The evolution and genetic control of stress tolerance in a complex world

by

Elizabeth R. Everman

B.A., William Jewell College, 2012

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

Abstract

Natural populations are highly complex and consist of genetically variable individuals that belong to continuously varying age classes. Genotype and age interact to determine how individuals respond to environmental stress, which ultimately determines the evolutionary trajectories and persistence of populations in variable environments. For small ectothermic species, seasonal and diurnal variation in temperature is an important source of environmental stress that impacts activity patterns and suites of phenotypes directly related to whole organism fitness. I used the genetic and ecological model *Drosophila melanogaster* to investigate the influence of seasonal and diurnal thermal variability on survival and reproduction in genetically diverse populations. First, I characterized changes in cold tolerance and phenotypic plasticity within a natural population as it responded to seasonal shifts in developmental and short-term acclimation and thermal selection. I found that seasonal variation in cold tolerance was significantly influenced by developmental acclimation that occurred in the field as well as in the lab, where flies that developed under warmer conditions had reduced cold tolerance relative to flies that developed under cooler conditions. Second, I characterized the effect of variation in age on stress response phenotypes in a genetically variable population. I measured genotype- and age-specific responses to multiple environmental stressors, and identified regions of the genome that were associated with age-specific stress tolerance. Genome-wide association mapping revealed that age-specific phenotypes were influenced by distinct sets of polymorphisms and genes, suggesting that the evolution of age-related decline in phenotypes is driven by mutation accumulation within phenotypes, but both mutation accumulation and antagonistic pleiotropy between phenotypes. Next, I characterized the costs and benefits of acclimation for survival and reproduction to understand how physiological and behavioral plasticity interact to determine

fitness. I found that phenotypic plasticity and the capacity for acclimation significantly influenced behavioral reproductive success, but the thermal cues that led to adaptive acclimation response in survival also led to decreased reproductive success. However, genotypes with the capacity to acclimate were more likely to survive thermal variation and more likely to reproduce, suggesting that genetic capacity for phenotypic plasticity has important implications for whole organism fitness. Finally, I measured the effect of acclimation on the induction of diapause and ability to survive cold stress in the recently introduced invasive species *Drosophila suzukii*. *D. suzukii* is endemic to Asia and was first detected in California in 2008 and in Topeka, KS in 2013. Its recent invasion history thus provides an interesting model to understand the role of plasiticy in the response to a novel and variable environment. I found that diapause was induced through a plastic response to acclimation and short photoperiod, though diapause was more drastically induced by acclimation. Overall, my research provides critical insights into how organisms respond to thermal variation by intergrating quantitative genetics, ecology, evolution, and life history tradeoffs. Collectively, my research demonstrates that the ability of organisms to survive thermal stress is a function of genetic capacity to tolerate stress, genetic capacity for phenotypic plasticity, prior exposure to thermal variation, and the age of the individual.

The evolution and genetic control of stress tolerance in a complex world

by

Elizabeth R. Everman

B.A., William Jewell College, 2012

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

Approved by:

Major Professor
Dr. Theodore J. Morgan

Copyright

Abstract

Natural populations are highly complex and consist of genetically variable individuals that belong to continuously varying age classes. Genotype and age interact to determine how individuals respond to environmental stress, which ultimately determines the evolutionary trajectories and persistence of populations in variable environments. For small ectothermic species, seasonal and diurnal variation in temperature is an important source of environmental stress that impacts activity patterns and suites of phenotypes directly related to whole organism fitness. I used the genetic and ecological model *Drosophila melanogaster* to investigate the influence of seasonal and diurnal thermal variability on survival and reproduction in genetically diverse populations. First, I characterized changes in cold tolerance and phenotypic plasticity within a natural population as it responded to seasonal shifts in developmental and short-term acclimation and thermal selection. I found that seasonal variation in cold tolerance was significantly influenced by developmental acclimation that occurred in the field as well as in the lab, where flies that developed under warmer conditions had reduced cold tolerance relative to flies that developed under cooler conditions. Second, I characterized the effect of variation in age on stress response phenotypes in a genetically variable population. I measured genotype- and age-specific responses to multiple environmental stressors, and identified regions of the genome that were associated with age-specific stress tolerance. Genome-wide association mapping revealed that age-specific phenotypes were influenced by distinct sets of polymorphisms and genes, suggesting that the evolution of age-related decline in phenotypes is driven by mutation accumulation within phenotypes, but both mutation accumulation and antagonistic pleiotropy between phenotypes. Next, I characterized the costs and benefits of acclimation for survival and reproduction to understand how physiological and behavioral plasticity interact to determine

fitness. I found that phenotypic plasticity and the capacity for acclimation significantly influenced behavioral reproductive success, but the thermal cues that led to adaptive acclimation response in survival also led to decreased reproductive success. However, genotypes with the capacity to acclimate were more likely to survive thermal variation and more likely to reproduce, suggesting that genetic capacity for phenotypic plasticity has important implications for whole organism fitness. Finally, I measured the effect of acclimation on the induction of diapause and ability to survive cold stress in the recently introduced invasive species *Drosophila suzukii*. *D. suzukii* is endemic to Asia and was first detected in California in 2008 and in Topeka, KS in 2013. Its recent invasion history thus provides an interesting model to understand the role of plasiticy in the response to a novel and variable environment. I found that diapause was induced through a plastic response to acclimation and short photoperiod, though diapause was more drastically induced by acclimation. Overall, my research provides critical insights into how organisms respond to thermal variation by intergrating quantitative genetics, ecology, evolution, and life history tradeoffs. Collectively, my research demonstrates that the ability of organisms to survive thermal stress is a function of genetic capacity to tolerate stress, genetic capacity for phenotypic plasticity, prior exposure to thermal variation, and the age of the individual.

Table of Contents

List of Figures

List of Tables

Acknowledgements

The research presented in my dissertation could not have been accomplished without the help and input of several people, and so I would like to acknowledge them here for their contributions, which range from showing me how to run screens, helping with data collection, co-authoring papers, to writing letters on my behalf as I applied to dozens of Post-doctoral research positions.

Thank you to Alison Gerken for showing me the ropes in fly maintenance and running phenotyping screens and for answering various random questions as I worked through data analysis, to Olivia Eller, Phil Freda, Mariah Brown, Jennifer Delzeit, Nick Ledbetter, Jackson Alex, Kate Hunter, and Chris Berger for their help in setting up and collecting data some of the 170,000 + experimental flies that went into this research, to Rex Rees from Rees' Fruit Farm and Cathy and Kenny from 86th St. Orchard for permitting me to traipse around their orchard and yards for four years to collect "wild" fruit flies, to Paula Roy and Kaila Colyott for facilitating screens and data collection at the University of Kansas, and to my co-authors Suegene Noh, Phil Freda, Jennifer Delzeit, Mariah Brown, Kate Hunter, Jennifer Gleason, Chris Berger, Raymond Cloyd, and Greg Ragland for their contributions to and feedback on the construction of manuscripts. Thank you to Tara Marriage for feedback on manuscripts, and thank you to Michi Tobler, Tony Joern, and Mike Herman for always being willing to write letters of recommendation. Thank you as well to my committee members Tony Joern, Mike Herman, Chis Toomajian, Mark Ungerer, and Mary Beth Kirkham.

I am also extremely grateful for the guidance, advice, and support of my advisor Ted Morgan. Thank you for your encouragement, for providing opportunities for me to cultivate my interests in a variety of areas from quantitative genetics to ecology, and for humoring my desire

to do “field work” with *Drosophila*. Thank you for reminding me that my research is interesting and not to be too humble when, after staring at data for weeks and weeks, I start to become desensitized to the broader implications. Thank you for your guidance in research and writing, and for facilitating presentation of my research at several conferences. Thank you for being an excellent mentor.

Thank you to my family for their patience and support when I become irritable, distant, and/or too busy to visit on weekends as a result of the stress of grad school or too much to do with too little time. Thank you to my significant other Paul Klawinski for his support and patience for the same, as well as his willingness to spend weekends helping me sort flies, to drive back and forth from field sites, to read various versions of manuscripts, to sit through and provide feedback on too many presentations to count, and to chat casually practically every night about statistical analyses. Thank you also for constantly trying to convince me that I do know what I’m doing and that I am a capable biologist.

Finally, thank you to several funding sources, including NSF, Kansas State University Graduate School, and the Biology Graduate Student Association. Thank you as well to my graduate student cohort, particularly Kirsten Grond, MK Mills, and Tori Rhodes—on the occasions I managed to find time for a social life, it was nice to find easy friendship among others who know exactly the ups and downs of grad school.

Introduction

Climate and thermal regime are important sources of environmental variation that influence species distributions, behavior and activity levels, performance, and ultimately the evolution and persistence of populations in changing environments (Andrewartha and Birch 1954; Cossins and Bowler 1987; Angilletta et al. 2002; Ayrinhac et al. 2004; Overgaard and Sørensen 2008; Angiletta 2009; Calosi et al. 2010; Dierks et al. 2012). Temperature is an especially critical component of the environment for ectothermic species for which temperature fluctuations have significant impacts on survival, reproduction, and behavior (Lee et al. 1987; Huey and Kingsolver 1989; Kelty and Lee 1999, 2001; Shreve et al. 2004; Geister and Fischer 2007; Kelty 2007; Bouazza et al. 2016; Westerman and Monteiro 2016). The capacity to tolerate thermal stress is highly variable among and within ectothermic species (Huey and Kingsolver 1989, 1993) and across age (Czajka and Lee 1990; Bowler and Terblanche 2008; Colinet et al. 2013, 2015), and it can be driven by shifts in basal tolerance, shifts in phenotypic plasticity, or a combination of both (Hoffmann et al. 2002, 2003c; Hoffmann and Sgrò 2011; Bergland et al. 2014b,a; Fallis et al. 2014; Seebacher et al. 2014; Gerken et al. 2015; Campbell-Staton et al. 2016). A growing body of evidence indicates that acclimation capacity and other forms of phenotypic plasticity are particularly important for the persistence of ectothermic populations in variable environments (Easterling et al. 2000; Kawecki 2000; Ayrinhac et al. 2004; Bale and Hayward 2010; Fallis et al. 2014; Gerken et al. 2015). Therefore, examination of the influence of genetic and age-related variation on acclimation capacity and basal levels of stress tolerance is critically important both in the context of climate change projections (Easterling et al. 2000; Kawecki 2000; Bale and Hayward 2010) and in a broader context of understanding multivariate evolution and whole organism fitness in variable environments.

Climate change models predict increases in average temperature as well as increases in the frequency of extreme weather events (Easterling et al. 2000; Jentsch et al. 2007), and it is likely that these changes will influence the evolution of ectotherm populations. Acclimation capacity is thought to be particularly important for persistence of ectothermic species under climate change (Kawecki 2000; Bale and Hayward 2010; Sørensen et al. 2016). There are three primary forms of acclimation through which organisms can respond to thermal variation: developmental acclimation, long-term acclimation or acclimatization, and short-term acclimation or hardening (Wilson and Franklin 2002; Bowler 2005; Angiletta 2009; Colinet and Hoffmann 2012). Developmental and long-term acclimation are most relevant in the context of seasonal variation in temperature, whereas short-term acclimation is most relevant in the context of diurnal temperature variation which can vary in magnitude through the season (Lee et al. 1987; Denlinger 1991; Moran 1992; Kelty and Lee 2001; Wilson and Franklin 2002; Bowler 2005; Loeschcke and Sørensen 2005; Teets and Denlinger 2013). These forms of acclimation have a genetic basis, and thus should evolve in response to climatic selection pressure (Fallis et al. 2014; Gerken et al. 2015); however, limited empirical evidence is available to support this hypothesis. This lack of evidence highlights a critical need to investigate how phenotypic plasticity of populations varies in response to shifts in thermal selection regime.

Although the evolutionary dynamics of phenotypic plasticity via acclimation are understudied, it has been well documented in a diverse group of species that various forms of acclimation tend to increase survival of individuals following exposure to stressful temperatures (Chen et al. 1987; Gilmour et al. 1988; Czajka and Lee 1990; Worland and Convey 2001; Wang and Kang 2003; Sinclair and Chown 2006; Ju et al. 2011; Gerken et al. 2015). However, the adaptive value of acclimation depends on the accuracy of the acclimation cue for the

environment experienced following acclimation (Moran 1992) and on the effects of the cue on other aspects of fitness, such as reproductive success (Shreve et al. 2004; Geister and Fischer 2007; Westerman and Monteiro 2016). For example, developmental acclimation to cool temperatures can lengthen development time and likely alter learning ability in various social contexts (Westerman et al. 2012; Westerman and Monteiro 2016), and long- and short-term acclimation can result in decreased mating success (Aspi and Hoikkala 1995; Hoikkala and Isoherranen 1997; Shreve et al. 2004). While it is important to understand the immediate effects of acclimation on multiple components of fitness, it is potentially more important to consider the role of capacity for phenotypic plasticity that is afforded through variation among genotypes. In particular, physiological and behavioral responses to thermal acclimation are likely genetically intertwined in ectothermic species as physiological tolerance is often linked to performance (Christian et al. 1983; Niehaus et al. 2012).

The ability of organisms to respond to thermal variation is clearly due in part to genetic capacity, acclimation, and to variation in thermal selection regime as a result of seasonal and diurnal temperature fluctuations. Furthermore, the effectiveness of natural selection for the evolution of tolerance and plasticity is also influenced by age (Bowler and Terblanche 2008; Colinet et al. 2013, 2015). As organisms age, natural selection becomes decreasingly effective, ultimately leading to age-related decline in fitness (Fisher 1930; Haldane 1941; Medawar 1952; Williams 1957; Hamilton 1966; Charlesworth and Hughes 1996; Charlesworth 2001). This decline in fitness can be driven by genetic mechanisms such as mutation accumulation and antagonistic pleiotropy, and through non-genetic mechanisms such as “wear and tear” on cellular repair machinery (Medawar 1952; Williams 1957; Ricklefs and Finch 1995). Genetic control of the decline has been consistently supported (Charlesworth and Hughes 1996; Tatar et al. 1996;

Curtsinger and Khazaeli 2002; Felix et al. 2012; Durham et al. 2014); however, limitations in genetic tools prior to the construction of large mapping panels of model organisms has reduced the ability of this previous research to fully understand and disseminate the genetic basis of age-related change (Schnebel and Grossfield 1988; Partridge and Barton 1993; Zwaan 1999; Linnen et al. 2001; Moorad and Promislow 2009). Reliance on changes in variance component analysis across age and inability to compare genetic patterns across phenotypes as well as within phenotypes with age has masked subtle genetic shifts in genetic architecture that are characteristic and diagnostic of mutation accumulation and antagonistic pleiotropy. It is probable that by taking full advantage of the genetic power of mapping panels and comparing patterns within and between phenotypes across age, we will begin to more fully understand the role of mutation accumulation, antagonistic pleiotropy, and natural selection in aging.

System and Objectives

Drosophila melanogaster is an excellent model for investigating the shifts in thermal tolerance and plasticity that occur across seasons and through aging. *D. melanogaster* evolved in equatorial Africa under tropical and seasonally constant thermal conditions (Begun and Aquadro 1993; Keller 2007; Duchen et al. 2013) and was subsequently introduced to more variable temperate regions, in the process experiencing a combination of severe bottlenecks and strong selection for wider thermal tolerances (Begun and Aquadro 1993; Lachaise and Silvain 2004; Keller 2007). As a result, evidence of adaptation to novel thermal regimes can be seen across clines (James et al. 1997; Hoffmann et al. 2002, 2003b) and in response to seasonal variation (Bergland et al. 2014a). Extensive evidence of acclimation capacity exists for this species as well, indicating that a combination of adaptation and phenotypic plasticity contributes to its success and persistence in thermally variable environments (Everman et al. In Press; Lee et al.

1987; Kelty and Lee 1999; Kelty 2007; Rajamohan and Sinclair 2009; Fallis et al. 2014; Gerken et al. 2015). A more recently introduced drosophilid, *Drosophila suzukii*, has also been shown to respond through adaptation and phenotypic plasticity to novel climatic regimes (Dalton et al. 2011; Jakobs 2014; Jakobs et al. 2015; Rossi-Stacconi et al. 2016; Shearer et al. 2016). Because of its recent introduction history, this species offers the opportunity to investigate the effects of adaptation to novel thermal regimes as they are currently occurring.

D. melanogaster has also served as an important model for understanding the evolution of aging (Rose and Charlesworth 1981; Hughes et al. 2002; Durham et al. 2014). Natural temperate populations of *D. melanogaster* age through the season such that populations are primarily composed of young individuals in the spring and summer and of older individuals in the fall, likely as a result of winter dormancy as populations experience a drop in temperature and reproductive activity during winter (Kimura 1988; Behrman et al. 2015). As a result, demographic structure is likely to have an important influence on stress tolerance (Bowler and Terblanche 2008; Colinet et al. 2015), though most research has focused on age-related decline in life history phenotypes (Rose 1985; Rose et al. 1992; Tatar et al. 1996; Durham et al. 2014; Curtsinger 2016). Because *D. melanogaster* is a well-developed genetic model, tools now exist for both a more thorough analysis of the influence of age on fitness phenotypes and for understanding the trajectory of the evolution of aging in combination with multivariate evolution. In particular, the *Drosophila* Genetic Reference Panel (DGRP; Mackay et al. 2012; Huang et al. 2014) and association mapping of age-specific phenotypes has enhanced our understanding of how genetic mechanisms (mutation accumulation and antagonistic pleiotropy) influence age-related decline in phenotypes.

D. melanogaster was first developed as an important genetic model organism in biology by Thomas Hunt Morgan, and has since been used to gain insight into a wide diversity of topics and processes (Fisher and De Beer 1947). I used *D. melanogaster* as a genetic and ecological model to understand evolutionary dynamics and genetic architecture of stress responses and phenotypic plasticity, addressing several critical questions in evolutionary biology, physiology, and age-related research:

(1) The evolution of phenotypic plasticity in variable environments is thought to be critically important for the persistence of ectothermic populations under long-range climate change predictions; however, it is not fully understood how phenotypic plasticity varies in response to shorter-term thermal variation that is characteristic of seasonality. Insight from investigating the influence of seasonality on the evolution of phenotypic plasticity is therefore especially useful for understanding how ectothermic organisms will respond to longer-term changes in thermal regime.

(2) Although the benefits of phenotypic plasticity have been consistently documented for ectothermic organisms, the influence of acclimation cues on other aspects of fitness requires additional research to understand the role of genetic variation and capacity for physiological and behavioral plasticity in response to environmental variation.

(3) While it is important to take variation in natural selection that occurs across seasons and years into account, it is also critical to consider variation in natural selection that occurs through the lifespan of individuals and the influence this has on the ability of organisms to survive and reproduce. It is likely that the age-related decline in fitness is influenced by a combination of natural selection acting on correlated phenotypes that have non-independent genetic architectures and phenotype-specific genetic

architectural shifts. Elucidation of these genetic architectural patterns across age will inform how evolution of aging and multivariate evolution are tightly intertwined.

These unanswered questions were addressed in four empirical chapters:

Chapter 1. Seasonal variation in temperature can influence the capacity of organisms to tolerate thermal stress. I collected wild flies from a population in Topeka, Kansas three to four times across the season from April to November over fours years to determine whether differences in cold tolerance were due to developmental acclimation of individuals at different points in the season. I addressed this question by measuring cold tolerance as chill coma recovery time and survival in these flies. Further, I investigated whether phenotypic plasticity varied through the season as well by rearing flies under two different developmental temperatures, and by exposing flies to short-term variation in temperature as adults.

Chapter 2. Natural populations consist of individuals that belong to continuously varying age classes, and age can significantly impact fitness of individuals through antagonistic pleiotropy or mutation accumulation. I used the *Drosophila* Genetic Reference Panel (DGRP) to measure age-related variation in stress response following exposure to non-nutritive media (starvation resistance), survival following cold shock without short-term acclimation, and survival following cold shock with short-term acclimation. I assessed the role of antagonistic pleiotropy and mutation accumulation in the decline in stress response using genome-wide association mapping and the calculation of additive effects of associated polymorphisms across age within and between stress phenotypes.

Chapter 3. Short-term cold acclimation is a process that usually leads to increased survival in flies that also experience cold shock, and is thus an adaptive response to diurnal thermal variation. However, the effect of short-term acclimation on reproductive success has not been

assessed in specific genotypes to understand how capacity for acclimation influences the effect of acclimation on reproductive fitness. I measured the effect of short-term acclimation on survival and reproductive behavior in the DGRP to investigate the role of acclimation and genetic variation in the capacity to acclimate on whole organism fitness.

Chapter 4. Seasonal variation in temperature and photoperiod can induce diapause phenotypes in drosophilids that overwinter as adults. A recently introduced species, *Drosophila suzukii*, has been shown to enter diapause upon long-term acclimation to cool temperature and exposure to short photoperiod; however previous research has focused on populations that have been established in northern United States and in Canada. Further, previous research suggests that this species does not benefit from short-term acclimation through increase in survival. I measured the effect of developmental and long-term acclimation on ovary development and the effect of short-term acclimation on survival in a low-diversity population established from a recently founded population in Topeka, Kansas to determine if these patterns hold for a population that experiences more moderate winters.

Synopsis

Persistence of populations in variable environments requires tolerance of a wide range of temperature stressors. Results from Chapter 1 indicated that the capacity of flies to acclimate depended on the temperature experienced during field development. Flies from each collection period were able to developmentally acclimate, but the benefit was greatest for flies that were collected during summer months, suggesting that a combination of mechanisms alter cold tolerance and that capacity for acclimation depends upon this capacity as well as previous temperature exposure. However, acclimation effects are not uniformly positive; genetic variation for the capacity to respond plastically to cold temperature stress can result in negative or

maladaptive acclimation responses (Geister and Fischer 2007; Gerken et al. 2015). In Chapter 2, I found that negative acclimation responses were more common in young individuals compared to old individuals of the same genotypes, and in Chapter 3, I found that exposure to the short-term acclimation cue that normally leads to increased survival had a negative effect on reproductive fitness. Interestingly, the negative effect of acclimation on reproductive fitness was only observed in genotypes for which the acclimation cue had a strong negative or strong positive influence on survival. Further, when reproductive fitness of control pairs was compared to the genetic capacity of males for acclimation, I found that more plastic genotypes mated more quickly. Overall, it appears that the roles of acclimation thermal cues and genetic capacity for an acclimation response have environment-dependent implications for reproductive success. In natural populations, exposure of small, ectothermic organisms to seasonal and diurnal fluctuations in temperature is therefore likely to have important consequences for both survival and reproduction.

Developmental acclimation and long-term acclimation have been previously demonstrated to influence cold stress tolerance in *D. suzukii* as well, but other studies have shown that short-term acclimation has a negative effect on survival. While Chapter 3 highlights that negative effects of short-term acclimation on fitness can occur, it is possible that the *D. suzukii* populations previously tested had low capacity for short-term acclimation by virtue of the selection regimes specific to the region from which they were collected. In Chapter 4, I demonstrated that short-term acclimation could result in dramatically increased adult survival in *D. suzukii* collected from Topeka, KS. Further, the induction of a diapause-like phenotype following developmental and long-term acclimation occurred at slightly warmer temperatures compared to populations from northern USA and southern Canada, suggesting that thermal

selection regimes between recently introduced populations can result in shifts in sensitivity of populations to environmental cues.

Natural selection for phenotypes that improve survival either through increased lifespan or through increased stress tolerance can have complex effects on multiple phenotypes that vary through an individual's lifespan. Generally, the effectiveness of natural selection declines with age, allowing for polymorphisms that have negative effects on phenotypes to become more common in populations when they have restricted ages of effect (Medawar 1952; Hamilton 1966; Maklakov et al. 2015). In Chapter 2, I demonstrated this was the case by showing the age-related decline in three stress phenotypes (starvation resistance, acclimation survival, and non-acclimation survival) was driven by changes in effects of associated polymorphisms across age. Mutation accumulation contributed to the decline in phenotypes across age, but antagonistic pleiotropy influenced age-related decline between phenotypes. I also found that the effects of natural selection on correlated phenotypes influenced the evolution of age-related change in stress responses. For example, the age-related starvation response was influenced by the effect of natural selection on polymorphisms that improve cold tolerance and by starvation-specific polymorphisms with negative additive effects that increased with age.

Results from my research provide insight into how organisms respond to thermal variation with implications for evolutionary biology, ecology, genetics, aging, and physiology by providing a treatment of the intersection between aging, genetics, and natural selection. Organisms experience thermal variability on multiple spatial and temporal scales, and this thermal variation can have a significant influence on the ability of individuals to survive and reproduce and for populations to persist over time. This is particularly important for populations that experience seasonal fluctuation in temperature, as the age structure of populations varies

through the season as well. The ability of an organism to survive thermal stress at multiple periods through a season is thus a function of genetic capacity to tolerate stress, genetic capacity for phenotypic plasticity, prior exposure to thermal variation, and the age of the individual.

Chapter 1 - Seasonal variation in basal and plastic cold tolerance: adaptation is influenced by both long- and short-term phenotypic plasticity[1]

Elizabeth R. Everman, Suegene Noh, Christopher M. Berger, and Theodore J. Morgan

Abstract

Understanding how thermal selection pressure across different time scales affects phenotypic distributions will allow us to better predict the effect of climate change on the fitness of ectotherms. We tested how seasonal thermal variation affects basal levels of cold tolerance and two types of phenotypic plasticity in *Drosophila melanogaster*. Developmental acclimation occurs as developmental stages of an organism are exposed to seasonal changes in temperature and its effect is irreversible, while reversible short-term acclimation can occur on a daily basis in response to diurnal changes in temperature. We collected wild flies from a temperate population across seasons and measured two cold tolerance metrics (chill coma recovery and survivorship) and their responses to developmental and short-term acclimation. Chill coma recovery responded to seasonal shifts in temperature, and phenotypic plasticity following both short-term and developmental acclimation improved cold tolerance. This improvement indicated that both types of plasticity are adaptive and that plasticity can compensate for genetic variation in basal cold tolerance during warmer parts of the season when flies tend to be less cold tolerant. We also observed a significantly stronger trade-off between basal cold tolerance and short-term acclimation during warmer months. For the longer-term developmental acclimation, a trade-off persisted regardless of season. A relationship between the two types of plasticity may provide

[1] Submitted as Noh, S., Everman, E. R., Berger, C. M. and Morgan, T. J. The first two authors Noh and Everman are co-first authors.

additional insight into why some measures of thermal tolerance are more sensitive to seasonal variation than others.

Introduction

Climate change is already impacting biological systems and affecting average population fitness by causing shifts in the phenologies and distributions of mobile and stationary species (Parmesan and Yohe 2003; Root et al. 2003; Kellermann et al. 2009). Significant effort in the scientific community to predict the magnitude and severity of the effects of changing temperatures on biodiversity has illustrated that, in order to understand how a species might respond to climate change, we need to know more about the dispersal ability, biotic and abiotic interactions, and, particularly, the adaptive potential of the species (reviewed in Lavergne et al. 2010). Because increases in climatic variability are predicted to accompany gross climate change, we now expect the impact of climate change to depend more on the variance rather than the mean of temperature change (Jentsch et al. 2007; Vasseur et al. 2014; Wang and Dillon 2014). This is in large part due to the nonlinear effects of temperature on various aspects of organismal biology, including biochemical processes, intraspecific traits such as physiological performance curves, and species interaction traits (Dell et al. 2011). In addition, thermal variation occurs at several time scales making it equally important to understand how population fitness will be affected by shorter-term fluctuations in temperature due to diurnal and seasonal changes, as well as longer-term gross fluctuations or extreme events (Marshall and Sinclair 2012). Understanding how different time scales of selection interact and affect phenotypic and allelic distributions will allow us to better predict the effect of climate change on the fitness of populations of many species.

Arthropods comprise a dominant proportion of global biomass and have key roles in many ecosystem processes (Wilson 1987; Miller 1993). Adapting to unpredictable change at multiple time scales may be particularly challenging for these ectotherms as their physiology is very sensitive to fluctuations in the thermal environment (Kawecki 2000; Deutsch et al. 2008; Foray et al. 2013). The implications of our changing climate for the survival of insects and other arthropods are often discussed in the context of basal stress tolerance and phenotypic plasticity (Sinclair et al. 2003; Danks 2005; Sinclair and Roberts 2005; Vesala and Hoikkala 2011). Basal thermal tolerance is a heritable phenotype (Hallas et al. 2002; Ayrinhac et al. 2004; Anderson et al. 2005; Gerken et al. 2015), and recent work has demonstrated that seasonal fluctuations in basal cold tolerance are linked to fluctuations in allele frequencies in *Drosophila melanogaster*, suggesting that seasonal changes can result in rapid genetic responses to varying environmental stress multiple times per year (Bergland et al. 2014a).

Phenotypic plasticity also significantly influences the survival of organisms following temperature stress (Kelty and Lee 2001; Ayrinhac et al. 2004; Geister and Fischer 2007; Kelty 2007; Deutsch et al. 2008; Gerken et al. 2015). Many phenotypes related to thermal stress tolerance are seasonally induced, including pigmentation (Shearer et al. 2016), levels of antifreeze proteins and cryoprotectants (Danks 2005), and reproductive diapause (Vesala and Hoikkala 2011; Wallingford et al. 2016). Phenotypic plasticity induced through both short-term acclimation and longer-term developmental acclimation have been repeatedly shown to increase survival in thermally variable environments in numerous organisms (Lee et al. 1987; Coulson and Bale 1990; Hoffmann et al. 2003a; Sinclair and Chown 2006; Geister and Fischer 2007; Basson et al. 2012). Short-term acclimation typically occurs following a brief (minutes to hours) exposure to a non-lethal cool temperature prior to a harsher thermal stress and has ephemeral

benefits on survival, wearing off after a few hours (Everman et al. In Press; Chen et al. 1987; Lee et al. 1987; Czajka and Lee 1990; Kelty and Lee 2001; Koveos 2001; Loeschcke and Sørensen 2005; Gerken et al. 2015). While short-term acclimation can occur across ontogeny, developmental acclimation occurs through exposure of organisms to conditions that alter development and is thus irreversible (Lee et al. 1987; Wilson and Franklin 2002; Teets and Denlinger 2013).

Despite this knowledgebase, we do not fully understand how basal and plastic responses to cold stress interact through seasonal temperature variation characteristic of temperate regions. In particular, a comprehensive understanding of the interaction between basal tolerance and short- and long-term acclimation responses to thermal stress is lacking for species that have complex life cycles (Kingsolver et al. 2011) or produce several generations per year (Bergland et al. 2014a). Theory predicts that adaptation to one set of conditions can result in mismatch between phenotype and environment when conditions shift; however, maintenance of the capacity to respond plastically to shifting environments can reduce this mismatch and facilitate survival of individuals and persistence of populations (Kawecki 2000; Gomez-Mestre and Jovani 2013; Lande 2014). In addition, it was recently suggested that phenotypic plasticity through short-term and developmental acclimation are evolutionarily linked more closely than was previously considered (Beaman et al. 2016). The capacity for plasticity following developmental acclimation (developmental plasticity), which generally leads to fixed phenotypic effects, should interact with the capacity for acclimation over short timescales (Beaman et al. 2016). The interaction between phenotypic plasticity following short-term and developmental acclimation is important because, acting together, they can reduce the probability that developmental acclimation will result in a mismatch between phenotype and environment due to unpredictable

environmental fluctuation. Essentially, if during development environmental cues indicate that future conditions are unpredictable, selection should favor acclimation capacity (Beaman et al. 2016).

We measured the influence of developmental and short-term acclimation on two measures of thermal tolerance as a natural population of *D. melanogaster* responded to seasonal changes in temperatures over multiple years. The cold tolerance metrics (chill coma recovery and survival) involve unique genetic mechanisms, and the different forms of acclimation represent specific temporal scales at which acclimation can occur and so are likely to be differentially affected by natural selection through the season (Rako and Hoffmann 2006; Colinet and Hoffmann 2012; Teets and Denlinger 2013; Gerken et al. 2015). Developmental acclimation models the impact of seasonal temperature variation experienced through early ontology on the response to thermal stress. The influence of developmental acclimation through the season on cold tolerance was assessed with chill coma recovery, which relates the time it takes for flies to regain neuromuscular control and flight capacity after exposure to mild cold temperatures (Gibert et al. 2001; MacMillan and Sinclair 2011), and with survival following cold shock, which involves a harsher stress that reflects the physiological limits of cold tolerance (Lee et al. 1987; Kelty and Lee 2001; Kelty 2007). Short-term acclimation models diurnal temperature variation, but also has a seasonal context because the magnitude of diurnal thermal variation fluctuates through the season (Kelty and Lee 2001; Colinet and Hoffmann 2012; Gerken et al. 2015). Short-term acclimation induces physiological changes that are relevant for survival, but has been previously shown to negatively impact chill coma recovery in *D. melanogaster* (Rako and Hoffmann 2006); therefore, we measured the effect of short-term acclimation on survival following cold stress. By measuring the effects of developmental and short-term acclimation on

two cold stress metrics, we aimed to determine the relative importance of long and short-term forms of phenotypic plasticity from season to season and year to year.

As it has been suggested that fluctuating selection should favor genes that modify basal cold tolerance rather than alter the response itself (Kawecki 2000), we expected cold stress survivorship to be less sensitive to developmental acclimation as a result of seasonal thermal variation compared to chill coma recovery. This is because cold stress survival tests the physiological limits of an organism and likely involves a unique set of genes and mechanisms compared to chill coma recovery (Rako and Hoffmann 2006). Next, we expected phenotypic plasticity due to both developmental and short-term acclimation to compensate for genetic differences in chill coma recovery and cold stress survivorship that resulted from seasonal selection. Specifically, we expected less cold tolerant flies from warmer months to still be able to resist cold temperature stress through phenotypic plasticity, despite having experienced weaker natural selection prior to collection. Finally, if a strong constraint exists between basal cold tolerance and plasticity, we expected this relationship to be differentially affected by seasonal temperature variation as well. Trade-offs between basal cold tolerance and plasticity are well documented (Hoffmann et al. 2003c; Kellett et al. 2005; Nyamukondiwa et al. 2011; Gerken et al. 2015), and seasonal variations in life history trade-offs are well known (Nylin and Gotthard 1998). Thus we hypothesized that the relationship between basal cold tolerance and phenotypic plasticity may constrain how organisms respond to seasonal variation. Specifically, chill coma recovery may show a consistent constraint between basal cold tolerance and plasticity because natural selection due to seasonal temperature variation should be relevant to the evolution of this phenotype. Because we expect cold stress survivorship and acclimation to be less closely related

to seasonal variation, the trade-off between these phenotypes may be maintained less consistently across seasons.

Methods

We collected flies through summer and fall of 2012 - 2015 from two commercial orchards in Topeka, KS (39.09 latitude, -95.59 longitude and 39.20 latitude, -95.74 longitude) that are 11 miles apart. In 2012 and 2013, we collected flies at three different times (July, September, October), in 2014 at five different times (June, July, August, September, November), and in 2015 at three different times (July, September, October). We attracted flies by placing fermented banana bait traps near or hanging onto apple trees at each orchard for 2-3 days. These traps were constructed from 1L plastic bottles with a single curved opening approximately 3 inches wide made on one side. All flies were combined as soon as they were brought into the lab, as the orchards were geographically close and grew similar types of fruit, and because the number of females collected at the two sites was often unequal. For each collection time, we isolated *D. melanogaster* or *D. simulans* females into individual vials with standard cornmeal-molasses food and allowed them to lay eggs. After one week in a vial, we removed these founder females. We identified to species the isofemale lines we established in this way by checking the genital morphology of male offspring. We retained only *D. melanogaster*, and maintained and inbred isofemale lines or outbred population cages established from ten isofemale lines each, depending on the experiment. More details are given below.

Chill coma recovery and developmental acclimation

We measured flies from 2012 – 2014 for chill coma recovery. Isofemale lines established in 2012 and 2013 were maintained for five to eight generations at 25°C prior to chill coma recovery phenotyping. Flies from 2014 isofemale lines were reestablished from outbred

populations that had been maintained in lab for three generations. The 2014 isofemale lines were then maintained for two more generations at 25°C prior to phenotyping. To determine the plastic effect of developmental acclimation on chill coma recovery, we reared the isofemale lines from 2012 and 2014 at 18°C for an additional three to five generations and phenotyped them once more.

We used an automated phenotyping technique to score up to 200 flies at a time for chill coma recovery as described in Crawford (2013). We placed a gridded phenotyping stage in an incubator set to 25°C. Above the stage, we positioned a digital SLR camera (Canon EOS Rebel T3) so that it captured all the grids within its view. We used camera software (DSLR Remote Pro for Windows) to automatically take photos at 60 sec intervals. We aimed to phenotype 40 females and males each from each line. To set up a phenotyping trial, we sexed flies of each line on a CO_2 stage and placed 8-11 flies of a single sex into an empty vial. Once 20 vials were full and all flies were awake, we placed a rack of vials into a refrigerator set to 0 °C for 3 hr. After 3 hr., we removed the rack from the refrigerator and emptied each vial into a cell of the gridded stage as quickly as possible. We then positioned the knocked out flies within the grid so that they were on their backs and sufficiently spaced so that none were touching each other. This usually took 3-4 min. We took photos every minute from 5 min post-removal from the refrigerator to 40 min post-removal. After 40 min, flies were removed from the staged incubator using a hand vacuum.

We used custom written code to score positions of flies at each minute interval using a fiji (ImageJ) script that directed the tool ParticleAnalyzer to report locations of flies. We scored the waking time of each fly by comparing the locations of flies from minute to minute. Any fly that shifted position between camera frames was considered awake. Any flies that had not moved

by the end of phenotyping were given ’41’ minutes for waking time because the vast majority of flies that were still immobile at this time would move once nudged with the hand vacuum used to clean the phenotyping stage.

Cold stress survivorship and short-term acclimation

We mass-reared flies collected in 2014 and 2015 for two generations at 25°C prior to cold stress survivorship phenotyping. We established four to six mass-reared population cages for each collection time from approximately 10 isofemale lines each.

To measure cold stress survivorship, we obtained flies from mass population cages two days post eclosion. From each population bottle we sorted flies by sex on a CO_2 stage into vials containing 20 individuals apiece. We allowed flies to recover and mature for five days prior to phenotyping. We measured cold stress survivorship by exposing one set of experimental flies to -6°C for one hour (non-acclimation treatment). We measured short-term acclimation through rapid cold-hardening by exposing a second set of the experimental flies first to 4°C for two hours, immediately followed by exposure to -6°C for one hour (acclimation treatment). Cold stress and acclimation temperatures were chosen following Gerken et al. (2015). We recorded survivorship per vial after a 24 hr recovery period at 25°C with access to food. We replicated each treatment twice per sex, per bottle for each of the collections.

Seasonal temperature variation and statistical analysis

To compare the effect of seasonal weather across years, we compiled data from degreedays.net regarding the cooling and heating degree days for the 14 days leading up to and including each collection date from the closest weather station location to the orchards we sampled: Topeka Billard Municipal Airport (KTOP: 39.07 N, 95.62 W, an average distance of 6 miles from the collection sites). Heating degree days are the cumulative degrees air temperatures

fell below a reference temperature (and thus required heating to maintain that temperature), while cooling degree days are the cumulative degrees air temperatures were above a reference temperature (and required cooling). We selected 25°C and 18°C for the cooling and heating degree day reference temperatures because these were the rearing temperatures selected to look at the effect of developmental temperature. We found that our collection dates over the three years were relatively evenly sampled and less skewed across the range of cooling degree days with a reference temperature of 18°C (CDD18 (skewness = 0.13)) compared to the alternatives (HDD25 (skewness = 1.11), HDD18 (skewness = 1.48), CDD25 (skewness = 1.08)). From here on, we use cumulative heat exposure above 18°C (CDD18) as a proxy for the seasonal weather experienced by the isofemale line founders. We imported these compiled data into R v.3.2.1 for statistical analysis (R Core Team 2015).

For chill coma recovery, we only included lines from which we were able to get data from at least 40 individuals total (both female and male). In 2012, we collected recovery time data from a total of 99 lines of flies (July – 33 lines, September – 35 lines, October – 31 lines). In 2013, we collected data from 89 lines of flies (July – 30 lines, September – 30 lines, October – 29 lines). In 2014, we collected data from 98 lines of flies (June – 30 lines, July – 30 lines, August – 20 lines, September – 18 lines).

Exploratory examination of our data suggested that chill coma recovery waking times were not normally distributed but instead fit a quasipoisson pattern, with mean and variance showing a positive linear relationship. Compared to data with a Poisson distribution, with quasipoisson data the variance increases at a rate above 1 as the mean increases. Therefore we chose to analyze our data with penalized quasi-likelihood generalized linear mixed models fit with a quasipoisson error distribution and its accompanying log link function using the R library

MASS v.7.3-44 (Venables and Ripley 2002). For cold stress survivorship, again we only included cages from which we were able to get data from at least 40 individuals total (both female and male) and the median number of flies that survived the cold survivorship assay across replicates was at least one individual. In 2014, we collected survivorship data from 14 cages of flies (August – 4 cages, September – 6 cages, November – 6 cages). In 2015, we collected data from 15 cages of flies (July – 5 cages, August – 4 cages, September – 6 cages). Because the response variable for cold stress survivorship is binary (alive, dead), we analyzed these data using a generalized linear mixed model fit with a binomial error distribution and its accompanying log link function using the R library lme4 v.1.1-10 (Bates et al. 2015). From hereon, we will refer to both types of models simply as mixed effects models.

We used the flies from the 2012 - 2014 25°C chill coma recovery experiments and 2014 - 2015 non-acclimated survival experiments to test the hypothesis: Seasonal temperature variation affects basal cold tolerance through natural selection. For the chill coma recovery data we fit a mixed model to waking time, with CDD18 as fixed factor and sex, nested in lines, nested in collection years as random factors. For the non-acclimation survival data we fit a mixed model to the binomial variable of flies alive vs. dead, with CDD18 as fixed factor and sex, nested in cages, nested in collection years as random factors. All models were fit with the continuous variable CDD18, but to make residual plots easier to visualize, we created a categorical variable from CDD18 (“low”, “mid”, and “high”) by simply dividing each range of collection dates into thirds.

Next we used the flies from the 2012 and 2014 25°C and 18°C chill coma recovery experiments and 2014 - 2015 short-term acclimation survival experiments to test the hypothesis: Developmental and short-term acclimation compensates for the genetic differences in cold tolerance. For the chill coma recovery data, we fit a mixed model to waking time with the

interaction between CDD18 and developmental temperature. For the cold stress survivorship data, we fit a mixed model to the binomial response variable with the interaction between CDD18 and acclimation treatment. The random effects were structured as with the previous model.

