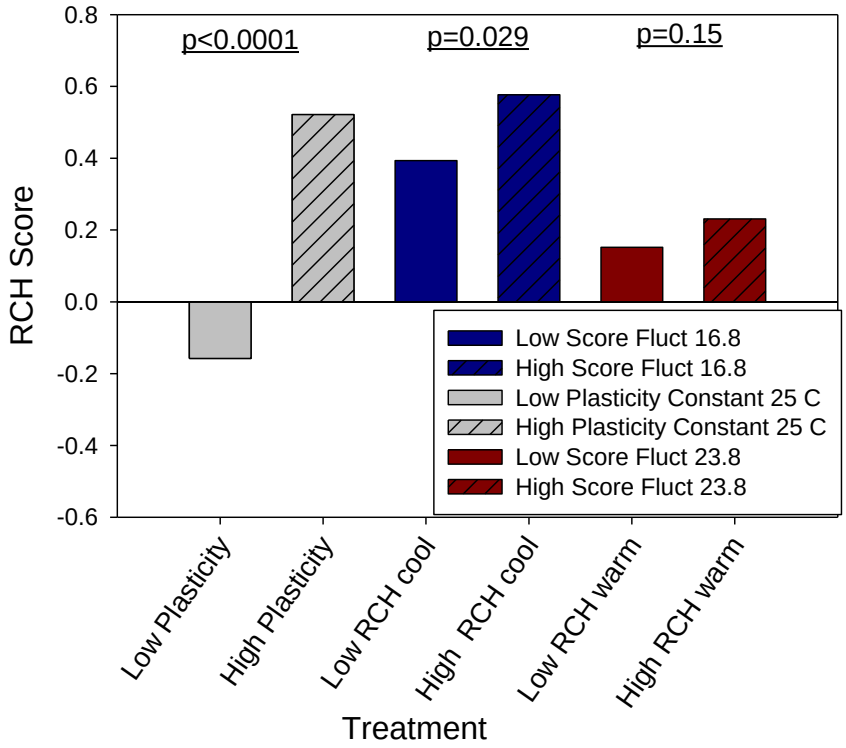

Figure 6.7. Significant associations with original high and low RCH scores for flies reared at 25° C constant. Constant reared RCH score was only significantly associated with RCH score for flies reared at cooler fluctuating temperatures but not at warmer fluctuating temperatures.

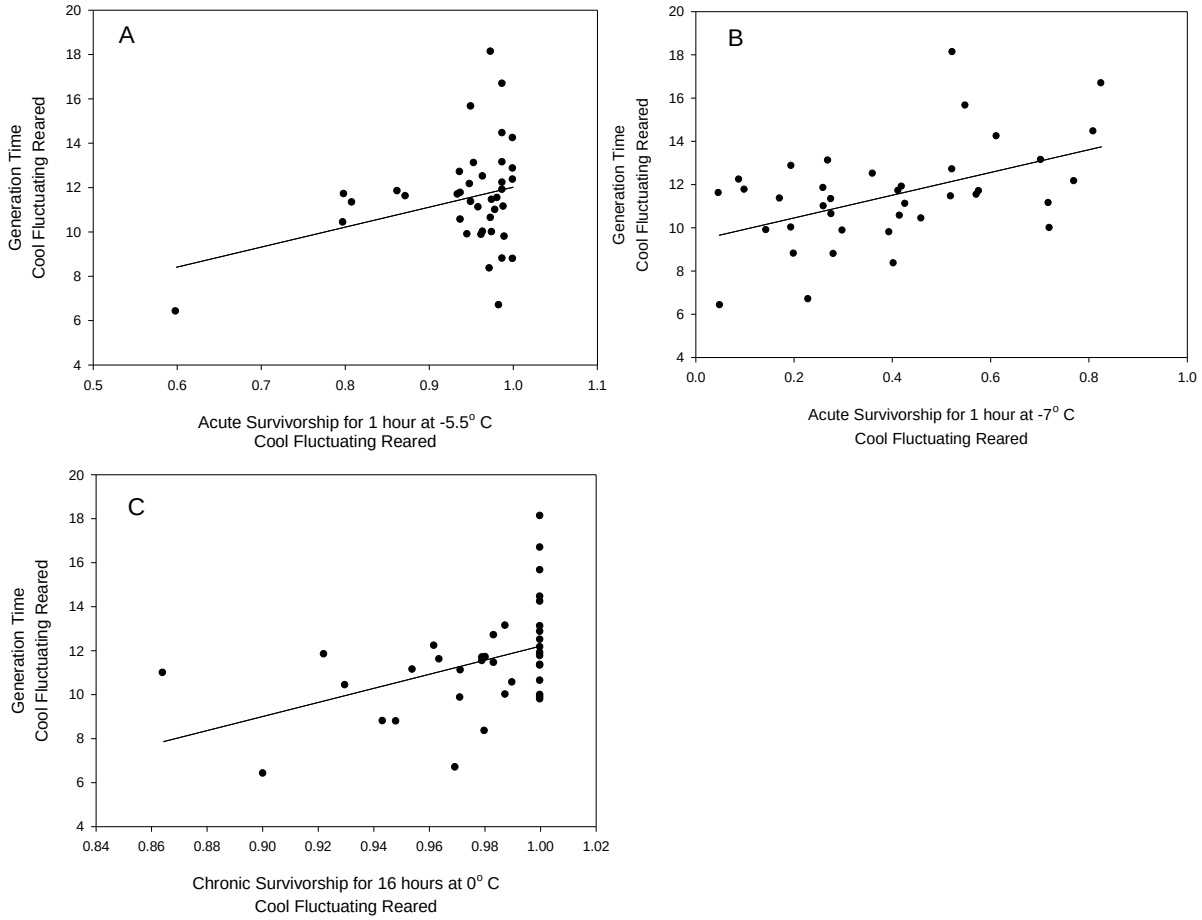

Figure 6.8. Generation time at cool fluctuating environment and correlations to survivorship. A) Correlation to acute survivorship for 1 hour at -5.5° C. B) Correlation to acute survivorship for 1 hour at -7° C. C) Correlation to chronic cold tolerance for 16 hours at 0° C.