Finally, we used flies from the 2012 and 2014 25°C and 18°C chill coma recovery experiments and 2014-2015 short-term acclimation experiments to test the hypothesis: Seasonal temperature variation affects the trade-off between basal cold tolerance and plasticity. We estimated developmental plasticity by taking the difference between recovery time in the two development treatments (development at 25°C vs. 18°C) for each line. We calculated an acclimation score by taking the difference between survivorship in the two cold-hardening treatments (acclimation and non-acclimation) for each population cage. We looked at the correlation between each type of cold tolerance metric and its corresponding plasticity metric within levels of CDD18. As described above, we simply divided collection times by CDD18 into thirds, so that for chill coma recovery the first third of collection times with the lowest CDD18 were put into the "low" category and so forth. If the total number of collection times was not a multiple of three, the excess times were included in the "mid" category. We used linear models to test whether the relationships between basal tolerance and plasticity were significantly different by CDD18 category.

Results

Evidence of seasonal variation in basal cold tolerance

We used cooling degree days with an 18°C reference (CDD18) as our proxy for the seasonal temperature experienced by founder females of the isofemale lines we tested in our experiment. Across the four collection years, we observed a wide range in thermal variation from

an average of 28.7°C each day over the two weeks preceding collection in the warmest month (July, 2012) to approximately 18°C over the two weeks preceding collection in the coolest month (September, 2013 and November, 2014; Fig. 1.1). A mixed effects model fitted to the chill coma recovery waking times of flies reared at 25°C indicated that an increase in cumulative heat exposure added to chill coma recovery time (β_{CDD18} = 0.001 +/- < 0.001, t = 5.53, P < 0.001; Fig. 1.2A). This effect was significant despite the 'common garden' rearing and maintenance of flies at 25°C for five or more generations in the lab. Males generally woke up slightly faster than females (SD = 0.10), and this difference was accounted for as a random intercept in our model. The variation among lines established from females collected at the same time (SD = 0.11) was similar to the variation among collection years (SD = 0.13). Visual examination of the random effects coefficients and boxplots of the residuals distributed across fixed and random effects (Fig. 1.3) did not show extreme outliers or potential issues due to variance heterogeneity.

Survivorship following short-term cold stress was not significantly influenced by cumulative heat exposure (β_{CDD18} = 0.007 +/- < 0.004, z = 1.60, P = 0.11; Fig. 1.4A). Males generally survived better than females (SD = 0.41) and this variation was similar in magnitude to the variation among population cages established from females collected at the same time (SD = 0.55). However, the variation among collection years was much larger (SD = 1.32) so that there was more variation between years than within (Fig. 1.5). As above, visual examination of the boxplots of the residuals distributed across fixed and random effects did not show extreme outliers or potential issues due to variance heterogeneity (Fig. 1.5).

Plasticity compensates for the seasonal genetic differences in cold tolerance

To determine the effect of developmental acclimation, flies collected in 2012 and 2014 were also reared and maintained at a constant 18°C 'common garden' environment for 3 or more

generations and tested once more for chill coma recovery. A mixed effects model fitted to fly phenotypes from these years indicated that this switch in developmental temperature from 25 °C to 18°C shortens waking times (β_{dev} = - 0.16 +/- 0.01, t = - 14.66, $P < 0.001$; Fig. 1.2B – D), and interacts with CDD18 to reduce the effect of CDD18 on waking time ($\beta_{CDD18:dev}$ = - 0.001 +/- < 0.001, t = - 9.16, P = < 0.001; Fig. 1.2B). As observed in the first hypothesis, an increase in cumulative heat exposure added to chill coma recovery time (β_{CDD18} = 0.001 +/- < 0.001, t = 6.58, P < 0.001; Fig. 1.2B). Again males generally woke faster (SD = 0.05). The variation among lines established from females collected at the same time (SD = 0.09) was similar to the variation among collection years (SD = 0.09). As above, visual examination of the random effects coefficients and boxplots of the residuals did not show extreme outliers or potential issue due to variance heterogeneity (Fig. 1.6).

To determine the effect of short-term acclimation, flies collected in 2014 and 2015 were exposed to a 4°C 'rapid cold-hardening' treatment and tested once more for cold stress survivorship. A mixed effects model fitted to fly phenotypes from these years indicated that this treatment significantly increases cold stress survivorship (β_{trt} = 1.99 +/- 0.05, z = 36.32, P < 0.001; Fig. 1.4B – D). Cumulative heat exposure also tended to improve cold stress survivorship (β_{CDD18} = 0.01 +/- 0.004, z = 2.47, P = 0.01; Fig. 1.4B). Again males had higher survivorship (SD = 0.29). The variation among cages established from females collected at the same time (SD = 0.56) and the variation among collection years (SD = 0.84) was large. As above, visual examination of the random effects coefficients and boxplots of the residuals did not show extreme outliers or potential issue due to variance heterogeneity (Fig. 1.7).

Evidence of seasonal variation in the trade-off between cold tolerance and acclimation

We found that for both chill coma recovery and cold stress survivorship, the basal cold tolerances and their respective plasticity measures showed significant relationships (Fig. 1.8). Flies with higher basal cold tolerance will have a shorter chill coma recovery time (note that the X-axis is flipped in orientation because of this in Fig. 1.8A – C). Thus the association between basal cold tolerance and developmental plasticity is negative, and flies with higher basal cold tolerance for chill coma recovery showed the least amount of developmental plasticity. This association was present regardless of the level of cumulative heat exposure, so that whether it was low or high, basal cold tolerance for chill coma recovery and developmental plasticity always showed a similar degree of association ($CDD18_{Level}$:CCR_{25}, $F_2 = 1.88$, $P = 0.15$; Fig. 1.8A – C). Cold stress survivorship also showed an overall negative relationship with short-term acclimation plasticity, and flies with higher basal cold tolerance for cold stress survivorship showed the least benefit from short-term acclimation. In contrast to chill coma recovery, this association was strongest in flies with a higher level of cumulative heat exposure than in flies with less cumulative heat exposure ($CDD18_{Level}$:CSS_{NON}, $F_2 = 6.20$, $P = 0.004$; Fig. 1.8D – F).

Discussion

Temperature fluctuations are effective sources of natural selection for small ectothermic organisms with short generation times (Kelty and Lee 2001; MacMillan and Sinclair 2011; Vesala and Hoikkala 2011; Bergland et al. 2014a). Over the four years that we sampled our natural population of *D. melanogaster*, the seasonal thermal variation experienced by founding females grew smaller with each successive year (Fig. 1.1). Despite the milder summer and fall temperatures in progressive years, we detected a significant effect of cumulative heat exposure in

flies that were reared at 25°C under common garden conditions and tested for chill coma recovery time (Fig. 1.2). Flies collected during warmer months typically took longer to wake from chill coma, suggesting that natural populations have decreased cold tolerance during this part of the season. Cold tolerance increased as cumulative heat exposure decreased, indicating that seasonal change in temperatures from summer to fall across the three years influenced cold tolerance in the expected direction as measured by chill coma recovery.

However, cold tolerance assessed through exposure to short-term cold stress did not recapitulate this pattern (Fig. 1.4). Flies collected during warmer months and during colder months were not different in their cold stress survivorship. It is important to note that the variance between collection years in cold stress survivorship was larger than the variance within each year for this metric. Larger differences in environmental conditions experienced by founding females may be necessary to elicit a change in this measure of cold stress survivorship. Thus it is quite possible that we were unable to detect a significant effect of season on cold stress survivorship due to the relatively small degree of temperature fluctuation across our collection dates during 2014 and 2015, as opposed to those in 2012 and 2013 (Fig. 1.1). This is especially true for females collected in 2015, where temperatures during the two weeks preceding each collection period were similar across a 4-month period of time (Fig. 1.1). At the same time, a lack of seasonal variation in extreme cold stress tolerance has been previously reported (Hoffmann and Watson 1993), suggesting that cold stress survivorship may not respond to seasonal thermal variation. Thus, our results may not be surprising because tolerance to short-term, severe temperature stress is expected to be important for surviving daily fluctuations in temperature (Lee et al. 1987; Hoffmann and Watson 1993; Kelty and Lee 2001).

The significant effect of cumulative heat exposure on chill coma recovery in flies reared at 25°C is a signal of genetic change as this natural population adapts to temperature variation throughout the seasons each year. Cyclical changes in selection pressure have repeatedly been shown to influence fitness and life history phenotypes (Bergland et al. 2014a; Betini et al. 2014; Behrman et al. 2015). Over short stretches of time, these cyclical selection pressures can cause high-frequency alleles that were beneficial earlier in the season to become less frequent when they are less beneficial. Bergland et al. (2014a) found evidence to support this pattern of allele frequency fluctuation and further linked specific fluctuating loci to chill coma recovery. Behrman et al. (2015) also observed oscillating cold tolerance phenotypes in both *D. simulans* and *D. melanogaster*. In addition to the positive effect of seasonal thermal variation on cold tolerance at least for chill coma recovery, both short-term acclimation and longer-term developmental acclimation improved cold tolerance (Fig. 1.2C – D, 1.4C – D). These results show that both types of phenotypic plasticity are adaptive because they allow warmer season flies to effectively recover the cold tolerance of cooler season flies that are selected by seasonal temperature variation to be more basally cold tolerant. The plasticity we observed in our population of flies is in line with previous reports of adaptive plasticity found for cold tolerance phenotypes, including chill coma recovery (Ayrinhac et al. 2004; Rako and Hoffmann 2006) and cold stress survivorship (Lee et al. 1987; Gerken et al. 2015).

Moreover, basal cold tolerance and adaptive plasticity for both types of acclimation showed a characteristic trade-off pattern, in which the capacity for phenotypic plasticity was greater for less basally cold tolerant lines or cages and vice versa (Fig. 1.8). Trade-offs between basal cold tolerance and plasticity have been reported before (Hoffmann et al. 2003c; Kellett et al. 2005; Nyamukondiwa et al. 2011; Gerken et al. 2015). While the relationship is naturally

biased toward a negative relationship, the slope describing this relationship within a single population over time provides insight into the dynamics of seasonality and cold tolerance (Sørensen et al. 2016). A previously untested but important aspect of the trade-off is whether the relationship between basal tolerance and adaptive plasticity constrains how individual organisms can respond to seasonal variation. With our two cold tolerance metrics, we were able to assess whether the capacity for adaptive plasticity related to short- and longer-term variation in temperatures differs by season. We found that seasonal variation in temperature significantly affected the trade-off for short-term acclimation but not developmental plasticity. We observed a significantly stronger trade-off between cold tolerance and acclimation plasticity for short-term cold stress survivorship during the warmest months. This trade-off was weaker during colder months, and in the coldest season sampled, all population cages had equally poor basal cold tolerance even though they retained a fairly large range of acclimation capacities (Fig. 1.8D – F). This was not the case for the longer-term developmental plasticity trade-off, whose relationship with basal cold tolerance persisted to a similar degree regardless of season (Fig. 1.8A – C).

This difference in the dynamics of the trade-off between developmental and short-term acclimation and their respective measures of basal tolerance may reflect a constraint that exists between these two types of phenotypic plasticity. Developmental acclimation results in an irreversible type of plasticity while short-term acclimation is generally reversible, wearing off after a few hours (Everman et al. In Press; Kelty and Lee 2001; Koveos 2001). Both types of plasticity are likely to evolve and interact in species that have short generation times and reproduce multiple times a year, because reversible acclimation can correct potential mismatches between basal tolerance and environment that occur as a result of irreversible developmental plasticity (Beaman et al. 2016). In the model presented by Beaman and colleagues, the relative

capacity of each type of acclimation depended on the evolutionary cost of maintaining acclimation capacity in particular. For seasonal traits such as chill coma recovery, the tight and non-fluctuating relationship between basal cold tolerance and developmental plasticity indicates that these insects will recover from chill coma fairly well regardless of season. However, during colder months, maintaining a higher acclimation capacity will be adaptive because the effect of an extended warm spell could otherwise be challenging due to the effect of developmental acclimation on basal levels of thermal tolerance. Thus this relationship between the two types of plasticity may constrain acclimation capacity to be maintained at a higher level during colder months, more so than the trade-off between short-term acclimation and basal cold tolerance. We are unable to conclusively test this relationship between developmental and short-term acclimation with our data because they were measured on two different cold tolerance phenotypes. However, the potential for this type of multivariate relationship between basal tolerance and phenotypic plasticity may provide additional insight into why some measures of thermal tolerance are more sensitive to seasonal variation than others, and could extend to broader spatial scales as well.

We expect natural thermal environments to fluctuate, and fluctuations that occur within the thermal performance range of an ectotherm typically increase its fitness (Colinet et al. 2015). We began our discussion by noting how much less variable each successive year from 2012 to 2015 was in terms of the metrics of thermal variation we used (Fig. 1.1). Year by year, the basal tolerances of flies increased for chill coma recovery from 2012 through 2014 and increased for cold stress survivorship from 2014 to 2015. If the reduced temperature variability and increase in basal tolerance over the last few years led to reduced allelic variation or capacity for plasticity, extended atypical weather, such as an extreme cold spell during a warmer season, would present

a serious challenge for this natural population. While plasticity has positive affects on cold tolerance within the range of thermal stresses we tested in this natural population of flies, the predictability and magnitude of climatic changes going forward is certain to influence the persistence of the population thanks to constraints such as the trade-offs we found that affect the evolution of plasticity.

Acknowledgments

We thank Ree's Fruit Farm and 86th Street Orchard for allowing us to collect the original *D. melanogaster* females to found laboratory cultures. This work was supported by the National Science Foundation awards NSF IOS-1051770 awarded to T.J.M. and a Biology Graduate Student Association Research Grant awarded to E.R.E.

Figures and Tables

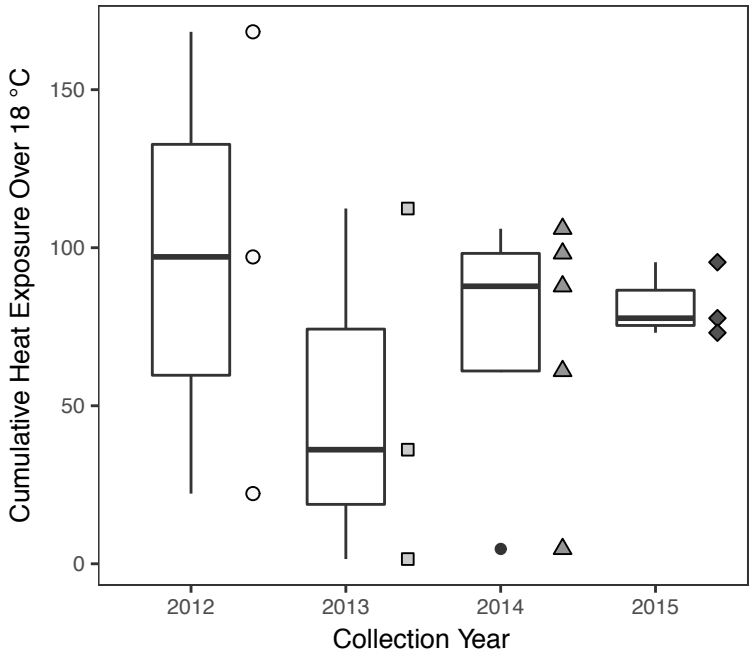

Figure 1.1 Cooling degree days above 18°C for the collection dates of the isofemale founders used in the experiments. Degree days are cumulative for the 14 days prior to and including the collection date, and were obtained from the nearest weather station located at Topeka Municipal Airport. Symbols indicate the average CDD18 for each collection day each year.

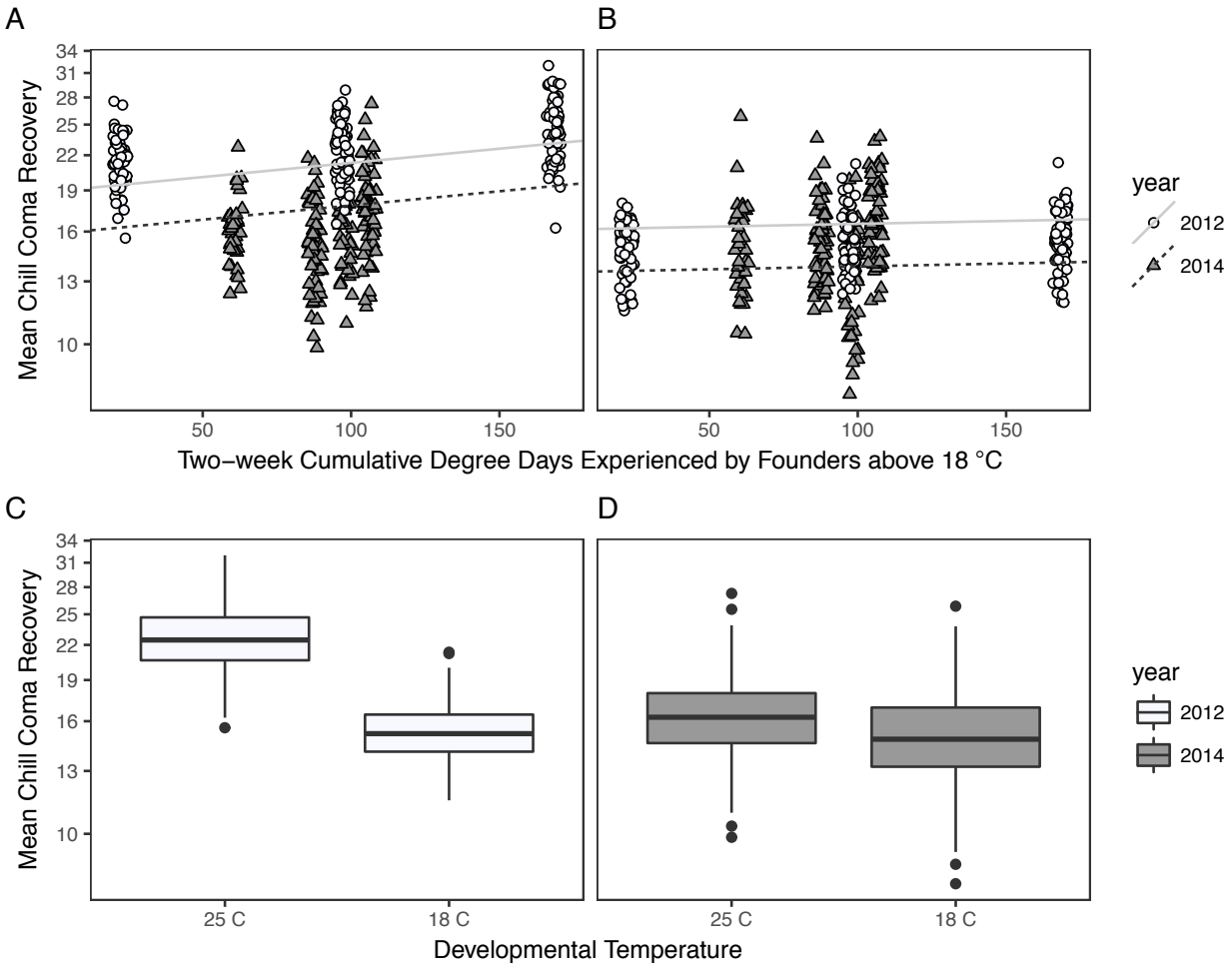

Figure 1.2 Mixed effects model fit to chill coma recovery times of flies founded from females collected at different times of the season and reared in the lab at 25°C (A) and subsequently at 18°C (B) to measure the effect of developmental acclimation. A. Offspring of collections made during cooler parts of the year woke more quickly from chill coma compared to offspring from collections made during warmer parts of the year. B. Developmental acclimation improved cold tolerance for all offspring, leading to a larger increase in cold tolerance for offspring of collections made during warmer parts of the year. C. Developmental acclimation lead to faster recovery times on average for offspring of flies collected during 2012. D. Cold tolerance was generally greater for offspring of flies collected in 2014; developmental acclimation improved cold tolerance in offspring of flies collected in 2014. Linear models shown incorporate both fixed and random effects coefficients obtained from the mixed effects model. Note that the axis is in log scale because Quasipoisson errors are log linked.

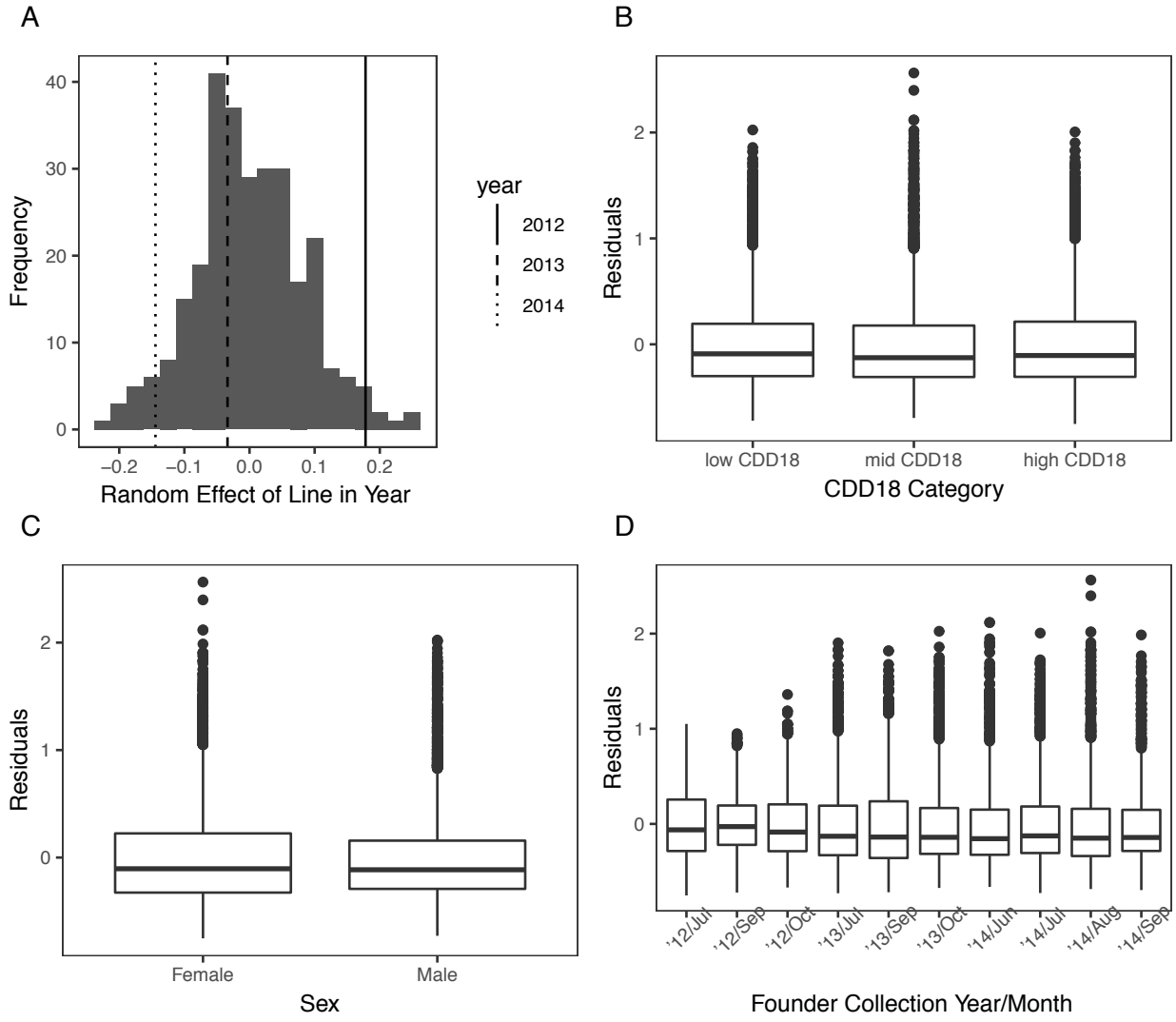

Figure 1.3 Analysis of residuals for chill coma recovery. A. Distribution of random effects fit to the waking times of flies reared at 25°C. the histogram is of the random effect intercepts fit to isofemale lines nested in year. Vertical lines are random effect intercepts fit to collection year. (B – D) Residuals of the mixed effects model fit. For convenient visualization, cooling degree days (CDD18) were divided into categories by dividing the range of the collection dates into three (low, mid, high).

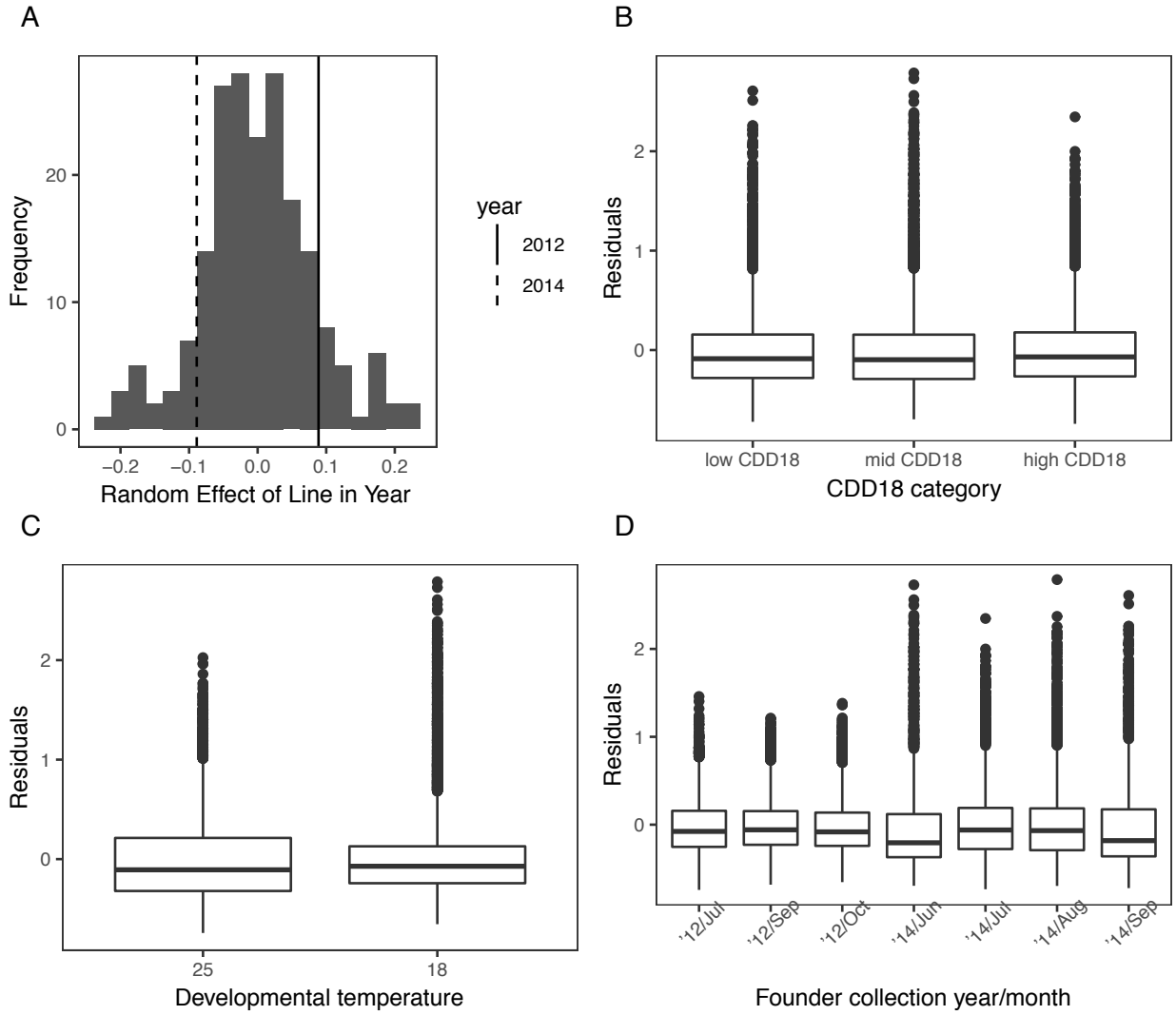

Figure 1.4 Analysis of developmental acclimation residuals. A. Distribution of random effects fit to chill coma recovery time of flies reared at 25°C and then 18°C. The histogram is of the random effect intercepts fit to isofemale lines nested in year. Vertical lines are random effect intercepts fit to collection year (B – D) Residuals of the mixed effects model.

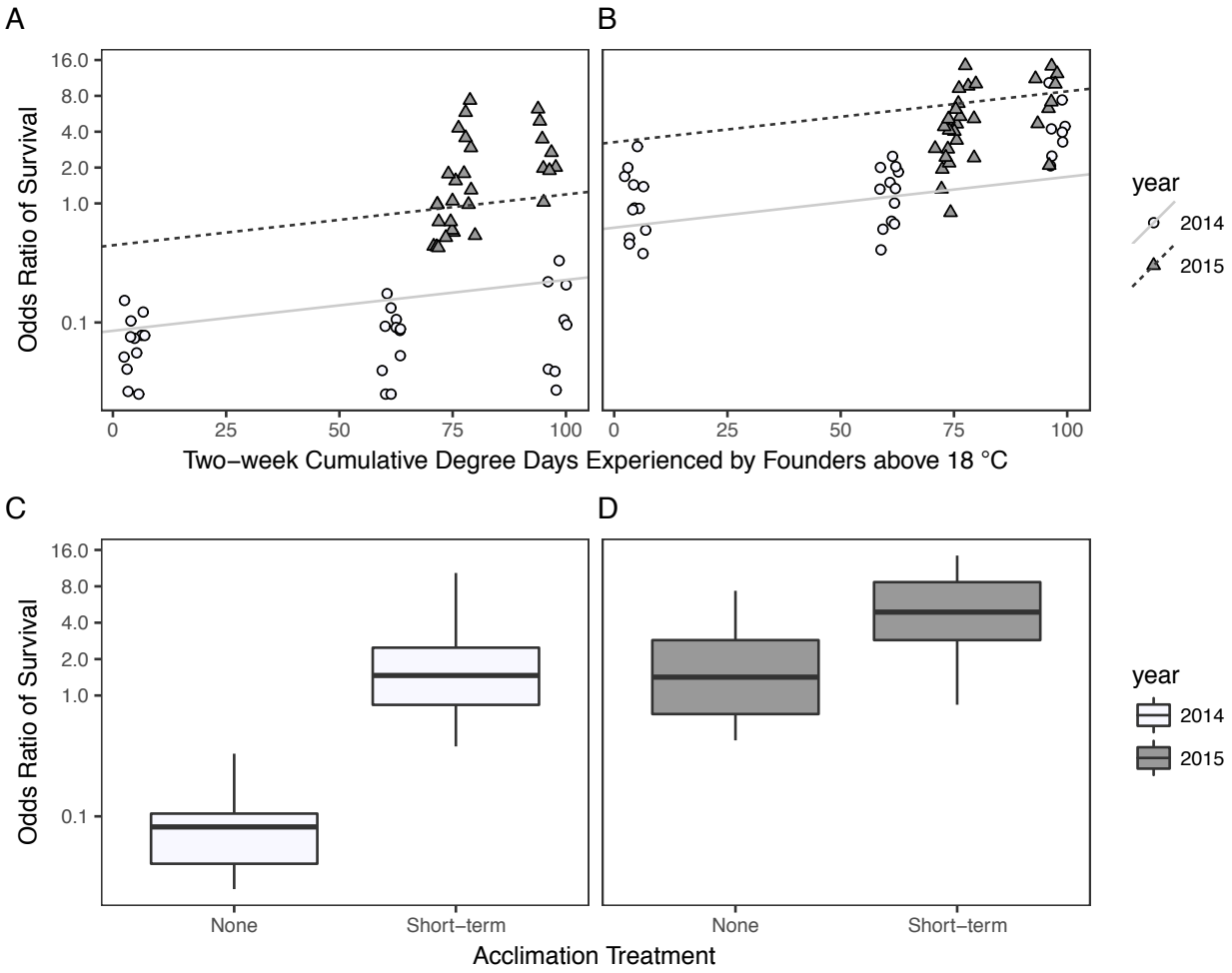

Figure 1.5 Mixed effects model fit to cold stress survivorship (alive vs. dead) from females collected at different times of the season and reared in the lab at 25°C. A. Cold tolerance of offspring of collected individuals did not vary significantly through the year due to seasonal variation in temperature. B. Short-term acclimation treatment at 4°C prior to the survivorship assay at -6°C improved cold tolerance; however, survival of offspring following acclimation was not influenced by seasonal variation in temperature. C. On average, short-term acclimation improved survival in offspring of 2014 collections. D. Offspring of flies collected in 2015 were more cold tolerance on average compared to 2014; short term acclimation improved survival of offspring of 2015 collections. Linear models shown incorporate both fixed and random effects coefficients obtained from the mixed effects model. Note that the y-axes are in log scale because binomial errors are log linked.

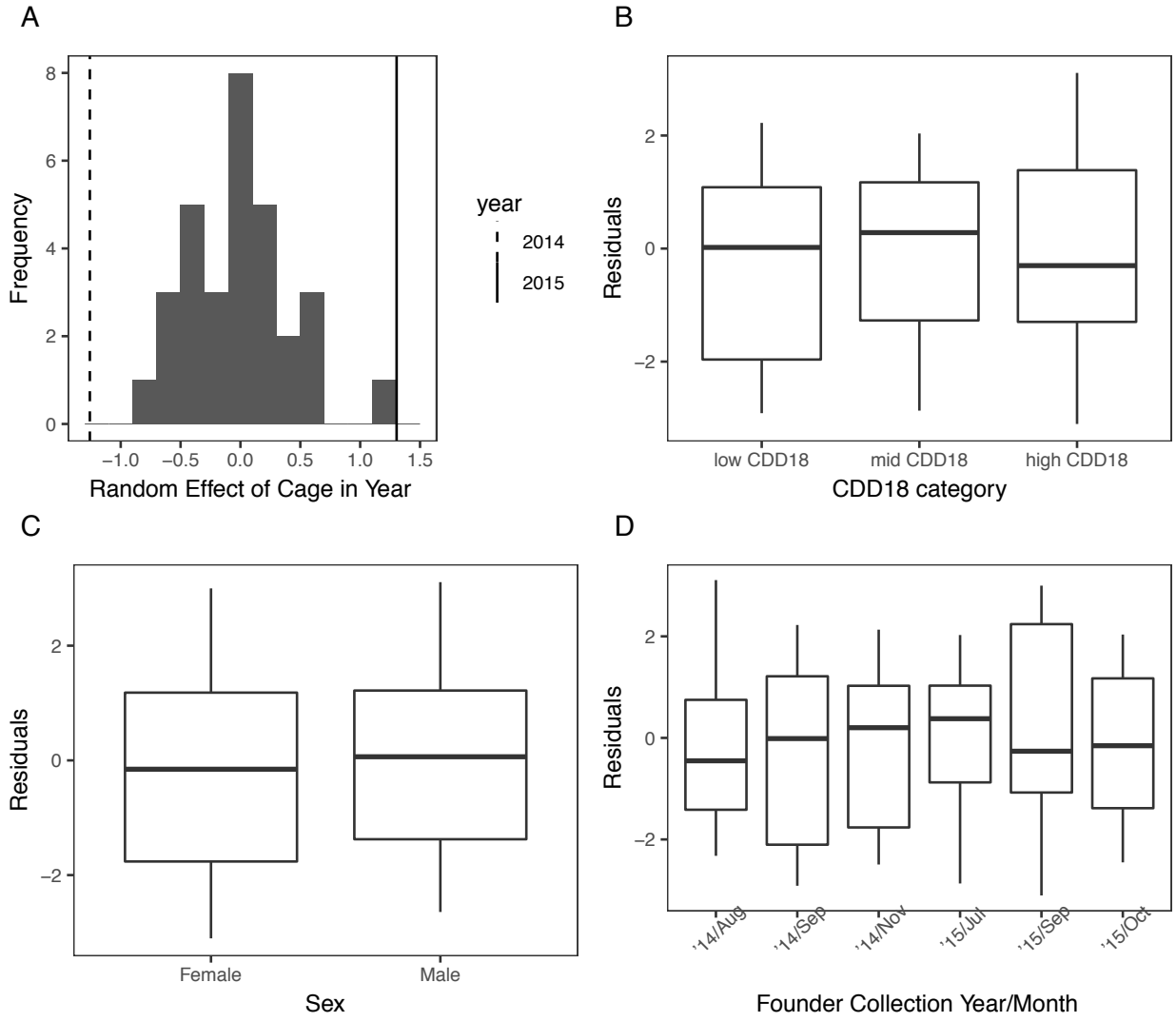

Figure 1.6 Analysis of residuals for short-term acclimation. A. Distribution of random effects fit to cold stress survivorship of flies directly exposed to cold stress at -6°C. The histogram is of the random effect intercepts fit to population cages nested in year. Vertical lines are random effect intercepts fit to collection year. (B – D) Residuals of the mixed effects model.

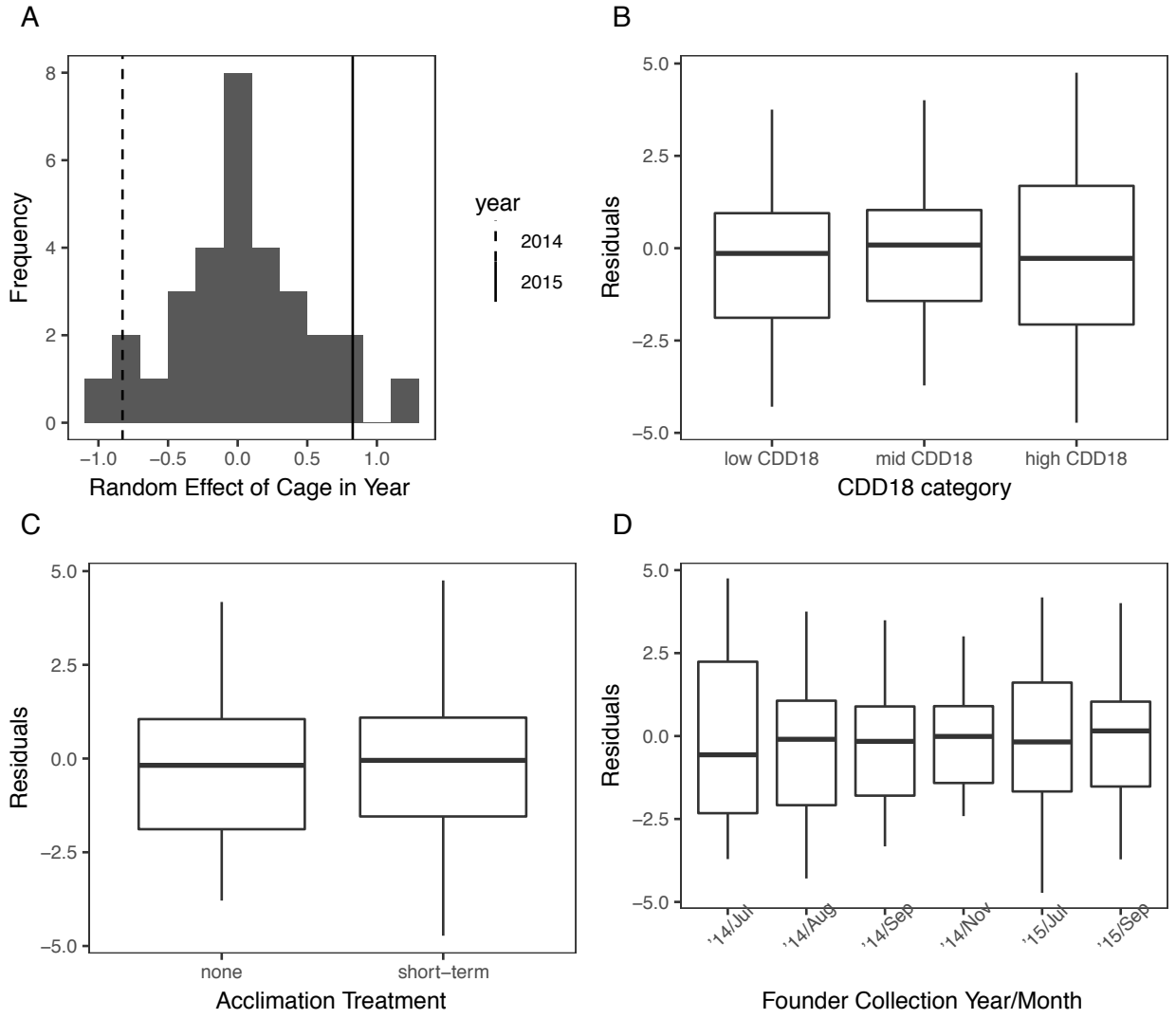

Figure 1.7 Analysis of short-term acclimation residuals. A. Distribution of random effects fit to cold stress survivorship of flies exposed to cold stress at -6°C with and without short-term acclimation at 4°C. The histogram is of the random effect intercepts fit to population cages nested in year. Vertical lines are random effect intercepts fit to collection year. (B – D) residuals of the mixed effects model.

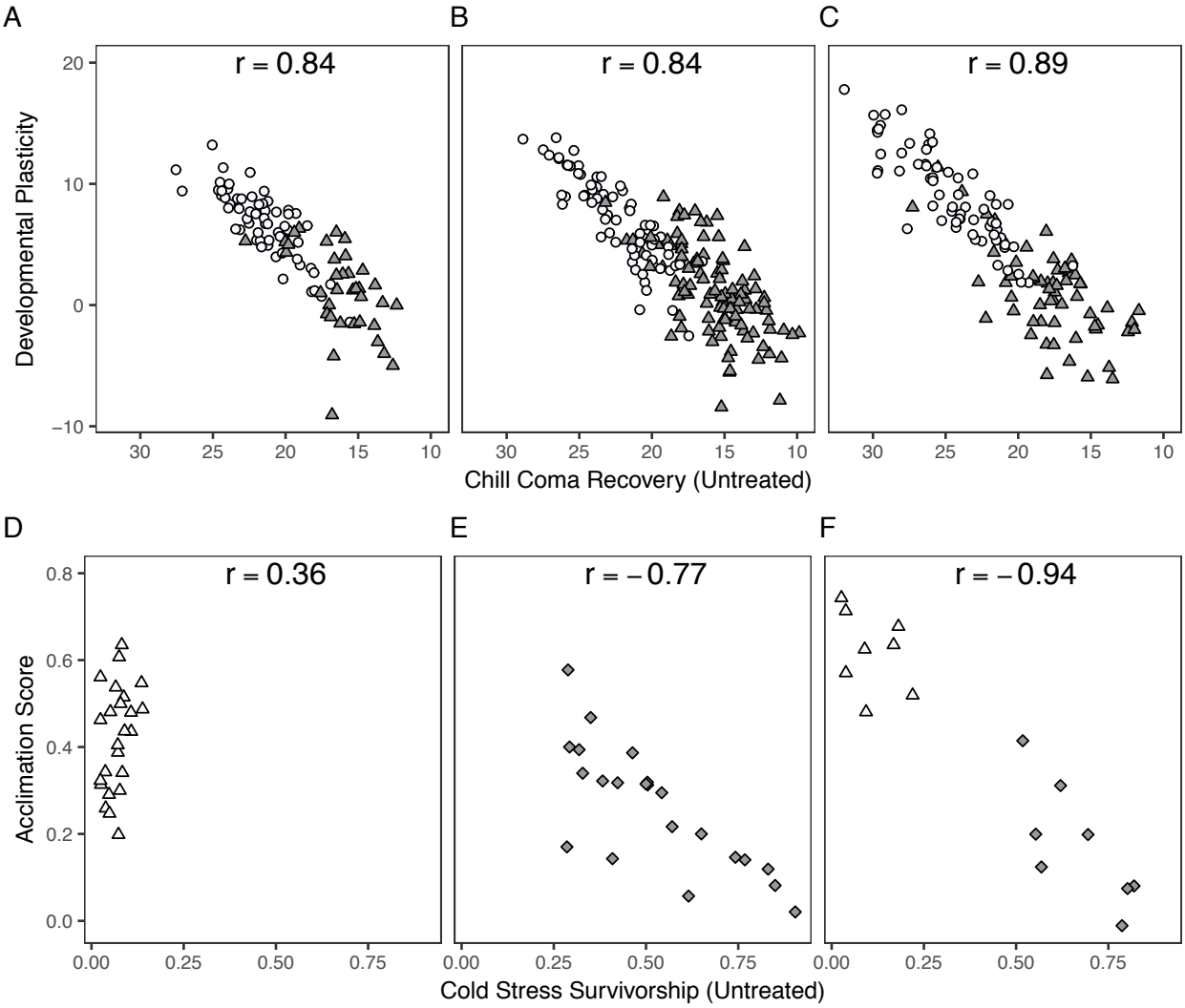

Figure 1.8 Seasonal variation in plasticity. (A – C) Basal chill coma recovery and its relationship with developmental plasticity across the range of cumulative heat exposure experienced by founder females of isofemale lines (individual points; A. low heat exposure, B. medium head exposure, C. high heat exposure). Note the reversed x-axis in plots A – C. (D – F) Basal cold stress survivorship and its relationship with short-term acclimation across the range of seasonal temperature variation experienced by founder females of population cages (individual points; D. low heat exposure, E. medium head exposure, F. high heat exposure). Circles indicate data from 2012, triangles indicate data from 2014, and diamonds indicate data from 2015. Correlations between the relevant metrics are shown individually for each panel.

Chapter 2 - Evolution of age-specific decline in stress phenotypes is driven by antagonistic pleiotropy and mutation accumulation

Elizabeth R. Everman and Theodore J. Morgan

Abstract

Efforts to more fully understand and test evolutionary theories of aging have produced distinct predictions for mutation accumulation (MA) and antagonistic pleiotropy (AP) mechanisms. We build on these predictions through the use of association mapping and investigation of the change in additive effects of polymorphisms across age and among traits for multiple stress response phenotypes. We found that cold stress survival with acclimation, cold stress survival without acclimation, and starvation resistance declined with age and that changes in the genetic architecture of each phenotype were consistent with MA predictions. We used a novel test for MA and AP by calculating the additive effect of polymorphisms across ages and found support for both MA and AP mechanisms in the age-related decline in stress tolerance. These patterns suggest both MA and AP contribute to age-related change in stress response and highlight the utility of association mapping to identify genetic shifts across age.

Introduction

The intensity of natural selection changes over an organism's lifespan, having greatest effect early in life, as individuals reach reproductive maturity, and smaller effect as organisms age (Fisher 1930; Haldane 1941; Medawar 1952; Williams 1957; Hamilton 1966; Charlesworth and Hughes 1996; Charlesworth 2001). Decreased effectiveness of natural selection at old age results in the accumulation of deleterious polymorphisms in populations and leads to decline in age-specific fitness, characteristic of senescence (Medawar 1952; Williams 1957; Hamilton 1966). Senescence is expected to negatively impact phenotypes related to fitness and is thought

to have evolved through two non-mutually exclusive genetic mechanisms (Charlesworth 1994; Ricklefs and Finch 1995; Bowler and Terblanche 2008). Under mutation accumulation (MA; Medawar 1952), decreased effectiveness of natural selection over lifespan allows the retention of deleterious polymorphisms that are only expressed later in life (Ricklefs and Finch 1995). Under antagonistic pleiotropy (AP; Williams 1957), genes that are expressed over a wide window of an individual's lifespan have positive effects on fitness at young age and negative effects on fitness at old age (Williams 1957; Ricklefs and Finch 1995; Charlesworth 2001; Maklakov et al. 2015). Both mechanisms rely on the relaxation of natural selection later in an organism's life but have unique predictions for how age-dependent genetic control of phenotypes changes.

Age-related declines and the influence of the MA and AP mechanisms have been well documented for life-history phenotypes such as mortality and fecundity (Rose 1984; Engström et al. 1989; Rose et al. 1992; Charlesworth and Hughes 1996; Promislow et al. 1996; Tatar et al. 1996; Pletcher et al. 1998; Hughes et al. 2002; Snoke and Promislow 2003; Bowler and Terblanche 2008; Durham et al. 2014), but far less is known about how stress response phenotypes change with age (Bowler and Terblanche 2008). Stress response over an organism's lifespan is a critical component of fitness, and is an important modulator of lifespan (Colinet et al. 2015). Variation in stress response can influence the persistence and evolution of populations over short time scales, especially in variable environments (Bergland et al. 2014a). In species that experience seasonal change in thermal regime, changes in the demographic structure of populations can also drastically influence the ability of individuals to tolerate stressful temperatures. For example, in *Drosophila melanogaster*, populations are primarily composed of young individuals in the spring when temperatures are increasing on average and primarily of older individuals in the fall when temperatures are decreasing on average (Behrman et al. 2015).

Thus, measures of thermal tolerance at one point in the season therefore do not reflect the influence of seasonal variation in age on thermal tolerance. Such shifts in the age structure of populations coupled with age related changes in the genetic control of fitness phenotypes have the potential to dramatically influence short- and long-term responses to environmental variation.

The MA and AP aging mechanisms make predictions about age-specific changes in multiple quantitative genetic parameters. Under MA, genetic variance is expected to increase with age because of the expression of age-restricted polymorphisms (Charlesworth and Hughes 1996; Charlesworth 2001; Hughes et al. 2002; Leips et al. 2006). These late acting polymorphisms are retained in the population because the individuals that possess them have successfully reproduced, allowing such alleles to evade natural selection (Haldane 1941; Charlesworth 2001; Maklakov et al. 2015). Additionally, because the genetic control of the phenotype across ages is independent, the genetic correlation of the phenotype between young and old individuals is expected to be non-negative (Charlesworth 2001; Reynolds et al. 2007; Maklakov et al. 2015). In contrast, under the AP hypothesis, age-related change in phenotypes is the result of a genetic trade-off, where polymorphisms that are beneficial early in life are detrimental late in life (Leips et al. 2006; Maklakov et al. 2015). These polymorphisms are retained in the population because of their beneficial effects on fitness at young age (Charlesworth and Hughes 1996; Leips et al. 2006; Maklakov et al. 2015). AP does not make clear predictions for changes in variance components with age; however, because the same polymorphisms are expected to influence the phenotype with opposite effects across age, the genetic correlation across ages is expected to be negative (Charlesworth and Hughes 1996; Hughes et al. 2002; Leips et al. 2006).

Despite these clear predictions, the influence of MA and AP and the theory behind these mechanisms does not fully explain age-related change in phenotypes. One reason for this is that the signature of AP may be lost because of small effects or fixation of loci involved in AP leading to ascertainment bias toward MA (Moorad and Promislow 2009; Maklakov et al. 2015). Further, calculations of genetic variance and correlations are indirect metrics of age-related change in the genetic control of phenotypes (Charlesworth 2001). In contrast, the use of association mapping allows us to extend these predictions to more explicitly evaluate subtle patterns predicted by the MA and AP theories of aging. Durham et al. (2014) previously used association mapping to identify and compare polymorphisms that are associated with variation in phenotypes measured at multiple ages. This use of association mapping can be extended in two important ways. First, sets of associated polymorphisms can be compared between two different phenotypes across age as well as within phenotypes across age. Second, even if sets of significantly associated polymorphisms are non-overlapping, association mapping allows the evaluation of age-related shifts in the additive effects of associated polymorphisms, thus facilitating the detection of weak antagonistic effects across age or phenotypes.

As an example, consider a hypothetical phenotype measured in young and old individuals that is associated with non-overlapping sets of polymorphisms at each age. Two different polymorphisms are associated with the hypothetical phenotype at young age and have positive additive effects on the young phenotype. The polymorphism that is consistent with MA will shift from a significant positive additive effect at young age to an effect that is near zero or of the same sign at old age. In contrast, the polymorphism that is consistent with AP will shift from a positive additive effect at young age to a negative additive effect at old age. Thus, even though association mapping may not detect the antagonistic polymorphism with small effect in old

individuals, calculation of additive effects of polymorphisms across age can be used to detect signals of AP (Fig. 2.1; Maklakov et al. 2015).

In the current study, we used a combination of association mapping and quantitative genetic analysis to dissect the variation in age-related changes in four environmental stress response phenotypes and tested the influence of MA and AP. To do this, we measured age-related survival after cold-stress with acclimation and without acclimation, thermal phenotypic plasticity, and starvation resistance in a genetically diverse *D. melanogaster* mapping population from the *Drosophila* Genetic Reference Panel (DGRP; Mackay et al. 2012; Huang et al. 2014). Starvation resistance and cold tolerance are quantitative genetic phenotypes, with heritable variation existing for each (Imasheva et al. 1998; Hoffmann et al. 2005; Morgan and Mackay 2006; Bowler and Terblanche 2008; Overgaard et al. 2010; Mackay et al. 2012; Schwasinger-Schmidt et al. 2012; Gerken et al. 2015). However, much of the previous work examining age-related change in cold tolerance has been done in a single genotype (Czajka and Lee 1990), and little research is available to inform how starvation resistance will change with age (but see Colinet et al. 2015). We predicted that starvation resistance and cold tolerance (measured as acclimation and non-acclimation survivorship) would decline with age.

Genetic variation has also been documented for various forms of thermal phenotypic plasticity (Fallis et al. 2014; Gerken et al. 2015). Short-term acclimation through rapid cold-hardening (acclimation score; Lee et al. 1987) is one form of plasticity that occurs when organisms are exposed to a mild thermal stress before experiencing more stressful conditions (Lee et al. 1987; Coulson and Bale 1990; Czajka and Lee 1990; Powell and Bale 2005; Gerken et al. 2015). In flies and other ectothermic species, this pre-treatment usually results in increased cold survivorship and provides a simple model of the physiological response of ectotherms as

they respond to episodic fluctuations in temperature (Coulson and Bale 1990; Bozinovic et al. 2011; Ju et al. 2011; Huey et al. 2012; Niehaus et al. 2012). In most cases, a strong, beneficial acclimation response is detected in young adult individuals (Powell and Bale 2005; Rajamohan and Sinclair 2009; Ju et al. 2011; Gerken et al. 2015), but age-related changes in this form of phenotypic plasticity have not been examined. We expected flies to lose the ability to survive acclimation and non-acclimation cold stress at a similar rate, resulting in acclimation scores (the difference in survival with and without cold acclimation) that would not change with age.

Methods

Fly stocks

The *Drosophila* Genetic Reference Panel (DGRP) was established as a set of natural isogenic lines founded from a single population in Raleigh, NC (Table A.1; Mackay et al. 2012). Stocks were obtained from Bloomington Stock Center and maintained at 25°C on a 12-hour light-dark cycle on cornmeal-molasses agar sprinkled with active yeast. The number of DGRP lines used in each experiment was determined based on logistical constraints of the experiment. Parents of experimental flies were sorted over light CO_2 anesthesia and placed into vials containing five individuals of each sex to establish the first experimental block. Females were allowed to mate and lay eggs for three days, after which the parents were transferred to a new set of vials. Egg laying continued in the new vials for three days to establish the second experimental block, and then parents were discarded. Experimental flies were collected on the third day of eclosion and sorted by sex to a density of 10 same-sex individuals per vial. Our experimental design measured responses in "young" and "old" cohorts of flies. Young flies were aged for 1 week (7 days) at 25°C, while old flies were aged for four weeks (28 days) at 25°C. The "old" cohort timing was selected because this was an advanced age time point, but prior to a

significant decline in average survivorship among lines (Ivanov et al. 2015). Previous research has also demonstrated reduced fecundity at this age (Tatar et al. 1996; Leips et al. 2006). Experimental flies in the "old" cohort were tipped every third day to new media until flies were tested at 28 days.

Age-related stress responses

Cold stress responses

We measured three cold stress responses on 101 DGRP lines at young and old age (Table A.1). We measured acclimation survival using a rapid cold hardening treatment that consisted of a two-hour exposure to 4°C immediately prior to cold shock at -6°C for one hour (Lee et al. 1987; Gerken et al. 2015). Following cold shock, the flies were transferred to fresh media and allowed to recover at 25°C for 24 hours (Fig. 2.2A). We also measured non-acclimation survival by transferring flies directly (without acclimation) to -6°C for one hour (Fig. 2.2B). As with the acclimation treatment, the non-acclimated flies were transferred to fresh media following cold stress and allowed to recover for 24 hours at 25°C. After 24 hours, the proportion of flies that had survived each treatment was recorded by counting the number of individuals in each vial that were capable of coordinated movement (flying or walking). Acclimation score (or rapid cold hardening capacity) was calculated by subtracting non-acclimation survivorship from acclimation survivorship (Gerken et al. 2015). A total of four replicates per sex, line, age, and cold stress treatment were measured in two experimental blocks with 10 individuals per vial replicate.

Starvation resistance

We measured starvation resistance in 164 DGRP lines, including the 101 lines used in the cold stress response experiments, at young and old age (Table A.1). Young and old flies were

maintained on standard media until they were one or four weeks of age, respectively. At one and four weeks of age, flies were transferred to starvation media (1.5% agarose) and maintained at 25°C. Vials were monitored every four hours, and average time of death per vial was recorded as the response. A total of three replicates per sex, line, and age were measured with 10 individuals per vial replicate.

Data analysis

Genetic variation

Genetic variation among all lines was analyzed via mixed-model ANOVA for the cold stress responses (acclimation survivorship, non-acclimation survivorship, and acclimation score) and starvation resistance. The model for each analysis included the main effects of age and sex, as well as interactions, with block (for cold stress responses only) and line as random effects. Specific effects of sex by age interactions were tested with Tukey's HSD post hoc test, with an experiment-wide $\alpha = 0.05$.

To assess the effect of variation in rate of aging on cold stress tolerance (i.e. physiological vs chronological cold stress phenotypes), linear regression was used to compare the mean cold stress responses of four-week-old flies with cold stress response of flies at their line specific Td50. However, because none of the physiologically-aged flies survived non-acclimated cold stress, we only compared acclimation survivorship in this analysis.

Genome-wide association analysis

We used association mapping to identify regions of the genome that were significantly associated with variation in acclimation survivorship, non-acclimation survivorship, acclimation score, and starvation resistance. Association mapping was performed on each age and phenotype separately, and significance was assigned at $-\log_{10}(5)$ (Mackay et al. 2012; Durham et al. 2014;

Gerken et al. 2015). Shifts in genetic architecture across age and phenotype were assessed by comparing the significant polymorphisms associated with each age-specific phenotype. We performed gene ontology (GO) enrichment analysis using FlyMine (Lyne et al. 2007) to determine whether specific classes of genes or pathways were overrepresented in the loci associated with each phenotype and age.

Quantitative genetic analyses

Heritability, variance components, and genetic correlations were estimated using the program H2boot, which applies bootstrap resampling to quantitative genetic data (Phillips 1998). Acclimation survivorship, non-acclimation survivorship, acclimation score, and starvation resistance for one- and four-week-old flies were treated as eight phenotypes. Data were analyzed using a one-way ANOVA, resampling lines 10,000 times with replacement. Because DGRP lines are inbred homozygous lines, reported heritability estimates are broad sense, and were estimated as:

$$H^2 = \sigma_L^2/(\sigma_L^2 + \sigma_E^2),$$

where σ_L^2 is the among line homozygous genetic variance component, and σ_E^2 is the environmental variance component. The coefficient of homozygous genetic variance was used to assess the effect of age on changes in homozygous genetic variance, and was estimated as:

$$CV_G = 100(\sqrt{\sigma_L^2})/\overline{z_l},$$

where $\overline{z_l}$ is the phenotype mean. Genetic correlations across ages were estimated as:

$$R_G = \sigma^2_{L1,4}/\sqrt{(\sigma^2_{L1}\sigma^2_{L4})},$$

where $\sigma^2_{L1,4}$ is the covariance component of the phenotype across the two ages tested, σ^2_{L1} is the among line homozygous genetic variance component of the phenotype for one-week-old flies, and σ^2_{L4} is the among line homozygous genetic variance component of the phenotype for four-week-old flies. Variance component and genetic correlation estimates were reported as the average of the 10,000 bootstraps, and the variation in the estimate was used to generate standard errors for each term.

The additive effect of an allele (α) for associated polymorphisms was calculated as one-half the difference in phenotypic mean of lines grouped according to homozygous genotype, corrected for *Wolbachia* infection and TE insertions (Falconer and Mackay 1996; Huang et al. 2014):

$$\alpha = (\alpha_1 - \alpha_2)/2.$$

Allele class was designated as major if allele frequency exceeded 50% in the experimental population. Standardized allele effects were calculated as the additive effect divided by the standard deviation of the phenotype:

$$a = \alpha/\sigma_P.$$

Evidence of MA and AP was examined four ways. First, if MA contributes to population-level decline in a phenotype, late acting alleles will inflate CV_G in four-week-old flies. Second, if MA is responsible for age-specific decline in phenotypes, unique regions of the genome should be associated with the phenotype at young and old age. Under AP, regions of the genome that are associated with the phenotype in young and old individuals should overlap. Third, under MA, we expect the genetic correlation between ages for each phenotype to be non-negative, due to the expectation that the additive effects at different ages are independent (Hughes et al. 2002; Leips et al. 2006), while under AP, we expect the genetic correlation between ages for each phenotype to be negative because regions of the genome associated with the phenotype in young and old individuals overlap (Charlesworth and Hughes 1996; Maklakov et al. 2015). Finally, under MA, the additive effects of polymorphisms that are associated with a phenotype in young individuals are expected to have additive effects on the phenotype in old individuals that are smaller but of the same sign (Fig. 2.1), while under AP, polymorphisms associated with a phenotype in young individuals are expected to have additive effects on the phenotype in old individuals that are of the opposite sign (Fig. 2.1).

In addition to testing these predictions of MA and AP within each phenotype across age, we also tested the role of MA and AP in age-related change between phenotypes. As above for each phenotype, we assessed the level of overlap of polymorphisms and genetic correlations between phenotypes and calculated the additive effects of polymorphisms associated with each phenotype on every other phenotype and age measured in our study. For example, the additive effects of polymorphisms associated with acclimation survival at one week were calculated for both the one and four-week non-acclimation survival and starvation resistance responses.

Tests for selection

We used the QTL sign test (QTLST) to the test the direction of the additive effects of associated polymorphisms identified through association mapping. The QTLST was developed by Orr 1998 to determine whether the signs of QTL effects were indicative of directional selection acting on a phenotype. The probability for rejecting the null hypothesis that selection does not influence the phenotype was calculated as in Orr (1998):

$$P = \sum_{i=n_{+obs}}^{n} Pr\{n_+ = i | 2\sum G_1 \geq R\},$$

In this study, we treated n as the number of associated polymorphisms detected for each phenotype, n_{+obs} as the number of these polymorphisms that had a positive additive effect, and G as a vector of all additive effects. Because association mapping does not involve generation of a mapping population from distinct lines, R was simply the standard deviation of the phenotype of the population. An exponential distribution of the polymorphism effects was assumed in our adaptation of this model as in applications of QTLST to QTL data. Because QTLST is sensitive to high variance among additive effects, we also performed the QTLST-EE, which assumes that each of the additive effects are equal in magnitude (Anderson and Slatkin 2003; Rice and Townsend 2012). Analyses were done in R using code adapted from Muir et al. (2014) (Wickham 2009; Hope 2013; Muir et al. 2014; R Core Team 2015).

Results

Phenotypic responses

All phenotypes measured in this study were variable across ages, lines, and sexes (Fig. 2.3; Table 2.1). Two-way interactions among these effects were also significant, except for age

by sex in non-acclimation survival (Table 2.1). The three-way interaction between age, line, and sex explained a significant amount of variation for acclimation survival and starvation resistance as well (Table 2.1). On average, acclimation and non-acclimation survival and starvation resistance decreased with age as expected (Fig. 2.3A, D, J; Table 2.1). However, age-related decline was stronger in non-acclimation survival compared to acclimation survival, resulting in an average acclimation score that increased significantly with age (Fig. 2.3G; Table 2.1). When the sex-specific cold tolerances were analyzed, male flies maintained their capacity to survive the acclimation treatment across age (Fig. 2.3B; adj. $P = 0.64$). However, this maintenance across age was not observed in the non-acclimation treatment (Fig. 2.3E; adj. $P < 0.001$). In females, flies tended to lose the survival capacity at an equal rate for both acclimation and non-acclimation treatments (Fig. 2.3B, E). As a result of these sex-specific age-related responses, female acclimation score did not change with age (adj. $P = 0.46$; Fig. 2.3H), but male acclimation score increased (adj. $P < 0.001$). Thus, the population-level increase in acclimation score was likely driven by retention of cold tolerance in the acclimated male flies. Post hoc comparisons of sex-specific starvation resistance revealed that the age-related average decrease in starvation resistance was primarily driven by a significant decrease in resistance in females (adj. $p < 0.001$; Fig. 2.3K).

Genotype-specific responses for each phenotype were highly variable (Fig. 2.3, right column; Table A.2); an age-related increase in stress resistance was observed for some lines, while responses in other lines remained constant or decreased (Fig. 2.3, right column; Table 2.1). Negative acclimation scores were obtained for several lines screened at one week of age, suggesting that the acclimation treatment had a detrimental effect on survival; however, the vast majority of lines responded positively to this treatment (Fig. 2.3I). When screened at four weeks,

the negative acclimation effect largely disappeared as only two lines had acclimation scores below 0. This change in the pattern of cold tolerance with age may have important implications for the role of plasticity in maintaining stress response with age.

Variation in senescence

To assess the relationship between chronological and physiological age, we measured acclimation survival, non-acclimation survival, and acclimation score on ten randomly selected lines from the DGRP (Table A.1). Non-acclimation survival of physiologically-aged flies was 0 in all lines, so only acclimation survival was compared between the physiologically-aged (Td50) flies and the chronologically-aged (four-week-old) flies. The average acclimation survival of the ten lines selected for the physiological aging experiment was comparable to that of the 101 lines (Table 2.2). The average proportion survived following acclimation for one-week old flies from the ten lines was 74.0 ± 8.1 S.E. compared to 63.4 ± 1.1 S.E. in 101 lines, while the average proportion survived following acclimation in four-week old flies was 52.2 ± 9.9 S.E. compared to 55.2 ± 1.2 S.E. in 101 lines. Regression analysis indicated that acclimation survival measured in four-week old flies was a good predictor of acclimation survival in flies that reached a similar physiological age ($R = 0.83$; $P < 0.003$; Fig. 2.4; Table 2.4). While longevity does vary among DGRP lines, variation in lifespan did not significantly alter the rank order of acclimation survival among the lines. This suggests that variation in longevity among the DGRP lines does not influence the age-related change in detected in phenotypes between young and old flies.

Genetic architecture

In one-week-old flies, association mapping identified 24 polymorphisms and 23 genes associated with acclimation survival, 22 polymorphisms and 14 genes associated with non-acclimation survival, 45 polymorphisms and 23 genes associated with acclimation score, and 20

polymorphisms and 9 genes associated with starvation resistance (Table 2.3). In four-week-old flies, association mapping identified 31 polymorphisms and 28 genes associated with acclimation survival, 69 polymorphisms and 48 genes associated with non-acclimation survival, 26 polymorphisms and 6 genes associated with acclimation score, and 27 polymorphisms and 22 genes associated with starvation resistance (Table 2.31). Surprisingly, no polymorphisms or genes were shared within phenotype across age or between phenotypes (Fig. 2.5; Table A.3).

Several polymorphisms were associated with genes that have been previously associated with cold-, starvation-, or age-related phenotypes, and were distributed across the phenotypes measured in this study (Table A.3 and references therein). Out of all genes identified in our study (Table 2.3, Table A.3), 54 have been previously associated with cold acclimation or with a cold-sensitive phenotype in *Drosophila*, 18 have been previously associated with starvation response or stress, and 59 have been previously associated with aging or lifespan. For example, *Cht2*, involved in chitin binding, has been previously associated with cold acclimation response (MacMillan et al. 2016) and was associated with four-week starvation resistance in this study. *Meltrin*, associated with one-week starvation resistance in our study, has been previously associated with cold acclimation response and age-specific fitness (Durham et al. 2014; MacMillan et al. 2016). *CG10916*, associated with four-week non-acclimation survival in our study, has previously been associated with determination of adult lifespan as well as cold acclimation (Paik et al. 2012; Vermeulen et al. 2013; MacMillan et al. 2016). Several genes (28) were also associated with oxidative stress resistance, which has been associated with aging and senescence (Schwarze et al. 1998). For example, *decay*, *rg*, and *Pde1c* have been previously associated with oxidative stress and were associated with four-week acclimation survival or one-week non-acclimation survival in our study (Table A.3). Additional details describing the

function of each gene and associated references are listed in Table A.3. Despite the previous reporting of genes that are associated with aging or stress phenotypes, no gene ontology (GO) categories were overrepresented following enrichment analysis.

Evolutionary theories of aging and the decline in stress response

Shifting genetic architecture within phenotypes across age

Each phenotype was associated with a unique set of polymorphisms across age and among phenotypes (Table 2.3, Fig. 2.5, Table A.3). Thus, the lack of overlap of associated polymorphisms across age within phenotypes suggests the genetic architecture shifted and that genetic control of the phenotypes was age-specific. MA not only predicts that associated polymorphisms at each age are unique, but also that associated polymorphisms have positive additive effects in young individuals that remain positive or approach 0 in older individuals. Conversely, AP predicts associated polymorphisms with positive additive effects on a phenotype in young individuals will have negative additive effects on a phenotype in old individuals. We calculated the additive effects of all significantly associated polymorphisms for the stress response phenotypes that declined with age (acclimation survival, non-acclimation survival, and starvation resistance; Fig. 2.6) for the one- and four-week response. When additive effects of the associated polymorphisms in one-week-old flies were calculated for the same phenotype in four-week-old flies, the additive effects were either closer to 0 or of the same sign (i.e. not antagonistic; Fig. 2.6; Table 2.4). The reverse comparison resulted in the same pattern; for example, additive effects of four-week acclimation survival polymorphisms on one-week acclimation survival were smaller and closer to 0 than the additive effects of four-week acclimation survival polymorphisms on four-week acclimation survival (Fig. 2.6; Table 2.4). This pattern of unique associated polymorphisms and additive effects that decrease with age was

observed for each of the phenotypes that declined with age (starvation resistance, acclimation survival, and non-acclimation survival) and is consistent with MA.

MA also predicts that, for phenotypes that decline with age, the coefficient of genetic variance (CV_G) will increase with age and that the genetic correlation between the phenotype in young individuals with the phenotype in old individuals will be non-negative (either not different from 0 or positively correlated). While AP does not have predictions specific to the coefficient of genetic variance (Partridge and Barton 1993; Houle et al. 1994), AP does predict a negative genetic correlation between the phenotype in young individuals and in old individuals. Variance component analysis provided further support for MA for each phenotype. We observed increase in CV_G for each phenotype, although the increase was slight for starvation resistance ($CV_{G,\ young}$ = 13.51 versus $CV_{G,\ old}$ 14; Table 2.3, Table 2.5). Also consistent with MA predictions, genetic correlations for each phenotype across age were either significantly positive (acclimation survival: $R_G = 0.70 \pm 0.3$ S.E.; starvation resistance: $R_G = 0.69 \pm 0.3$ S.E.), or not statistically different from 0 (non-acclimation survival: $R_G = 0.43 \pm 0.3$ S.E.; Table 2.6). The lack of negative genetic correlations for each phenotype between young and old individuals combined with the increase in the coefficient of genetic variance with age provides additional evidence that supports the MA mechanism.

Shifting genetic architecture between phenotypes with age

Comparisons of associated polymorphisms for each phenotype measured in this study demonstrated unique genetic architecture for each phenotype across age. However, the lack of overlap in associated polymorphisms does not necessarily mean that the polymorphisms detected for each phenotype do not influence variation in other phenotypes at other ages, but rather that the additive effect was too small to be detected by association mapping. Polymorphisms

associated with one phenotype (e.g. one-week acclimation survival) may have small but important additive effects on other phenotypes (e.g. four-week starvation resistance), and if this is the case, the interpretation is similar to the comparison of additive effects within phenotypes across age reported above (e.g. Fig. 2.1). If the additive effect is close to 0 and of the same sign, this supports the MA mechanism; if the additive effect is different from 0 and of the opposite sign, this supports the AP mechanism (Fig. 2.1).

To test for the presence of pleiotropic effects of associated polymorphisms on other phenotypes measured in this study, we calculated the average standardized additive effect of associated polymorphisms for each phenotype on every other phenotype and age (Fig. 2.6; Table 2.4). For example, the additive effects of the set of associated polymorphisms with acclimation survival in one-week-old flies were calculated for one- and four-week non-acclimation survival and one- and four-week starvation resistance (Fig. 2.6A). Confidence intervals were used to determine if the calculated average additive effects were different from 0 (Table 2.4).

Approximately half of the calculated average effects were not different from 0 (55.6%), and 27.8% of the comparisons resulted in average effects with a sign opposite that of the average effect of the polymorphisms in the phenotype with which they were significantly associated (suggesting an antagonistic relationship; Table 2.4). The antagonistic effects of polymorphisms between phenotypes were often, but not always, reciprocal (Fig. 2.6). For example, polymorphisms associated with one-week acclimation survival had an average antagonistic additive effect on starvation resistance at both ages (Fig. 2.6A), while polymorphisms associated with four-week starvation resistance had an average antagonistic additive effect on only one-week acclimation survival (Fig. 2.6F). In an aging context, evidence from additive effect comparisons support both AP and MA across age, depending on the phenotypes being compared;

however, MA was more common based on apparent independence of additive effects (average additive effects were not different from 0).

Phenotypes that appeared to be antagonistically pleiotropic such that polymorphisms increased the phenotype in young flies but decreased the phenotype in old flies include acclimation survival and starvation resistance (Fig. 2.6A, B, F) and non-acclimation survival and starvation resistance (Fig. 2.6C, D, F). Polymorphisms associated with one-week starvation resistance did not have an antagonistic effect on average on any other phenotype, although several individual polymorphisms did have antagonistic effects on other phenotypes (Fig. 2.6E; Table 2.4). The antagonistic relationship between one-week acclimation survival and four-week starvation resistance was further supported by a significant negative genetic correlation between traits across ages (R_G = -0.47 ± 0.2 S.E.; Table 2.6). However, all other combinations of phenotypes involved effects and genetic correlations that were not different from 0 (Fig. 2.6; Table 2.4, 2.6), and were thus more consistent with the predictions of MA.

Evidence of selection and phenotypic trade-offs

With the exception of acclimation score, the additive effects of the majority of polymorphisms significantly associated with the phenotypes measured in our study were of the same sign (i.e. most additive effects were positive or negative; Fig. 2.6 and 2.7, Table A.3). To determine if more additive effects of positive sign were associated with the phenotype than expected by chance, we used the QTLST to test for evidence of selection. More positive additive effects than expected by chance were observed for one-week acclimation survival, non-acclimation survival, and acclimation score suggesting selection increased these phenotypes in the founding population of the DGRP (Fig. 2.6 and Fig. 2.7A, C, E). The QTLST was also significant for four-week acclimation survival, non-acclimation survival, and both one- and four-

week starvation resistance, suggesting selection has acted to decrease these phenotypes (Fig. 2.6 and Fig. 2.7B, D, G, H). However, because the effectiveness of natural selection is expected to decline with age, this significant result is likely the result of a correlated response resulting from selection on young phenotypes. The signs of the additive effects of polymorphisms associated with acclimation score in four-week-old flies were more mixed than any other phenotype, leading to a non-significant QTLST for this phenotype (Fig. 2.6 and Fig. 2.7F).

Discussion

Genetic variation in age-specific decline in stress tolerance

As expected, the average phenotypic responses for most phenotypes declined with age, with the exception of plasticity measured as acclimation score (Fig. 2.3). For each phenotype, we observed significant genetic variation across ages, with some genotypes exhibiting increased stress resistance with age. We investigated variation in longevity among DGRP lines by comparing cold tolerance responses measured at four weeks to those in physiologically aged flies at the point when the population reached Td50. We know that lifespan for virgin female flies varies from approximately 20 days to approximately 80 days in the DGRP (Ivanov et al. 2015). This variation could result in an inequivilence of line comparisons at four weeks. Based on Ivanov et al. (2015) longevity estimates, some lines may be only half way through their lifespan while others may be closer to the end of their lifespan at four weeks of age. Furthermore, genetic shifts known to be associated with senescence would be further advanced in lines that age more quickly (Pletcher et al. 2002). The significant positive correlation in acclimation survival between chronologically aged (four-week) flies and physiologically aged (Td50) flies suggests that, despite variation in the rate of senescence among lines, the four-week point is representative of how an “old” fly responds to cold stress. Additionally, this subset of the DGRP lines reached

Td50 well after four weeks of age (Table 2.2), indicating that the four-week aging period does not lead to many lines entering a late-life plateau in mortality and that our lines were likely in the aging phase (Charlesworth 2001; Curtsinger 2016).

The only phenotype in our study that behaved unexpectedly and did not decline with age was plasticity measured as acclimation score. Acclimation score was significantly higher in four-week-old flies (Fig. 2.3G), where we expected this phenotype to remain constant across age. The age-related response in acclimation score was driven by the male response. Four-week-old male flies had a stronger age-related decline in non-acclimation survival compared to acclimation survival. Therefore, the observed increase in acclimation score for our population has at least two interpretations. First, plasticity at the population level may increase with age, potentially as a compensatory mechanism to overcome the overall loss of basal cold tolerance (Fig. 2.3F). Throughout the season, natural populations of *D. melanogaster* are expected to be composed of increasingly old individuals such that by the time temperatures begin to cool at the beginning of the fall season, a greater proportion of populations is composed of older individuals (Behrman et al. 2015). If older individuals are less cold tolerant, they may still be able to tolerate cold temperature exposures through increased capacity for adaptive plasticity through acclimation. Second, acclimation pretreatment appears to have had a less detrimental effect on survival in four-week-old flies compared to one-week-old flies (Fig. 2.3I). In one-week-old flies, acclimation improved survival in the majority of lines; however, several lines (14%) had negative acclimation scores, indicating that exposure to 4°C prior to the -6°C exposure was more damaging than the -6°C exposure alone (Fig. 2.3I; Gerken et al. 2015). Only 2% of lines tested had negative acclimation scores at four weeks suggesting that acclimation may be more likely to either improve or not affect survival following cold stress at four weeks. Shifts in the effect of

acclimation on survival were observed when genotypes were reared under different developmental conditions or experienced altered thermal regimes (Kelty and Lee 2001; Gerken et al. 2015); our results suggest that the age of the individual has an important influence on the effect of acclimation on survival as well.

MA describes age-related change within individual phenotypes

When each phenotype was considered separately across age, we found support for MA, satisfying predictions based on analysis of quantitative genetic parameters (Charlesworth and Hughes 1996; Charlesworth 2001; Hughes et al. 2002; Leips et al. 2006; Reynolds et al. 2007). First, the coefficient of genetic variance increased with age for each phenotype (Table A.1). The increase in CV_G in starvation resistance was less drastic than other phenotypes, but when sexes were analyzed separately, the increase was more dramatic (Table 2.7). An increase in CV_G indicates that a greater proportion of the phenotypic variance can be explained genetically at four weeks of age (Houle et al. 1994; Charlesworth and Hughes 1996; Charlesworth 2001) and this increase is consistent with the hypothesis that the age-related decline in the phenotype is the result of the accumulation of deleterious age-specific polymorphisms that influence variation in the phenotype (Engström et al. 1989; Charlesworth and Hughes 1996; Charlesworth 2001).

Second, MA predicts that a phenotype is controlled by unique sets of genes across age (Medawar 1952; Rose 1991; Partridge and Barton 1993; Charlesworth 1994, 2001; Charlesworth and Hughes 1996; Maklakov et al. 2015). We detected unique sets of polymorphisms that were associated with each phenotype across age. This is consistent with age-specific association patterns presented by (Durham et al. 2014) who determined age-related change in fecundity was also influenced by MA. The genetic independence of phenotypes across age was further supported by the non-significant or significantly positive genetic correlations for each phenotype

across age (Table 2.6). While positive genetic correlations suggest that the genetic control of the phenotype across age is not independent (inconsistent with MA), Charlesworth (2001) and Maklakov et al. (2015) suggest that positive pleiotropy can lead to significantly positive genetic correlations across age under MA.

Positive pleiotropy (Maklakov et al. 2015) expands on MA predictions in that, instead of limiting polymorphisms to very narrow windows of effect, polymorphisms can influence a wider window of ages but with lower additive effects (Maklakov et al. 2015). Thus, the positive genetic correlations across age are the result of the associated polymorphisms having a slightly wider window of age-specific effects that ultimately influence the phenotype at other ages. This pattern was observed for acclimation and non-acclimation survival and starvation resistance across age; for all phenotypes, the four-week associated polymorphisms had negative additive effects on the one-week phenotype that were smaller than the effect of the polymorphisms on the four-week phenotype. This suggests that four-week polymorphisms that led to decline in each phenotype do have small pleiotropic effects (in the same direction) at one week of age. The failure of natural selection to remove polymorphisms that have small negative effects early in life and larger negative effects later in life is one way in which senescence can evolve and is consistent with expectations of positive pleiotropy under MA (Wachter et al. 2013, 2014; Maklakov et al. 2015).

Our third piece of evidence to support MA comes from our novel approach of calculating the additive effects of polymorphisms across age. We calculated the additive effects of polymorphisms associated with the one-week phenotype in the four-week phenotype data (and vice versa). Under MA, we expected the additive effect of the one-week polymorphisms to be smaller and in the same direction (i.e. they have the same sign) when the additive effects were calculated for the four-week response data (Maklakov et al. 2015). If the sign of a particular

polymorphism had flipped (a polymorphism with a positive effect in one age had a negative effect in the other age), this would have suggested that an antagonistic relationship existed and would have supported AP (Maklakov et al. 2015). For all phenotypes, the calculated additive effects of age-specific polymorphisms across age were either closer to 0 and/or in the same direction, providing definitive support for the role of MA in the age-related decline in the stress responses measured (Fig. 2.6).

MA and AP describe age-related variation between phenotypes

Our novel extension of association mapping through the calculation of additive effects of polymorphisms across phenotypes allowed us to investigate the role MA and AP on age-specific responses between phenotypes as well. Though each phenotype and age was associated with a unique set of polymorphisms (consistent with predictions for MA), we found support for AP between several phenotypes (Fig. 2.6). We observed a significantly negative phenotypic correlation between one-week acclimation survival and both one- and four-week starvation resistance, corroborating a pattern reported by Hoffmann et al. (2005) (Table 2.6). We also observed a significantly negative genetic correlation between one-week acclimation survival and four-week starvation resistance (Table 2.6), suggesting that AP (between one-week acclimation survival and four-week starvation resistance) influenced age-related change in these phenotypes. When the additive effects of polymorphisms associated with four-week starvation were calculated for both one-week cold tolerance phenotypes (Table 2.4), all four-week starvation resistance associated polymorphisms, which had negative additive effects on four-week starvation resistance, had positive additive effects on one-week acclimation survival and one-week non-acclimation survival (Fig. 2.6F). The change in sign of the additive effects of four-week starvation resistance associated polymorphisms on both of the one-week cold tolerance

phenotypes is strong evidence to support the role of AP in age-related decline in starvation resistance.

Additional examples of AP existed between phenotypes in our data as well and were identified through confidence interval analysis of additive effects (Fig. 2.6; Table 2.4). Specifically, one-week acclimation and non-acclimation survival polymorphisms had positive effects on their respective phenotypes across age but negative additive effects on four-week starvation resistance (Fig. 2.6A – D; Table 2.4). This pattern is consistent with that discussed above and again suggests that age-related change in starvation resistance is influenced by AP with cold tolerance. Interestingly, four-week acclimation and non-acclimation survival polymorphisms had largely positive additive effects on one-week starvation resistance (Fig. 2.6B, D; Table 2.4). This pattern suggests that AP may also be contributing to age-related decline in acclimation and non-acclimation survival. With evidence from our examination of acclimation and non-acclimation survival across age (discussed above), and the apparent role of MA and positive pleiotropy for age-related change within these phenotypes, it is evident that it may not be possible to fully disentangle the roles of MA and AP on the age-related decline in phenotypes. In essence, polymorphisms that increase one phenotype in young individuals and decrease another phenotype in old individuals through AP may also contribute to age-related change within the phenotype through positive pleiotropy under MA. These results demonstrate the need for caution in interpreting the lack of overlap in significant associated polymorphisms as support for MA in isolation of other evidence because AP may still be playing an important role in the age-related change in phenotypes.

We also found support for the role of MA between phenotypes across age. Age-related change in acclimation survival and non-acclimation survival appears to be evolving largely

independently of the other phenotype under MA as we found either non-significant or significantly positive phenotypic and genetic correlations between all age combinations of these phenotypes (Table 2.4, 2.6). The calculated additive effects of polymorphisms between phenotypes and ages uphold this interpretation to a large degree as well (Fig. 2.6A – D; Table 2.4). One-week acclimation survival polymorphisms, which had positive effects on one-week acclimation survival, all had positive additive effects when calculated for both one- and four-week non-acclimation survival (Fig. 2.6A). On average, additive effects of polymorphisms associated with four-week acclimation survival had additive effects that were not different from zero, although some individual polymorphisms did have antagonistic effects on one- and four-week non-acclimation survival. All but two polymorphisms associated with one-week non-acclimation survival had additive effects of the same sign on one- and four-week acclimation survival, and all polymorphisms associated with four-week non-acclimation survival had additive effects of the same sign on one- and four-week acclimation survival. While the small number of individual polymorphisms with antagonistic additive effects across phenotype may impact age-related change in these cold tolerance phenotypes, it likely that this impact is small in comparison to the role of MA and positive pleiotropy.