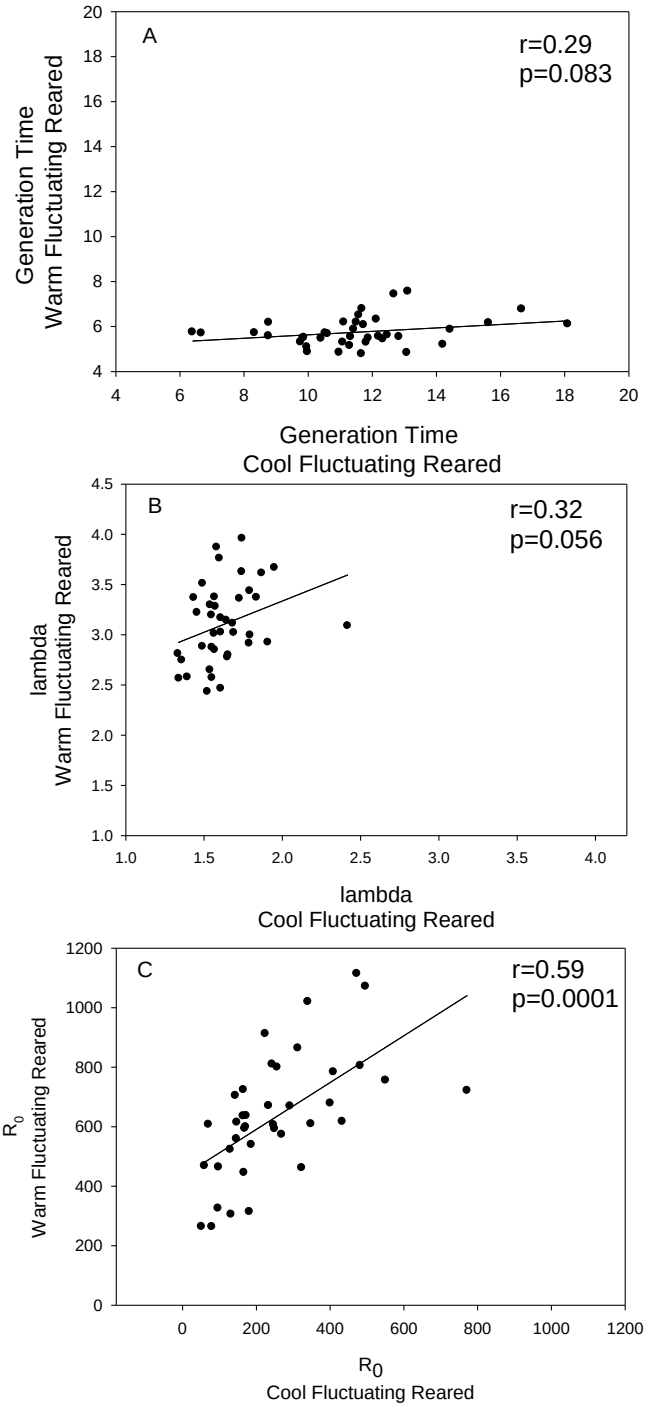

Figure 6.9. Demographic correlations between two rearing environments, 16.8±6° C and 23.8±6° C. A) Generation time in days. B) λ, or intrinsic rate of population increase. C) R_0, or net reproductive rate.

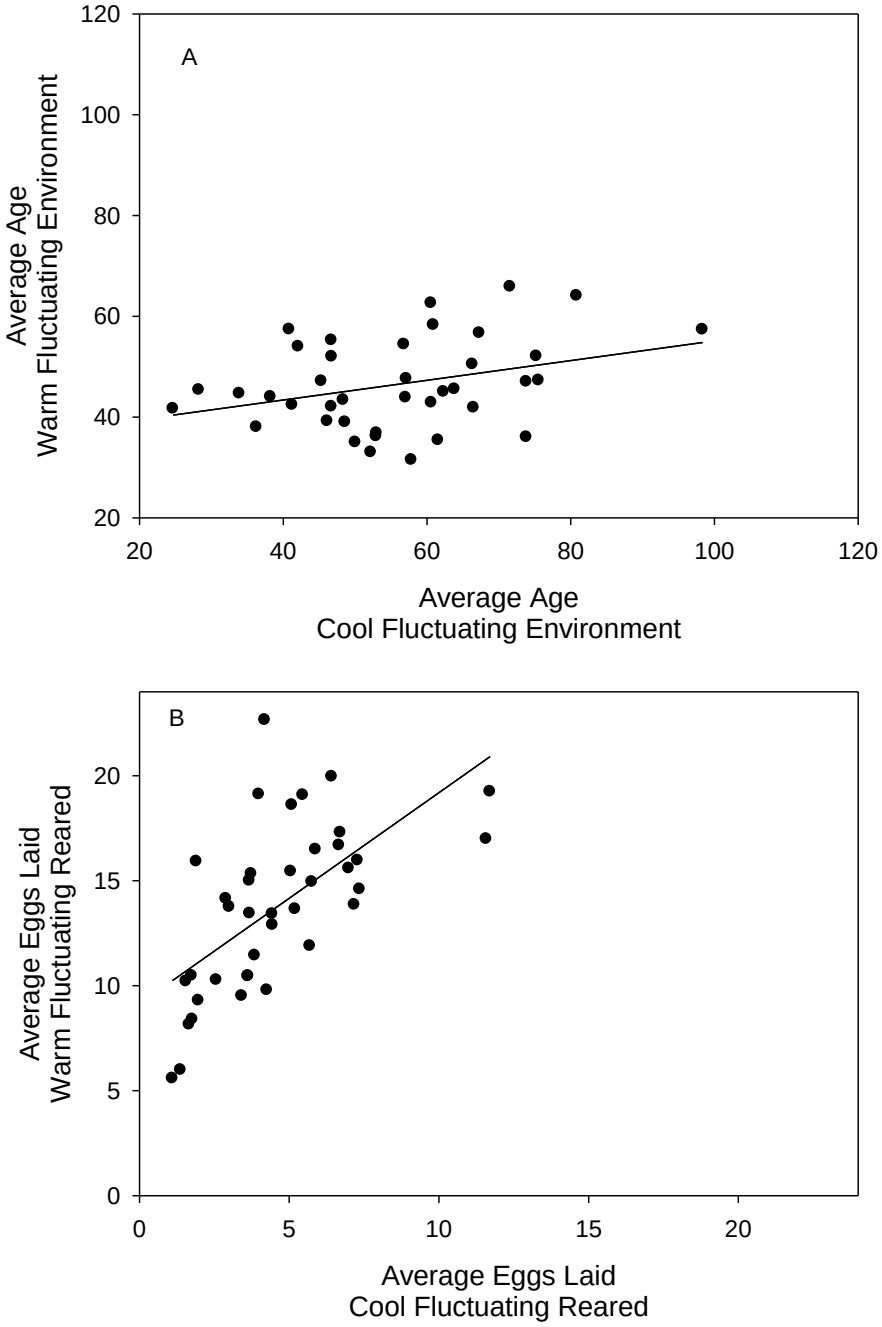

Figure 6.10. Fitness and survivorship correlations for two rearing environments, 16.8±6° C and 23.8±6° C. A) Average age for genotypes in days. B) Average number of eggs laid for genotypes.