Natural selection shapes phenotypic variation across age

Our data recapitulate previously reported relationships between different measures of cold tolerance and starvation resistance (Table 2.6; Hoffmann et al. 2005; Sinclair and Roberts 2005; Gerken et al. 2015); however, we have added another layer to these relationships by considering the influence of age and natural selection on these phenotypes. Specifically, the direction of the additive effects of polymorphisms associated with each phenotype may reflect the role natural selection has played in the evolution of the phenotype. Through applying a basic

sign test (QTLST probabilities) to the additive effects of the associated age-specific polymorphisms, it is evident that the signs of the effects are not randomly associated with the frequency of the allele (Fig. 2.7A – E, G, H). If a phenotype is evolving neutrally, polymorphisms that influence the phenotype are equally likely to have positive or negative effects (Orr 1998; Anderson and Slatkin 2003; Rice and Townsend 2012). Signatures of directional selection are detected when this null hypothesis is rejected (Orr 1998; Rieseberg et al. 2002; Anderson and Slatkin 2003; Rice and Townsend 2012; Muir et al. 2014). Because we observed an overabundance of major alleles with positive effects on one-week acclimation and non-acclimation survival, this suggests that natural selection favored polymorphisms that increase cold tolerance phenotypes in the population from which the DGRP was established (Fig. 2.6A, C and 2.7A, C; Table A.3). This finding is consistent with evidence of selection for cold tolerance in natural populations of *D. melanogaster* (Bergland et al. 2014a), as well as previous reports of majority positive additive effects of polymorphisms associated with chill coma recovery (Mackay et al. 2012).

Conversely, most of the polymorphisms associated with cold tolerance at four weeks of age were negative (Fig. 2.6B, D), indicating that the major alleles decreased survival following cold stress. While the QTLST was significant for these late-acting polymorphisms, it is very unlikely that natural selection directly led to this pattern. Instead, polymorphisms associated with acclimation and non-acclimation survival in old individuals likely arose through mutation and were maintained in the population because their negative effect on survival in young individuals was small relative to the four-week additive effects (Houle et al. 1994; Charlesworth 2001; Maklakov et al. 2015; Fig. 2.6B, D). Similarly, in both young and old individuals, most of the additive effects of starvation resistance associated polymorphisms were negative (Fig. 2.6E, F).

This pattern suggests that age-related change in starvation resistance is influenced by positive selection on acclimation and non-acclimation survival in young individuals. Alternatively, the effectiveness of natural selection on starvation resistance may be constrained by pleiotropy between one-week acclimation survival and one-week starvation resistance (many one-week acclimation and non-acclimation polymorphisms had negative effects on one-week starvation resistance; Fig. 2.6A, C). Some positive selection on starvation resistance in young individuals may provide a mechanism for the retention of four-week acclimation and non-acclimation survival associated polymorphisms that have increasingly negative effects with age.

Implications for evolutionary theories of aging

Efforts to more fully understand and test evolutionary theories of aging have encouraged expansion and clarification of predictions of both MA and AP mechanisms (Houle et al. 1994; Charlesworth 2001; Reynolds et al. 2007; Wachter et al. 2013, 2014; Maklakov et al. 2015). Our novel extension of existing methods not only revealed the relative importance of MA and AP for age-related change in stress response, but also verified recent hypotheses that present expansions on the theory of MA. When originally formulated, the MA mechanism predicted that fitness was controlled by polymorphisms that had very narrow windows of effect (Medawar 1952; Rose 1991; Charlesworth and Hughes 1996), but evidence from several studies has indicated that it is more likely that polymorphisms which contribute to late-life decline in fitness and age-related change in phenotypes have wide windows and increasingly large effects across age (Houle et al. 1994; Charlesworth 2001; Maklakov et al. 2015). Patterns of additive effects of associated polymorphisms identified in our study align well these elaborations of MA predictions and provide empirical support for positive pleiotropy. In addition, we provide evidence supporting the potential for positive genetic correlations between young and old phenotypes under MA,

demonstrating through patterns of additive effects of polymorphisms that, while the size of the effect may change across age, the direction of the effect is often preserved (Reynolds et al. 2007; Maklakov et al. 2015).

Our use of calculated additive effects also allowed us to overcome difficulties in characterizing the role of AP in age-related change in phenotypes. The isolated analysis of the coefficient of genetic variance and genetic correlation is problematic because the signature of AP may be too small to detect due to near fixation of segregating alleles at the antagonistic loci (Schnebel and Grossfield 1988; Partridge and Barton 1993; Moorad and Promislow 2009). For this reason, association mapping may be particularly biased against the detection of AP loci. However, by comparing the effects of polymorphisms calculated for each age and phenotype pair, we are able to overcome this bias against polymorphisms that have small effects.

Very few studies present convincing evidence of the influence of both MA and AP on age-related change within and among phenotypes (but see Leips et al. 2006), but we have demonstrated that both mechanisms contribute to age-related change in stress response. It is clear from our results that individual polymorphisms that are significantly associated with phenotypes at different ages can contribute to age-related decline within and among phenotypes in patterns that are consistent with both MA and AP. Thus, the evolution of senescence and associated decline in fitness is influenced by a combination of natural selection acting on correlated phenotypes that have non-independent antagonistic genetic architectures, as well as the accumulation of polymorphisms with negative effects that strengthen with age. It is likely that similar patterns will be observed for other phenotypes related to fitness as well, adding to our understanding of how evolution of aging and multivariate evolution are tightly intertwined.

Acknowledgments

We thank Jennifer L. Delzeit, Olivia Eller, Mariah Brown, and Paul Klawinski for assistance with experimental fly set up and maintenance. We also thank Michi Tobler and Phil Freda for comments on earlier versions of this manuscript. The National Science Foundation awards NSF1051770 and NSF1156571 to T.J.M supported this work.

Figures and Tables

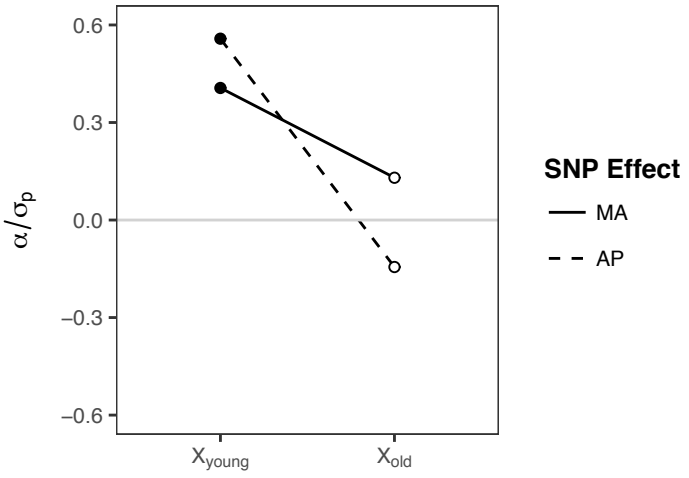

Figure 2.1 An example of how calculated additive effects (α/σ_p) of polymorphisms identified through association mapping can be used to characterize the role of mutation accumulation (MA) and antagonistic pleiotropy (AP) on the age-related change in hypothetical phenotype *X*. Two polymorphisms that are associated with phenotype *X* in young individuals (X_{young}) but not in old individuals (X_{old}). The additive effects of the associated polymorphisms at young ages are represented by the closed symbols. The open symbols indicate the additive effects of X_{young} polymorphisms on the phenotype at old age (X_{old}). The polymorphism connected with a solid line demonstrates the shifting effect across age consistent with MA, where the polymorphism has a small additive effect of the same sign (positive) on the phenotype at old age (X_{old}) as the effect of the polymorphism on X_{young}. The polymorphism connected with a dashed line illustrates an AP pattern, where the polymorphism has an additive effect that is of the opposite sign (negative) on X_{old} compared to the effect of the polymorphism on X_{young}.

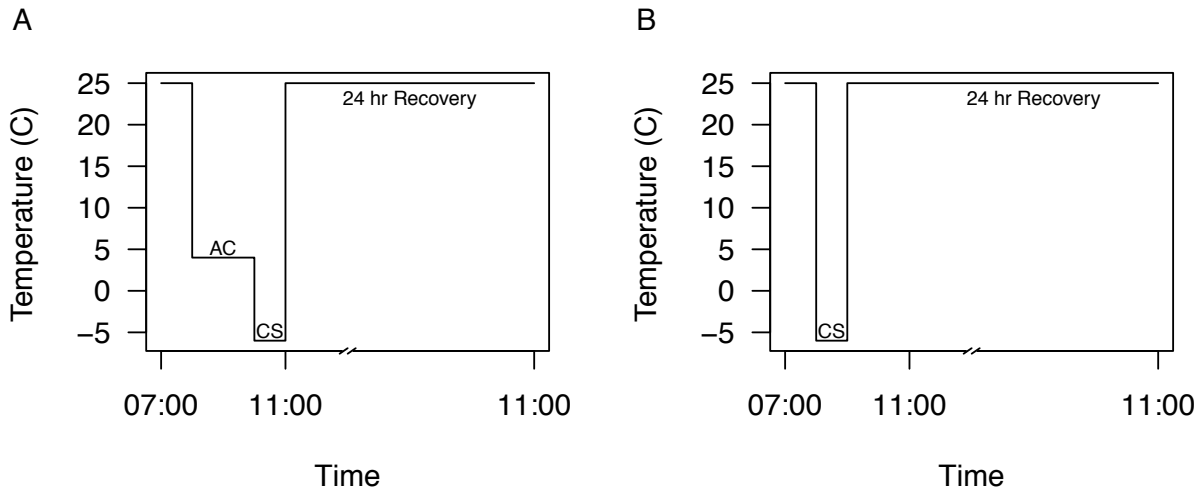

Figure 2.2 Graphical representation of treatments: acclimation (A) and non-acclimation treatment (B). Flies were maintained at 25°C during rearing and recovery, and lights on occurred at 07:00hrs. A. Flies were transferred from 25°C to 4°C for two hours for the acclimation (AC) treatment and then were transferred immediately to -6°C for one hour for the cold shock treatment (CS). Flies were placed on fresh media and allowed to recover for 24 hours at 25°C. B. Flies were transferred to -6°C for one hour for the cold shock treatment (CS) and were allowed to recover at 25°C for 24 hours on fresh media.

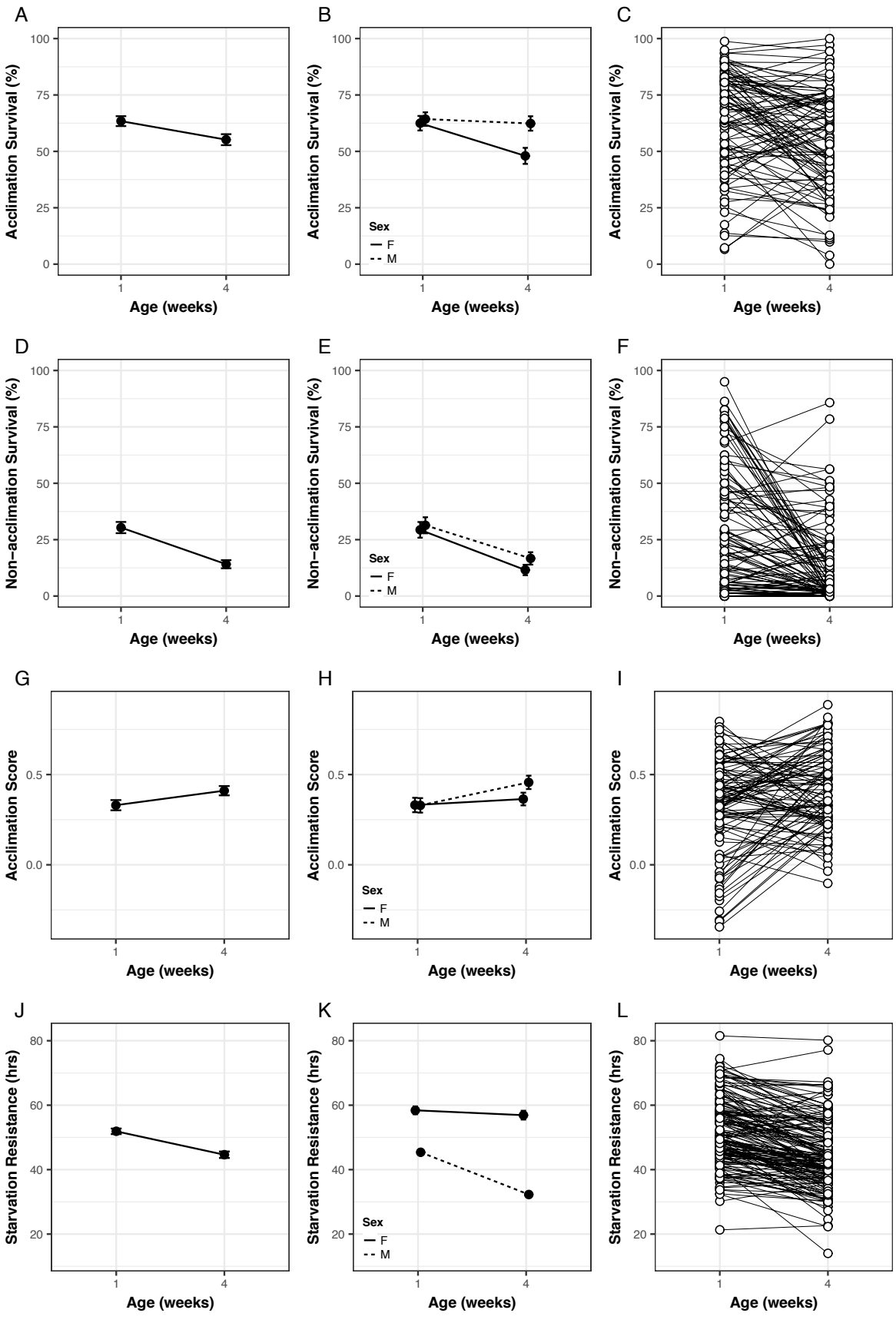
A
B
C
D
E
F
G
H
I
J
K
L
Acclimation Survival (%)
Non−acclimation Survival (%)
Acclimation Score
Starvation Resistance (hrs)
Age (weeks)
Sex
F
M

Figure 2.3 Plots of mean phenotypes across age are shown with 95% CI for the total population (left column) and by sex (middle columns); among-line variation observed for each phenotype is shown as line averages (right column). A. Average acclimation survival declined significantly with age ($F_{1,1212}$ = 49.5, P < 0.001). B. Males retained their ability to survive the acclimation treatment, while acclimation survival significantly declined in females (age by sex: $F_{1,1212}$ = 28.9, P < 0.001). C. Acclimation survival significantly varied among the 101 DGRP lines (age by line: $F_{100,1212}$ = 3.21, P < 0.001). D. Average non-acclimation survival declined significantly with age ($F_{1,1212}$ = 215.4, P < 0.001). E. Non-acclimation survival significantly declined in both females and males to a similar degree (age by sex: $F_{1,1212}$ = 1.98, P = 0.16). F. Non-acclimation survival significantly varied among the 101 DGRP lines (age by line: $F_{100,1212}$ = 5.88, P < 0.001). G. Average acclimation score increased significantly with age ($F_{1,1212}$ = 25.13, P < 0.001). H. Acclimation score significantly increased in males, but remained consistent across age for females (age by sex: $F_{1,1212}$ = 8.68, P < 0.01). I. Acclimation score significantly varied among the 101 DGRP lines (age by line: $F_{100,1212}$ = 3.18, P < 0.001). J. Average starvation resistance decreased significantly with age ($F_{1,1623}$ = 893.0, P < 0.001). K. Starvation resistance significantly decreased in both sexes, but to a larger degree in females (age by sex: $F_{1,1623}$ = 567.0, P < 0.001). L. Starvation resistance significantly varied among the 164 DGRP lines (age by line: $F_{163,1623}$ = 6.0, P < 0.001).

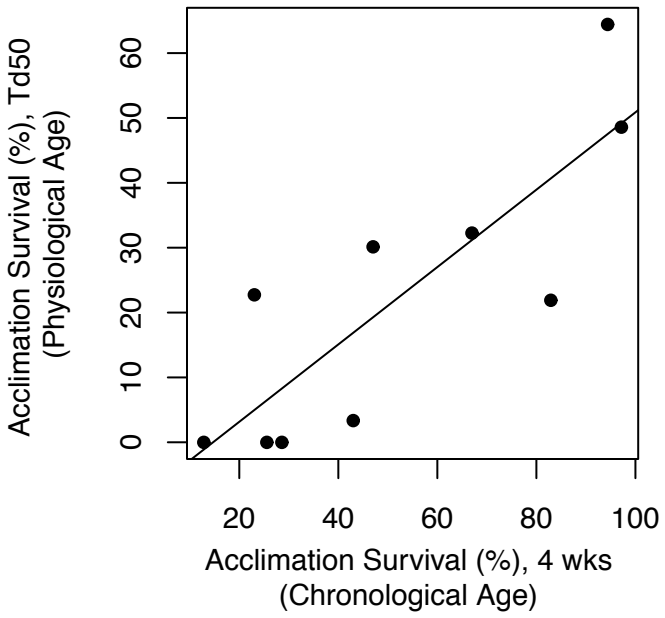

Figure 2.4 Physiological versus chronological age. Stress tolerance of 4-week-old flies was correlated with stress tolerance of flies that were a similar physiological age (Td50). Acclimation and non-acclimation survival was measured for 10 DGRP lines that were aged to four weeks (chronological age) and until populations in experimental vials reached 50% of the starting population (physiological age). Physiologically-aged flies did not survive the non-acclimation treatment. Acclimation survival in four-week old flies is a good predictor of acclimation survival in flies that are approximately the same physiological age ($R = 0.833$, $P < 0.003$). Points shown indicate average acclimation survival for the 10 DGRP lines.

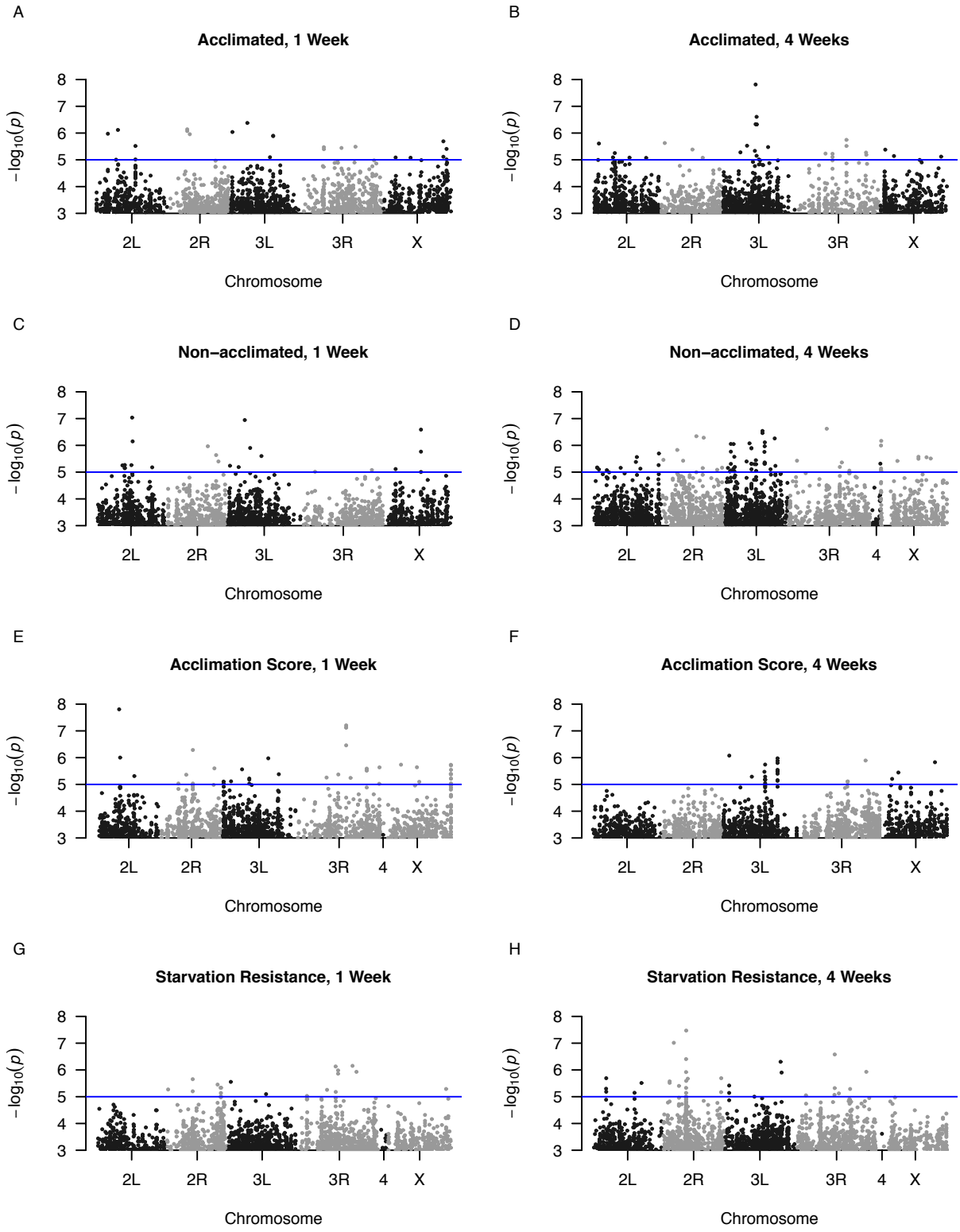

Figure 2.5 Manhattan plots of each phenotype. The significance threshold is indicated by the horizontal line at –log(5).

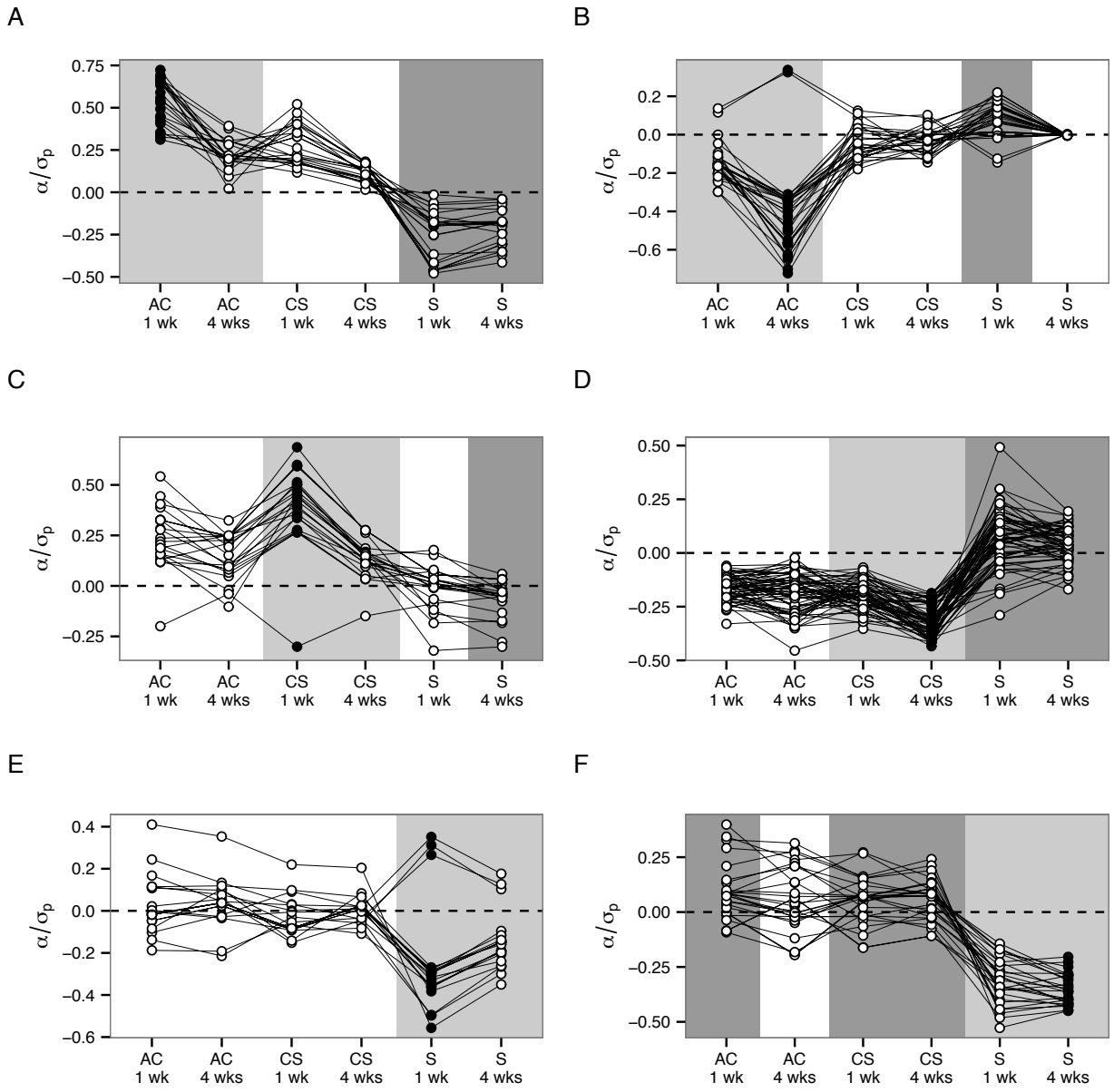

Figure 2.6 Standardized additive effects (α / σ_p) of polymorphisms associated with each phenotype across age (A – F). In each plot, black solid points indicate which age and phenotype the polymorphisms were significantly associated with. Open points indicate the calculated additive effects of those polymorphisms on each of the other phenotypes. The light grey shading highlights the 'within phenotype' change in the additive effect of polymorphisms across age. Darker grey shading highlights 'between phenotype' comparisons where antagonistic effects of polymorphisms were found based on analysis of 95% confidence interval around the average additive effect (Table B.6). Unshaded sections of each plot indicate calculated additive effects that are consistent with MA relative to the additive effects of the polymorphisms on their associated phenotype. In each plot, AC indicates acclimation survival, CS indicates non-acclimation survival, and S indicates starvation resistance. A. Polymorphisms associated with acclimation survival of one-week-old flies all had positive additive effects on the phenotype that

became smaller when the additive effects were calculated for four-week-old acclimation survival and non-acclimation survival of one- and four-week-old flies. Polymorphisms had antagonistic effects on starvation resistance at both ages. B. Polymorphisms associated with acclimation survival of four-week-old flies had smaller additive effects on every other phenotype except one-week starvation resistance, where the effects were largely antagonistic. C. Polymorphisms associated with non-acclimation survival of one-week-old flies had smaller additive effects on every other phenotype except four-week starvation resistance, where the effects were largely antagonistic. D. Polymorphisms associated with non-acclimation survival of four-week-old flies had smaller additive effects on every other phenotype except starvation resistance at both ages, where the effects were largely antagonistic. E. Polymorphisms associated with one-week starvation resistance had smaller additive effects on every other phenotype. F. Polymorphisms associated with four-week starvation resistance had antagonistic effects on every other phenotype except acclimation survival in four-week-old flies and starvation resistance in one-week-old flies.

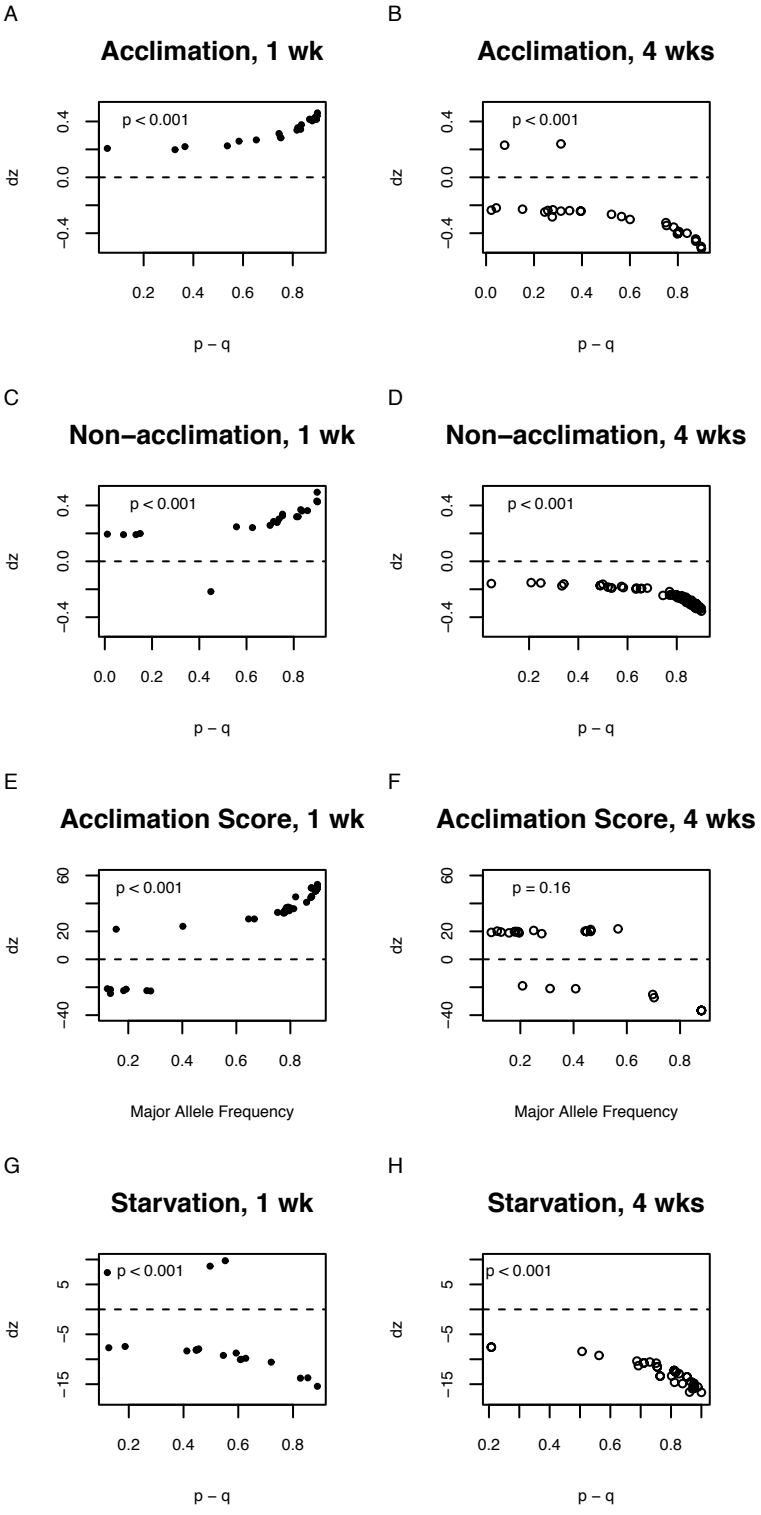

Figure 2.7 Plots of allele frequency (p – q) against the effect of significant polymorphisms reflected as change in phenotype (dz). Inset numbers are QTLST probabilities that directional selection influenced the phenotype.

Table 2.1 Mixed-model ANOVA testing the effect of DGRP line, age, and sex on each phenotype measured.

Trait	Source	df	MS	SS	*F*	*P*
Acclimation	Age	1	2.72	2.72	49.50	< 0.001
	Line	100	62.80	0.63	11.40	< 0.001
	Sex	1	2.66	2.66	48.40	< 0.001
	Age x Line	100	17.60	0.18	3.21	< 0.001
	Age x Sex	1	1.59	1.59	28.90	< 0.001
	Line x Sex	100	17.40	0.17	3.17	< 0.001
	Age x Line x Sex	100	13.00	0.13	2.37	< 0.001
	Residuals	1212	0.06	67.38		
Non-Acclimation	Age	1	10.60	10.60	215.40	< 0.001
	Line	100	55.70	0.56	11.30	< 0.001
	Sex	1	0.50	0.50	10.20	< 0.01
	Age x Line	100	29.00	0.29	5.88	< 0.001
	Age x Sex	1	0.10	0.10	1.98	0.16
	Line x Sex	100	7.63	0.08	1.55	< 0.001
	Age x Line x Sex	100	5.86	0.06	1.19	0.11
	Residuals	1212	0.05	60.50		
Acclimation Score	Age	1	2.60	2.60	25.13	< 0.001
	Line	100	61.75	0.62	5.97	< 0.001
	Sex	1	0.85	0.85	8.19	< 0.01
	Age x Line	100	32.90	0.33	3.18	< 0.001
	Age x Sex	1	0.90	0.90	8.68	< 0.01
	Line x Sex	100	14.43	0.14	1.40	< 0.01
	Age x Line x Sex	100	11.94	0.12	1.16	0.15
	Residuals	1212	0.10	125.40		
Starvation Resistance	Age	1	29559	29559	893	< 0.001
	Line	163	224084	1375	42	< 0.001
	Sex	1	219551	219551	6632	< 0.001
	Age x Line	163	32158	197	6	< 0.001
	Age x Sex	1	18756	18756	567	< 0.001
	Line x Sex	163	63716	391	12	< 0.001
	Age x Line x Sex	163	20806	128	4	< 0.001
	Residuals	1623	33	53732		

Table 2.2 Average acclimation and non-acclimation % (S.E.) survival for 10 DGRP lines at four weeks (chronological age) and Td50 (physiological age).

	Chronological Age			Physiological Age		
Line	**Age**	**AC**	**CS**	**Age**	**AC**	**CS**
RAL_304	4 wks	97.2 (1.9)	50.2 (13.4)	72.5 days	48.6 (10.8)	0
RAL_774	4 wks	28.6 (5.0)	1.25 (1.3)	79.5 days	0.00 (0.0)	0
RAL_153	4 wks	47.0 (14.3)	2.81 (1.9)	55.0 days	30.1 (11.3)	0
RAL_177	4 wks	94.4 (3.7)	15.6 (12.4)	44.5 days	64.4 (9.9)	0
RAL_336	4 wks	23.1 (9.4)	2.81 (1.9)	39.0 days	22.7 (14.9)	0
RAL_359	4 wks	82.9 (3.4)	1.25 (1.3)	63.0 days	21.91 (8.6)	0
RAL_361	4 wks	43.0 (13.5)	7.78 (5.4)	69.0 days	3.33 (2.5)	0
RAL_367	4 wks	12.8 (3.6)	0.00 (0.00)	63.5 days	0.00 (0.0)	0
RAL_440	4 wks	25.6 (10.7)	0.00 (0.00)	69.0 days	0.00 (0.0)	0
RAL_406	4 wks	67.0 (8.6)	42.7 (10.9)	65.5 days	32.3 (11.5)	0

AC = Acclimation Survival

CS = Non-acclimation Survival

Table 2.3 Quantitative genetic estimates (± S.E.) for all phenotypes as they vary with age and the number of polymorphisms (generalized as SNPs) and genes significantly associated with each phenotype identified by GWAS with a threshold of –log10(5). All heritabilities reported are broad-sense and are greater than 0.

Phenotype	No. Lines	Age (weeks)	Mean	H^2	CV_G	CV_E	No. SNPs	No. Genes
Acclimation	101	1	63.4 (1.1)	0.26 (0.04)	22.68	38.58	24	23
Acclimation	101	4	55.2 (1.2)	0.19 (0.04)	25.60	52.70	31	28
Non-acclimation	101	1	30.4 (1.3)	0.33 (0.04)	58.76	84.52	22	14
Non-acclimation	101	4	14.1(0.9)	0.26 (0.05)	83.86	141.09	69	48
Acclimation Score	101	1	33.03 (1.4)	0.20 (0.03)	50.53	101.67	45	23
Acclimation Score	101	4	41.1 (1.3)	0.14 (0.02)	31.94	79.03	26	6
Starvation	164	1	51.89 (0.4)	0.34 (0.03)	13.51	18.66	20	9
Starvation	164	4	44.62 (0.5)	0.13 (0.02)	14.00	36.45	27	22

Table 2.4 Mean standardized additive effects (95% CI) of polymorphisms significantly associated with their respective phenotype are indicated in green. Calculated mean effects are presented for each set of polymorphisms in all other phenotypes. Red boxes indicate comparisons for which the polymorphisms associated with the phenotype in the left column had an opposite mean effect that excludes 0 based on 95% CI of the mean effect.

	AC, 1 wk	**AC, 4 wks**	**CS, 1 wk**	**CS, 4 wks**	**S, 1 wk**	**S, 4 wks**
AC, 1 wk	0.54 (0.06)	0.22 (0.04)	0.28 (0.05)	0.12 (0.02)	-0.26 (0.07)	-0.21 (0.05)
AC, 4 wks	-0.15 (0.04)	-0.41 (0.09)	-0.05 (0.03)	-0.03 (0.02)	0.08 (0.03)	0.00 (0.00)
CS, 1 wk	0.24 (0.07)	0.14 (0.05)	0.40 (0.09)	0.13 (0.04)	-0.01 (0.05)	-0.07 (0.04)
CS, 4 wks	-0.16 (0.01)	-0.18 (0.02)	-0.20 (0.02)	-0.32 (0.02)	0.07 (0.03)	0.04 (0.02)
S, 1 wk	0.02 (0.06)	0.03 (0.05)	-0.03 (0.04)	0.00 (0.03)	-0.25 (0.1)	-0.14 (0.06)
S, 4 wks	0.09 (0.05)	0.05 (0.06)	0.05 (0.04)	0.06 (0.04)	-0.32 (0.04)	-0.35 (0.03)

AC = Acclimation Survival

CS = Non-acclimation Survival

S = Starvation Resistance

Table 2.5 Quantitative genetic estimates (± S.E.) for all phenotypes as they vary with age. All heritabilities reported are broad-sense and are greater than 0.

MALES ONLY						
Phenotype	**# Lines**	**Age (weeks)**	**Mean**	**H^2**	**CV_G**	**CV_E**
Acclimation Survivorship	101	1	0.667 (0.02)	0.25 (0.05)	20.14	14.71
Acclimation Survivorship	101	4	0.564 (0.02)	0.26 (0.05)	27.96	10.82
Non-acclimation Survivorship	101	1	0.361 (0.02)	0.62 (0.05)	68.16	16.60
Non-acclimation Survivorship	101	4	0.124 (0.01)	0.30 (0.06)	91.67	11.46
Acclimation Score	101	1	0.329 (0.02)	0.21 (0.04)	51.44	101.16
Acclimation Score	101	4	0.457 (0.02)	0.17 (0.03)	31.57	69.43
Starvation Resistance	164	1	45.37 (0.5)	0.71 (0.03)	14.79	36.58
Starvation Resistance	164	4	32.28 (0.3)	0.63 (0.03)	15.41	39.04
FEMALES ONLY						
Phenotype	**# Lines**	**Age (weeks)**	**Mean**	**H^2**	**CV_G**	**CV_E**
Acclimation Survivorship	101	1	0.601 (0.02)	0.27 (0.04)	25.31	12.43
Acclimation Survivorship	101	4	0.540 (0.02)	0.15 (0.03)	23.76	7.60
Non-acclimation Survivorship	101	1	0.246 (0.02)	0.29 (0.05)	59.04	9.38
Non-acclimation Survivorship	101	4	0.159 (0.01)	0.27 (0.05)	82.29	9.03
Acclimation Score	101	1	0.331 (0.02)	0.20 (0.04)	38.86	101.44
Acclimation Score	101	4	0.364 (0.02)	0.19 (0.04)	40.23	82.50
Starvation Resistance	164	1	58.41 (0.6)	0.73 (0.03)	15.36	25.73
Starvation Resistance	164	4	56.91 (0.7)	0.66 (0.03)	19.79	11.83

Table 2.6 Genetic (upper diagonal) and phenotypic (lower diagonal) correlations for all phenotypes (S.E.).

BOTH SEXES						
	AC, 1 wk	AC, 4 wks	CS, 1 wk	CS, 4 wks	S, 1 wk	S, 4 wks
AC, 1 wk	--	0.70 (0.3)	0.50 (0.2)	0.44 (0.3)	-0.23 (0.2)	-0.47 (0.2)
AC, 4 wks	0.20 (0.07)	--	0.26 (0.2)	0.55 (0.4)	0.19 (0.3)	-0.16 (0.3)
CS, 1 wk	0.22 (0.08)	0.12 (0.07)	--	0.43 (0.3)	-0.36 (0.2)	-0.081 (0.3)
CS, 4 wks	0.084 (0.1)	0.25 (0.1)	0.11 (0.1)	--	-0.088 (0.3)	-0.014 (0.4)
S, 1 wk	-0.20 (0.1)	-0.036 (0.08)	-0.14 (0.1)	0.037 (0.2)	--	0.69 (0.3)
S, 4 wks	-0.21 (0.07)	-0.061 (0.07)	-0.17 (0.8)	0.055 (0.1)	0.74 (0.2)	--
MALES ONLY						
	AC, 1 wk	AC, 4 wks	CS, 1 wk	CS, 4 wks	S, 1 wk	S, 4 wks
AC, 1 wk	--	0.76 (0.4)	0.28 (0.3)	0.41 (0.4)	-0.13 (0.3)	0.17 (0.3)
AC, 4 wks	0.21 (0.1)	--	0.10 (0.2)	0.45 (0.4)	0.17 (0.3)	-0.13 (0.4)
CS, 1 wk	0.15 (0.09)	0.071 (0.08)	--	0.39 (0.3)	-0.23 (0.3)	-0.12 (0.4)
CS, 4 wks	0.13 (0.1)	0.22 (0.1)	0.13 (0.1)	--	-0.21 (0.3)	0.032 (0.5)
S, 1 wk	-0.18 (0.2)	0.015 (0.2)	-0.11 (0.2)	-0.071 (0.3)	--	0.81 (0.3)
S, 4 wks	-0.20 (0.2)	-0.071 (0.2)	-0.040 (0.2)	0.095 (0.4)	0.56 (0.2)	--
FEMALES ONLY						
	AC, 1 wk	AC, 4 wks	CS, 1 wk	CS, 4 wks	S, 1 wk	S, 4 wks
AC, 1 wk	--	0.57 (0.4)	0.72 (0.3)	0.40 (0.3)	-0.33 (0.2)	-0.40 (0.1)
AC, 4 wks	0.22 (0.08)	--	0.35 (0.4)	0.56 (0.4)	0.12 (0.4)	-0.13 (0.3)
CS, 1 wk	0.29 (0.1)	0.22 (0.1)	--	0.34 (0.3)	-0.37 (0.2)	-0.18 (0.2)
CS, 4 wks	0.089 (0.1)	0.30 (0.1)	0.16 (0.2)	--	-0.0051 (0.3)	-0.003 (0.2)
S, 1 wk	-0.12 (0.1)	-0.0033 (0.2)	-0.0082 (0.1)	0.045 (0.2)	--	0.74 (0.3)
S, 4 wks	-0.17 (0.09)	-0.043 (0.1)	-0.011 (0.1)	-0.037 (0.2)	0.51 (0.2)	--

AC = Acclimation Survival

CS = Non-acclimation Survival

S = Starvation Resistance

yellow = significant positive correlations

orange = significant negative correlations

Chapter 3 - Costs and benefits of cold acclimation on survival and reproductive behavior in *Drosophila melanogaster*

Elizabeth R. Everman, Jennifer L. Delzeit, F. Kate Hunter, Jennifer M. Gleason,

and Theodore J. Morgan

Abstract

Fitness is determined by the ability of an organism to survive and to reproduce. However, the mechanisms that produce increased survival may not be identical to those that increase reproductive success. We used nineteen natural *Drosophila melanogaster* genotypes from the *Drosophila* Genetic Reference Panel to determine if adaptive plasticity following short-term acclimation through rapid cold-hardening (RCH) affects mating behavior and success. We confirmed that exposure to the acclimation temperature is beneficial to survival following cold stress; however, we found that this same acclimation temperature exposure led to less efficient male courtship and a significant decrease in the likelihood of mating. Genotypic variation in RCH capacity was correlated with variation in courtship duration of males not exposed to the acclimation temperature, indicating that the capacity to acclimate can positively influence reproductive fitness, but only in constant environmental conditions. Finally, we tested if the exposure of males to the acclimation temperature influenced courtship song. While exposure to the acclimation temperature again significantly increased courtship duration courtship song was unchanged. These results illustrate a balance between costs and benefits of short-term acclimation on survival and reproductive components of fitness and demonstrate the short-term acclimation environment can have a pronounced effect on reproductive success.