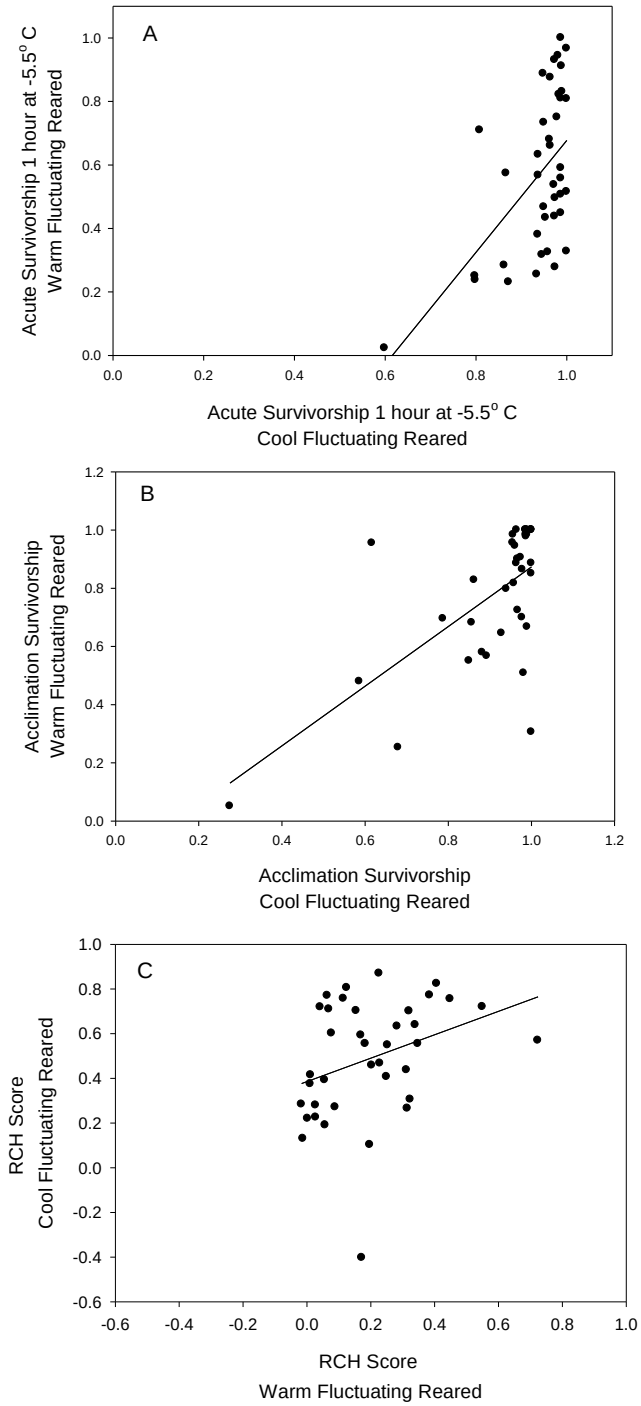

Figure 6.11. Correlations between survivorships from 2 rearing environments, 16.8±6° C and 23.8±6° C. A) Acute survivorships at 1 hour -5.5° C. B) Acclimation survivorship for 2 hours at 4° C pretreatment followed by 1 hour at -7° C for cool fluctuating environment and -5.5° C for warm fluctuating environment.

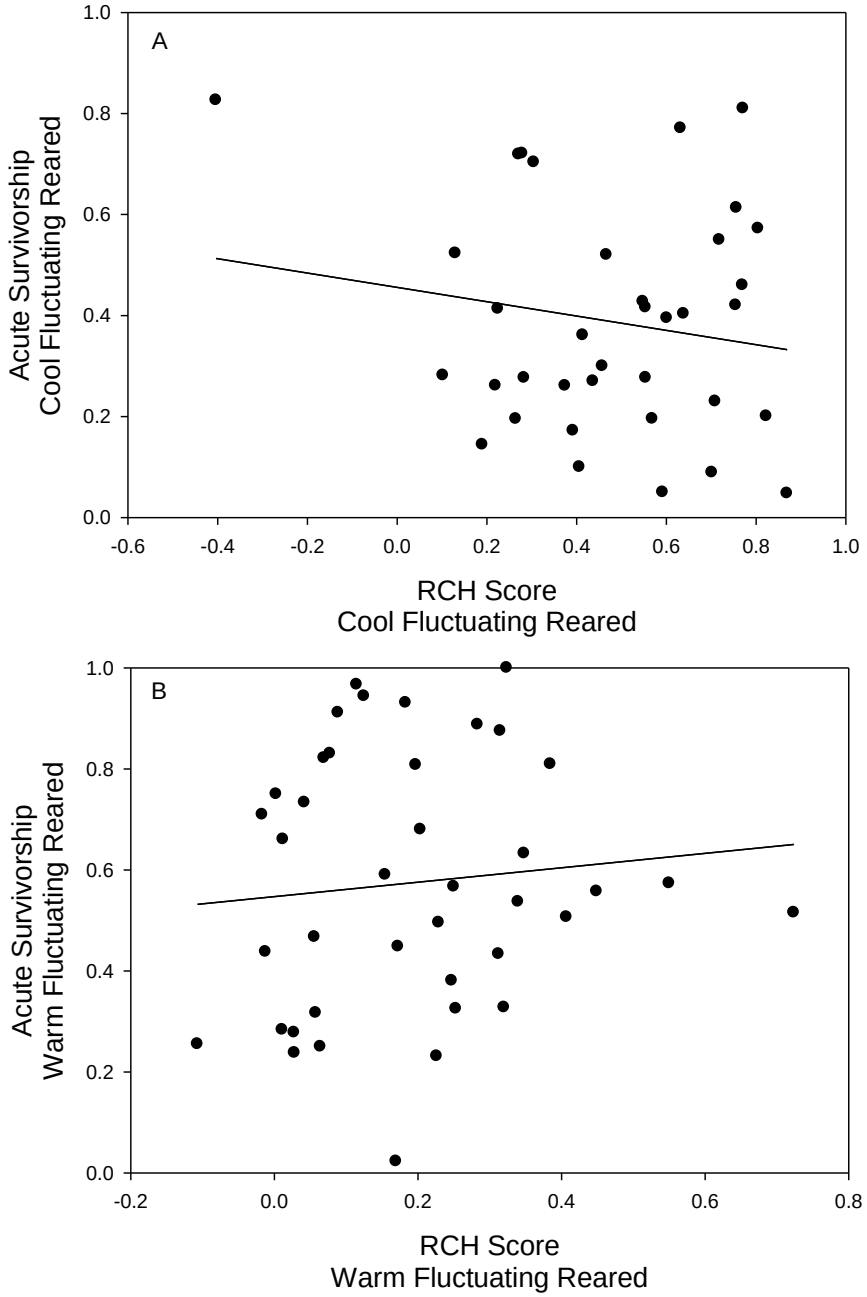

Figure 6.12.Trade-offs between basal tolerance and plasticity for RCH score at A) 16.8±6° C and acute survivorship at -7° C for 1 hour and B) 23.8±6° C and acute survivorship at -5.5° C for 1 hour.

Table 6.1. Average age, average eggs, lambda, R_0, and generation time for 39 genotypes in 2 different environments. For environments, 16.8= 16.8±6° C and 23.8=23.8±6° C. Average age and generation time are in days.

DGRP Line	Average eggs (16.8)	Average eggs (23.8)	Average age (16.8)	Average age (23.8)	Generation time (16.8)	Generation Time (23.8)	R_0 (16.8)	R_0 (23.8)	Lambda (16.8)	Lambda (23.8)
25176	3.84	11.43	60.90	58.25	12.47	5.61	233.51	669.62	1.54	3.19
25179	1.74	10.47	33.90	44.65	6.39	5.74	60.06	467.56	1.90	2.92
25184	3.68	13.44	45.33	47.11	11.42	5.86	164.90	635.12	1.56	3.00
25187	5.69	11.89	71.60	65.85	12.68	7.43	409.48	783.07	1.60	2.46
25198	7.288	15.96	66.35	50.45	11.33	5.53	482.33	804.61	1.72	3.35
25199	1.76	8.39	73.85	36.00	12.33	5.43	131.88	304.61	1.49	2.88
25200	3.62	10.45	67.31	56.65	16.66	6.76	250.13	592.07	1.39	2.57
25209	3.63	10.46	46.73	55.22	11.68	6.77	169.13	593.17	1.55	2.56
25445	4.25	9.78	75.55	47.25	12.20	5.55	323.65	461.07	1.60	3.02
28138	11.70	19.24	40.85	57.35	9.84	5.47	473.08	1113.60	1.86	3.61
28143	1.55	10.2	63.85	45.50	8.76	5.57	98.25	463.91	1.69	3.01
28146	4.18	22.65	61.57	35.39	9.98	4.86	256.84	799.33	1.74	3.95
28154	5.20	13.64	28.26	45.37	11.08	5.29	147.55	614.00	1.56	3.37
28160	5.88	16.48	60.58	62.60	11.73	6.06	340.27	1019.37	1.64	3.14
28166	11.58	16.98	66.50	41.84	9.96	5.08	772.10	720.57	1.95	3.66
28168	5.76	14.94	42.10	53.95	13.11	7.55	243.38	809.52	1.52	2.43
28176	1.10	5.58	73.84	47.00	11.66	4.77	79.80	263.09	1.45	3.21
28183	3.67	14.99	46.73	42.05	11.50	6.17	173.05	636.14	1.56	2.84
28192	3.98	19.11	57.15	47.58	9.76	5.30	224.70	911.83	1.74	3.62
28194	3.73	15.32	46.15	39.17	10.96	4.83	171.44	598.54	1.59	3.75
28204	7.35	14.58	75.25	52.05	10.40	5.46	551.00	755.47	1.83	3.36
28206	2.56	10.27	56.80	54.40	11.58	6.50	146.72	558.64	1.53	2.64
28227	4.43	13.41	60.63	42.85	11.11	6.18	269.40	573.11	1.65	2.79
28233	7.17	13.85	62.33	45.00	12.13	6.31	433.46	616.51	1.65	2.77
28236	6.70	17.29	24.68	41.63	8.77	6.17	165.65	723.39	1.78	2.91
28239	1.96	9.29	50.05	34.95	15.63	6.15	96.64	325.03	1.34	2.56
28244	1.90	15.91	36.28	38.00	11.81	5.28	70.57	606.65	1.43	3.36
28257	5.05	15.43	48.60	38.95	10.60	5.66	247.16	605.57	1.68	3.11
28258	1.37	5.98	38.25	44	12.83	5.54	51.76	263.49	1.35	2.74
28260	3.41	9.51	52.20	33	11.87	5.47	181.57	313.29	1.55	2.87
28261	6.42	19.95	48.36	43.35	9.86	5.50	313.23	863.28	1.79	3.43
28262	5.09	18.6	98.35	57.32	14.43	5.86	496.53	1071.02	1.54	3.29
28263	4.44	12.89	41.27	42.40	18.10	6.09	186.88	539.28	1.33	2.80
28264	6.66	16.68	52.94	36.16	6.67	5.69	348.70	608.50	2.41	3.08
28278	6.98	15.58	57.05	43.85	13.08	4.82	401.05	678.19	1.58	3.86
29655	2.89	14.13	57.85	31.47	11.30	5.14	166.93	445.03	1.57	3.27

29656	5.45	19.07	53.00	36.78	14.20	5.19	291.78	668.52	1.49	3.50
29657	3.00	13.75	46.76	51.95	10.53	5.70	143.76	703.99	1.60	3.16
29660	1.66	8.14	80.84	64.05	8.32	5.70	129.76	522.53	1.79	2.99

Table 6.2. Survivorship averages for flies reared at 23.8±6° C fluctuating and constant 25° C RCH score.

DGRP Line	1 hour at -5.5° C	16 hours at 0° C	2 h at 4° C +1 h at -5.5° C	RCH score	RCH score Constant 25° C
25176	0.66	1.00	1.00	0.33	0.66
25179	0.02	0.06	0.05	0.02	-0.17
25184	0.49	0.55	0.69	0.20	-0.14
25187	0.38	0.35	0.69	0.31	-0.09
25198	0.46	0.45	0.47	0.01	-0.40
25199	0.32	0.10	0.57	0.24	-0.30
25200	0.44	0.55	0.64	0.19	-0.11
25209	0.23	0.86	0.55	0.31	0.55
25445	0.59	0.66	0.81	0.22	0.46
28138	0.67	0.98	1.00	0.32	-0.18
28143	0.80	0.47	0.85	0.04	0.46
28146	0.87	0.50	1.00	0.12	-0.05
28154	0.32	0.46	0.50	0.18	0.49
28160	0.56	0.45	0.68	0.11	0.57
28166	0.27	0.84	0.30	0.02	-0.06
28168	1.00	0.66	0.98	-0.01	0.57
28176	0.25	0.60	0.57	0.32	-0.06
28183	0.94	0.75	1.00	0.05	-0.19
28192	0.83	0.68	1.00	0.16	-0.32
28194	0.75	0.84	0.82	0.07	-0.05
28204	0.25	0.73	0.25	0.002	-0.04
28206	0.23	0.40	0.95	0.72	0.49
28227	0.911	0.50	1.00	0.08	0.46
28233	0.88	0.57	0.94	0.05	0.53
28236	0.50	0.02	0.95	0.44	0.51
28239	0.73	0.81	0.98	0.25	-0.08
28244	0.28	0.42	0.56	0.28	0.46
28257	0.93	0.50	1.00	0.06	0.55
28258	0.51	0.28	0.90	0.38	0.53
28259	0.57	0.75	0.46	-0.10	-0.05
28260	0.55	0.98	0.90	0.34	0.65
28261	0.31	0.31	0.72	0.40	0.46
28262	0.80	0.78	0.79	-0.01	0.54
28263	0.43	0.51	0.66	0.22	-0.31
28264	0.82	0.61	0.88	0.06	0.43
28278	0.43	0.95	0.98	0.55	0.50
29655	0.70	0.60	0.86	0.15	-0.17
29656	0.96	0.43	0.97	0.01	0.47
29657	0.63	0.61	0.88	0.25	-0.09
29660	0.53	0.68	0.70	0.17	-0.18