Introduction

Survival and reproduction are the primary determinants of fitness, and they are jointly influenced by a suite of environmental factors. Organisms have evolved diverse sets of mechanisms that allow the maintenance of survival and reproduction in response to environmental stress. Generally, organisms use basal tolerance and the capacity to respond to environmental stress through phenotypic plasticity to facilitate fitness gain (Kingsolver and Huey 1998; Basson et al. 2012; Franks and Hoffmann 2012). One widely studied source of environmental stress is the thermal environment. Fluctuations in temperature can occur on a daily and seasonal scale, and these fluctuations can influence activity levels (Ayrinhac et al. 2004; Overgaard and Sørensen 2008) and contribute to species distributions and the evolution of thermal response (Andrewartha and Birch 1954; Ayrinhac et al. 2004; Dierks et al. 2012). Variation in temperature can negatively impact the survival and reproduction of ectothermic organisms as a result of their sensitivity to small changes in temperature (Kelty and Lee 1999, 2001; Shreve et al. 2004; Kelty 2007). Phenotypic plasticity through acclimation to thermal variation is one mechanism by which ectotherms can overcome these negative fitness impacts.

The benefits of phenotypic plasticity in variable thermal environments have been established through studies that demonstrate increased survival results when exposure to a fluctuating thermal environment precedes temperature stress (Lee et al. 1987; Sinclair and Roberts 2005; Kelty 2007; Gerken et al. 2015). However, the primary focus has been on survival benefits leaving the influences of the temperature fluctuation on other components of fitness understudied (Best et al. 2012). For example, one process that usually results in a survival benefit is rapid cold-hardening (RCH; Lee et al. 1987). In insects and other ectotherms, RCH is initiated by a shift to a mild acclimation temperature prior to a harsher stress exposure (Terblanche et al.

2007; Overgaard et al. 2011). The large increase in survival of RCH induced individuals, compared to individuals exposed to harsh temperatures without acclimation, suggests that the RCH response is adaptive (Leroi et al. 1994). The capacity to respond to acclimation through RCH is a form of phenotypic plasticity and can be quantified as RCH capacity. The effects of RCH are not necessarily uniformly positive across all genotypes, environments, or components of fitness. Furthermore, exposure to non-lethal temperature influences reproductive fitness in a diverse group species (Hawley and Aleksiuk 1975; Leroi et al. 1994; Scott et al. 1997; Patton and Krebs 2001; Fasolo and Krebs 2004; Geister and Fischer 2007; Best et al. 2012; Dick et al. 2013; Schou et al. 2015). Thus, individuals that have increased survival induced by RCH may experience lower reproductive success or negative effects on other components of fitness from the same exposure to the acclimation temperature (Leroi et al. 1994; Geister and Fischer 2007; Schou et al. 2015). Finally, it is also not known how genotype-specific capacity for plastic response to temperature stress influences the effect of temperature on reproductive success.

Drosophila melanogaster is an excellent model for studying the influence of temperature on mating behavior because males have predictable, complex courtship behaviors that consist of several elements that can be altered by the environment. During male courtship, the male follows the female, orients near the female's head, and produces courtship song (Bastock and Manning 1955). Courtship song is produced when the male extends a wing so that it is perpendicular to his body and vibrates it (Bastock and Manning 1955; Bennet-Clark and Ewing 1967). These wing movements produce an audible, species-specific song, which in *D. melanogaster* consists of pulse and sine elements that are known to impact female receptivity (Ewing and Bennet-Clark 1968; Bennet-Clark and Ewing 1969; Von Schilcher 1976).

Temperature influences pulse song and other aspects of male courtship behavior in many drosophilid species, both at the time of courtship and as a result of acclimation to seasonal and short-term fluctuations in temperature (Aspi and Hoikkala 1995; Ritchie and Gleason 1995; Hoikkala and Isoherranen 1997; Patton and Krebs 2001; Fasolo and Krebs 2004; Shreve et al. 2004; Best et al. 2012). Seasonal acclimation to stressful temperatures can result in decreased mating success (Aspi and Hoikkala 1995; Hoikkala and Isoherranen 1997). For example, male *D. montana* and *D. littoralis* collected during warmer months produce higher frequency pulse song compared to males collected immediately following the overwintering period (Hoikkala and Isoherranen 1997). Summer-collected males have higher mating success than winter-collected males because females of these species prefer high frequency pulse song (Hoikkala and Isoherranen 1997). Short-term temperature stress can have a similar negative influence on mating success in *Drosophila* and other invertebrates (Shreve et al. 2004; Dick et al. 2013). For example, a drop in temperature of 7°C negatively affected mating success in *D. melanogaster* (Shreve et al. 2004). Allowing males to acclimate to the lower temperature mitigated this negative effect, suggesting that plastic response to the thermal environment can benefit reproduction. This implies that a larger temperature shift, such as acclimation prior to harsh cold stress, will have a significant effect on mating behavior.

In this study, we address several open questions by measuring the costs and benefits of RCH on two components of fitness: survival and reproductive success. Overall, we expect short-term thermal acclimation through RCH to have a positive effect on survivorship following cold stress and to have a negative effect on mating behavior and success. Because levels of basal cold tolerance, RCH capacity, and mating behavior are genetically controlled (Gaertner et al. 2015;

Gerken et al. 2015), we also expect that genotype-specific levels of temperature tolerance will interact with the negative impact of exposure to acclimation temperature on mating success.

We test these genotypic effects by measuring thermal tolerance, plasticity, and reproductive behavior in nineteen unique, naturally derived *D. melanogaster* genotypes from the *Drosophila* Genetic Reference Panel (DGRP). The DGRP consists of multiple inbred genotypes and provides a powerful tool for measuring identical genotypes across environments and traits. This panel was recently used to characterize the heritability of courtship behavior (Gaertner et al. 2015) as well as the genetic control of the effect of RCH on survival (Gerken et al. 2015). We build on the existing knowledge of RCH capacity and mating behavior provided by these studies by using genotypes from the DGRP to investigate how the genetic capacity for plasticity and fitness are related under multiple environments. Finally, we address potential mechanisms by which exposure to acclimation temperature influences mating success by examining the effect of acclimation on specific components of mating behavior. Because previous research has shown that courtship song can be altered by long-term temperature fluctuations, we expect specific elements of courtship behavior, including pulse song, to be negatively impacted by exposure to the short-term thermal acclimation temperature that leads to the RCH response.

Methods

Fly stocks

The DGRP is a collection of 205 inbred and fully-sequenced *D. melanogaster* lines established by sampling naturally segregating genetic variation in a single population in Raleigh, North Carolina (Mackay et al. 2012; Huang et al. 2014). Nineteen lines were selected from the DGRP that had a range of positive and negative responses to acclimation (Fig. 3.1B). We chose a subset of nineteen lines from the DGRP to perform highly detailed analyses of courtship

behavior and song (discussed below). All flies were reared on standard cornmeal/molasses/agar media at 25 ± 1°C in narrow polystyrene vials (25 x 95mm) on a 12-hour light:dark cycle.

All experimental flies were reared at moderate larval density by placing five males and five females in each vial and allowing females to lay eggs for three days to control larval density. After three days, the parents were transferred to new vials. In the new vials, females were allowed to continue oviposition for another three days before all parents were discarded. Experimental flies were obtained from both sets of vials. For mating experiments, virgin males and females were collected from each DGRP line and were sorted and maintained individually in vials. Non-virgin individuals were collected for cold tolerance assays (as in Morgan and Mackay 2006; Gerken et al. 2015). Experimental flies for cold tolerance assays were sorted by sex, with 10 individuals per vial, before they were tested at five to seven days of age.

Normality and homoscedasticity of all response variables were assessed via the Shapiro Wilk test and the Levene test, respectively. All variables recorded in this study deviated from the assumptions of parametric tests, and the issues with normality remained following data transformation. Because parametric tests are generally robust to violations of normality and homoscedasticity (Glass et al. 1972), we proceeded cautiously with parametric tests of our data, as many models were too complicated to be tested non-parametrically, and type I error is prone to inflation when the number of observations are high in non-parametric tests (Rogan and Keselman 1977; Zimmerman 1998). As a result, our analyses are conservative assessments of the influence of genotype and treatment on survival and behavior. All analyses were performed in R (Wickham 2009; Kuznetsova et al. 2015; R Core Team 2015) unless otherwise indicated. All data are archived with Dryad.

Cold tolerance assay and analysis

To characterize the response to cold stress for each isogenic line, males and females of each DGRP line were separated into two groups. We chose our temperatures following Gerken et al. (2015). Briefly, to measure basal cold tolerance, cohorts of 10 individuals from each line and sex were exposed to -6°C for one hour (Fig. 3.2A). After a recovery period of 24 hours at 25°C, percent survival of each vial was measured. To measure acclimated cold tolerance, cohorts from each line and sex received a mild cold stress at 4°C for two hours before they were transferred to -6°C for an hour (Fig. 3.2B). After a recovery period of 24 hours at 25°C, percent survival of each vial was measured. Measures of basal and acclimated cold tolerance were replicated four times for each sex in each of the nineteen lines.

The effects of acclimation and DGRP line on the proportion of flies that survived cold stress at -6°C were tested with a three-way mixed-model ANOVA, where line was a random effect and sex and acclimation treatment were fixed effects. Rapid cold-hardening (RCH) capacity was calculated as the difference between the mean proportion of flies that survived the acclimation cold tolerance assay and the mean proportion that survived the basal cold tolerance assay (Gerken et al. 2015).

Mating latency assay and analysis

To investigate the effect of exposure to the acclimation temperature for inducing RCH on reproductive fitness, we quantified two metrics of *D. melanogaster* mating behavior in groups of flies that were either exposed to the acclimation temperature or flies maintained at 25°C. Three hours prior to lights on, virgin flies in the acclimation treatment were transferred to 4°C for two hours (Fig. 3.2C). These flies were then transferred to fresh media and allowed to recover from acclimation temperature-induced coma for an hour at 25°C. Control flies were maintained at

25°C prior to the mating assay. Because two-hour acclimation at 4°C has been shown to wear off after four hours in these DGRP genotypes such that survivorship is no longer benefited by acclimation (Everman et al. In Press) and to ensure that the acclimation treatment applied in the survivorship assay aligned temporally with the acclimation treatment used in this mating assay, mating latency was assessed following a one-hour recovery period. At lights on, male and female flies from each treatment were paired within and between the acclimation and control treatments (i.e., acclimated male x acclimated female; control male x acclimated female; acclimated male x control female; control male x control female) for each DGRP line. Each pair was observed for four hours at 25°C in vials containing media and activated yeast. During the four-hour screen, we measured courtship latency (the time until males started courtship behavior) and courtship duration (the time from the start of courtship to the time when the male successfully began copulation). Males that did not engage in courtship were not included in the calculation of courtship duration and were given a courtship latency of 14400 seconds (4 hours), because, when left in pairs over one week, every pair eventually mated and produced offspring. Courtship duration for males that did not mate but engaged in courtship behavior within the four-hour screen was calculated by assigning time of copulation as 14400 seconds (4 hours) for the same reason given above, before subtracting the time of courtship initiation. Data were collected with five to eight replicates per line and treatment combination (Table 3.1).

Two three-way mixed-model ANOVAs were used to test the effect of acclimation treatment and DGRP line on courtship latency and courtship duration. In both analyses, line was a random effect and the sex-specific acclimation treatments were fixed effects. We ran these analyses first only including males that mated during the screen and then including all males that initiated courtship behavior (who may or may not have mated). The results of both analyses were

highly consistent, so we only present the results of the latter analysis. The effect of acclimation on the probability of mating was analyzed with a chi-square test.

We used regression analysis to determine the effect of genotype-specific levels of basal cold tolerance and RCH capacity on courtship latency and courtship duration. Because mixed model ANOVA results indicated that only males were negatively impacted by the acclimation treatment prior to mating, we used male-specific measures of RCH capacity and basal cold tolerance averaged by DGRP line and compared these responses to courtship latency and duration measured for the control pair of flies and for the pair in which only the male was exposed to the acclimation temperature.

Behavioral plasticity following acclimation temperature exposure was calculated by subtracting the average courtship duration of acclimated males from the average courtship duration of control males. As before, we only included the pairs with control females. We analyzed these data using linear and quadratic regression with and without an outlier genotype (DGRP line RAL-367; see results) to test the relationship between behavioral and physiological plasticity measured as RCH capacity.

Courtship song assay and analysis

Five lines with the highest positive RCH capacities (RAL-362, RAL-517, RAL-365, RAL-153, RAL-195), which also had among the most negative male behavioral plasticity scores, were selected to test the effect of acclimation on courtship song. Only a subset of the nineteen lines was used for this experiment as we were not able to collect song data in a high-throughput manner and collection of song data was a severely limiting step in analysis. We chose these five lines because survivorship and courtship behavior were most differentially affected by acclimation. Therefore, further examination of these five lines provided the opportunity to

examine costs of acclimation on courtship in lines that have an adaptive survival response to the treatment. Experimental flies for the courtship song assay were reared and collected in an identical manner as the mating latency assay. Males were divided evenly among the acclimation temperature exposure and control treatments; the acclimation temperature exposure treatment was the same as above for the mating latency experiments. Females were maintained at 25°C in this experiment because the primary effect of acclimation temperature exposure on mating behavior was in the males; therefore, females were standardized to determine the effect of exposure to the acclimation temperature on male song. Over the five DGRP lines, 15.6 ± 2.70 S.D. males per line received the acclimation treatment and 14.2 ± 2.05 S.D. received the control treatment (Table 3.1).

To record song, a virgin male and a virgin female were paired in a clear circular mating chamber (25 mm diameter, 12 mm height) with a clear plastic bottom. The mating chamber was placed directly over an Insectavox microphone (Gorczyca and Hall 1987). Video and sound recording continued until copulation or fifteen minutes had elapsed if the pair did not copulate. Because we had only one microphone, recordings of pairs could only be done one at a time. Recordings were made up to four hours post lights on each day. The order of DGRP lines and treatment of the males was randomized each recording day. Ambient temperature was recorded at the start and end of the recording window. Song and video recordings were captured using EZGrabber (Geniatech).

To measure the song characters, MP4 video files were first converted to MP3 audio files with MacX Free MP3 Video Converter Version 4.1.8 (Digitary Software). MP3 sound files were filtered with a high pass frequency of 100 Hz and a low pass frequency of 1000 Hz and saved as WAV files in Audacity® 2.1.1 (Audacity Team 2015). Pulses were identified on oscillograms

and the time between pulses, the interpulse interval (IPI), was measured manually in all bursts of at least three pulses (Fig. 3.3). In total, 24,305 pulses were analyzed. Because IPI varies with temperature, regression analysis was used to determine whether recording temperature variation significantly influenced IPI. The slope of this regression (-1.0437) was used to correct all IPI data to a common temperature (25°C) using the equation:

$$corrected\ IPI = -1.0437(25°C - recording\ temperature) + measured\ IPI.$$

The mean of the temperature at the start and end of the recording was used as the recording temperature.

We used a custom bootstrapping procedure to identify the minimum number of IPI needed to accurately estimate the mean IPI for each song following temperature correction. To identify this cut-off, we selected the top 5% of songs with the most IPI per song and randomly sampled 3, 5, 10, 15, 20, 25, and 30 IPI from each song with 10,000 iterations. The average IPI of each iteration was used to calculate the mean IPI and standard deviation for each cut-off, which was then compared to the mean IPI for the entire song. Visual examination of the standard deviation of the estimates for each cut-off and song demonstrated that the variation around the estimated mean decreased substantially when the cutoff was set to 15 IPI (Fig. 3.4). The variation was not greatly improved when more than 15 IPI were used as the cut-off (Fig. 3.4); therefore, to calculate the mean IPI for songs recorded in our study, only songs with at least 15 IPI were retained in subsequent analyses. Mean IPI was calculated for each individual across the entire courting period. In addition, we calculated the mean IPI of the first and last minute of song for individuals with at least 15 IPI in each of those minutes. In males that mated, the last minute

of courtship occurred immediately before copulation. For males that did not mate, the last minute of the recording was the last minute of song.

From the videos, we manually recorded mating success, courtship duration, courtship index (the proportion of the courtship duration in which the male was actively courting), and song index (the proportion of the courtship duration in which the male was actively singing). Song index was determined by adding together all IPIs for a song to calculate the total time males spent singing and dividing that number by the courtship duration. Both song parameters and courtship parameters were scored blindly with respect to line and treatment.

To assess the broad effect of exposure to the acclimation temperature on courtship behavior and likelihood of mating, we tested the effect of male treatment on mating success, courtship occurrence, and song occurrence with chi-square tests. We then tested the effect of DGRP line, male treatment, and mating status (whether males mated or not) on mean IPI, courtship index, song index, and courtship duration individually with mixed-model three-way ANOVAs. We used a repeated-measures ANOVA to test the effects of DGRP line, male treatment, and mating status on song consistency by comparing the mean IPI of the first minute of song to the last minute of song.

Results

The effect of acclimation and genetic variation on survivorship

Survivorship following exposure to cold stress was influenced both by treatment and genetic variation. The acclimation treatment significantly improved survivorship compared to the basal cold tolerance treatment ($F_{1,228}$ = 123.9, P < 0.001; Fig. 3.1A) indicating most individuals had positive RCH capacity. DGRP line also significantly influenced survivorship following the acclimation and basal cold tolerance treatments ($F_{18,228}$ = 9.52, P < 0.001; Table 3.2). Treatment

and DGRP line interacted to influence survivorship following the acclimation and basal cold tolerance treatments as well, indicating that the genotypes included in this study responded differentially to the cold stress treatments (Fig. 3.1B; $F_{18,228} = 9.43$, $P < 0.001$) and that the DGRP lines had different levels of cold tolerance and RCH capacity. Genotype-specific responses to cold with and without acclimation and RCH capacity point to genetically determined differences in physiological response to cold stress. The lack of a sex-specific response suggests that male and female survival was influenced by treatment in similar ways.

The effect of acclimation and genetic variation on mating behavior

The exposure of males and females to the cold acclimation temperature did not delay the initiation of courtship behavior (male treatment: $F_{1,458} = 0.79$, $P = 0.3$, female treatment: $F_{1,458} = 0.03$, $P = 0.86$, male by female treatment interaction: $F_{1,458} = 0.0009$, $P = 1$; Fig. 3.5A, Table 3.2). However, exposure of males to the cold acclimation temperature significantly increased courtship duration, indicating that exposure to the acclimation temperature decreased the efficiency of mating ($F_{1,458} = 28.9$, $P < 0.001$; Fig. 3.5B, Table 3.2). Males that received the acclimation treatment were also significantly less likely to mate than control males ($\chi^2 = 20.2$, $P < 0.0001$). The acclimation temperature exposure of females did not influence courtship duration ($F_{1,458} = 0.40$, $P = 0.5$; Table 3.2). Courtship duration was also not influenced by an interaction between male and female acclimation temperature treatment, indicating that the negative effect of cold acclimation on courtship duration was driven by the ability of males to court, but not the female's ability to accept the male's courtship ($F_{1,458} = 0.29$, $P = 0.6$; Fig. 3.5, Table 3.2).

Significant variation among DGRP lines was detected for both behavioral responses measured (courtship latency: $F_{18,458} = 4.15$, $P < 0.001$; courtship duration: $F_{18,458} = 9.06$, $P < 0.001$; Fig. 3.6, Table 3.2). The male treatment by DGRP line interaction had a significant effect

on courtship latency ($F_{18,458}$ = 1.08, P < 0.05), but all other interactions were not significant. Collectively, these results show that the cold acclimation temperature exposure negatively affected mating latency and that there was significant genetic variation in both courtship latency and courtship duration.

We tested the effect of genotypic variation in male-specific basal cold tolerance and RCH capacity on mating behavior as well. Neither basal cold tolerance (Fig. 3.7A and B) nor RCH capacity (Fig. 3.8A and B) was correlated with courtship latency. RCH capacity was negatively correlated with control male courtship ($F_{1,17}$ = 8.44, P < 0.01, R^2 = 0.33; Fig. 3.8C); however when males were exposed to the acclimation temperature, the relationship became non-significant (Fig. 3.8D). Because we observed a significant relationship between courtship duration and RCH capacity under control conditions, we conclude that genetic variation in the acclimation response influences mating behavior as well.

For each genotype, the change in courtship behavior between the control and acclimation temperature treatment is a measure of behavioral plasticity. Because RCH capacity influenced mating behavior, we also analyzed the effect of RCH capacity on behavioral plasticity to understand how the capacity for a physiological response to the acclimation temperature correlated with a behavioral response to the acclimation temperature. The relationship between mating behavioral plasticity and RCH capacity (physiological plasticity) was not significant when analyzed with a linear ($F_{1,17}$ = 2.60, P = 0.13) or quadratic ($F_{2,16}$ = 1.56, P = 0.24) regression analysis (Fig. 3.9). While most genotypes had negative behavioral plasticity (courtship duration was longer for acclimated males), genotype RAL-367 had an unusually large, positive behavioral response to acclimation, and unusually low RCH capacity (outlier in Fig. 3.9). Genotype RAL-367 also had unusually long courtship latency when exposed to the

acclimation temperature (Fig. 3.6). The relatively longer recovery period that resulted from this courtship latency may have allowed RAL-367 males to court more effectively once they started courting females. If this were the case, the acclimation treatment may not have influenced courtship behavior for RAL-367 males the same way that it influenced courtship behavior in males of other genotypes exposed to acclimation temperature. When the outlier was excluded from the analysis, the quadratic relationship between behavioral plasticity and RCH capacity (physiological plasticity) was significant ($F_{2,15}$ = 8.02, P < 0.01; Fig. 3.9). Overall, the acclimation exposure at 4°C improved survivorship for most genotypes but negatively affected courtship behavior (Fig. 3.9). For genotypes that had either negative or very high positive levels of RCH capacity, the negative effect of the acclimation temperature on behavioral plasticity was large (Fig. 3.9). Courtship in genotypes with intermediate levels of RCH capacity was less negatively affected by acclimation temperature; in two cases exposure to the acclimation temperature decreased courtship duration leading to positive behavioral plasticity (Fig. 3.9). The complex relationship between the physiological and behavioral response to the acclimation temperature illustrates that it is possible for fitness to be gained via shifts in survival and reproductive behavior in genotypes with an intermediate physiological capacity to respond to acclimation temperature.

The effect of acclimation temperature on male courtship song

Because the five genotypes used to test the effect of acclimation on male courtship song had high positive RCH capacities and similar negative behavioral responses to the acclimation temperature treatment (Tukey's HSD; all comparisons > 0.05), we did not analyze each genotype separately. As with the mating latency assay, the male acclimation temperature treatment had a significant negative effect on mating success (χ^2 = 6.92, P < 0.01); 72.5% of the control males

successfully mated, whereas only 51.3% of the acclimation temperature exposed males mated within the fifteen-minute recording and observation period. Exposure to the acclimation temperature did not influence song occurrence as all but two males across the song assay experiment engaged in courtship behavior (χ^2 = 0.4, P = 0.53). Of the males that courted in this experiment, 83.3% of the acclimated temperature exposed males and 78.6% of the control males produced pulse song (χ^2 = 0.28, P = 0.60). Therefore, the negative effect of the acclimation temperature treatment on mating success was not the result of an inability of acclimated males to court or sing.

The songs and mating behavior of the males exposed to the acclimation temperature were very similar to the distribution of IPI produced by control males (Fig. 3.10A). Exposure of males to the acclimation temperature did not influence mean IPI of the entire song ($F_{1,73}$ = 0.73, P = 0.40; Table 3.3, Fig. 3.10B), courtship index ($F_{1,128}$ = 0.0015, P = 0.97; Table 3.3, Fig. 3.10C), or song index ($F_{1,73}$ = 0.21, P = 0.65; Table 3.3, Fig. 3.10D). However, males exposed to the acclimation temperature did have marginally longer courtship duration ($F_{1,128}$ = 3.89, P = 0.05; Table 3.3, Fig. 3.10E). When all males (mated and unmated) were considered together, the mean IPI in the last minute of song of males exposed to the acclimation temperature tended to be slightly shorter than that of control males ($F_{1,69}$ = 2.99, P = 0.09; Table 3.3, Fig. 3.10F, left panel). When only males that successfully mated were considered, variation in song consistency was further reduced (mated males: $F_{1,24}$ = 0.38, P = 0.55; Fig. 3.10F, middle panel); however when only males that did not mate during the recording period were considered, the difference in song consistency between control males and males exposed to the acclimation temperature became more pronounced ($F_{1,24}$ = 4.1, P = 0.05; Fig. 3.5F, right panel). Although there are no striking changes in song due to exposure to the acclimation temperature, the negative effect of

acclimation on mating success may be related to inconsistent song produced by males exposed to the acclimation temperature that court for a longer period of time before mating.

Discussion

Cold-acclimation capacity has costs and benefits on survivorship and mating success

The ability of an organism to thrive in a thermally variable environment influences both the persistence of the population and the reproductive potential of the organism. In a simple scenario, an organism that survives an exposure to cold temperature fluctuation can reproduce in the future; in this way, the survival benefit of acclimation RCH would also indirectly benefit reproductive success. However, non-lethal temperature exposure can directly alter reproductive success as well (Shreve et al. 2004; Geister and Fischer 2007), and while acclimation can greatly benefit survival, this mild form of cold stress can be very costly for reproductive success. The presence of benefits and costs for survival and reproductive success is clearly demonstrated in our study.

Survival following cold stress without acclimation (basal cold tolerance) varied, but when flies were exposed to the acclimation pre-treatment before cold stress, average survival significantly increased among the DGRP lines (Fig. 3.1B). In most cases (14 of 19 DGRP lines; Fig. 3.9), RCH capacity was positive, providing evidence of adaptive physiological plasticity and indicating that acclimation has a beneficial effect on survival. Although this is a relatively simple assay of cold acclimation, multiple studies with ecologically relevant thermal periodicity and cooling rates yield results consistent with the simplified assay used in this study (Chen et al. 1987; Kelty and Lee 2001), and similar benefits of acclimation for survival have been

demonstrated in multiple species (Lee et al. 1987; Coulson and Bale 1992; Kelty and Lee 2001; Kristensen et al. 2008; Gerken et al. 2015; Schou et al. 2015).

While the 4°C acclimation treatment significantly improved survival following -6°C cold stress in most DGRP lines (Fig. 3.1A), the exposure of males to the acclimation temperature had a significant negative effect on courtship duration (Fig. 3.5B) as well as the likelihood of copulation ($\chi^2 = 6.92$, $P < 0.01$). Courtship duration increased when males, but not females, were exposed to the acclimation temperature suggesting that mild cold exposure resulted in a reduction in attractiveness of male courtship behavior. Exposure to the acclimation temperature did not significantly impact the female's ability to mate; combined with absence of an effect of acclimation temperature exposure on courtship latency, this implies that the acclimation temperature-induced increase in courtship duration in males is driven by female choice against acclimated males. The negative effect of exposure to the acclimation temperature on courtship duration observed in our study is consistent with the decrease in mating success of *D. montana* males exposed to chronic winter-like temperatures (Hoikkala and Isoherranen 1997) and short-term exposure without acclimation of *D. melanogaster* to temperatures that varied by 7°C from rearing temperature (Shreve et al. 2004). Our results demonstrate that fitness effects following exposure to 4°C acclimation are context-dependent, and that courtship behavior in *D. melanogaster* is sensitive to short-term temperature fluctuations that can occur over a period of a few hours.

Survival and reproductive behavior varied significantly across the nineteen DGRP lines in our study (Table 3.1, Fig. 3.1B, Fig. 3.6). The correlation between the physiological and behavioral responses to the acclimation temperature (Fig. 3.9) indicates that genetic variation in RCH capacity can influence more than survival alone. Males from genotypes with low RCH

capacity are not likely to survive in an environment in which fluctuation to cold temperatures is likely or common, nor are they likely to mate quickly, regardless of variation in the thermal environment (Fig. 3.8C and D). Low probability of survival in thermally variable environments combined with lower reproductive success suggests these low RCH capacity genotypes would have poor overall fitness in natural populations. Males from genotypes with high RCH capacity survived well under acclimation temperature conditions and mated quickly under control conditions (Fig. 3.8C) and so experienced a benefit from acclimation for survival and future reproduction. However, when exposed to the acclimation temperature prior to engaging in courtship behavior, males with high RCH capacity achieved copulation slowly and thus experienced a large reproductive cost (Fig. 3.8D, Fig. 3.9). This implies that, for these genotypes, exposure to the acclimation temperature that improved survival was stressful and negatively impacts reproduction, a critical component of fitness. In contrast, males from genotypes with intermediate RCH capacity retained the ability to survive cold stress and had the least reproductive cost of exposure to acclimation temperature as measured by mating behavior. Thus total fitness may be best achieved through moderate capacity to survive temperature fluctuations. Moderate levels of plasticity can reduce the probability of phenotypic mismatch between basal tolerance and thermal environmental variability (Kawecki 2000; Gomez-Mestre and Jovani 2013; Bergland et al. 2014a; Beaman et al. 2016). Together, environmental context and genotypic dependence of the cost and benefits of exposure to the acclimation temperature exposure yields insights into whole organism fitness by highlighting the fact that exposure to the acclimation temperature is nearly always beneficial for survival and may be benefit reproductive fitness as well under certain environmental conditions for certain genotypes.

Decreased mating success following exposure to acclimation temperature is not caused by changes in courtship song

We observed a strong negative effect of exposure to acclimation temperature on courtship duration when the male, but not female, of each line received the cold treatment, suggesting that male courtship behavior was altered by cold exposure (Fig. 3.5B). Measuring courtship behavior and courtship song of individual males allowed us to assess the effect of the acclimation treatment in much greater detail than that afforded by our mating latency assay. We expected mean IPI to be influenced by acclimation, given the previous report of temperature-induced changes to pulse song in overwintered *D. montana* males (Hoikkala and Isoherranen 1997). However, when we tested the effect of exposure to acclimation temperature on mean IPI of the male song, courtship index, and song index, we found that none of these variables responded in a way that provided a mechanistic explanation for the negative effect of acclimation on mating success (Fig. 3.10B-D). Exposure to the acclimation temperature over short time periods did not appear to alter *D. melanogaster* courtship song in a manner that sufficiently explained the drastic reduction in mating success or the increase in courtship duration of males that were exposed to the acclimation temperature (Fig. 3.5B and Fig. 3.10E).

Although courtship song produced by males exposed to acclimation temperature did not differ from that of control males, exposure to the acclimation temperature may have influenced the consistency of courtship song production. For example, mean IPI may be similar among males, but when considered on an individual basis, IPI may vary over the duration of courtship. We therefore assessed the effect of exposure to the acclimation temperature on the variability of mean IPI by comparing mean IPI during the first and last minute of song. Here we found a slight effect of exposure to the acclimation temperature: in the last minute, males exposed to the

acclimation temperature had shorter mean IPI than control males and a greater difference between the first and last minute of song (Fig. 3.10F) than control males. These subtle differences in mean IPI through an individual's song are unlikely to be a contributing factor to the decreased mating success of males exposed to the acclimation temperature. Genetic analyses (Ritchie and Kyriacou 1996; Gleason et al. 2002; Turner and Miller 2012) have implied the presence of directional selection for short IPI, which may be an indicator of male fitness, assuming fast song (short IPI) is more difficult to produce. Our results are contradictory to this assumption and imply that a longer IPI is preferred by the females from genotypes used in our study. While our result should be considered carefully and warrants further investigation, this may imply that female *D. melanogaster* do not always prefer shorter IPI, but rather that they prefer a specific range of IPI that may depend on the genotype of the female (Yadav and Yadav 2012; Gaertner et al. 2015)

IPI is one of many song parameters that may contribute to male attractiveness during courtship. Other song parameters may play a role in mating success, though this has not been demonstrated in *D. melanogaster*. Other mating signals that may be affected by temperature include cuticular hydrocarbons (CHCs) that act as pheromones, which are important for female response to males (Grillet et al. 2006). Heat shock can affect CHC composition (Savarit and Ferveur 2002) and social context affects levels of pheromones (Krupp et al. 2008); thus acclimation stress as applied this context might detrimentally affect male mating success, though this remains to be measured.

Further testing is required to identify the mechanism through which acclimation impacts mating success. However, testing the physiological limits of outbred genotypes under multiple conditions is difficult, as is correlating physiological response of outbred genotypes to behavioral

response in subsequent assays. Our use of inbred genotypes from the DGRP was strategic in that it allows this study to overcome these significant difficulties and to assess genotype-specific patterns in behavior and physiology across multiple environments with replicated measures of the same genotype. Although they are inbred, the DGRP is an excellent tool for investigating the relationship between genetically controlled phenotypes in the context of natural genetic variation. The lines are derived from a natural population and thus represent a snapshot of standing genetic variation. Furthermore, because they are inbred genotypes, we can examine effects of recessive polymorphisms in the population that influence phenotypic responses in multiple environments in genetic backgrounds that represent natural genetic variation. While it is possible that the inbred nature of each DGRP line could alter male courtship behavior independently of the acclimation treatment, the understanding we gain through the use of these naturally derived lines provides insight into how genetic variation in physiology and behavior interacts with short-term environmental variation to determine whole organism fitness.

Conclusions

We have addressed several significant aspects of the effect of exposure to 4°C acclimation on survival and reproductive behaviors in *D. melanogaster*. We have demonstrated that cold tolerance and the capacity to acclimate vary among several unique, naturally derived genotypes from the DGRP and that different levels of cold tolerance and plasticity influence the effect of exposure to acclimation temperature on behaviors relevant to reproductive fitness. Consistent with the research available on the effects of temperature on mating behavior, we demonstrate that mildly cold stressing males through exposure to 4°C acclimation decreases their likelihood of mating and cautiously suggest that an underlying mechanism for this pattern may relate to subtle changes in mean IPI over the course of a male's song. Because populations of *D.*

melanogaster are likely to experience mild cold stress similar to that used in our study, for example during the early morning hours of a spring or fall day, we hypothesize the significantly decreased mating success of males exposed to the acclimation temperature have biologically important consequences for fitness and the evolution of plasticity and cold tolerance in natural populations.

Acknowledgments

We thank Ken Ratzlaff of the University of Kansas Instrument Design Laboratory for redesigning our Insectavox. This work was supported by the National Science Foundation award NSF IOS-1051770 to T.J.M. Two undergraduate scientists, J.D. and F.K.H., were supported by the NSF REU-Site award (NSF DBI-1156571). J.D. was also supported by an Undergraduate Research Mentoring in Ecological Genomics Grant (NSF DBI-1041199).

Figures and Tables

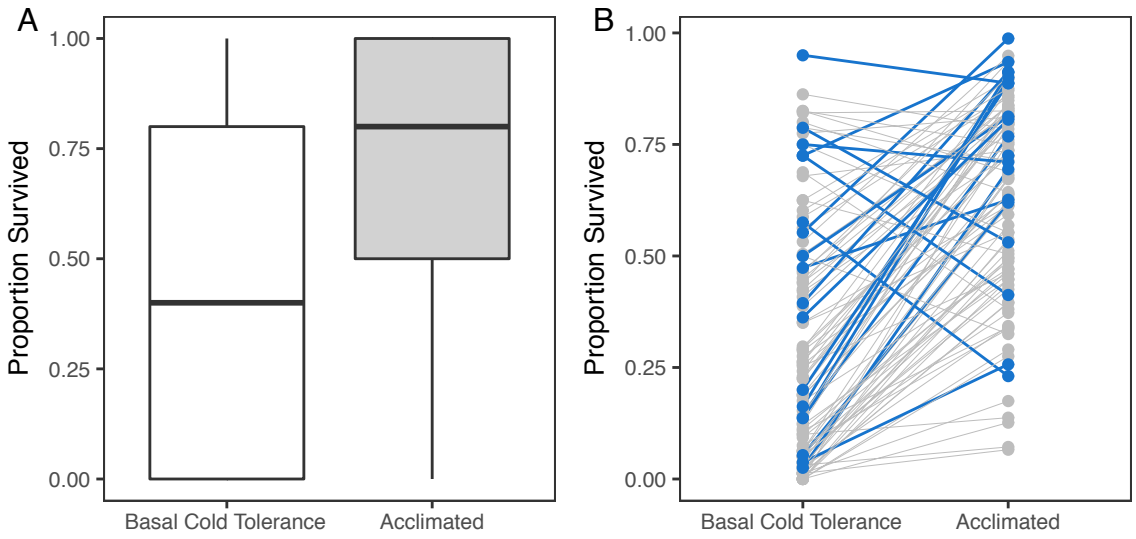

Figure 3.1 Survival following cold stress with and without the acclimation pre-treatment. A. Flies that were acclimated had higher survival than flies that did not receive the acclimation treatment prior to cold stress ($F_{1,228}$ = 123.9, P < 0.001). B. Genotype significantly influenced survival following the basal cold tolerance and acclimation treatments ($F_{18,228}$ = 9.52, P < 0.001). Each point and connecting line represents the change in a genotype's average survival between the basal cold tolerance and acclimation treatments. This change (acclimation survival – basal survival) is RCH capacity, one measure of phenotypic plasticity. Variation in change in survival between the two treatments led to genotype-specific variation in RCH capacity.

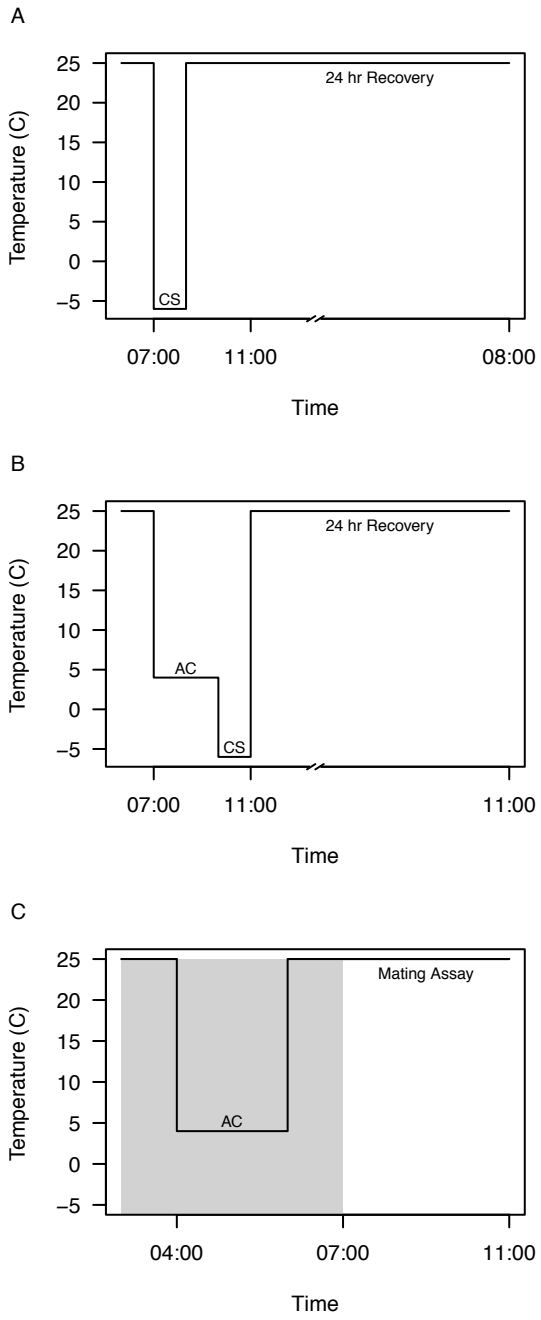

Figure 3.2 Temperature treatments for the cold tolerance and mating assays. Flies were exposed to either (A) a basal cold tolerance treatment for 1 hour at -6°C or (B) an acclimation treatment for two hours at 4°C followed by -6°C for 1 hour to determine the level of cold tolerance for each genotype. C. Flies were exposed to either the acclimation treatment temperature (4°C) for 2 hours or were held at 25°C for the mating latency and song assays. Shading in C indicates the timing of lights on (07:00 hrs) for experimental flies used in mating assays.

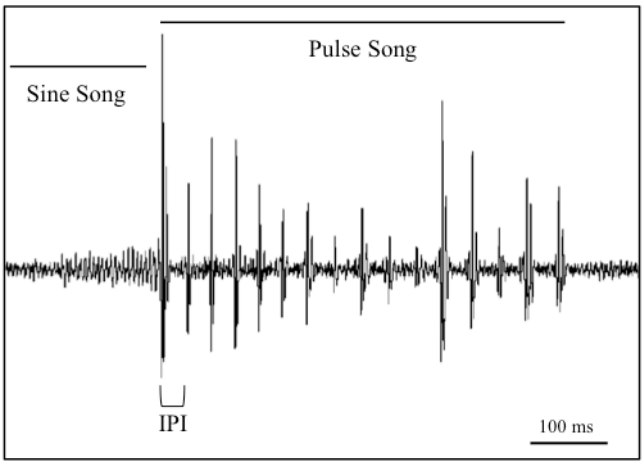

Figure 3.3 Oscillogram presenting an example of song produced by *D. melanogaster* males. Pulse and sine song was recorded for acclimated and control males from five DGRP lines. Interpulse Interval (IPI) denotes the interval between two pulses. IPI was measured for sets of pulses that occurred in bursts of 3 or more pulses. Figure S3. Estimation of the mean IPI using cut-offs ranging from 5 – 30 IPI per song following bootstrapping with 10,000 iterations. We selected the top 5% of songs (a113, a124, 125, a128, a131, a 154, a42, and a44) with the most IPI per song and randomly sampled 3, 5, 10, 15, 20, 25, and 30 IPI from each song with 10,000 iterations. The average IPI of each iteration was used to calculate the mean IPI and standard deviation (left panel) and 95% CI (right panel) for each cut-off, which was then compared to the mean IPI for the entire song. Variation around the mean estimate was not greatly improved when more than 15 IPI were used as the cut-off; therefore, to calculate the mean IPI for songs recorded in our study, only songs with at least 15 IPI were retained in subsequent analyses. Each graph shows the sampling for one individual.