Table 6.3. Survivorship averages for flies reared at 16.8±6° C. Blank boxes are missing data.

DGRP Line	1 hour at -5.5° C	1 hour at -7° C	16 hours at 0° C	2 h at 4° C + 1 h at -7° C	RCH score
25176	0.96	0.36	1.00	1.00	0.63
25179	0.59	0.05	0.90	0.27	0.22
25184	0.97	0.52	0.98	0.97	0.45
25187	0.93	0.52	0.98	0.78	0.26
25198	0.95	0.17	1.00	0.58	0.41
25199	1				
25200	0.98	0.82	1.00	0.92	0.10
25209	0.79	0.41	0.98	0.85	0.43
25445	0.98	0.08	0.96	0.95	0.86
28138	0.96	0.29	0.97	1.00	0.70
28143	1.00	0.28	0.94	1.00	0.71
28146	0.96	0.19	0.98	1.00	0.80
28154	0.95	0.42	0.97	0.98	0.55
28160	0.93	0.10	1.00	0.85	0.75
28166	0.97	0.72	1.00	1.00	0.27
28168	0.98	0.70	0.98	0.98	0.28
28176	0.93	0.57	0.97	0.88	0.30
28183	0.98	0.57	0.97	0.96	0.39
28192	0.99	0.39	1.00	0.98	0.59
28194	0.97	0.26	0.86	0.86	0.60
28204	0.79	0.45	0.92	0.67	0.21
28206	0.87	0.04	0.96	0.61	0.56
28227	0.98	0.71	0.95	0.99	0.27
28233	0.94	0.77	1.00	0.96	0.19
28236	0.98	0.20	0.94	0.95	0.75
28239	0.95	0.54	1.00	0.95	0.40
28244	0.86	0.26	0.92	0.89	0.63
28257	0.97	0.27	1.00	0.98	0.70
28258	1.00	0.19	1.00	0.96	0.77
28259	0.86		0.97		
28260	0.98	0.42	1.00	0.97	0.55
28261	0.94	0.14	1.00	0.96	0.82
28262	0.98	0.81	1.00	0.94	0.13
28263	0.97	0.52	1.00	0.99	0.46
28264	0.98	0.22	0.96	1.00	0.77
28278	0.95	0.27	1.00	0.99	0.72
29655	0.80	0.27	1.00	0.97	0.70
29656	1.00	0.61	1.00	0.98	0.37
29657	0.93	0.41	0.99	0.96	0.54
29660	0.97	0.40	0.98		-0.40

Table 6.4. Pearson's correlations and p-values for demographic and fitness parameters compared to RCH score for flies reared at constant 25° C.

Fitness Parameter	Correlation (r)	p-value
Generation time (16.8±6° C)	-0.02	0.90
Generation time (23.8±6° C)	0.12	0.46
λ (16.8±6° C)	-0.05	0.76
λ (23.8±6° C)	-0.17	0.30
R_0 (16.8±6° C)	-0.08	0.63
R_0 (23.8±6° C)	-0.07	0.69
Average Eggs (16.8±6° C)	-0.05	0.78
Average Eggs (23.8±6° C)	-0.08	0.61
Average Age (16.8±6° C)	-0.05	0.77
Average Age (23.8±6° C)	0.01	0.93

Table 6.5. Pearson's correlations and p-values for demographic parameters and RCH scores for flies reared at 16.8±6° C fluctuating. *=statistically significant

Fitness Parameter	Correlation (r) RCH score (16.8±6° C)	p-value (16.8±6° C)
Generation time (16.8±6° C)	-0.33	0.047 *
λ (16.8±6° C)	0.19	0.26
R_0 (16.8±6° C)	-0.25	0.14
Average Eggs (16.8±6° C)	-0.04	0.80
Average Age (16.8±6° C)	-0.33	0.043 *
RCH score (constant 25° C)	0.27	0.10

Table 6.6. Pearson's correlations and p-values for demographic parameters and RCH scores for flies reared at 23.8±6° C fluctuating.

Fitness Parameter	Correlation (r) RCH score (23.8±6° C)	p-value (23.8±6° C)
Generation time (23.8±6° C)	0.03	0.85
λ (23.8±6° C)	-0.12	0.49
R_0 (23.8±6° C)	-0.19	0.25
Average Eggs (23.8±6° C)	-0.08	0.083
Average Age (23.8±6° C)	-0.29	0.53
RCH score (constant 25° C)	0.22	0.18

Table 6.7. One-way ANOVA p-values and means associated with original "high" and "low" RCH scores for flies reared at constant 25° C. *= statistically significant.

Phenotypic Value	p-value	"High" Mean ± Standard Deviation	"Low" Mean ± Standard Deviation
RCH score (constant 25° C)	<2.2e-16	0.52 ± 0.06	-0.15 ± 0.10
RCH score (16.8±6° C)	0.029 *	0.57 ± 0.22	0.39 ± 0.27
RCH score (23.8±6° C)	0.15	0.23 ± 0.20	0.15 ± 0.12
Generation Time (16.8±6° C)	0.98	11.50 ± 1.87	11.51 ± 2.77
Generation Time (23.8±6° C)	0.41	5.83 ± 0.62	5.66 ± 0.66
λ (16.8±6° C)	0.92	1.63 ± 0.21	1.63 ± 0.18
λ (23.8±6° C)	0.41	3.05 ± 0.35	3.16 ± 0.43
R_0 (16.8±6° C)	0.83	248.67 ± 121.61	259.20 ± 190.01
R_0 (23.8±6° C)	0.86	639.04 ± 198.37	627.45 ± 216.22
1 hour at -5.5° C (16.8±6° C)	0.23	0.95 ± 0.05	0.92 ± 0.09
1 hour at -5.5° C (23.8±6° C)	0.28	0.61± 0.26	0.53 ± 0.24
1 hour at -7° C (16.8±6° C)	0.38	0.36 ± 0.23	0.42 ± 0.19
16 hours at 0° C (16.8±6° C)	0.60	0.98 ± 0.02	0.97 ± 0.03
16 hours at 0° C (23.8±6° C)	0.67	0.56 ± 0.25	0.60 ± 0.23
2 hours at 4° C + 1 hour at -7° C (16.8±6° C)	0.15	0.94 ± 0.08	0.87 ± 0.19
2 hours at 4° C + 1 hour at -5.5° C (23.8±6°)	0.025 *	0.84 ± 0.16	0.68 ± 0.27
Average Eggs (16.8±6° C)	0.81	4.48 ± 3.05	4.67 ± 1.81
Average Eggs (23.8±6° C)	0.76	13.54 ± 4.28	13.92 ± 3.73
Average Age (16.8±6° C)	0.34	53.37 ± 16.36	58.06 ± 13.98
Average Age (23.8±6° C)	0.84	46.17 ± 8.22	46.73 ± 9.68

Table 6.8. Pearson's correlations for fitness, survivorship, and RCH scores for flies reared at 16.8±6° C. Correlation (p-value). *=statistically significant

	Generation time	λ	R_0	Average Age	Average Eggs Laid
1 hour at -5.5° C	0.32 (0.05) *	-0.14 (0.40)	0.16 (0.33)	0.14 (0.41)	0.21 (0.21)
1 hour at -7° C	0.50 (0.0015) *	-0.28 (0.08)	0.27 (0.10)	0.32 (0.053) *	0.14 (0.39)
16 hours at 0° C	0.44 (0.0065) *	-0.22 (0.19)	0.24 (0.14)	0.25 (0.12)	0.18 (0.29)
2 hours at 4° C + 1 hour at -7° C	0.27 (0.11)	-0.14 (0.39)	0.04 (0.79)	-0.01 (0.93)	0.15 (0.37)
RCH score	-0.33 (0.047) *	0.19 (0.26)	0.32 (0.052) *	-0.33 (0.043) *	-0.04 (0.80)

Table 6.9. Pearson's correlations for fitness, survivorship, and RCH scores for flies reared at 23.8±6° C. Correlation (p-value). *=statistically significant

	Generation time	λ	R_0	Average Age	Average Eggs Laid
1 hour at -5.5° C	0.05 (0.77)	0.04 (0.80)	0.17 (0.30)	-0.23 (0.16)	0.35 (0.034) *
16 hours at 0° C	-0.17 (0.30)	0.23 (0.16)	0.10 (0.56)	0.06 (0.74)	0.01 (0.94)
2 hours at 4° C + 1 hour at -7° C	0.08 (0.65)	-0.04 (0.82)	0.05 (0.77)	-0.17 (0.31)	0.17 (0.31)
RCH score	0.03 (0.18)	-0.12 (0.49)	-0.19 (0.25)	0.12 (0.49)	-0.29 (0.083)

Table 6.10. Pearson's correlations for fitness parameters between 16.8±6° C and 23.8±6° C. Values are reported as Correlation or r (p-value). *=statistically significant

	Generation time (23.8±6° C)	λ (23.8±6° C)	R_0 (23.8±6° C)	Average age (23.8±6° C)	Average eggs (23.8±6° C)
Generation time (16.8±6° C)	0.29 (0.083)	-0.3 (0.069)	-0.11 (0.50)	-0.15 (0.36)	-0.24 (0.15)
λ (16.8±6° C)	-0.18 (0.29)	0.32 (0.056) *	0.33 (0.043) *	-0.09 (0.61)	0.44 (0.006) *
R_0 (16.8±6° C)	-0.03 (0.83)	0.34 (0.042) *	0.59 (0.0001) *	0.27 (0.10)	0.47 (0.003) *
Average age (16.8±6° C)	-0.03 (0.85)	0.1 (0.56)	0.18 (0.29)	0.33 (0.048) *	-0.01 (0.93)
Average eggs (16.8±6° C)	-0.02 (0.90)	0.35 (0.032)	0.65 (<0.0001) *	0.19 (0.26)	0.59 (0.0001) *

Table 6.11. Pearson's correlations for cold stress survivorship between 16.8±6° C and 23.8±6° C. Values are reported as Correlation or r (p-value). *=statistically significant

	1 hour at -5.5° C (23.8±6° C)	16 hours at 0° C (23.8±6° C)	2 h at 4° C + 1 h at -5.5° C (23.8±6° C)	RCH score (23.8±6° C)	RCH score (constant 25° C)
1 h at -5.5° C (16.8±6° C)	0.59 (0.0001) *	0.23 (0.165)	0.63 (<0.0001) *	0.00 (0.98)	0.19 (0.26)
1 h at -7° C (16.8±6° C)	0.29 (0.085)	0.33 (0.049) *	0.04 (0.79)	-0.37 (0.0067) *	-0.09 (0.58)
16 h at 0° C (16.8±6° C)	0.23 (0.16)	0.20 (0.22)	0.32 (0.050) *	0.1 (0.54)	0.07 (0.67)
2 h at 4° C + 1 h at -7° C (16.8±6° C)	0.58 (0.0002) *	0.38 (0.018)	0.64 (<0.0001) *	0.03 (0.14)	0.26 (0.11)
RCH score (16.8±6° C)	0.1 (0.54)	-0.07 (0.68)	0.39 (0.016) *	0.39 (0.016) *	0.27 (0.10)

Table 6.12. Pearson's correlations between plasticity score and basal tolerance within rearing environment. Reported at correlation or r (p-value). NA= not applicable comparison. *=statistically significant

	RCH score (23.8±6° C)	RCH score (16.8±6° C)
1 hour at -5.5 C (23.8±6° C)	-0.44 (0.0067) *	NA
1 hour at -5.5 C (16.8±6° C)	NA	0.2 (0.22)
1 hour at -7 C (16.8±6° C)	NA	-0.77 (<0.0001) *
16 hours at 0 C (23.8±6° C)	-0.03 (0.85)	NA
16 hours at 0 C (16.8±6° C)	NA	-0.01 (0.95)

Chapter 7- Conclusions and Thoughts

This dissertation work had several different facets, which allowed me to explore the genetics of cold tolerance with several different methods and tools. This work significantly advanced our understanding of the evolution and genetics of artificial selection as well as population-wide naturally segregating population genomics. These broad questions allowed me to take on a variety of analyses, including microarray, association mapping, functional mutant analyses, and evolutionary components of fitness and phenotypes of cold tolerance. We can make several conclusions based on the work within these chapters and here I will briefly highlight them.