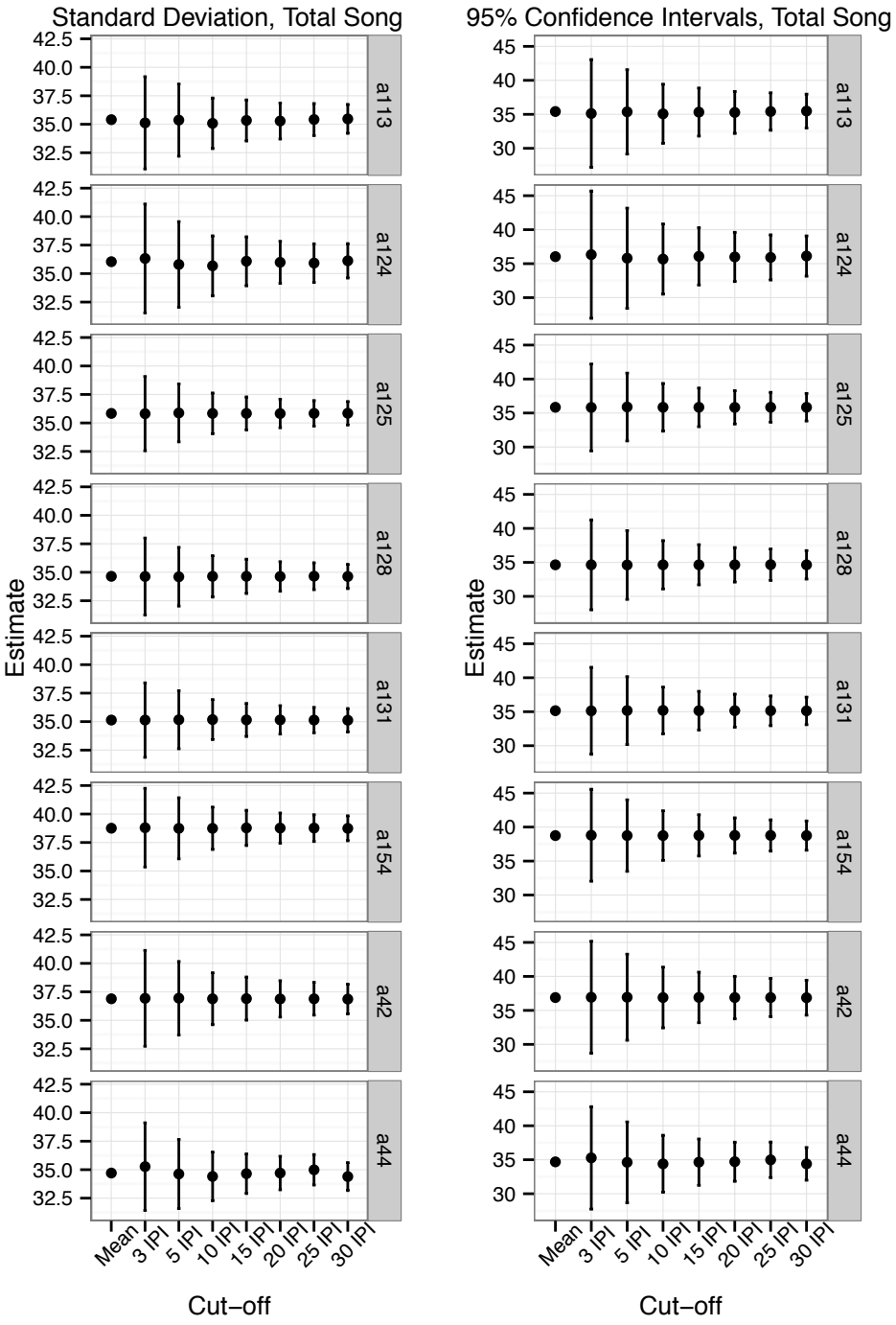

Figure 3.4 Estimation of the mean IPI using cut-offs ranging from 5 – 30 IPI per song following bootstrapping with 10,000 iterations. We selected the top 5% of songs (a113, a124, 125, a128, a131, a 154, a42, and a44) with the most IPI per song and randomly sampled 3, 5, 10, 15, 20, 25, and 30 IPI from each song with 10,000 iterations. The average IPI of each iteration was used to calculate the mean IPI and standard deviation (left panel) and 95% CI (right panel) for each cut-off, which was then compared to the mean IPI for the entire song. Variation around the mean estimate was not greatly improved when more than 15 IPI were used as the cut-off; therefore, to

calculate the mean IPI for songs recorded in our study, only songs with at least 15 IPI were retained in subsequent analyses. Each graph shows the sampling for one individual.

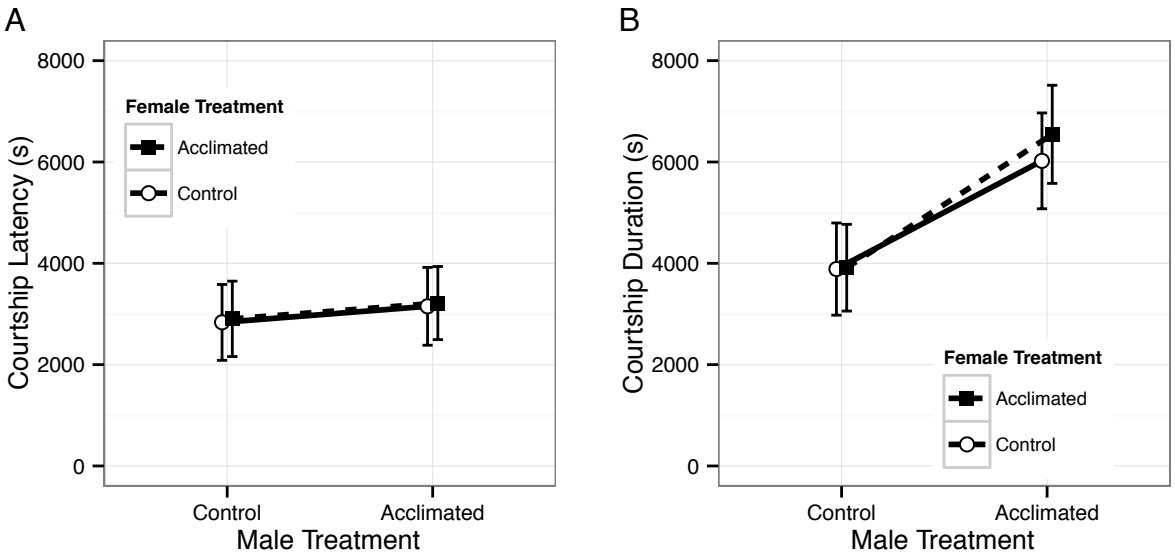

Figure 3.5 Courtship latency and courtship duration with and without acclimation. A. Exposure to the acclimation treatment did not impact courtship latency when either the male or female received the acclimation treatment (male treatment: $F_{1,458} = 0.79$, $P = 0.3$, female treatment: $F_{1,458} = 0.03$, $P = 0.86$, male by female treatment interaction: $F_{1,458} = 0.0009$, $P = 1$). B. Males exposed to the acclimation temperature had significantly longer courtship duration ($F_{1,458} = 28.9$, $P < 0.001$) independent of female treatment ($F_{1,458} = 0.40$, $P = 0.5$). Means are shown with 95% confidence intervals; symbols indicate female treatment (filled = acclimated, open = control).

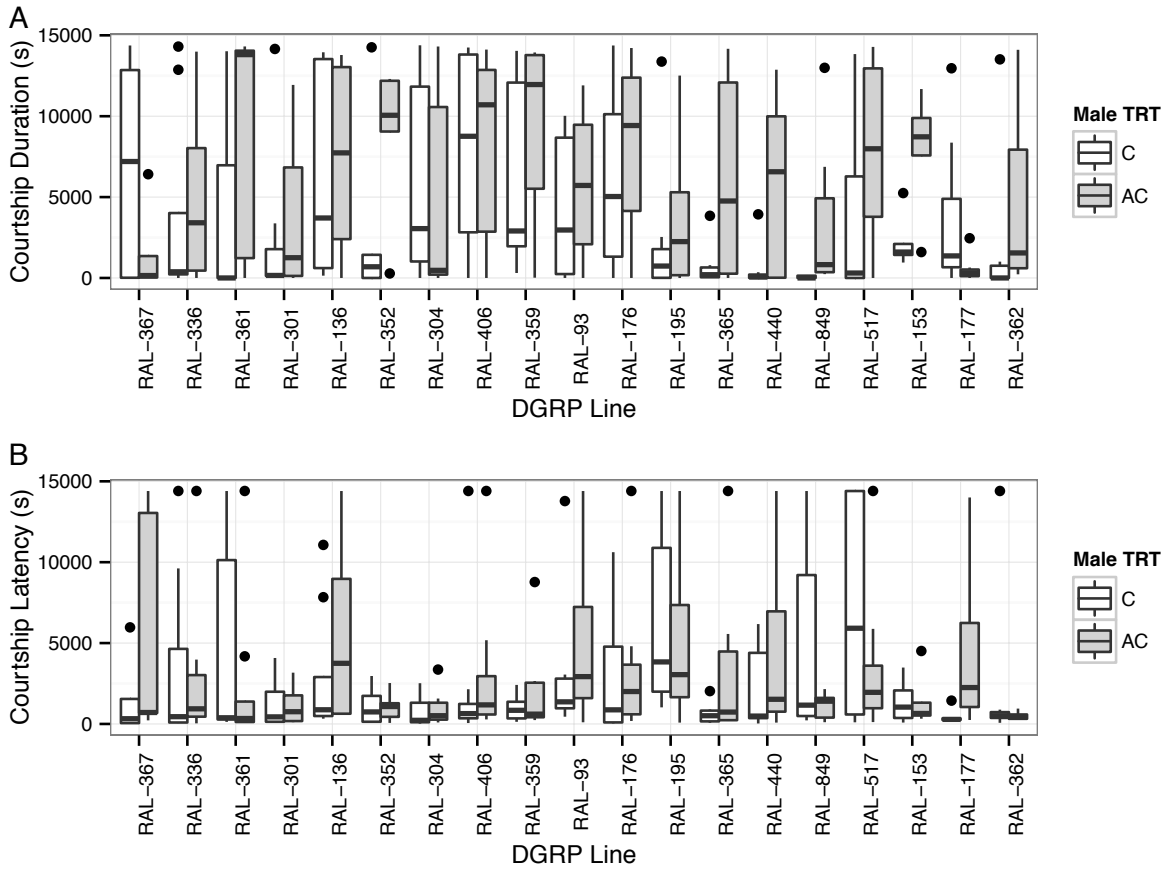

Figure 3.6 Boxplots of mating latency, showing courtship duration (A) and courtship latency (B) measured in 19 lines. In both plots, males that were exposed to the acclimation temperature (AC) are indicated by shaded boxplots; control males (C) are indicated by non-shaded boxplots. Behavioral response was highly variable among the DGRP lines regardless of whether the males received the acclimation treatment. For most genotypes, males exposed to the acclimation temperature spent more time courting females compared to control males but courtship latency was not different between treatments.

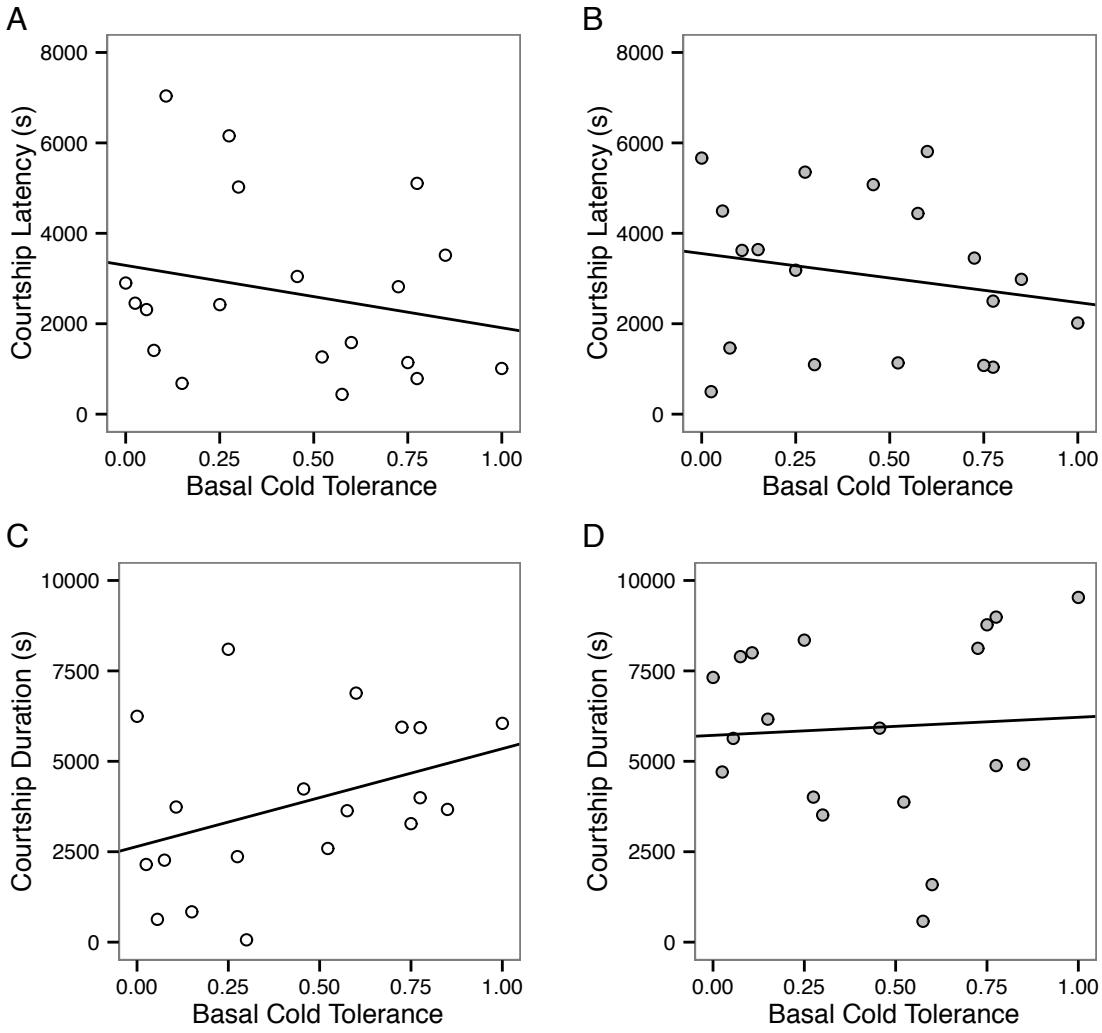

Figure 3.7 Correlations between basal cold tolerance and courtship traits in control males (open symbols; A and C) and males exposed to acclimation temperature (shaded symbols; B and D). Each dot represents the mean for a genotype. Basal cold tolerance was not correlated with either courtship latency (A: $F_{1,17} = 0.96$, $P = 0.34$, $R^2 = 0.05$; B: $F_{1,17} = 0.71$, $P = 0.41$, $R^2 = 0.04$) or courtship duration (C: $F_{1,17} = 3.02$, $P = 0.10$, $R^2 = 0.15$; D: $F_{1,17} = 0.068$, $P = 0.80$, $R^2 = 0.004$).

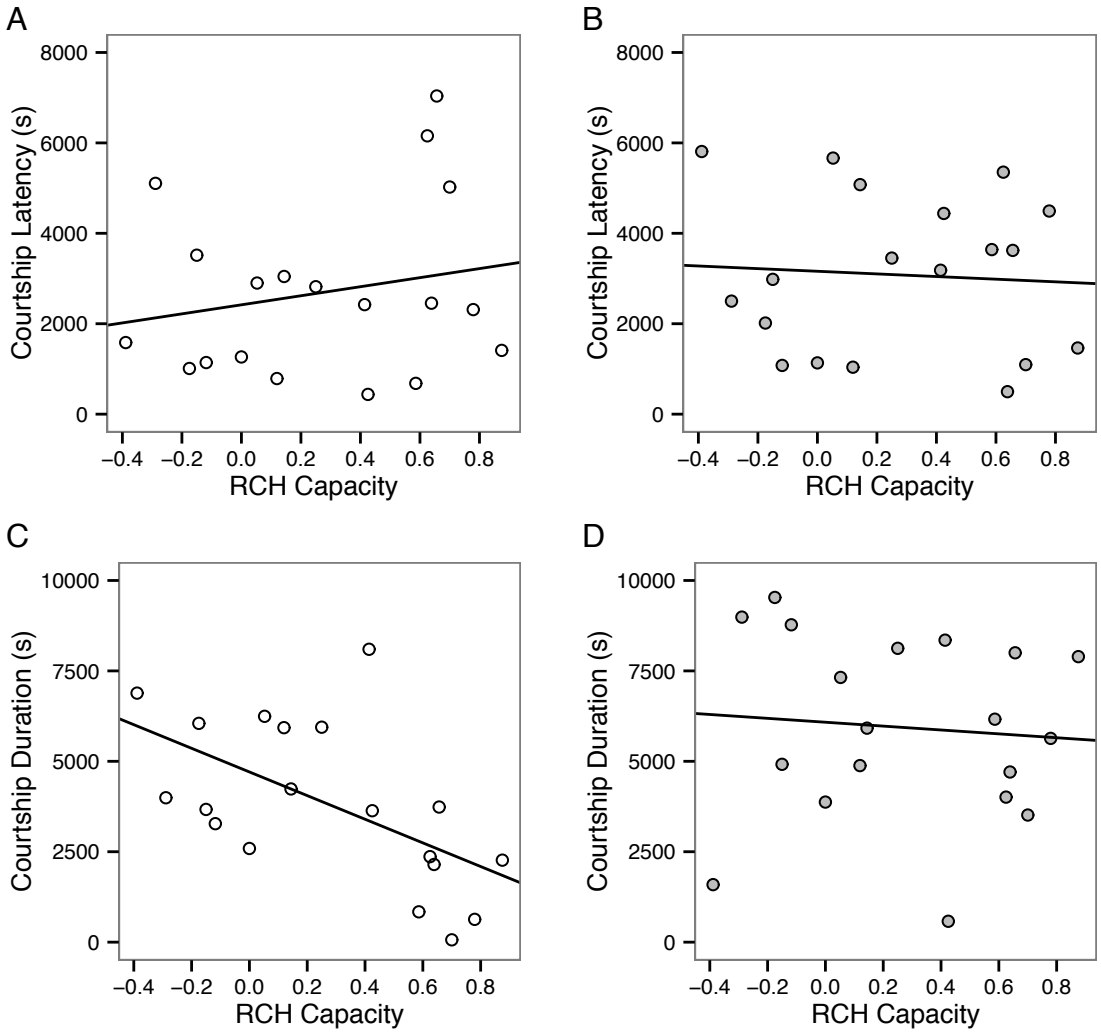

Figure 3.8 Correlations between RCH capacity and courtship traits in control males (open symbols; A and C) and males exposed to acclimation temperature (shaded symbols; B and D). Each dot represents the mean for a genotype. RCH capacity was not correlated with courtship latency (A: $F_{1,17}$ = 0.75, P = 0.40, R^2 = 0.04; B: $F_{1,17}$ = 0.075, P < 0.79, R^2 = 0.004). RCH capacity was significantly correlated with courtship duration (C: $F_{1,17}$ = 8.44, P < 0.01, R^2 = 0.33), but only when males did not receive the acclimation treatment. When males were exposed to the acclimation temperature, the relationship between RCH capacity and courtship duration became non-significant (D: $F_{1,17}$ = 0.12, P = 0.74, R^2 = 0.007; D). The non-significant result in D is primarily due to a change in the slope of the relationship compared to C as the y-intercept of the relationship does not change between treatments. This suggests the non-significant result was driven by a relatively larger increase in courtship duration following acclimation in genotypes with high RCH capacity.

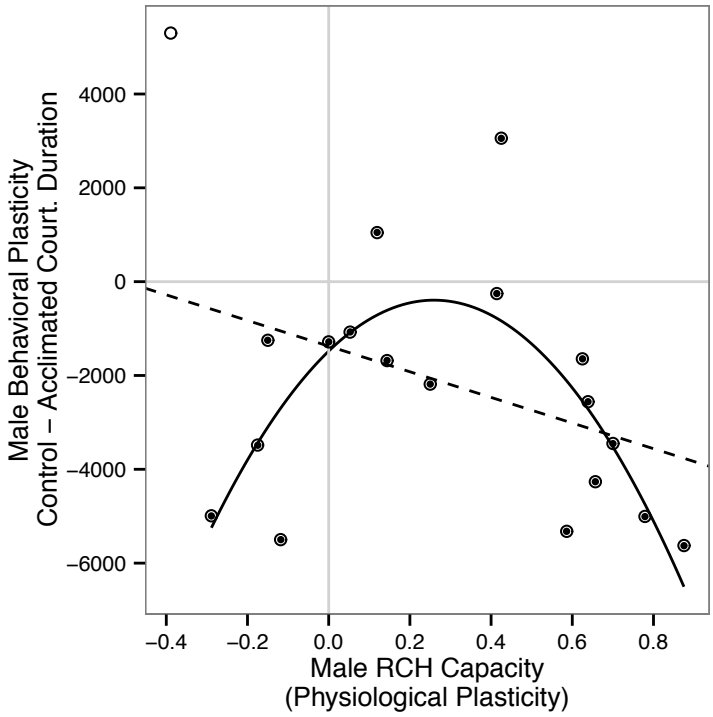

Figure 3.9 Relationship between male physiological and behavioral plasticity for the nineteen genotypes. Negative and very high positive male RCH capacity was correlated with a large negative effect of acclimation on behavioral plasticity (solid curve; $F_{2,15} = 8.02$, $P < 0.01$) when the outlier (indicated as an open symbol) was excluded from the analysis. The relationship was not significant when the outlier was included for either the quadratic (not shown; $F_{2,16} = 1.56$, $P = 0.24$) or linear analysis (dotted line; $F_{1,17} = 2.60$, $P = 0.13$). Overall, most genotypes responded positively to acclimation through increased survival (positive RCH capacity), while behavioral plasticity was negative for most genotypes.

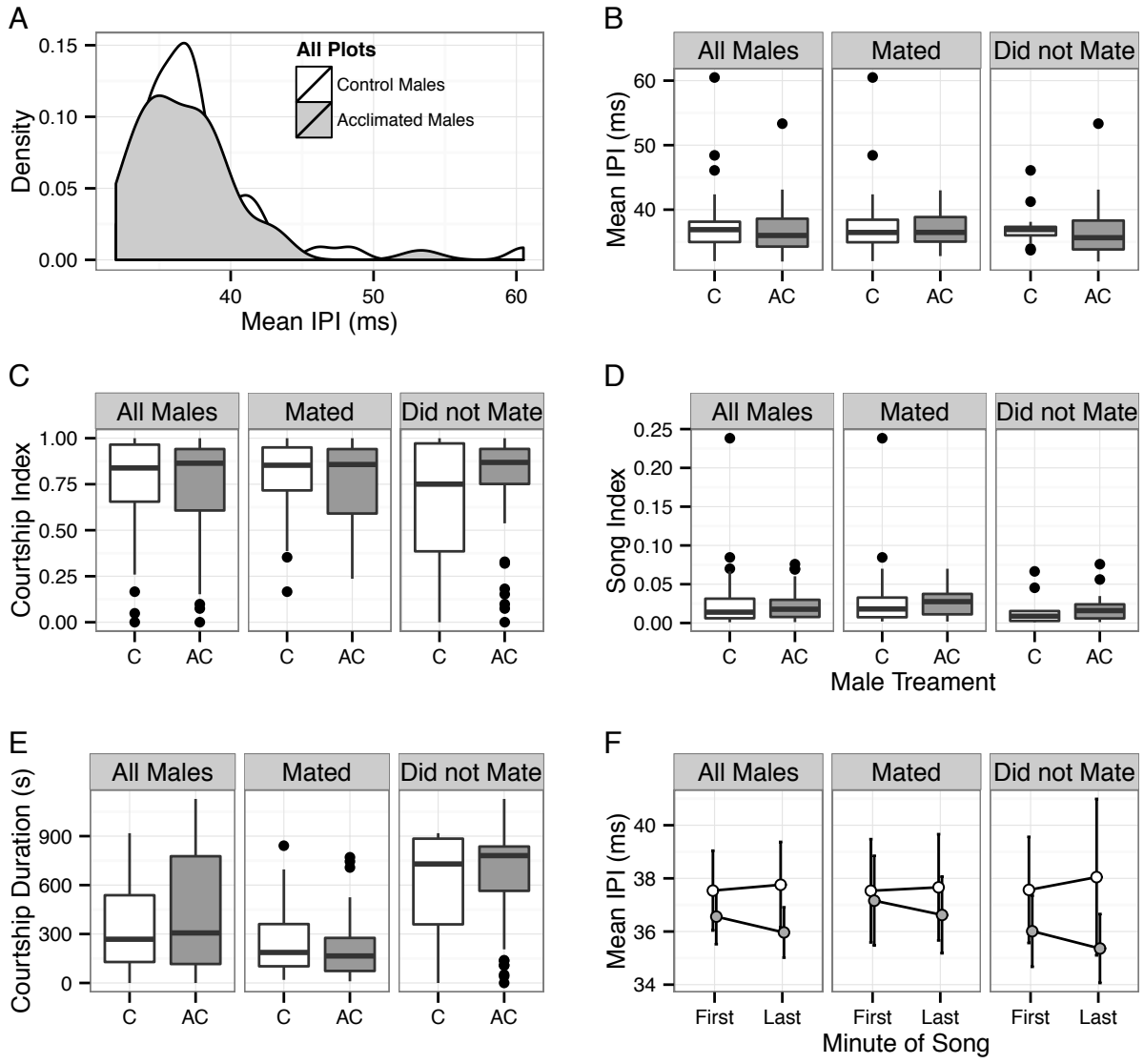

Figure 3.10 Effect of exposure of males to acclimation temperature on courtship parameters measured in five genotypes. A. The distribution of IPI produced by males that were exposed to the acclimation treatment (shaded distribution) was very similar to that produced by control males (non-shaded distribution). In figures B – D, boxplots show males that were exposed to the acclimation temperature (AC) were not significantly different from control (C) males when mean IPI (B: $F_{1,73} = 0.73$, $P = 0.40$), courtship index (C: $F_{1,128} = 0.0015$, $P = 0.97$), or song index (D: $F_{1,73} = 0.21$, $P = 0.65$) was compared. E. Males exposed to the acclimation temperature had slightly longer courtship duration compared to control males ($F_{1,128} = 3.89$, $P = 0.05$). F. The mean IPI in the first and last minute of song of all males exposed to the acclimation temperature tended to be slightly shorter than that of control males (plotted as means with 95% confidence intervals; $F_{1,69} = 2.99$, $P = 0.09$). This difference between treatments was more pronounced when only males that did not mate were considered ($F_{1,24} = 4.1$, $P = 0.05$). In all plots, shading indicates males that were exposed to the acclimation temperature; lack of shading indicates control males.

Table 3.1 Sample sizes for the cold tolerance assay, mating latency assay, and courtship song assays.

DGRP Line	Cold Tolerance Assay (per Sex)	Mating Latency Assay No. per Treatment Combination	Courtship Song Assay (No. Acclimated, No. Control)
RAL_304	4	6	
RAL_362	4	7	11,16
RAL_517	4	8	18,14
RAL_365	4	6	17,16
RAL_93	4	8	
RAL_101	4	7	
RAL_136	4	8	
RAL_153	4	5	16,13
RAL_176	4	8	
RAL_177	4	7	
RAL_195	4	8	16,11
RAL_336	4	8	
RAL_352	4	5	
RAL_359	4	8	
RAL_361	4	8	
RAL_367	4	5	
RAL_440	4	7	
RAL_849	4	7	
RAL_406	4	8	

Table 3.2 Analysis of variance of survival following cold stress, courtship latency, and courtship duration with and without exposure to the acclimation treatment in nineteen DGRP lines.

Response	Source	df	MS	*F*	*P*
Survival following cold stress	Sex	1	0.01	0.19	0.66
	TRT	1	7.02	123.94	**< 0.001**
	Line	18	0.54	9.52	**< 0.001**
	Sex x TRT	1	0.08	1.47	0.23
	Sex x Line	18	0.13	2.36	**< 0.01**
	TRT x Line	18	0.53	9.43	**< 0.001**
	Sex x TRT x Line	18	0.04	0.78	0.72
	Error	228	0.06		
Courtship Latency	Line	18	69636290	4.15	**< 0.001**
	Male TRT	1	13291995	0.79	0.37
	Female TRT	1	501153	0.03	0.86
	Line x Male TRT	18	30178234	1.8	**< 0.05**
	Line x Female TRT	18	22242282	1.33	0.17
	Male x Female TRT	1	14599	0.0009	0.98
	Line x Male TRT x Female TRT	18	9736530	0.58	0.91
	Residuals	458	16780770		
Courtship Duration	Line	18	223395907	9.06	**< 0.001**
	Male TRT	1	744302220	28.91	**< 0.001**
	Female TRT	1	10482539	0.41	0.52
	Line x Male TRT	18	27778314	1.08	0.37
	Line x Female TRT	18	24325841	0.94	0.52
	Male x Female TRT	1	7339810	0.29	0.59
	Line x Male TRT x Female TRT	18	17975976	0.7	0.81
	Residuals	458	25746853		

TRT = Treatment; significant results are in bold

Table 3.3 Analysis of variance of all song recording variables including mean IPI, courtship index, song index, courtship duration, and repeated measures analysis of variance of the first and last minute of song with and without exposure to the acclimation treatment in five DGRP lines.

Response	Source	df	MS	*F*	*P*
Mean IPI	Male TRT	1	11.56	0.78	0.4
	Line	4	98.31	6.6	**< 0.001**
	Mated vs. Not	1	2.13	0.13	0.71
	Male TRT x Line	4	11.9	0.75	0.56
	Male TRT x Mated vs. Not	1	6.87	0.43	0.51
	Line x Mated vs. Not	4	1.95	0.12	0.97
	Male TRT x Line x Mated vs. Not	3	15.42	0.97	0.41
	Residuals	73	15.84		
Courtship Index	Male TRT	1	0	0.002	0.97
	Line	4	0.083	1.33	0.26
	Mated vs. Not	1	0.07	1.13	0.29
	Male TRT x Line	4	0.12	1.97	0.1
	Male TRT x Mated vs. Not	1	0.043	0.7	0.4
	Line x Mated vs. Not	4	0.1	1.68	0.16
	Male TRT x Line x Mated vs. Not	4	0.026	0.42	0.8
	Residuals	128	0.062		
Song Index	Male TRT	1	0	0.21	0.65
	Line	4	0	2.61	**< 0.05**
	Mated vs. Not	1	0	0.02	0.9
	Male TRT x Line	4	0	0.67	0.61
	Male TRT x Mated vs. Not	1	0	0.02	0.88
	Line x Mated vs. Not	4	0	0.92	0.46
	Male TRT x Line x Mated vs. Not	3	0	0.55	0.65
	Residuals	73	0		
Courtship Duration	Male TRT	1	226604	3.89	**0.05**
	Line	4	572675	9.83	**< 0.001**
	Mated vs. Not	1	3005640	51.58	**< 0.001**
	Male TRT x Line	4	66112	1.13	0.34
	Male TRT x Mated vs. Not	1	31874	0.55	0.46
	Line x Mated vs. Not	4	211828	3.64	**< 0.01**
	Male TRT x Line x Mated vs. Not	4	38747	0.66	0.62
	Residuals	128	58272		

Song Consistency	Male TRT	1	84.66	2.99	0.09
	Line	4	153.36	5.41	**< 0.001**
	Mated vs. Not	1	0.37	0.01	0.91
	Male TRT x Line	4	44.99	1.59	0.19
	Male TRT x Mated vs. Not	1	1.18	0.042	0.84
	Line x Mated vs. Not	4	12.05	0.43	0.79
	Male TRT x Line x Mated vs. Not	3	18.45	0.65	0.58
	Residuals	69	28.32		

TRT = Treatment; significant results are in bold

Chapter 4 - Overwintering of the invasive pest *Drosophila suzukii* Matsumura in the central plains is facilitated by thermal acclimation

Elizabeth R. Everman, Philip J. Freda, Mariah Brown, Gregory Ragland,

and Theodore J. Morgan

Abstract

In temperate regions, seasonal and diurnal temperature variation presents novel challenges to small invasive ectotherms; however, phenotypic plasticity can facilitate survival and persistence. We tested the role of developmental acclimation, adult long-term acclimation, and photoperiod on the induction of overwintering phenotypes including reproductive diapause and adult survival in a low genetic diversity population of *D. suzukii* cultured from a recently established population in Topeka, Kansas (USA). We found that both temperature and photoperiod resulted in reduced ovary size and level of development relative to control females. Reduction in ovary development was observed in response to each acclimation temperature, with adult long-term acclimation at 11°C resulting in the largest reduction in ovary development and size. Additionally, reduction in ovary development was observed at warmer temperatures relative to previous reports of the induction of diapause in populations sampled in the northern USA and southern Canada. We also provide evidence that *D. suzukii* is capable of short-term hardening, contrary to previous reports. Our study highlights the central role of phenotypic plasticity in response to winter-like laboratory conditions and provides an essential geographic comparison to previously published assessments of diapause and short-term hardening survival response for *D. suzukii* collected in southern Canada.

Introduction

The ability for invasive species to persist in novel environments despite limited genetic variation within the founding population remains an evolutionary paradox (Frankham 2005), but hypothesized mechanisms for successful invasion into a novel environment following a founding event are diverse (Dlugosch and Parker 2008; Stapley et al. 2015). One mechanism that can facilitate invasion is phenotypic plasticity for fitness-related traits, which can allow a small number of genotypes to thrive in a novel environment. Davidson et al. (2011) illustrated that invasive plants have greater phenotypic plasticity for many fitness related traits when compared to their non-invasive congeners. Similar results have also been documented in arthropods (Lardies and Bozinovic 2008; Kleinteich and Schneider 2011). Taken together, these results suggest that plastic species are able to respond to a range of environmental stressors and more successfully survive and persist in novel environments.

Temperature fluctuations occur daily and seasonally in temperate habitats, regularly dropping below freezing between fall and spring. The ability of organisms to tolerate these changes in temperature is often predictive of geographic distribution and activity (Addo-Bediako et al. 2000; Kellermann et al. 2012; Overgaard et al. 2014; Andersen et al. 2015) as environmental temperature is a strong source of selective pressure (Huey and Kingsolver 1993). In small ectothermic species, exposure to temperatures below freezing can result in chilling injury and ultimately death (mechanisms reviewed in Teets and Denlinger 2013); however, these consequences can be avoided through short-term hardening, long-term acclimation, and developmental acclimation (Wilson and Franklin 2002; Bowler 2005; Angiletta 2009; Colinet and Hoffmann 2012; Fallis et al. 2014; Gerken et al. 2015).

Short-term hardening, one form of which is rapid cold-hardening (RCH), is a reversible process that occurs over very short timescales (minutes to hours) and has been demonstrated to increase survival in diverse species following brief exposure to a nonlethal temperature prior to harsher cold stress (Chen et al. 1987; Lee et al. 1987; Czajka and Lee 1990; Worland and Convey 2001; Wang and Kang 2003; Bowler 2005; Loeschcke and Sørensen 2005; Sinclair and Chown 2006). Long-term acclimation or seasonal cold hardening (SCH) is also reversible but occurs over longer timescales (weeks to months) and can be induced by shifts in photoperiod as well as temperature variation associated with seasonal change (Denlinger 1991; Bowler 2005). Developmental acclimation is an irreversible process that is induced by environmental conditions experienced during development (Lee et al. 1987; Wilson and Franklin 2002; Teets and Denlinger 2013). Long-term and developmental acclimation are directly related to seasonal shifts in average temperature, but short-term hardening occurs as a result of diurnal thermal variation. In *Drosophila melanogaster*, developmental acclimation, long-term acclimation, and short-term hardening can act synergistically; for example, short-term hardening of developmentally acclimated individuals can increase survival relative to individuals that are not developmentally acclimated (Kelty and Lee 2001; Gerken et al. 2015). Understanding how an invasive species employs all three strategies will elucidate their overall potential for range expansion in a novel environment as temperature tolerance is directly related to an organism's overall fitness and reproductive success.

Drosophila suzukii (Matsumura), an invasive pest species endemic to Asia, was first detected in the continental USA in California in 2008 (Walsh et al. 2011). Within three years of this initial detection, specimens were collected throughout the west and east coasts of the United States and in Canada (Burrack et al. 2012; Freda and Braverman 2013) and recently, stable

populations have been identified in central plains (Everman et al. 2015). Concurrently, *D. suzukii* was introduced and expanded its range throughout Europe from 2008 to 2012 (Cini et al. 2012). These introductions are significant because unlike the vast majority of drosophilids, *D. suzukii* is an agricultural pest. Female *D. suzukii* have a serrated ovipositor allowing eggs to be laid in un-ripened, soft-skinned fruits including a variety of berries, stone fruits, and grapes (Walsh et al. 2011; Everman et al. 2015). However, what makes *D. suzukii* of interest from an evolutionary and ecological standpoint is its ability to successfully and rapidly invade and thrive within novel environments.

For invasive species like *D. suzukii*, the ability to respond to diverse thermal regimes through phenotypic plasticity via short-term hardening, long-term acclimation, and developmental acclimation can facilitate overwintering in novel temperate environments. Overwintering can be achieved via a combination of increasing basal levels of cold tolerance (Bergland et al. 2014a; Shearer et al. 2016) and/or limiting reproduction by entering diapause (Kimura 1988; Hoffmann et al. 2003b) where ovaries are reduced in size and contain few to no mature eggs (Saunders et al. 1989; Denlinger 1991). Female drosophilids such as *D. pseudoobscura*, *D. melanogaster*, and *D. suzukii* can store sperm until thermal conditions become permissive for development (Denlinger 1991; Price et al. 1999; Giraldo-Perez et al. 2016; Ryan et al. 2016). Thus, as average temperature rises, ovarian development continues, and eggs are fertilized and oviposited. *D. suzukii* likely employs a similar strategy in North America and Europe (Ryan et al. 2016).

Ovarian diapause phenotypes can be induced via long-term and developmental acclimation by reducing the photoperiod and/or the overall temperature that females experience (Kimura 1988; Zhai et al. 2016). These environmental cues can induce diapause in *D. suzukii* as

well; however, much of this insight comes from studies that tested individuals collected from populations established in Canada, New York, Oregon and Michigan (Jakobs et al. 2015; Stephens et al. 2015; Wallingford and Loeb 2016; Wallingford et al. 2016) where selection pressure on overwintering strategies is constant and strong. Recently, *D. suzukii* was detected in Kansas, where populations experience milder winters and more variable seasonal shifts in temperature compared to more northern populations (Everman et al. 2015). As this invasive pest becomes established across an increasingly broad geographic range, it is essential to understand the phenotypic response of *D. suzukii* to winter conditions. Study of a recently established *D. suzukii* population facilitates both a more comprehensive understanding of its success as an invasive species and the prediction of the degree to which overwintering strategies determine geographic boundaries of this species.

In this study we tested the ability of a *D. suzukii* population from the central plains (Topeka, Kansas, USA) that was established in 2013 to plastically respond to diverse environmental conditions including photoperiod and temperature. First, we tested whether long-term and developmental acclimation at low, non-lethal temperatures and alteration to photoperiod induced female reproductive diapause. Second, we determined effect of short-term hardening (RCH) to non-lethal temperature on adult cold tolerance. We hypothesized that short photoperiod, adult long-term acclimation, and developmental acclimation would induce a diapause phenotype in female *D. suzukii* and that adult short-term hardening to non-lethal temperature prior to cold shock would increase overall survival in both male and female adult *D. suzukii*. Because we used a low genetic diversity population following several generations of inbreeding, changes to reproductive and cold tolerance phenotypes induced by experimentally altered environmental conditions illustrate the capacity of *D. suzukii* to respond plastically to all

three acclimation regimes. Such plastic responses to these treatments further illustrate the importance of phenotypic plasticity in invasive species survival and population persistence in geographically diverse novel environments.

Methods

Stock maintenance

Adult female *D. suzukii* were captured from an orchard in Topeka, Kansas (39°12'10.4" N, 95°44'31.4" W) in July, 2014. A low genetic diversity population culture was initiated with four females and was maintained at 23°C on a 12-hour light:dark cycle for 30 generations prior to experiments. Flies were maintained in 8 oz polypropylene stock bottles on standard cornmeal-molasses-yeast agar supplemented with raspberries. While food was still warm and unset, a frozen raspberry was added to the media. When food cooled, the raspberry was thawed and embedded in the media.

Effect of photoperiod and long-term acclimation on ovary development

Experimental *D. suzukii* individuals were obtained by placing five male and five female parents *D. suzukii* on raspberry supplemented media in polystyrene vials at 23°C on a 12-hour light:dark cycle and allowing females to lay eggs for 48 hours before being removed. Experimental flies were collected at a maximum of 8 hours post eclosion by discarding any hatched experimental flies at 8 am and removing any flies a total of 8 hours later on the same day (Saunders et al. 1989; Zhai et al. 2016). The experimental flies were briefly anesthetized with CO_2 and sorted by sex and males were discarded.

Experimental females were transferred to vials grouped by eclosion day to treatment incubators set at 15°C and were maintained for 21 days. We measured the effect of photoperiod using two treatments. The long-photoperiod treatment consisted of a 14:10 hour light:dark cycle,

and the short-photoperiod treatment consisted of a 10:14 hour light:dark cycle (Fig. 4.1A). Long- and short-photoperiod treatments were conducted in separate incubators. To ensure conditions were comparable between the two incubators, a light-proof control box was built and placed in the short-photoperiod incubator. Females in the control box experienced a long-photoperiod treatment. Each treatment received an equal number of experimental females per eclosion day. A total of 32 females were exposed to the long-photoperiod treatment; 13 females were exposed to the short-photoperiod treatment. After the incubation period, all experimental females were preserved at -80°C until ovaries were dissected.

Dissections were done using 1 x PBS/0.14% Triton solution with Dumont No. 5 Inox alloy forceps. Ovary width and length was measured using an ocular micrometer, and ovary development was classified according to three categories. Immature ovaries were 0 - 0.40 mm wide and 0 - 0.50 mm long with fewer than two maturing eggs visible within the ovarioles. Developing ovaries were 0.41 - 0.55 mm wide and 0.51 - 0.70 mm long with more than two maturing eggs. Developed ovaries were 0.56 - 0.95 mm wide and 0.71 - 1.1 mm long and nearly all ovarioles contained maturing eggs.

Effect of temperature on ovary development

To determine the effect of developmental and long-term acclimation on ovary development independent of photoperiod, parent flies were set up as described above and all experimental females were maintained on a 12-hour light:dark cycle. Ten vials of approximately 20 eggs each were allowed to develop at either 11°C, 15°C, or 23°C. Once eclosion occurred, the flies were briefly anesthetized with CO_2 before being sorted by sex and males were discarded. Experimental females were transferred to vials grouped by eclosion day. Females of the same eclosion day that developed at 11 or 15°C were maintained at their developmental temperature

for 21 days to test the effect of developmental acclimation on ovary development (Fig. 4.1B). Females of the same eclosion day that were reared at 23°C were shifted within eight hours of emergence to 11°C for 21 days to test the effect of long-term adult acclimation on ovary development. A control set of females was maintained at 23°C during both development and 21 days post eclosion. After the 21-day incubation period, all of the females were preserved at -80°C until ovaries were dissected. Ovaries were dissected, measured, and classified according to developmental level as described above. A total of 17 females were developmentally acclimated at 11°C, 19 females were developmentally acclimated at 15°C, 20 females were long-term acclimated at 11°C, and 20 females were maintained at the control temperature of 23°C.

Effect of temperature on adult survival

To determine the effect of cold temperature stress on survival, five males and females were allowed to lay eggs on media supplemented with raspberries for four days at 23°C. After this period parents were removed, experimental flies were reared at 23°C, and adults were collected on the third day of eclosion. Experimental flies were sorted by sex with light CO_2 anesthesia. Flies were allowed to recover and mature for five days at 23°C with 10 same-sex individuals per vial.

Survivorship was measured following exposure to one of two experimental treatments: Acclimated flies were exposed to 4°C for two hours immediately prior to a one hour -6°C cold stress (Fig. 4.1C). Non-acclimated flies were exposed to the one-hour -6°C cold stress without a pretreatment (Fig. 4.1D). Temperature treatments were used following Gerken et al. (2015). Once the cold stress treatment was completed, flies were transferred to fresh raspberry supplemented media and allowed to recover for 24 hours at 23°C, at which point survivorship was determined as the proportion of flies alive in each vial. Flies were determined to be alive if

they were capable of walking and/or flying. A total of 106 vials were tested (53 acclimated and non-acclimated vials).

Data Analysis

Both ovaries were measured in each female and used to calculate the average ovary length and width for each female. To summarize the variation in ovary length and width, we calculated the product of these measures to approximate ovary size. We assessed normality and homoscedasticity for ovary size and survival following temperature stress. Residuals of ovary size were not normally distributed ($W = 0.91$, $P < 0.001$) but were homoscedastic ($F_{5,115} = 0.83$, $P = 0.53$). We used a square-root transformation to improve the violation of normality for ovary size ($W = 0.97$, $P = 0.01$; Fig. 4.2). Because the ovaries of females from the photoperiod control box were not different from ovaries of females treated in the long photoperiod incubator ($F_{1,30} = 0.13$, $P = 0.72$), these ovaries were combined for analysis of the effect of photoperiod on ovary size. The effects of photoperiod and temperature on ovary size were analyzed with one-way analyses of variance, and post hoc comparisons were performed to compare the influence of each treatment on ovary size.

Because quantitative differences in ovary size due to photoperiod and temperature treatment may not fully reflect the level of ovary development under the treatment conditions, we also compared the frequency of ovaries classified as developed, developing, or immature following each photoperiod and temperature treatment. Differences in frequency of ovaries classified as developed, developing, and immature were tested using a contingency table analysis for each experiment. To test the effect of photoperiod on ovary development when females were long-term acclimated at 15°C, we performed a two (long versus short photoperiod) by three (developed, developing, and immature ovaries) contingency table analysis. To test the effect of

acclimation treatments without altered photoperiod on ovary development versus the control females reared and maintained at 23°C, we performed a four (dev. acclimation at 15°C, dev. acclimation at 11°C, long-term acclimation at 11°C, and control) by three (ovary development) contingency table analysis. To determine if differences in the frequency of developed, developing, and immature ovaries existed between different types of acclimation and versus the control, we used post hoc pairwise comparisons to compare each acclimation treatment to the control, and between the different types of acclimation (Table 4.1). The test statistic for pairwise post hoc analyses was adjusted for multiple comparisons using the Bonferroni correction (α = 0.0083). All analyses were conducted in R (Wickham 2009; Hope 2013; R Core Team 2015).

Residuals of survival following the acclimated and non-acclimated cold stress treatments were also not normally distributed ($W = 0.92$, $P < 0.001$) but were homoscedastic ($F_{1,104} = 2.45$, $P = 0.14$). Arcsine transformation of the proportion of individuals that survived per vial did not improve the violation of normality. Therefore, we analyzed the effect of sex and treatment on the survival data (analyzed as alive or dead for each individual) with a general linear model with a binomial error distribution and logit link function in the R package MASS (Venables and Ripley 2002). Post-hoc comparisons were assessed using Tukey's HSD with an experiment-wide α = 0.05.

Results

Photoperiod and acclimation (developmental and long-term) significantly influenced ovary size ($F_{5,115} = 32.94$, $P > 0.001$; Fig. 4.3A). Post hoc comparisons of the treatments indicated that reduction in ovary development and size was observed as a result of photoperiod and acclimation treatments relative to control females that had been maintained at 23°C on a

12:12 light:dark cycle. While the control treatment was not directly consistent with natural, summer-like conditions, it is consistent with the conditions experienced by females during the 30-generation period post collection, and so reflects ovary development in females experiencing non-stressful, permissive conditions.

Effect of photoperiod on ovary development

Females reared at 23°C and subsequently long-term acclimated at 15°C for 21 days under different photoperiods had significantly reduced ovary size relative to the control (adj. $P < 0.001$, Fig. 4.3A). Variation in photoperiod also resulted in a significant difference in ovary size for females that were reared under long or short photoperiods (adj. $P < 0.05$; Fig. 4.3A). On average, ovaries were significantly larger in females reared under a long photoperiod that was more similar to summer conditions (Fig. 4.3A). When females were reared under the short photoperiod more consistent with winter conditions, ovaries were significantly smaller (adj. $P < 0.05$; Fig. 4.3A). Consistent with our quantitative estimate of ovary size, contingency table analysis showed that exposure of females to a long photoperiod with long-term acclimation at 15°C significantly increased the frequency of developed and developing ovaries, while exposure to the short photoperiod under the same thermal conditions increased the frequency of immature or developing ovaries ($X^2 = 15.38$, $df = 2$, $P < 0.001$; Table 4.1, Fig. 4.3B). Because experimental females were long-term acclimated under both the long and short photoperiods at a constant, relatively cool temperature (15°C) and 50% of females had developed ovaries under long day conditions (Fig. 4.3B), the resulting difference in ovary size and level of development indicates that variation in photoperiod interacts with exposure to cool temperature and has the potential to reverse the effects of long-term acclimation to a cool temperature on reduced ovary development. Further, because these responses were examined in a low genetic diversity

population, the shift in ovary size following changes in the photoperiod suggests that ovary development responded plastically to the photoperiodicity of the environment experienced by the female.

Effect of temperature on ovary development

We tested the effect of developmental acclimation at 11°C, developmental acclimation at 15°C, and long-term acclimation at 11°C (reared at 23°C, adults shifted to 11°C) on ovary development under 12:12 hour photoperiod. Both developmental and long-term acclimation in the absence of altered photoperiod significantly influenced ovary size relative to the control (adj. $P < 0.001$; Fig. 4.3A). When ovary size was considered as a quantitative variable, there were no differences in ovary size between the developmentally acclimated and long-term acclimated females (all adj. $P > 0.05$; Fig. 4.3A). Similarly, when the developmental category of the ovaries was considered via contingency table analysis, we found that level of ovary development differed among the control, developmentally acclimated, and long-term acclimated females ($X^2 = 76.98$, $df = 6$, $P < 0.0001$; Fig. 4.3B; Table 4.1). Pairwise comparisons of each temperature treatment showed that developmental acclimation at 15 and 11°C resulted in significantly reduced ovary development relative to control females (15°C dev. acclimation: $X^2 = 31.72$, $df = 2$, $P < 0.0001$; 11°C dev. acclimation: $X^2 = 37.0$, $df = 2$, $P < 0.0001$; Fig. 4.3B; Table 4.1) as did the long-term acclimation treatment at 11°C ($X^2 = 36.19$, $df = 2$, $P < 0.0001$; Fig. 3B; Table 1). Developmental acclimation at 15°C did not significantly alter ovary development relative to developmental acclimation at 11°C ($X^2 = 5.02$, $df = 2$, $P = 0.08$; Fig. 4.3B; Table 4.1), nor did developmental acclimation at 11°C relative to long-term acclimation at 11°C ($X^2 = 3.39$, $df = 2$, $P = 0.18$; Fig. 4.3B; Table 4.1). However, developmental acclimation at 15°C did result in more ovary development relative to long-term acclimation at 11°C ($X^2 = 10.56$, $df = 2$, $P = 0.005$; Fig.

4.3B; Table 4.1). Overall, these results suggest that developmentally acclimated females were more likely to have some developing or developed ovaries while long-term acclimated females had almost exclusively immature ovaries.

The significant effect of acclimation on ovary development indicated that both developmental and long-term exposure to cool temperatures of 11° and 15°C in the absence of variation in photoperiod was sufficient to stunt ovary development. Further, it is clear that long-term acclimated females that were reared from egg to adult at 23°C and then transferred to 11°C for 21 days were capable of a more drastic plastic response to a shift in temperature compared to females that were developmentally acclimated. This suggests that both gradual and abrupt changes in environmental temperature have the potential to induce quiescence of ovary development. Finally, when ovary size in females exposed to a short photoperiod and long-term acclimated at 15°C was compared to that of thermally acclimated females (developmentally or long-term) without variation in photoperiod, reduction in ovary size was similar (adj. $P > 0.05$; Figure 4.3A) suggesting that change in temperature was sufficient to induce a diapause-like phenotype without reduced photoperiod.

Effect of temperature on survival

The effect of cold stress treatment on survival was highly significant; the non-acclimation cold stress resulted in poor survivorship, while the acclimation cold stress significantly improved survival (β_{TRT} = 1.38 +/- 0.2, z = 8.6, $P < 0.001$; Fig. 4.4). While the effect of sex was significant (β_{SEX} = 1.50 +/- 0.4, z = 4.0, $P < 0.001$; Fig. 4.4), there was no sex by treatment interaction ($\beta_{TRT:SEX}$ = -0.66 +/- 0.5, z = -1.4, P = 0. 15; Fig. 4.4). Thus, *D. suzukii* adults, when reared at 23°C similar to summer conditions, had very low cold tolerance; however, the marked increase

in survival following the short-term hardening treatment demonstrates that adults have the capacity to respond plastically to cold temperature variation.

Discussion

Temperature fluctuations that occur through seasonal shifts can limit the spread of invasive species that are sensitive to thermal stress. Therefore, phenotypic plasticity is a critically important mechanism that can facilitate the spread of invasive species into temperate habitats (Richards et al. 2006; Lardies and Bozinovic 2008; Davidson et al. 2011; Kleinteich and Schneider 2011; Lamarque et al. 2013). Natural populations of *D. suzukii* in the northern USA and southern Canada respond to seasonal drops in temperature and shortening of photoperiod through diapause and increased cold tolerance, suggesting that *D. suzukii* spread through temperate North America has been facilitated by the capacity to respond to cold temperature (Jakobs 2014; Jakobs et al. 2015; Stephens et al. 2015; Ryan et al. 2016; Shearer et al. 2016; Wallingford and Loeb 2016; Wallingford et al. 2016). In *D. melanogaster*, the induction of diapause varies with latitude through the USA, with flies collected from Florida being generally less sensitive to diapause-inducing temperatures and photoperiod compared to flies collected from Maine (Schmidt et al. 2005). The recent introduction and rapid spread of *D. suzukii* through the temperate USA and Canada makes this species an interesting model to understand the role of variable thermal regime in successful establishment and persistence of populations across a range of latitude.

The purpose of our study was to determine the relative importance of variation in photoperiod and acclimation to cool temperatures on the reduction in ovary development. Our study is unique in that we focus on a recently established population from Topeka, Kansas (USA), where *D. suzukii* had not been detected until 2013 and has since been sustained

(Everman et al. 2015). Our study is also unique in that we used a low genetic diversity population that allowed us to specifically test the capacity of *D. suzukii* to respond plastically to rearing and adult treatment conditions. As a result, we provide insight into the capacity of a recently established population to adapt and respond plastically to seasonal and daily temperature variation in a mid-latitude temperate region.

We found that both ovary size and development were significantly influenced by photoperiod and both developmental and long-term acclimation to cool (11°C) and moderately cool (15°C) temperatures. Because we were examining these responses in a low genetic diversity population, it is clear that phenotypic plasticity contributed to these photoperiod- and temperature-induced changes in ovary development. When females were developmentally acclimated and maintained at 11° or 15°C, ovary size was greatly reduced (Fig. 4.3A); however, while the quantitative reduction in size was comparable between females developmentally acclimated at 11° and 15°C, the proportion of immature, developing, and developed ovaries was marginally different (Fig. 4.3B). We found that females reared and maintained at 11°C tended to have a greater proportion of immature ovaries and no developed ovaries compared to the females reared and maintained at 15°C (Fig. 4.3B). This increase in the proportion of immature ovaries in 11°C developmentally acclimated females is consistent with other reports that diapause in *D. suzukii* is closely tied to environmental temperature in natural populations (Wallingford et al. 2016; Zhai et al. 2016).

A similar temperature-dependent change in ovary development was observed in long-term acclimated *D. suzukii* originating from native populations in China (Zhai et al. 2016). Zhai et al. (2016) demonstrated that the proportion of females with immature ovaries increased when females were long-term acclimated at gradually decreasing temperatures. In this native

population, long-term acclimation of adult females to 15°C under a 12:12 photoperiod resulted in levels of delayed ovary development that are similar to that of females developmentally acclimated at 15°C in our study. In contrast, *D. suzukii* originating from populations in New York and Oregon at higher latitude did not exhibit a marked reduction in ovary development when females were developmentally acclimated at 15°C relative to controls (Wallingford and Loeb 2016; Wallingford et al. 2016), nor did females collected in Ontario, Canada that were exposed to long-term acclimation or fluctuating environment conditions that simulated fall temperatures and photoperiod (Jakobs 2014). The degree to which ovary reduction varies among the invasive populations sampled suggests that, while diapause is likely to occur across latitude when females experience cooler temperatures and shorter photoperiod, the temperatures at which the initial shift occurs are influenced by latitude.

In our study, long-term acclimation at 11°C with a 12:12 photoperiod resulted in the greatest reduction in ovary development, leading to a significantly higher proportion of immature ovaries than in females that were developmentally acclimated at 15°C. Exposure of newly eclosed adults to a shift to cold temperature is a strong cue to induce diapause (Saunders et al. 1989), and a strong effect of long-term acclimation on ovary development is consistent with results previously reported for New York and China populations of *D. suzukii* (Wallingford and Loeb 2016; Wallingford et al. 2016; Zhai et al. 2016). This suggests that exposure of maturing adult females to cool temperatures is sufficient to impair ovary development across a range of latitudes. The sharp reduction in ovary development in females that were reared at 23°C and then maintained at 11°C may be relevant for understanding how natural populations respond to the transition between summer and fall. For example, females in natural populations during this transition would likely have developed under warm summer-like conditions, but may be faced

with much cooler temperatures as adults. The ability to plastically alter reproductive strategy would allow these females to survive increased fluctuation in daily temperature and potentially overwinter as diapausing adults (Stephens 2015; Shearer et al. 2016; Wallingford and Loeb 2016; Wallingford et al. 2016).

Photoperiod also plays an important role in the induction of diapause in nature in response to seasonal change (Kimura 1988; Saunders et al. 1989; Vesala and Hoikkala 2011; Wallingford et al. 2016; Zhai et al. 2016). In our study, females exposed to long-term acclimation at 15°C under a short photoperiod had remarkably similar levels of reduced ovary development compared to females that were developmentally acclimated at 15°C under a 12:12 hour photoperiod (Fig. 4.3B). Though there was a significant difference between the control females and females that experienced the long photoperiod, that 50% of the long photoperiod females had fully developed ovaries suggests that the significant difference may not be biologically meaningful. The tendency for a larger proportion of developed ovaries in this treatment might be indicative of a benefit for females in populations at the beginning of spring. In invasive populations of *D. suzukii* in North America, more females are observed early in spring when temperatures are gradually warming and photoperiod is lengthening (Dalton et al. 2011), whereas in Italy, invasive populations are composed primarily of males early in the spring (Rossi-Stacconi et al. 2016). The increase in the proportion of developed ovaries that appears to accompany longer photoperiod in long-term acclimated females could therefore facilitate early reproduction of this species in North America during the spring, especially given that *D. suzukii* females may store sperm and are capable of ovipositing viable eggs following an extended cooling period (Ryan et al. 2016). The ability of females to respond plastically to environmental

variation in terms of both thermal acclimation and photoperiod has likely aided in their invasion of North American habitat.

Capacity for diapause alone is likely not sufficient population persistence following seasonal temperature changes. An increase in cold tolerance accompanies most examples of reproductive senescence in *D. suzukii* (Shearer et al. 2016; Wallingford and Loeb 2016; Wallingford et al. 2016; Zhai et al. 2016), and it is probable that this increase in cold tolerance is due to a combination of acclimation and adaptation to seasonal shifts in temperature (Bergland et al. 2014a). Developmental acclimation, long-term acclimation, and short-term hardening have been repeatedly shown to increase survival following cold stress in many ectothermic species (Kimura 1988; Coulson and Bale 1992; Hoffmann et al. 2003c; Sinclair and Roberts 2005; Geister and Fischer 2007; Basson et al. 2012; Jakobs et al. 2015; Stephens 2015). However, when Jakobs and colleagues (2015) examined the short-term hardening response in *D. suzukii* in flies collected from Ontario, Canada, exposure to a short hardening treatment did not improve survival following cold stress. Their data suggest *D. suzukii* has little capacity for acclimation at the level of daily temperature fluctuations. However, in our study, *D. suzukii* individuals exposed to a very similar short-term hardening treatment had significantly higher survival following cold stress relative to non-acclimated individuals (Fig. 4.4). The lack of a short-term hardening response in a northern population and presence of a strong acclimation response coupled with low cold tolerance in our central plains population may by indicative of latitudinal variation in the capacity to plastically respond to short-term cold acclimation.

It is possible that the differences in short-term hardening capacity between flies collected in Canada and Kansas are due to a trade-off between cold tolerance and plasticity. Comparisons of plasticity and basal levels of cold tolerance often result in a negative correlation between these

two traits, where cold tolerant individuals have low capacity for plasticity and vice versa (Kellett et al. 2005; Nyamukondiwa et al. 2011; Gerken et al. 2015). Overall, in Kansas *D. suzukii* populations typically experience milder winters and longer, warmer summers relative to populations established in New York, Michigan, or Canada where flies are generally more cold tolerant (Jakobs et al. 2015; Stephens et al. 2015; Wallingford and Loeb 2016). If there is a constraint between maintaining the capacity for plasticity and basal levels of cold tolerance, the weaker selection pressure on basal cold tolerance may allow greater capacity for plasticity to be maintained, as the ability to respond positively to short-term hardening can have positive effects on reproductive fitness (Shreve et al. 2004). The effects of developmental and long-term acclimation and photoperiod on ovary development tie into this relationship between increased capacity for plastic responses in Kansas females as we show that relatively mild temperature (15°C) is sufficient to induce a diapause-like phenotype, and the degree of development can influenced by a shift in photoperiod alone.

Conclusions

Responses to thermal variation are not constant over geographic distributions of species (Hoffmann and Watson 1993; Hoffmann et al. 2002), and have been shown to be particularly variable for insect species (Sinclair et al. 2012). *D. suzukii* has become a highly successful invasive pest that appears to have established populations throughout the United States and Canada that survive overwintering periods of the temperate seasons. For these populations to persist, individuals must either adapt to novel thermal environments that vary in seasonally predictable ways, respond through phenotypic plasticity, or use a combination of these strategies. While the region affected by the invasive distribution of *D. suzukii* can be broadly described as primarily temperate, it is important to note that within the latitudinal gradient of the temperate

region that spans from Canada through the United States, a gradient of seasonal and daily variation in temperature exists. In order to fully understand the success of invasive species that are hypothesized to be temperature-limited (Kimura 1988; Stephens et al. 2015), it is important to account for variation in the adaptive and phenotypic plasticity strategies that characterize populations over the span of their distribution.

Acknowledgments

We thank Ree's Fruit Farm and 86th Street Orchard for allowing us to collect the original *D. suzukii* females to found laboratory cultures. This work was supported by the National Science Foundation award NSF IOS-1051770 awarded to T.J.M. and a Biology Graduate Student Association Research Grant awarded to E.R.E.

Figures and Tables

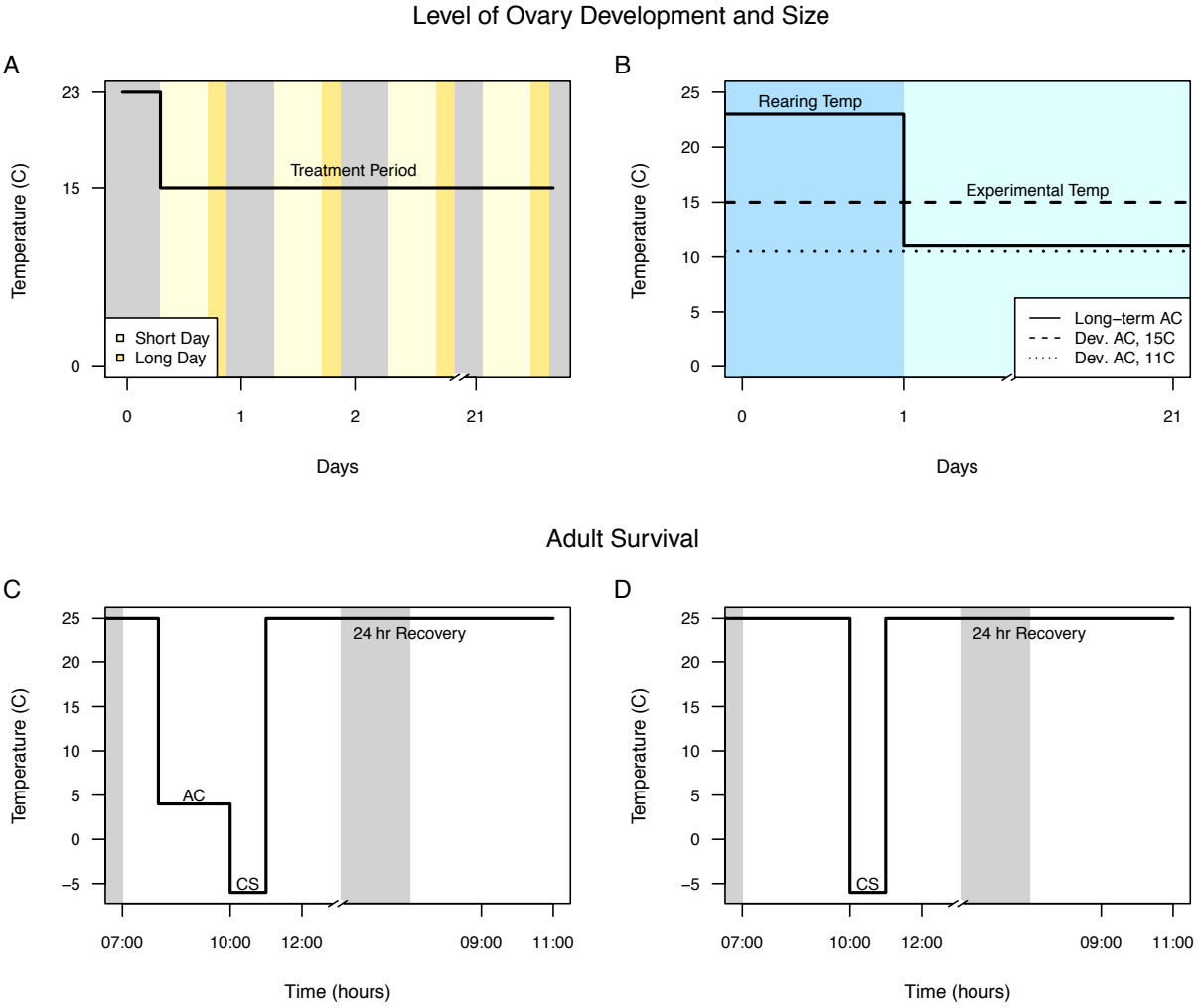

Figure 4.1 Diapause and survival treatments used to test the effect of (A) photoperiod and (B) developmental and long-term acclimation on ovary development and to test the effect of (C) acclimation and (D) cold shock without acclimation on survival. A. Females were exposed to a short (10 hour; light yellow shading) or long (14 hour; light and dark yellow shading combined) photoperiod while being long-term acclimated as adults at 15°C for 21 days post eclosion. Grey shading indicates "lights off". "Lights on" occurred each day at 07:00 hrs. B. Females were either developmentally acclimated at 15°C (dashed line) or 11°C (dotted line), or were long-term acclimated as adults at 11°C (solid line) for 21 days after development at 23°C. Dark blue shading indicates the rearing conditions; light blue indicates the post-eclosion conditions. Females experienced a 12:12 hr photoperiod that began at 07:00 hrs each day. C. Adult flies were exposed to an acclimation treatment (4°C) for two hours prior to cold shock (-6°C) for one hour and were allowed to recover for 24 hours at the rearing temperature (23°C) before survival was assessed. D. Adult non-acclimated flies were exposed to the cold shock temperature (-6°C)

for one hour and were allowed to recover for 24 hours at the rearing temperature (23°C) before survival was assessed. In both C and D, shading indicates the timing of the 12:12 photoperiod.

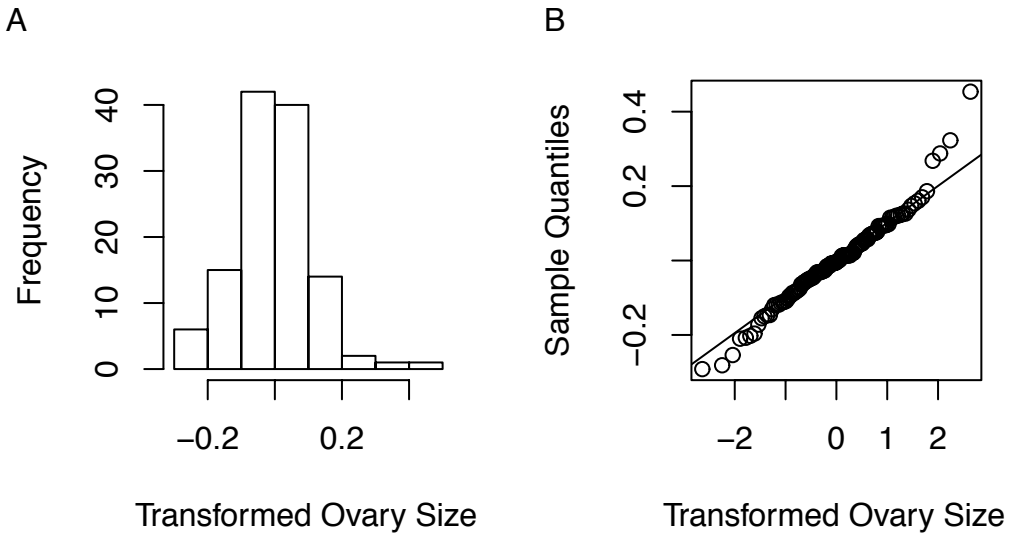

Figure 4.2 Residuals of ovary size shown as a histogram (A) and QQplot (B) following square root transformation.

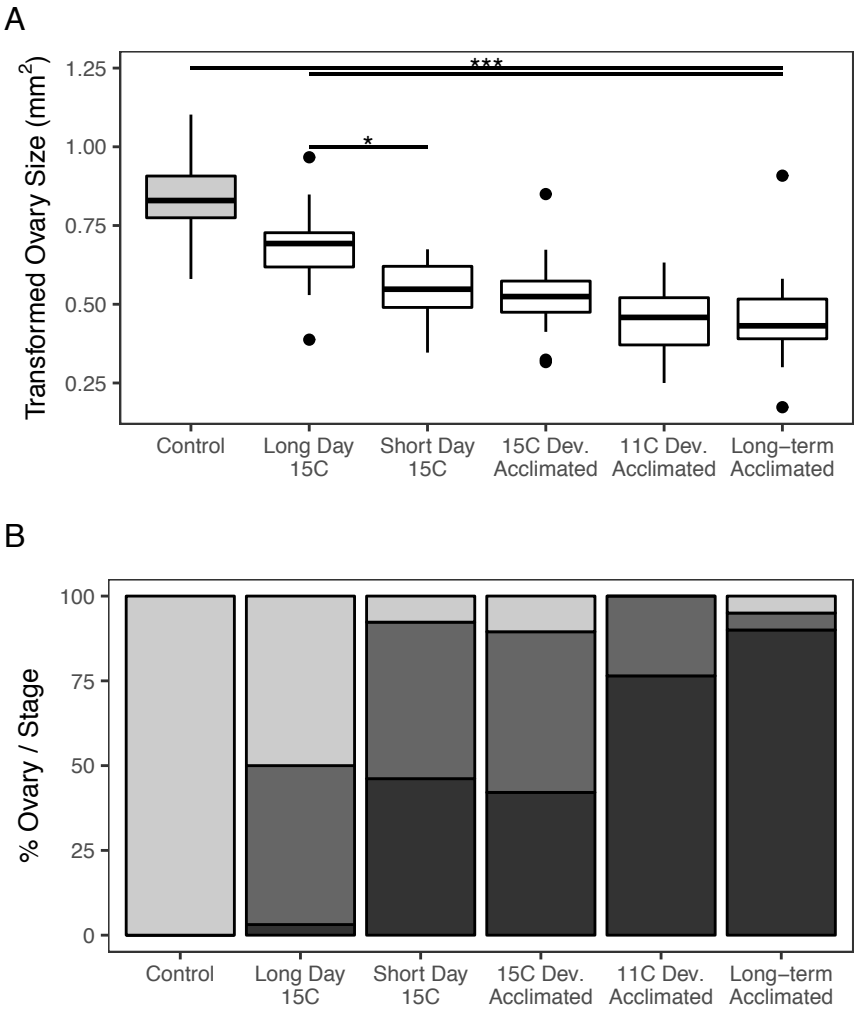

Figure 4.3 Ovary size and developmental stage were influenced by photoperiod variation and developmental and long-term acclimation. A. *D. suzukii* females that experienced a summer-like photoperiod had larger ovaries than females that experienced a winter-like photoperiod (asterisks indicate significant comparisons, adj. $P < 0.05$). Females that experienced either developmental or long-term acclimation without a modified photoperiod (15C Dev. Acclimated, 11C Dev. Acclimated, and Long-term Acclimated) had ovaries that were significantly smaller than those of control females (adj. $P < 0.001$). Developmentally and long-term acclimated females did not differ in ovary size in the absence of an altered photoperiod (adj. $P > 0.05$). Long-term acclimated females at 15°C under a long day photoperiod had ovaries that were larger than all other treated females (adj. $P < 0.001$). All measurements are given in mm^2 following square root transformation. The filled box indicates the control treatment. B. Ovaries of females were designated as immature (dark grey), developing (medium grey), or developed (light grey) varied among the treatments. Short photoperiod variation combined with long-term acclimation at 15°C resulted in a higher proportion of immature ovaries compared to females that experienced the long day (adj. $P < 0.0001$). Acclimation in the absence of photoperiod variation influenced ovary development compared to control as well (adj. $P < 0.001$).

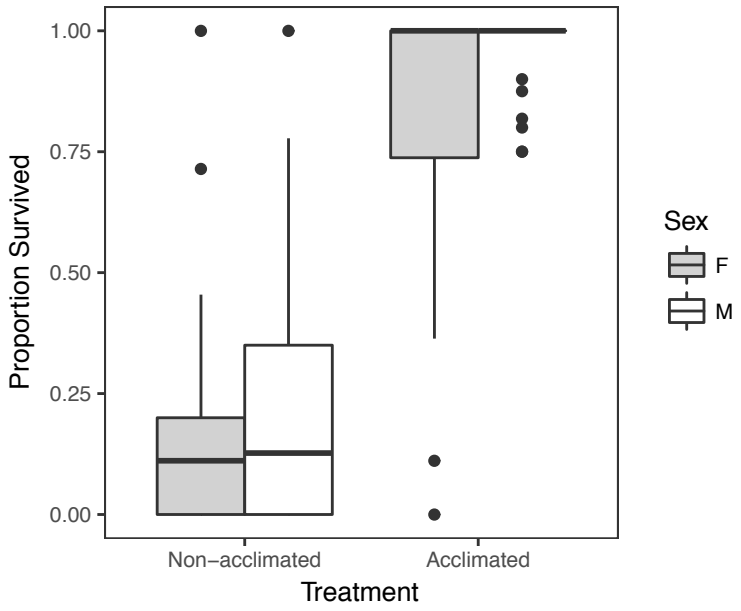

Figure 4.4. The proportion survived of adult *D. suzukii* reared and maintained at 25°C was significantly influenced by acclimation and non-acclimation cold stress treatments. While flies were generally highly susceptible to the non-acclimation stress, acclimation greatly improved survival for both sexes. The y-axis shows the average proportion of the ten individuals per vial that survived. Females are shown as filled boxplots; males are shown as open boxplots.

Table 4.1 Chi-square post hoc comparisons of ovary developmental stage (family-wise α = 0.05).

Experiment	Comparison	X^2	df	*P* value
Photoperiod	Long x Short photoperiod	15.38	2	**0.0005**
Temperature	Control x 15 DA x 11 DA x 11 LT	76.98	6	**< 0.0001**
	Control x 15 DA	31.72	2	**< 0.0001**
	Control x 11 DA	37.0	2	**< 0.0001**
	Control x 11 LT	36.19	2	**< 0.0001**
	15 DA x 11 DA	5.02	2	0.08
	15 DA x 11 LT	10.56	2	**0.005**
	11 DA x 11 LT	3.39	2	0.18

DA = Developmental Acclimation

LT = Long-term Acclimation

Chapter 5 - Synthesis

Each of the studies presented in this dissertation are quite different in topic and approach, but they are all tied together in that they each provide a depiction of how natural, diverse populations respond to the complexities of environmental variation. The usefulness of *Drosophila melanogaster* and similar species for understanding short and long term evolutionary processes and the genetic architectures of complex traits is abundantly clear through these studies, and these findings certainly apply to *D. melanogaster* in natural temperate populations. At the same time, these findings have much broader applications and implications for natural communities of diverse species. The thermal environment has broad influences on how organisms behave, reproduce, forage, and survive, and this research reveals that these responses are further influenced by age and genetic capacity to tolerate thermal stress.

A major theme throughout my research is the influence of seasonal variation in temperature on fitness. Cyclical patterns in selection pressure as a result of seasonal change in temperature have measurable effects on thermal tolerance of wild flies that are predicable and consistent across years. The degree to which average cold tolerance varies through the season seems directly tied to the average temperatures of the season. For example, during more variable years, the change in thermal tolerance through the season was greater compared to less variable seasons. Average thermal tolerance was influenced in similar ways as well. These data provide clear evidence that mismatch between phenotype and environment is likely through the season, especially as adult individuals adapted for one thermal environment experience the transition between late summer and fall. However, accompanying variation in phenotypic plasticity appears to be important for reducing this mismatch.

For *D. melanogaster* populations, the capacity for acclimation to compensate for seasonal change likely increases the probability that populations will persist over multiple years. It is also likely that variation in the genetic capacity for acclimation played an important role in the spread and persistence of *D. melanogaster* in temperate regions. These hypotheses and observations readily apply to other systems of small ectothermic species—especially those that have multiple generations per season and year. Native species must respond to and cope with thermal variation as well, and in the broader context of climate change, native and invasive populations alike will be faced with novel and increasingly variable thermal regimes. The capacity for short-lived organisms to respond to variation in selection pressure from the thermal environment with respect to tolerance and plasticity will likely be important as populations experience predicted changes in climate. As indicated in Chapter 4, we can gain insight into these patterns by taking advantage of the recent invasion history of *D. suzukii* in North America. As *D. suzukii* becomes established across a broad latitudinal gradient, variation in thermal regime will likely result in adaptation and shifts in capacity for phenotypic plasticity in this species that mirror the processes observed in *D. melanogaster* and other similar ectothermic species.

Seasonal variation can also lead to interesting patterns in the demographic structure of populations. For those species that experience reproductive quiescence during winter months, the warming period experienced during the spring can result in a synchronized increase in reproduction. My research indicates clearly that age typically has a negative influence on fitness when assessed through stress tolerance, and many others have demonstrated similar patterns with respect to other aspects of fitness. The combined influences of seasonal variation in thermal selection pressure and age pose potential challenges for adult individuals as they experience the transition between summer and fall. However, I also found that phenotypic plasticity increases

with age, and similar to our observations that acclimation capacity can compensate for seasonal change, this finding suggests that plasticity can also compensate for age-related decline in stress tolerance.

When the genetic control of stress tolerance was examined across age, it became evident that age-related decline in stress tolerance is influenced by a combination of polymorphisms that have increasingly negative effects across age as well as non-independent genetic architectures with a range of pleiotropic effects. In light of these data, the previous consideration of mutation accumulation and antagonistic pleiotropy as a dichotomy appears less useful—age-related change in genetic control of phenotypes may ultimately be more accurately described by varying degrees of pleiotropy. Furthermore, the effects of polymorphisms on a phenotype are influenced by environment through phenotypic plasticity, and so these patterns suggest that the specific age-related changes in polymorphisms that were observed in Chapter 2 are subject to environmental variability. One hypothesis that therefore emerges from this research is that the specific polymorphisms and effects will vary across seasons, populations, and environments. However, the broad-scale patterns observed with regard to pleiotropy and shifts in genetic architecture are likely to be upheld and present a hypothesis of interest for future research.

My research also makes important contributions to the broader understanding of how thermal variation influences reproductive components of fitness. Organisms must be able to function beyond survival in variable environments, and reproductive behavior is an important component of fitness that should be taken into account. Environmental cues that lead to physiological plasticity can have diverse influences on behavioral plasticity as well. Of particular interest are cues that have positive effects on some components of fitness and negative effects on other components. Acclimation, which usually increases survival in thermally stressful

environments tends to have a negative effect on reproductive behavior of males. While it is unclear which aspect of male courtship behavior is influenced by stress, it is clear that genetic capacity for physiological response influences the behavioral response to thermal cues. As with patterns observed in terms of survival following seasonal thermal variation and age-related change, capacity for plasticity seems to lead to increased fitness under certain environmental conditions. In essence, when flies experience constant conditions similar to warmer months when daily variation in temperature is reduced, flies with greater genetic capacity for acclimation mate more quickly. Looking back to patterns observed in Chapter 1, it is possible that the increase in developmental acclimation observed for individuals collected during warmer months is in part influenced by increased reproductive success of males that have increased capacity for acclimation. This tie between seasonal patterns acclimation capacity and reproductive fitness is likely much more complicated, but because the data presented herein span a great breadth of genetic and environmental variability, these broad patterns are observable and can be used to motivate future research.

My dissertation research was motivated by an interest in understanding how complex populations of diverse individuals respond to environmental variability. Each chapter provides insight into a unique aspect of this general goal, and while providing illuminating answers to important questions, more questions arise in the wake of these studies. I look forward to using this insight to pursue related research in the future.

References

Addo-Bediako, A., S. L. Chown, and K. J. Gaston. 2000. Thermal tolerance, climatic variability and latitude. Proc. R. Soc. Lond. B Biol. Sci. 267:739–745.

Andersen, J. L., T. Manenti, J. G. Sørensen, H. A. MacMillan, V. Loeschcke, and J. Overgaard. 2015. How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. Funct. Ecol. 29:55–65.

Anderson, A. R., A. A. Hoffmann, and S. W. McKechnie. 2005. Response to selection for rapid chill-coma recovery in *Drosophila melanogaster*: physiology and life-history traits. Genet. Res. 85:15–22.

Anderson, E. C., and M. Slatkin. 2003. Orr's quantitative trait loci sign test under conditions of trait ascertainment. Genetics 165:445–446.

Andrewartha, H. G., and L. C. Birch. 1954. The distribution and abundance of animals. Chicago University Press, Chicago.

Angiletta, M. J. 2009. Thermal adaptation: A theoretical and empirical synthesis. Oxford University Press, Oxford.

Angilletta, M. J., P. H. Niewiarowski, and C. A. Navas. 2002. The evolution of thermal physiology in ectotherms. J. Therm. Biol. 27:249–268.

Aspi, J., and A. Hoikkala. 1995. Male mating success and survival in the field with respect to size and courtship song characters in *Drosophila littoralis* and *D. montana* (Diptera: Drosophilidae). J. Insect Behav. 8:67–87.

Audacity Team. 2015. Audacity (R). Version 2.1.1. Audio editor and recorded.

Ayrinhac, A., V. Debat, P. Gibert, A.-G. Kister, H. Legout, B. Moreteau, R. Vergilino, and J. R. David. 2004. Cold adaptation in geographical populations of *Drosophila melanogaster*: phenotypic plasticity is more important than genetic variability. Funct. Ecol. 18:700–706.

Bale, J. S., and S. A. L. Hayward. 2010. Insect overwintering in a changing climate. J. Exp. Biol. 213:980–994.

Basson, C. H., C. Nyamukondiwa, and J. S. Terblanche. 2012. Fitness costs of rapid cold-hardening in *Ceratitis capitata*. Evolution 66:296–304.

Bastock, M., and A. Manning. 1955. The courtship of *Drosophila melanogaster*. Behaviour 8:85–110.

Bates, D., M. Mæchler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67:1–48.

Beaman, J. E., C. R. White, and F. Seebacher. 2016. Evolution of plasticity: mechanistic link between development and reversible acclimation. Trends Ecol. Evol. 31:237–249.

Begun, D. J., and C. F. Aquadro. 1993. African and North American populations of *Drosophila melanogaster* are very different at the DNA level. Nature 365:548–550.

Behrman, E. L., S. S. Watson, K. R. O'Brien, M. S. Heschel, and P. S. Schmidt. 2015. Seasonal variation in life history traits in two *Drosophila* species. J. Evol. Biol. 28:1691–1704.

Bennet-Clark, H. C., and A. W. Ewing. 1969. Pulse interval as a critical parameter in the courtship song of Drosophila melanogaster. Anim. Behav. 17:755–759.

Bennet-Clark, H. C., and A. W. Ewing. 1967. Stimuli provided by courtship of male *Drosophila melanogaster*. Nature 215:699–671.

Bergland, A. O., E. L. Behrman, K. R. O'Brien, P. S. Schmidt, and D. A. Petrov. 2014a. Genomic evidence of rapid and stable adaptive oscillations over seasonal time scales in *Drosophila*. PLoS Genet. 10:e1004775.

Bergland, A. O., R. Tobler, J. Gonzalez, P. Schmidt, and D. Petrov. 2014b. Secondary contact and local adaptation contribute to genome-wide patterns of clinal variation in Drosophila melanogaster.

Best, A. R., Z. Lewis, G. D. D. Hurst, and A. Lizé. 2012. Thermal environment during and outside courtship jointly determine female remating rate in *Drosophila melanogaster*. Anim. Behav. 83:1483–1490.

Betini, G. S., C. K. Griswold, L. Prodan, and D. R. Norris. 2014. Body size, carry-over effects and survival in a seasonal environment: consequences for population dynamics. J. Anim. Ecol. 83:1313–1321.