In Chapter 2, we discussed the implications of artificial selection on chill-coma recovery time. We hypothesized that several other stress traits would show correlation with recovery time; however, we found that none of our stresses correlated, and if they did it was primarily because of strong differences among all the lines and not directly associated with selection regime. We did however, discuss that chill-coma recovery is a good quantitative trait to study because it is highly heritable and non-lethal.

Chapter 3 used the selection lines again to distinguish gene expression differences between the direction of selection as well as over a time series of before, during, and after cold stress. We found that many genes were involved primarily in the recovery phase, which could be due to the fact that chill-coma recovery is a recovery trait and selection on gene expression before or during cold stress is not a direct target of selection. We found that resistant to cold lines also had higher enrichments of genes associated with metabolism maintenance and that susceptible lines had strong associations with general transcription processes as well as an up-regulation in reproductive genes during recovery from cold stress. This up-regulation could be

maladaptive as it could use energy in egg production, rather than in basic biological functions for recovery from cold.

In Chapter 4 we examined fitness patterns across a broad range of temperatures to test if artificial selection had a negative impact on reproduction and survival in our selection lines. We found that λ and generation time were significantly different between susceptible and resistant lines only at cool temperatures. This suggests that susceptible lines do not perform poorly overall of the fitness temperatures, but instead their manifestation of the chill-coma recovery response reflects itself at cooler, more stressful temperatures.

Chapter 5 used a different population of interest, this time focusing on using natural genetic variation to assess phenotypic and genetic differences. We asked whether long-term and short-term acclimation had genes in common for their traits and they did not. These two acclimation pretreatments are, much like physiological research suggests, quite different in their genetic regions associated with long- and short-term response. However, candidate genes showed overlap in biological and molecular function between the two acclimation phenotypes. We also tested functional mutants and found two genes associated with autophagy being significantly associated with decreased ability to respond to acclimation. This might suggest that blocking autophagy causes a build-up of damaged cells and ultimately leads to decreased ability to survive.

The final chapter addressed the linkage between plasticity in constant environments and fitness ability in fluctuating environments, based on the hypothesis that plasticity ability should correlate with fitness ability in fluctuating environments if these two responses are on the same plasticity level. We found that there were no correlations between these two traits for flies tested in both warm and cool fluctuating environments. We were also able to compare genetic

correlations between demographic parameters and found that genotypes are significantly correlated in their demographic parameters as well as survivorship probabilities. This suggests that although there is an environmental response to shifts in temperature, a given genotype is constrained within a given number of eggs and survivorship. This could have implications for identifying "poor" genotypes as temperatures shift.

This research has broadened my understanding of evolutionary genetics, genomics approaches, evolutionary ecology, and thermal biology. Understanding the broader implications of thermal biology in an ecological, genetic, physiological, and evolutionary perspective requires utilization of several different approaches, which is what I have done here. Understanding how traits are influenced by one another as well as the environment is important for understanding how traits can evolve and how organisms and populations can persist through time.

Appendix A- Supplemental Table and Figures

Table 5.S1. Correlations for male and female survivorship for each treatment. Sexes were strongly and significantly correlated for all survivorships. Survivorship proportions were then pooped for both association mapping analyses and RCH score calculation to give a picture of population-wide association of acclimation.

Male/Female Comparison (Rearing temp_Test)	t	df	p-value	Correlation (Pearson's r)
18°C_-6 °C for 1 hour	5.252	180	4.21×10^{-7}	0.364
18°C _-8°C for 1 hour	7.660	181	1.08×10^{-12}	0.494
18°C _RCH + -8°C for 1 hour	8.043	177	1.21×10^{-13}	0.517
25°C _-6 °C for 1 hour	6.048	180	8.28×10^{-9}	0.410
25°C _RCH + -6°C for 1 hour	8.544	184	4.89×10^{-15}	0.532
25°C _0 °C for 16 hours	9.787	164	2.20×10^{-16}	0.607

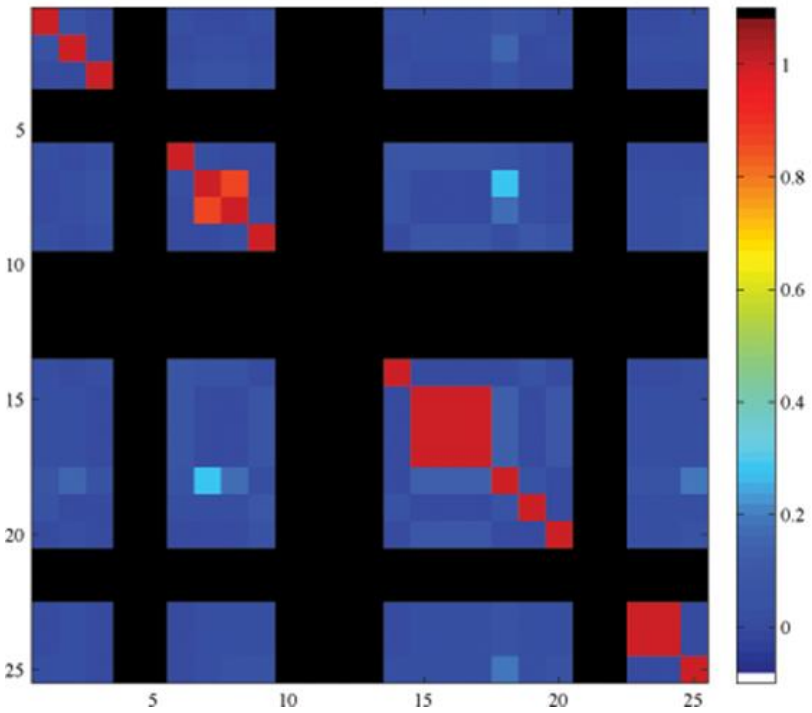

Figure 5.S1. Linkage disquilibrium mapping for association mapping of chronic cold tolerance for flies reared at 25° C.