Bouazza, A., T. Slimani, H. El Mouden, G. Blouin-Demers, and O. Lourdais. 2016. Thermal constraints and the influence of reproduction on thermoregulation in a high-altitude gecko (*Quedenfeldtia trachyblepharus*). J. Zool. 300:36–44.

Bowler, K. 2005. Acclimation, heat shock and hardening. J. Therm. Biol. 30:125–130.

Bowler, K., and J. S. Terblanche. 2008. Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? Biol. Rev. 83:339–355.

Bozinovic, F., P. Calosi, and J. I. Spicer. 2011. Physiological correlates of geographic range in animals. Annu. Rev. Ecol. Evol. Syst. 42:155–179.

Burrack, H. J., J. P. Smith, D. G. Pfeiffer, G. Koeher, and J. Laforest. 2012. Using volunteer-based networks to track *Drosophila suzukii* (Diptera: Drosophilidae) an invasive pest of fruit crops. J. Integr. Pest Manag. 3:1–5.

Calosi, P., D. T. Bilton, J. I. Spicer, S. C. Votier, and A. Atfield. 2010. What determines a species' geographical range? Thermal biology and latitudinal range size relationships in European diving beetles (Coleoptera: Dytiscidae). J. Anim. Ecol. 79:194–204.

Campbell-Staton, S. C., S. V. Edwards, and J. B. Losos. 2016. Climate-mediated adaptation after mainland colonization of an ancestrally subtropical island lizard, *Anolis carolinensis*. J. Evol. Biol. 29:2168–2180.

Charlesworth, B. 1994. Evolution in age-structured populations. 2nd ed. Cambridge University Press, New York, New York.

Charlesworth, B. 2001. Patterns of age-specific means and genetic variances of mortality rates predicted by the mutation-accumulation theory of ageing. J. Theor. Biol. 210:47–65.

Charlesworth, B., and K. A. Hughes. 1996. Age-specific inbreeding depression and components of genetic variance in relation to the evolution of senescence. Proc. Natl. Acad. Sci. 93:6140–6145.

Chen, C.-P., D. L. Denlinger, and R. E. Lee Jr. 1987. Cold-shock injury and rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. Physiol. Zool. 297–304.

Christian, K., C. R. Tracy, and W. P. Porter. 1983. Seasonal shifts in body temperature and use of microhabitats by Galapagos land iguanas (*Conolophus Pallidus*). Ecology 64:463–468.

Cini, A., C. Ioriatti, G. Anfora, and others. 2012. A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. Bull. Insectology 65:149–160.

Colinet, H., T. Chertemps, I. Boulogne, and D. Siaussat. 2015. Age-related decline of abiotic stress tolerance in young *Drosophila melanogaster* adults. Gerontol. Soc. Am. 0:1–7.

Colinet, H., and A. A. Hoffmann. 2012. Comparing phenotypic effects and molecular correlates of developmental, gradual and rapid cold acclimation responses in *Drosophila melanogaster*. Funct. Ecol. 26:84–93.

Colinet, H., D. Siaussat, F. Bozzolan, and K. Bowler. 2013. Rapid decline of cold tolerance at young age is associated with expression of stress genes in *Drosophila melanogaster*. J. Exp. Biol. 216:253–259.

Cossins, A. R., and K. Bowler. 1987. Temperature biology of animals. Chapman and Hall, New York.

Coulson, S. C., and J. S. Bale. 1990. Characterisation and limitations of the rapid cold-hardening response in the housefly *Musca domestica* (Diptera: Muscidae). J. Insect Physiol. 36:207–211.

Coulson, S. C., and J. S. Bale. 1992. Effect of rapid cold hardening on reproduction and survival of offspring in the housefly *Musca domestica*. J. Insect Physiol. 38:421–424.

Crawford, P. 2013. The genetic basis of variation in thermal plasticity in *Drosophila melanogaster*. Kansas State University.

Curtsinger, J. W. 2016. Retired flies, hidden plateaus, and the evolution of senescence in *Drosophila melanogaster*: MORTALITY PLATEAUS. Evolution 70:1297–1306.

Curtsinger, J. W., and A. A. Khazaeli. 2002. Lifespan, QTLs, age-specificity, and pleiotropy in Drosophila. Mech. Ageing Dev. 123:81–93.

Czajka, M. C., and R. E. Lee. 1990. A rapid cold-hardening response protecting against cold shock injury in *Drosophila melanogaster*. J. Exp. Biol. 148:245–254.

Dalton, D. T., V. M. Walton, P. W. Shearer, D. B. Walsh, J. Caprile, and R. Isaacs. 2011. Laboratory survival of Drosophila suzukii under simulated winter conditions of the Pacific Northwest and seasonal field trapping in five primary regions of small and stone fruit production in the United States. Pest Manag. Sci. 67:1368–1374.

Danks, H. V. 2005. Key themes in the study of seasonal adaptations in insects I. Patterns of cold hardiness. Appl. Entomol. Zool. 40:199–211.

Davidson, A. M., M. Jennions, and A. B. Nicotra. 2011. Do invasive species show higher phenotypic plasticity than native species and, if so, is it adaptive? A meta-analysis: Invasive species have higher phenotypic plasticity. Ecol. Lett. 14:419–431.

Dell, A. I., S. Pawar, and V. M. Savage. 2011. Systematic variation in the temperature dependence of physiological and ecological traits. Proc. Natl. Acad. Sci. 108:10591–10596.

Denlinger, D. L. 1991. Relationship between cold hardiness and diapause. Pp. 174–198 *in* R. E. Lee and D. L. Denlinger, eds. Insects at Low Temperature. Springer US, Boston, MA.

Deutsch, C. A., J. J. Tewksbury, R. B. Huey, K. S. Sheldon, C. K. Ghalambor, D. C. Haak, and P. R. Martin. 2008. Impacts of climate warming on terrestrial ectotherms across latitude. Proc. Natl. Acad. Sci. 105:6668–6672.

Dick, C. A., N. E. Rank, M. McCarthy, S. McWeeney, D. Hollis, and E. P. Dahlhoff. 2013. Effects of temperature variation on male behavior and mating success in a montane beetle. Physiol. Biochem. Zool. 86:432–440.

Dierks, A., N. KöLzow, K. Franke, and K. Fischer. 2012. Does selection on increased cold tolerance in the adult stage confer resistance throughout development?: Correlated responses to selection across life stages. J. Evol. Biol. 25:1650–1657.

Dlugosch, K. M., and I. M. Parker. 2008. Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. Mol. Ecol. 17:431–449.

Duchen, P., D. Zivkovic, S. Hutter, W. Stephan, and S. Laurent. 2013. Demographic inference reveals African and European admixture in the North American *Drosophila melanogaster* Population. Genetics 193:291–301.

Durham, M. F., M. M. Magwire, E. A. Stone, and J. Leips. 2014. Genome-wide analysis in *Drosophila* reveals age-specific effects of SNPs on fitness traits. Nat. Commun. 5.

Easterling, D. R., G. A. Meehl, C. Parmesan, S. A. Changnon, T. R. Karl, and L. O. Mearns. 2000. Climate extremes: observations, modeling, and impacts. science 289:2068–2074.

Engström, G., L. E. Liljedahl, M. Rasmuson, and T. Björdrklund. 1989. Expression of genetic and environmental variation during ageing. Theor. Appl. Genet. 77:119–122.

Everman, E. R., R. A. Cloyd, C. Copland, and T. J. Morgan. 2015. First report of Spotted Wing Drosophila, *Drosophila suzukii* Matumura (Diptera: Drosophilidae) in Kansas. J. Kans. Entomol. Soc. 88:128–133.

Everman, E. R., N. Ledbetter, and T. J. Morgan. In Press. The persistence of short-term cold acclimation in *Drosophila melanogaster* (Diptera: Drosophilidae). Physiol. Entomol., doi: 10.1111/phen.12191.

Ewing, A. W., and H. C. Bennet-Clark. 1968. The courtship songs of *Drosophila*. Behaviour 31:288–301.

Falconer, D. S., and T. F. C. Mackay. 1996. Quantitative genetics. 4th ed. Longman Group Ltd., Essex, England.

Fallis, L. C., J. J. Fanara, and T. J. Morgan. 2014. Developmental thermal plasticity among *Drosophila melanogaster* populations. J. Evol. Biol. 27:557–564.

Fasolo, A. G., and R. A. Krebs. 2004. A comparison of behavioural change in *Drosophila* during exposure to thermal stress. Biol. J. Linn. Soc. 83:197–205.

Felix, T. M., K. A. Hughes, E. A. Stone, J. M. Drnevich, and J. Leips. 2012. Age-specific variation in immune response in *Drosophila melanogaster* has a genetic basis. Genetics 191:989–1002.

Fisher, R. A. 1930. The genetical theory of natural selection. Oxford University Press, Chicago.

Fisher, R. A., and G. R. De Beer. 1947. Thomas Hunt Morgan. 1866-1945. Obit. Not. Fellows R. Soc. 5:451–466.

Foray, V., E. Desouhant, Y. Voituron, V. Larvor, D. Renault, H. Colinet, and Gibert, P. 2013. Does cold tolerance plasticity correlate with the thermal environment and metabolic profile of a parasitoid wasp? Comp. Biochem. Physiol. Part A 164:77–83.

Frankham, R. 2005. Resolving the genetic paradox in invasive species. Heredity 94:385–385.

Franks, S. J., and A. A. Hoffmann. 2012. Genetics of climate change adaptation. Annu. Rev. Genet. 46:185–208.

Freda, P. J., and J. M. Braverman. 2013. *Drosophila suzukii*, or spotted wing Drosophila, recorded in Southeastern Pennsylvania, USA. Entomol. News 123:71–75.

Gaertner, B. E., E. A. Ruedi, L. J. McCoy, J. M. Moore, M. F. Wolfner, and T. F. C. Mackay. 2015. Heritable variation in courtship patterns in *Drosophila melanogaster*. G3 5:531–539.

Geister, T. L., and K. Fischer. 2007. Testing the beneficial acclimation hypothesis: temperature effects on mating success in a butterfly. Behav. Ecol. 18:658–664.

Gerken, A. R., O. C. Eller, D. A. Hahn, and T. J. Morgan. 2015. Constraints, independence, and evolution of thermal plasticity: Probing genetic architecture of long- and short-term thermal acclimation. Proc. Natl. Acad. Sci. 112:4399–4404.

Gibert, P., B. Moreteau, G. Pétavy, D. Karan, and J. R. David. 2001. Chill-coma tolerance, a major climatic adaptation among *Drosophila* species. Evolution 55:1063–1068.

Gilmour, S. J., R. K. Hajela, and M. F. Thomashow. 1988. Cold acclimation in *Arabidopsis thaliana*. Plant Physiol. 87:745–750.

Giraldo-Perez, P., P. Herrera, A. Campbell, M. L. Taylor, A. Skeats, R. Aggio, N. Wedell, and T. A. R. Price. 2016. Winter is coming: hibernation reverses the outcome of sperm competition in a fly. J. Evol. Biol. 29:371–379.

Glass, G. V., P. D. Peckham, and J. R. Sanders. 1972. Consequences of failure to meet assumptions underlying the fixed effects analyses of variance and covariance. Rev. Educ. Res. 42:237.

Gleason, J. M., S. V. Nuzhdin, and M. G. Ritchie. 2002. Quantitative trait loci affecting a courtship signal in *Drosophila melanogaster*. Heredity 89:1–6.

Gomez-Mestre, I., and R. Jovani. 2013. A heuristic model on the role of plasticity in adaptive evolution: plasticity increases adaptation, population viability and genetic variation. Proc. R. Soc. B Biol. Sci. 280:20131869–20131869.

Gorczyca, M., and J. C. Hall. 1987. The Insectavox, an integrated device for recording and amplifying courtship songs of *Drosophila*. Drosoph. Inf. Serv. 66:157–160.

Grillet, M., L. Dartevelle, and J.-F. Ferveur. 2006. A *Drosophila* male pheromone affects female sexual receptivity. Proc. R. Soc. B Biol. Sci. 273:315–323.

Haldane, J. B. S. 1941. New paths in genetics. Allen and Unwin, London.

Hallas, R., M. Schiffer, and A. A. Hoffmann. 2002. Clinal variation in *Drosophila serrata* for stress resistance and body size. Genet. Res. 79:141–148.

Hamilton, W. D. 1966. The moulding of sensecence by natural selection. J. Theor. Biol. 12:12–45.

Hawley, A. W., and M. Aleksiuk. 1975. Thermal regulation of spring mating behavior in the red-sided garter snake (*Thamnophis sirtalis parietalis*). Can. J. Zool. 53:768–776.

Hoffmann, A. A., A. Anderson, and R. Hallas. 2002. Opposing clines for high and low temperature resistance in Drosophila melanogaster. Ecol. Lett. 5:614–618.

Hoffmann, A. A., R. Hallas, A. R. Anderson, and M. Telonis-Scott. 2005. Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in Drosophila melanogaster. J. Evol. Biol. 18:804–810.

Hoffmann, A. A., R. J. Hallas, J. A. Dean, and M. Schiffer. 2003a. Low potential for climatic stress adaptation in a rainforest *Drosophila* species. Science 301:100–102.

Hoffmann, A. A., M. Scott, L. Partridge, and R. Hallas. 2003b. Overwintering in *Drosophila melanogaster*: outdoor field cage experiments on clinal and laboratory selected populations help to elucidate traits under selection. J. Evol. Biol. 16:614–623.

Hoffmann, A. A., and C. M. Sgrò. 2011. Climate change and evolutionary adaptation. Nature 470:479–485.

Hoffmann, A. A., J. G. Sørensen, and V. Loeschcke. 2003c. Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. J. Therm. Biol. 28:175–216.

Hoffmann, A. A., and M. Watson. 1993. Geographical variation in the acclimation response of *Drosophila* to temperature extremes. Am. Nat. 142:S93–S113.

Hoikkala, A., and E. Isoherranen. 1997. Variation and repeatability of courtship song characters among wild-caught and laboratory-reared *Drosophila montana* and *D. littoralis* males (Diptera: Drosophilidae). J. Insect Behav. 10:193–202.

Hope, R., M. 2013. Rmisc: Ryan Miscellaneous.

Houle, D., K. A. Hughes, D. K. Hoffmaster, J. Ihara, and S. Assimacopoulos. 1994. Effects of spontaneous mutation on quantitative traits. I. Variances and covariances of life history traits. Genetics 138:773–785.

Huang, W., A. Massouras, Y. Inoue, J. Peiffer, M. Ramia, A. M. Tarone, L. Turlapati, T. Zichner, D. Zhu, R. F. Lyman, M. M. Magwire, K. Blankenburg, M. A. Carbone, K. Chang, L. L. Ellis, S. Fernandez, Y. Han, G. Highnam, C. E. Hjelmen, J. R. Jack, M. Javaid, J. Jayaseelan, D. Kalra, S. Lee, L. Lewis, M. Munidasa, F. Ongeri, S. Patel, L. Perales, A. Perez, L. Pu, S. M. Rollmann, R. Ruth, N. Saada, C. Warner, A. Williams, Y.-Q. Wu, A. Yamamoto, Y. Zhang, Y. Zhu, R. R. H. Anholt, J. O. Korbel, D. Mittelman, D. M. Muzny, R. A. Gibbs, A. Barbadilla, J. S. Johnston, E. A. Stone, S. Richards, B. Deplancke, and T. F. C. Mackay. 2014. Natural variation in genome architecture among

205 *Drosophila melanogaster* Genetic Reference Panel lines. Genome Res. 24:1193–1208.

Huey, R. B., M. R. Kearney, A. Krockenberger, J. A. M. Holtum, M. Jess, and S. E. Williams. 2012. Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. Philos. Trans. R. Soc. B Biol. Sci. 367:1665–1679.

Huey, R. B., and J. G. Kingsolver. 1993. Evolution of resistance to high temperature in ectotherms. Am. Nat. 142:S21–S46.

Huey, R. B., and J. G. Kingsolver. 1989. Evolution of thermal sensitivity of ectotherm performance. Trends Ecol. Evol. 4:131–135.

Hughes, K. A., J. A. Alipaz, J. M. Drnevich, and R. M. Reynolds. 2002. A test of evolutionary theories of aging. Proc. Natl. Acad. Sci. 99:14286–14291.

Imasheva, A. G., V. Loeschcke, L. A. Zhivotovsky, and O. E. Lazebny. 1998. Stress temperatures and quantitative variation in *Drosophila melanogaster*. Heredity 81:246–253.

Ivanov, D. K., V. Escott-Price, M. Ziehm, M. M. Magwire, T. F. C. Mackay, L. Partridge, and J. M. Thornton. 2015. Longevity GWAS using the *Drosophila* Genetic Reference Panel. J. Gerontol. A. Biol. Sci. Med. Sci. 70:1470–1478.

Jakobs, R. 2014. Low temperature tolerance of adult *Drosophila suzukii* (Diptera: Drosophilidae). The University of Western Ontario.

Jakobs, R., T. D. Gariepy, and B. J. Sinclair. 2015. Adult plasticity of cold tolerance in a continental-temperate population of *Drosophila suzukii*. J. Insect Physiol. 79:1–9.

James, A. C., R. B. R. Azevedo, and L. Partridge. 1997. Genetic and environmental responses to temperature of *Drosophila melanogaster* from a latitudinal cline. Genetics 146:881–890.

Jentsch, A., K. Jurgen, and C. Beierkuhnlein. 2007. A new generation of climate-change experiments: Events, not trends. Front. Genet. Environ. 5:365–374.

Ju, R.-T., Y.-Y. Xiao, and B. Li. 2011. Rapid cold hardening increases cold and chilling tolerances more than acclimation in the adults of the sycamore lace bug, *Corythucha ciliata* (Say) (Hemiptera: Tingidae). J. Insect Physiol. 57:1577–1582.

Kawecki, T. J. 2000. The evolution of genetic canalization under fluctuating selection. Evolution 54:1–12.

Keller, A. 2007. *Drosophila melanogaster*'s history as a human commensal. Curr. Biol. 17:R77–R81.

Kellermann, V., V. Loeschcke, A. A. Hoffmann, T. N. Kristensen, C. Fløjgaard, J. R. David, J.-C. Svenning, and J. Overgaard. 2012. Phylogenetic constraints in key functional traits

behind species' climate niches: patterns of desiccation and cold resistance across 95 *Drosophila* species: phylogenetic constraints in stress resistance. Evolution 66:3377–3389.

Kellermann, V., B. van Heerwaarden, C. M. Sgro, and A. A. Hoffmann. 2009. Fundamental evolutionary limits in ecological traits drive *Drosophila* species distributions. Science 325:1244–1246.

Kellett, M., A. A. Hoffmann, and S. W. Mckechnie. 2005. Hardening capacity in the *Drosophila melanogaster* species group is constrained by basal thermotolerance. Funct. Ecol. 19:853–858.

Kelty, J. 2007. Rapid cold-hardening of *Drosophila melanogaster* in a field setting. Physiol. Entomol. 32:343–350.

Kelty, J. D., and R. E. Lee. 1999. Induction of rapid cold hardening by cooling at ecologically relevant rates in Drosophila melanogaster. J. Insect Physiol. 45:719–726.

Kelty, J., and R. E. Lee. 2001. Rapid cold-hardening of *Drosophila melanogaster* (Diptera: Drosophilidae) during ecologically based thermoperiodic cycles. J. Exp. Biol. 204:1659–1666.

Kimura, M. T. 1988. Adaptations to temperate climates and evolution of overwintering strategies in the *Drosophila melanogaster* species group. Evolution 42:1288.

Kingsolver, J. G., H. Arthur Woods, L. B. Buckley, K. A. Potter, H. J. MacLean, and J. K. Higgins. 2011. Complex life cycles and the responses of insects to climate change. Integr. Comp. Biol. 51:719–732.

Kingsolver, J. G., and R. B. Huey. 1998. Evolutionary analyses of morphological and physiological plasticity in thermally variable environments. Am. Zool. 38:545–561.

Kleinteich, A., and J. M. Schneider. 2011. Developmental strategies in an invasive spider: constraints and plasticity. Ecol. Entomol. 36:82–93.

Koveos, D. S. 2001. Rapid cold hardening in the olive fruit fly *Bactrocera oleae* under laboratory and field conditions. Entomol. Exp. Appl. 101:257–263.

Kristensen, T. N., A. A. Hoffmann, J. Overgaard, J. G. Sørensen, R. Hallas, and V. Loeschcke. 2008. Costs and benefits of cold acclimation in field-released *Drosophila*. Proc. Natl. Acad. Sci. 105:216–221.

Krupp, J. J., C. Kent, J.-C. Billeter, R. Azanchi, A. K.-C. So, J. A. Schonfeld, B. P. Smith, C. Lucas, and J. D. Levine. 2008. Social experience modifies pheromone expression and mating behavior in Male *Drosophila melanogaster*. Curr. Biol. 18:1373–1383.

Kuznetsova, A., B. Brockhoff, and H. Bojesen Christensen. 2015. lmerTest: Tests in linear mixed effects models.

Lachaise, D., and J.-F. Silvain. 2004. How two Afrotropical endemics made two cosmopolitan human commensals: the *Drosophila melanogaster-D. simulans* palaeogeographic riddle. Pp. 17–39 *in* Drosophila melanogaster, Drosophila simulans: So Similar, So Different. Springer.

Lamarque, L. J., A. J. Porté, C. Eymeric, J.-B. Lasnier, C. J. Lortie, and S. Delzon. 2013. A test for pre-adapted phenotypic plasticity in the invasive tree *Acer negundo L.* PLoS ONE 8:e74239.

Lande, R. 2014. Evolution of phenotypic plasticity and environmental tolerance of a labile quantitative character in a fluctuating environment. J. Evol. Biol. 27:866–875.

Lardies, M. A., and F. Bozinovic. 2008. Genetic variation for plasticity in physiological and life-history traits among populations of an invasive species, the terrestrial isopod *Porcellio laevis*. Evol. Ecol. Res. 10:747–762.

Lavergne, S., N. Mouquet, W. Thuiller, and O. Ronce. 2010. Biodiversity and climate change: integrating evolutionary and ecological responses of species and communities. Annu. Rev. Ecol. Evol. Syst. 41:321–350.

Lee, R. E., C. Chen, and D. L. Denlinger. 1987. A rapid cold-hardening process in insects. Science 238:1415–1417.

Leips, J., P. Gilligan, and T. F. C. Mackay. 2006. Quantitative trait loci with age-specific effects on fecundity in *Drosophila melanogaster*. Genetics 172:1595–1605.

Leroi, A. M., A. F. Bennett, and R. E. Lenski. 1994. Temperature acclimation and competitive fitness: an experimental test of the beneficial acclimation assumption. Proc. Natl. Acad. Sci. 91:1917–1921.

Linnen, C., M. Tatar, and D. Promislow. 2001. Cultural artifacts: a comparison of senescence in natural, laboratory-adapted and artificially selected lines of *Drosophila melanogaster*. Evol. Ecol. Res. 3:877–888.

Loeschcke, V., and J. G. Sørensen. 2005. Acclimation, heat shock and hardening—a response from evolutionary biology. J. Therm. Biol. 30:255–257.

Lyne, R., R. Smith, K. Rutherford, M. Wakeling, A. Varley, F. Guillier, H. Janssens, W. Ji, P. Mclaren, P. North, D. Rana, T. Riley, J. Sullivan, X. Watkins, M. Woodbridge, K. Lilley, S. Russell, M. Ashburner, K. Mizuguchi, and G. Micklem. 2007. FlyMine: an integrated database for *Drosophila* and *Anopheles* genomics. Genome Biol. 8:R129.

Mackay, T. F. C., S. Richards, E. A. Stone, A. Barbadilla, J. F. Ayroles, D. Zhu, S. Casillas, Y. Han, M. M. Magwire, J. M. Cridland, M. F. Richardson, R. R. H. Anholt, M. Barrón, C. Bess, K. P. Blankenburg, M. A. Carbone, D. Castellano, L. Chaboub, L. Duncan, Z. Harris, M. Javaid, J. C. Jayaseelan, S. N. Jhangiani, K. W. Jordan, F. Lara, F. Lawrence, S. L. Lee, P. Librado, R. S. Linheiro, R. F. Lyman, A. J. Mackey, M. Munidasa, D. M. Muzny, L. Nazareth, I. Newsham, L. Perales, L.-L. Pu, C. Qu, M. Ràmia, J. G. Reid, S.

M. Rollmann, J. Rozas, N. Saada, L. Turlapati, K. C. Worley, Y.-Q. Wu, A. Yamamoto, Y. Zhu, C. M. Bergman, K. R. Thornton, D. Mittelman, and R. A. Gibbs. 2012. The *Drosophila melanogaster* Genetic Reference Panel. Nature 482:173–178.

MacMillan, H. A., J. M. Knee, A. B. Dennis, H. Udaka, K. E. Marshall, T. J. S. Merritt, and B. J. Sinclair. 2016. Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. Sci. Rep. 6:28999.

MacMillan, H. A., and B. J. Sinclair. 2011. Mechanisms underlying insect chill-coma. J. Insect Physiol. 57:12–20.

Maklakov, A. A., L. Rowe, and U. Friberg. 2015. Why organisms age: Evolution of senescence under positive pleiotropy? BioEssays 37:802–807.

Marshall, K. E., and B. J. Sinclair. 2012. The impacts of repeated cold exposure on insects. J. Exp. Biol. 215:1607–1613.

Medawar, P. B. 1952. An unsolved problem of biology. London: H.K. Lewis.

Miller, J. C. 1993. Insect natural history, multi-species interactions and biodiversity in ecosystems. Biodivers. Conserv. 2:233–241.

Moorad, J. A., and D. E. L. Promislow. 2009. What can genetic variation tell us about the evolution of senescence? Proc. R. Soc. B Biol. Sci. 276:2271–2278.

Moran, N. A. 1992. The evolutionary maintenance of alternative phenotypes. Am. Nat. 139:971–989.

Morgan, T. J., and T. F. C. Mackay. 2006. Quantitative trait loci for thermotolerance phenotypes in *Drosophila melanogaster*. Heredity 96:232–242.

Muir, C. D., J. B. Pease, and L. C. Moyle. 2014. Quantitative genetic analysis indicates natural selection on leaf phenotypes across wild tomato species (*Solanum sect*. *Lycopersicon*; Solanaceae). Genetics 198:1629–1643.

Niehaus, A. C., M. J. Angilletta, M. W. Sears, C. E. Franklin, and R. S. Wilson. 2012. Predicting the physiological performance of ectotherms in fluctuating thermal environments. J. Exp. Biol. 215:694–701.

Nyamukondiwa, C., J. S. Terblanche, K. E. Marshall, and B. J. Sinclair. 2011. Basal cold but not heat tolerance constrains plasticity among *Drosophila* species (Diptera: Drosophilidae). J. Evol. Biol. 24:1927–1938.

Nylin, S., and K. Gotthard. 1998. Plasticity in life-history traits. Annu. Rev. Entomol. 43:63–83.

Orr, H. A. 1998. Testing natural selection vs. genetic drift in phenotypic evolution using quantitative trait locus data. Genetics 149:2099–2104.

Overgaard, J., A. A. Hoffmann, and T. N. Kristensen. 2011. Assessing population and environmental effects on thermal resistance in Drosophila melanogaster using ecologically relevant assays. J. Therm. Biol. 36:409–416.

Overgaard, J., M. R. Kearney, and A. A. Hoffmann. 2014. Sensitivity to thermal extremes in Australian *Drosophila* implies similar impacts of climate change on the distribution of widespread and tropical species. Glob. Change Biol. 20:1738–1750.

Overgaard, J., and J. G. Sørensen. 2008. Rapid thermal adaptation during field temperature variations in *Drosophila melanogaster*. Cryobiology 56:159–162.

Overgaard, J., J. G. Sørensen, L. T. Jensen, V. Loeschcke, and T. N. Kristensen. 2010. Field tests reveal genetic variation for performance at low temperatures in *Drosophila melanogaster*. Funct. Ecol. 24:186–195.

Paik, D., Y. G. Jang, Y. E. Lee, Y. N. Lee, R. Yamamoto, H. Y. Gee, S. Yoo, E. Bae, K.-J. Min, M. Tatar, and J.-J. Park. 2012. Misexpression screen delineates novel genes controlling *Drosophila* lifespan. Mech. Ageing Dev. 133:234–245.

Parmesan, C., and G. Yohe. 2003. A globally coherent fingerprint of climate change impacts across natural systems. Nature 421:37–42.

Partridge, L., and N. H. Barton. 1993. Optimality, mutation and the evolution of ageing. Nature 362:305–311.

Patton, Z. J., and R. A. Krebs. 2001. The effect of thermal stress on the mating behavior of three *Drosophila* species. Physiol. Biochem. Zool. 74:783–788.

Phillips, P. C. 1998. H2boot: bootstrap estimates and tests of quantitative genetic data. University of Texas at Arlington.

Pletcher, S. D., D. Houle, and J. W. Curtsinger. 1998. Age-specific properties of spontaneous mutations affecting mortality in *Drosophila melanogaster*. Genetics 148:287–303.

Pletcher, S. D., S. J. Macdonald, R. Marguerie, U. Certa, S. C. Stearns, D. B. Goldstein, and L. Partridge. 2002. Genome-wide transcript profiles in aging and calorically restricted *Drosophila melanogaster*. Curr. Biol. 12:712–723.

Powell, S. J., and J. S. Bale. 2005. Low temperature acclimated populations of the grain aphid *Sitobion avenae* retain ability to rapidly cold harden with enhanced fitness. J. Exp. Biol. 208:2615–2620.

Price, C. S. C., K. A. Dyer, and J. A. Coyne. 1999. Sperm competition between *Drosophila* males involves both displacement and incapacitation. Nature 400:449–452.

Promislow, D. E. L., M. Tatar, A. A. Khazaeli, and J. W. Curtsinger. 1996. Age-specific patterns of genetic variance in *Drosophila melanogaster*. I. Mortality. Genetics 143:839–848.

R Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Rajamohan, A., and B. J. Sinclair. 2009. Hardening trumps acclimation in improving cold tolerance in *Drosophila melanogaster* larvae. Physiol. Entomol. 34:217–223.

Rako, L., and A. A. Hoffmann. 2006. Complexity of the cold acclimation response in *Drosophila melanogaster*. J. Insect Physiol. 52:94–104.

Reynolds, R. M., S. Temiyasathit, M. M. Reedy, E. A. Ruedi, J. M. Drnevich, J. Leips, and K. A. Hughes. 2007. Age Specificity of Inbreeding Load in *Drosophila melanogaster* and Implications For the Evolution of Late-Life Mortality Plateaus. Genetics 177:587–595.

Rice, D. P., and J. P. Townsend. 2012. Resampling QTL effects in the QTL sign test leads to incongruous sensitivity to variance in effect size. GenesGenomesGenetics 2:905–911.

Richards, C. L., O. Bossdorf, N. Z. Muth, J. Gurevitch, and M. Pigliucci. 2006. Jack of all trades, master of some? On the role of phenotypic plasticity in plant invasions. Ecol. Lett. 9:981–993.

Ricklefs, R. E., and C. E. Finch. 1995. Aging: A natural history. Scientific American Library, New York.

Rieseberg, L. H., A. Widmer, A. M. Arntz, and J. M. Burke. 2002. Directional selection is the primary cause of phenotypic diversification. Proc. Natl. Acad. Sci. 99:12242–12245.

Ritchie, M. G., and J. M. Gleason. 1995. Rapid evolution of courtship song pattern in *Drosophila willistoni* sibling species. J. Evol. Biol. 8:463–479.

Ritchie, M. G., and C. P. Kyriacou. 1996. Artificial selection for a courtship signal in *Drosophila melanogaster*. Anim. Behav. 52:603–611.

Rogan, J. C., and H. J. Keselman. 1977. Is the ANOVA F-Test robust to variance heterogeneity when sample sizes are equal?: An investigation via a coefficient of variation. Am. Educ. Res. J. 14:493.

Root, T. R., J. T. Price, K. R. Hall, S. H. Schneider, C. Rosenzweig, and J. A. Pounds. 2003. Fingerprints of global warming on wild animals and plants. Nature 421:54–57.

Rose, M. R. 1984. Laboratory evolution of postponed senescence in *Drosophila melanogaster*. Evolution 38:1004–1010.

Rose, M. R. 1985. Life history evolution with antagonistic pleiotropy and overlapping generations. Theor. Popul. Biol. 28:342–358.

Rose, M. R. 1991. The evolutionary biology of aging. Oxford University Press, Oxford.

Rose, M. R., and B. Charlesworth. 1981. Genetics of life history in *Drosophila melanogaster*. II. Exploratory selection experiments. Genetics 97:187–196.

Rose, M. R., L. N. Vu, S. U. Park, and J. L. Graves, Jr. 1992. Selection on stress resistance increases longevity in *Drosophila melanogaster*. Exp. Gerontol. 27:214–250.

Rossi-Stacconi, M. V., R. Kaur, V. Mazzoni, L. Ometto, A. Grassi, A. Gottardello, O. Rota-Stabelli, and G. Anfora. 2016. Multiple lines of evidence for reproductive winter diapause in the invasive pest *Drosophila suzukii*: useful clues for control strategies. J. Pest Sci. 89:689–700.

Ryan, G. D., L. Emiljanowicz, F. Wilkinson, M. Kornya, and J. A. Newman. 2016. Thermal tolerances of the Spotted-Wing Drosophila *Drosophila suzukii* (Diptera: Drosophilidae). J. Econ. Entomol. 109:746–752.

Saunders, D. S., V. C. Henrich, and L. I. Gilbert. 1989. Induction of diapause in *Drosophila melanogaster*: Photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. Proc. Natl. Acad. Sci. 86:3748–3752.

Savarit, F., and J.-F. Ferveur. 2002. Temperature affects the ontogeny of sexually dimorphic cuticular hydrocarbons in *Drosophila melanogaster*. J. Exp. Biol. 205:3241–3249.

Schmidt, P. S., L. Matzkin, M. Ippolito, W. F. Eanes, and J. Hey. 2005. Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. Evolution 59:1721–1732.

Schnebel, E. M., and J. Grossfield. 1988. Antagonistic pleiotropy: An interspecific *Drosophila* comparison. Evolution 42:306.

Schou, M. F., V. Loeschcke, and T. N. Kristensen. 2015. Strong costs and benefits of winter acclimatization in *Drosophila melanogaster*. PloS One 10:e0130307.

Schwarze, S. R., R. Weindruch, and J. M. Aiken. 1998. Oxidative stress and aging reduce COX I RNA and cytochrome oxidase activity in *Drosophila*. Free Radic. Biol. Med. 25:740–747.

Schwasinger-Schmidt, T. E., S. D. Kachman, and L. G. Harshman. 2012. Evolution of starvation resistance in *Drosophila melanogaster*: Measurement of direct and correlated responses to artificial selection. J. Evol. Biol. 25:378–387.

Scott, M., D. Berrigan, and A. A. Hoffmann. 1997. Costs and benefits of acclimation to elevated temperature in *Trichogramma carverae*. Entomol. Exp. Appl. 85:211–219.

Seebacher, F., C. R. White, and C. E. Franklin. 2014. Physiological plasticity increases resilience of ectothermic animals to climate change. Nat. Clim. Change 5:61–66.

Shearer, P. W., J. D. West, V. M. Walton, P. H. Brown, N. Svetec, and J. C. Chiu. 2016. Seasonal cues induce phenotypic plasticity of *Drosophila suzukii* to enhance winter survival. BMC Ecol. 16.

Shreve, S. M., J. Kelty, and R. E. Lee Jr. 2004. Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response. J. Exp. Biol. 207:1797–1802.

Sinclair, B. J., and S. L. Chown. 2006. Rapid cold-hardening in a Karoo beetle, *Afrinus sp.* Physiol. Entomol. 31:98–101.

Sinclair, B. J., and S. P. Roberts. 2005. Acclimation, shock and hardening in the cold. J. Therm. Biol. 30:557–562.

Sinclair, B. J., P. Vernon, C. Jaco Klok, and S. L. Chown. 2003. Insects at low temperatures: an ecological perspective. Trends Ecol. Evol. 18:257–262.

Sinclair, B. J., C. M. Williams, and J. S. Terblanche. 2012. Variation in thermal performance among insect populations. Physiol. Biochem. Zool. 85:594–606.

Snoke, M. S., and D. E. L. Promislow. 2003. Quantitative genetic tests of recent senescence theory: age-specific mortality and male fertility in Drosophila melanogaster. Heredity 91:546–556.

Sørensen, J. G., T. N. Kristensen, and J. Overgaard. 2016. Evolutionary and ecological patterns of thermal acclimation capacity in *Drosophila*: is it important for keeping up with climate change? Curr. Opin. Insect Sci. 17:98–104.

Stapley, J., A. W. Santure, and S. R. Dennis. 2015. Transposable elements as agents of rapid adaptation may explain the genetic paradox of invasive species. Mol. Ecol. 24:2241–2252.

Stephens, A. R. 2015. Cold tolerance of *Drosophila suzukii*. University of Minnesota.

Stephens, A. R., M. K. Asplen, W. D. Hutchison, and R. C. Venette. 2015. Cold hardiness of winter-acclimated *Drosophila suzukii* (Diptera: Drosophilidae) adults. Environ. Entomol. 44:1619–1626.

Tatar, M., D. E. L. Promislow, A. A. Khazaeli, and J. W. Curtsinger. 1996. Age-specific patterns of genetic variance in *Drosophila melanogaster*. II. Fecundity and its genetic covariance with age-specific mortality. Genetics 143:849–858.

Teets, N. M., and D. L. Denlinger. 2013. Physiological mechanisms of seasonal and rapid cold-hardening in insects: Seasonal and rapid cold-hardening in insects. Physiol. Entomol. 38:105–116.

Terblanche, J. S., J. A. Deere, S. Clusella-Trullas, C. Janion, and S. L. Chown. 2007. Critical thermal limits depend on methodological context. Proc. R. Soc. B Biol. Sci. 274:2935–2943.

Turner, T. L., and P. M. Miller. 2012. Investigating natural variation in *Drosophila* courtship song by the evolve and resequence approach. Genetics 191:633–642.

Vasseur, D. A., J. P. DeLong, B. Gilbert, H. S. Greig, C. D. G. Harley, K. S. McCann, V. Savage, T. D. Tunney, and M. I. O'Connor. 2014. Increased temperature variation poses a greater risk to species than climate warming. Proc. R. Soc. B Biol. Sci. 281:20132612–20132612.

Venables, W. N., and B. D. Ripley. 2002. Modern applied statistics. Springer, New York.

Vermeulen, C. J., P. Sørensen, K. Kirilova Gagalova, and V. Loeschcke. 2013. Transcriptomic analysis of inbreeding depression in cold-sensitive *Drosophila melanogaster* shows upregulation of the immune response. J. Evol. Biol. 26:1890–1902.

Vesala, L., and A. Hoikkala. 2011. Effects of photoperiodically induced reproductive diapause and cold hardening on the cold tolerance of *Drosophila montana*. J. Insect Physiol. 57:46–51.

Von Schilcher, F. 1976. The function of pulse song and sine song in the courtship of *Drosophila melanogaster*. Anim. Behav. 24:622–625.

Wachter, K. W., S. N. Evans, and D. Steinsaltz. 2013. The age-specific force of natural selection and biodemographic walls of death. Proc. Natl. Acad. Sci. 110:10141–10146.

Wachter, K. W., D. Steinsaltz, and S. N. Evans. 2014. Evolutionary shaping of demographic schedules. Proc. Natl. Acad. Sci. 111:10846–10853.

Wallingford, A. K., J. C. Lee, and G. M. Loeb. 2016. The influence of temperature and photoperiod on the reproductive diapause and cold tolerance of spotted-wing drosophila, *Drosophila suzukii*. Entomol. Exp. Appl. 159:327–337.

Wallingford, A. K., and G. M. Loeb. 2016. Developmental acclimation of *Drosophila suzukii* (Diptera: Drosophilidae) and its effect on diapause and winter stress tolerance. Environ. Entomol. 0.

Walsh, D. B., M. P. Bolda, R. E. Goodhue, A. J. Dreves, J. Lee, D. J. Bruck, V. M. Walton, S. D. O'Neal, and F. G. Zalom. 2011. *Drosophila suzukii* (Diptera: Drosophilidae): invasive pest of ripening soft fruit expanding its geographic range and damage potential. J. Integr. Pest Manag. 2:1–7.

Wang, G., and M. E. Dillon. 2014. Recent geographic convergence in diurnal and annual temperature cycling flattens global thermal profiles. Nat. Clim. Change 4:988–992.

Wang, X., and L. Kang. 2003. Rapid cold hardening in young hoppers of the migratory locust *Locusta migratoria* L. (Orthoptera: Acridiidae). CryoLetters 24:331–340.

Westerman, E. L., A. Hodgins-Davis, A. Dinwiddie, and A. Monteiro. 2012. Biased learning affects mate choice in a butterfly. Proc. Natl. Acad. Sci. 109:10948–10953.

Westerman, E., and A. Monteiro. 2016. Rearing temperature influences adult response to changes in mating status. PLOS ONE 11:e0146546.

Wickham, H. 2009. ggplot2: elegant graphics for data analysis. Springer New York.

Williams, G. C. 1957. Pleiotropy, natural selection, and the evolution of senescence. Evolution 11:398.

Wilson, E. O. 1987. The little things that run the world (The importance and conservation of invertebrates). Conserv. Biol. 1:344–346.

Wilson, R. S., and C. E. Franklin. 2002. Testing the beneficial acclimation hypothesis. Trends Ecol. Evol. 17:66–70.

Worland, M. R., and P. Convey. 2001. Rapid cold hardening in Antarctic microarthropods. Funct. Ecol. 15:515–524.

Yadav, S., and A. K. Yadav. 2012. Effect of temperature on behavioral isolation: A study with *Drosophila ananassae* populations. J. Exp. Biol. 50:366–371.

Zhai, Y., Q. Lin, J. Zhang, F. Zhang, L. Zheng, and Y. Yu. 2016. Adult reproductive diapause in *Drosophila suzukii* females. J. Pest Sci. 89:679–688.

Zimmerman, D. W. 1998. Invalidation of parametric and nonparametric statistical tests by concurrent violation of two assumptions. J. Exp. Educ. 67:55–68.

Zwaan, B. J. 1999. The evolutionary genetics of ageing and longevity. Heredity 82:589–597.

Appendix A - Chapter 2 Supplemental Table Legends

Note: Tables are available as external supplemental files.

Table A.1 DGRP lines included in each experiment.

Table A.3 Mean responses for each phenotype by DGRP line, age, and sex.

Table A.5 Data from GWAS, generated by DGRP Freeze 2.0 pipeline, based on Flybase release 5.49. Functions are highlighted in colors to indicate previous associations: yellow = age or lifespan-related genes; blue = cold response-related genes; red = starvation response or sensitivity related genes; orange = age and starvation; green: age and cold; purple: starvation and cold; grey: starvation, age, and cold. Unless otherwise noted functions were provided by FlyBase Curators et al. 2004.