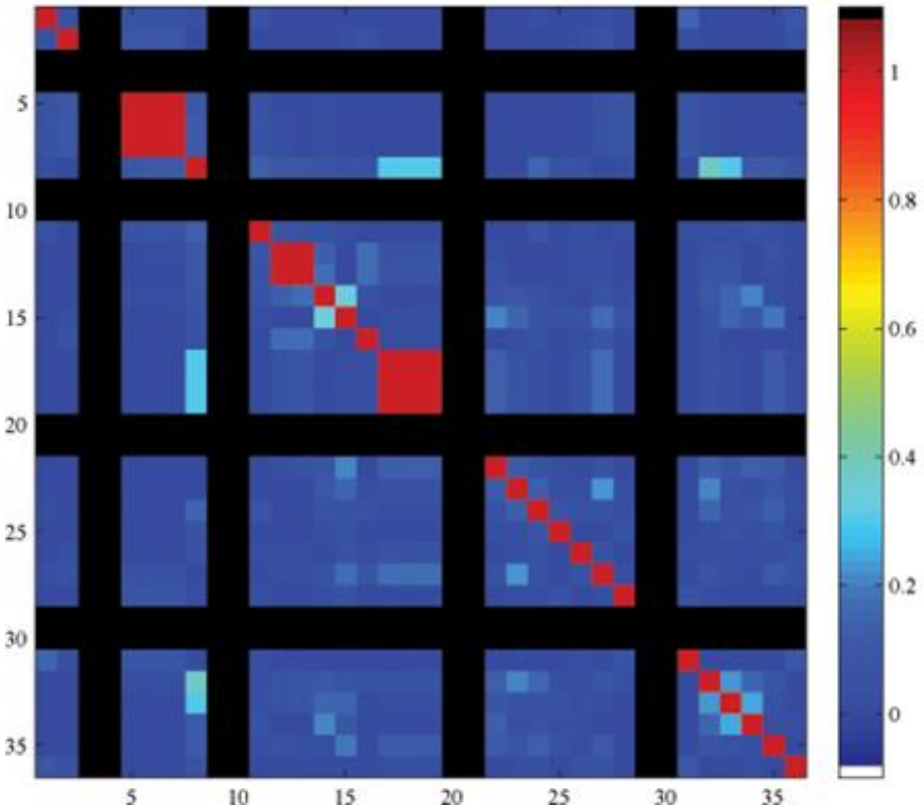

Figure 5.S2. Linkage disquilibrium mapping for association mapping of RCH score for flies reared at 25° C.

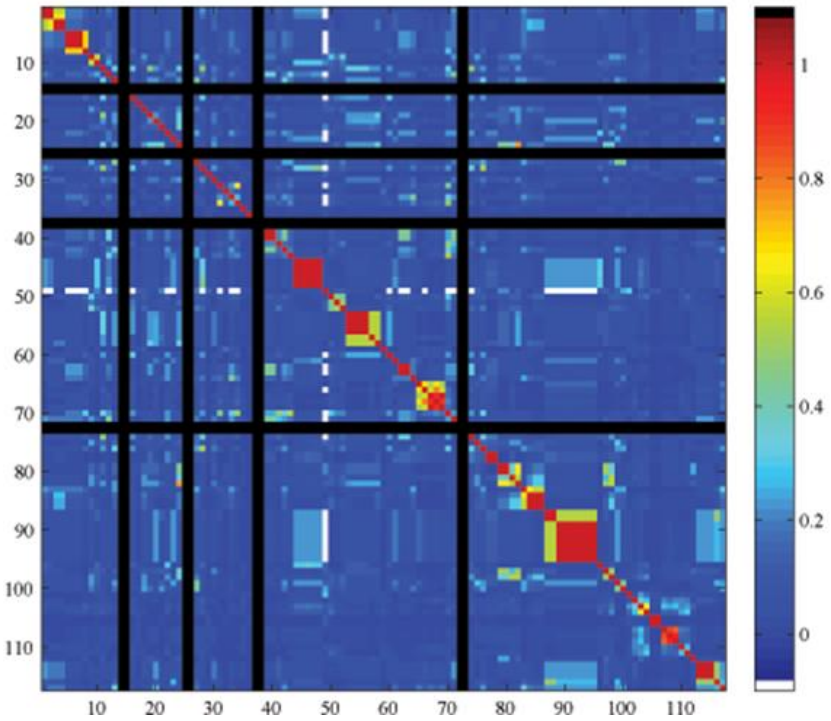

Figure 5.S3. Linkage disquilibrium mapping for association mapping of DACC score for flies reared at 25° C compared to flies reared at 18° C.

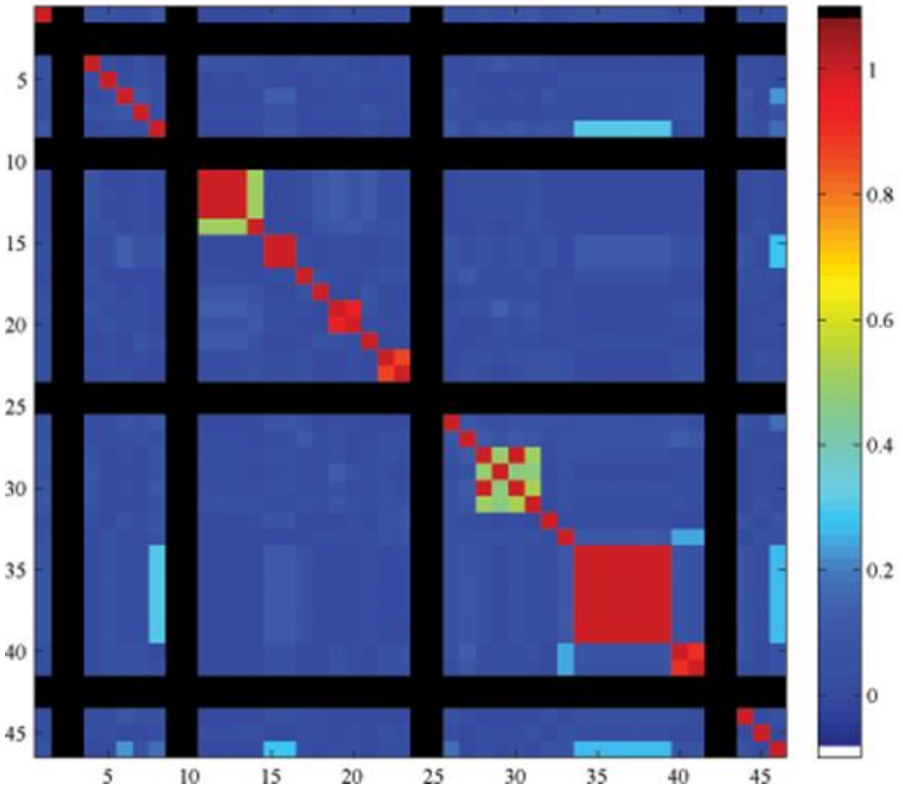

Figure 6.S4. Linkage disquilibrium mapping for association mapping of RCH score for flies reared at 18° C.