THE GENETIC BASIS OF VARIATION IN THERMAL PLASTICITY IN *DROSOPHILA MELANOGASTER*

by

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Abstract

The organismal response to temperature represents one of the most ubiquitous processes that occur in the natural world, and this response is critical for survival in most habitats. Increased attention should be focused on how organisms cope with temperature extremes, either through adaptation, plasticity, or a combination of both, as climate models predict increased variations in temperature accompanied by novel thermal extremes. *Drosophila melanogaster* is an excellent resource for answering questions pertaining to how organisms persist in environmental extremes because they originated in central tropical Africa and have since colonized nearly the entire globe, exposing them to many novel thermal stressors. In this work I elucidated regions of the genome contributing to phenotypic variation in cold tolerance and thermal plasticity. A quantitative trait locus (QTL) approach was used, which involved phenotyping roughly 400 recombinant inbred lines (RILs) of *D. melanogaster* from the *Drosophila* Synthetic Population Resource (DSPR). The DSPR captures genetic variation from around the globe, allowing for precision mapping of cold tolerance and thermal plasticity QTL, while simultaneously determining the frequency of the QTL alleles. Upon development at both 18°C and 25°C, RILS were measured for a common cold tolerance metric, chill-coma recovery time (CCR), and a plasticity value was derived as the change in CCR between environments. Analysis of variance revealed significant effects of sex, line (RIL), treatment (temperature), and line by treatment interaction (GxE). Mapped QTL for chill-coma recovery time at 18°C and 25°C spanned the same regions as several studies previously reported, validating the automated phenotyping method used and the mapping power of the DSPR. QTL between CCR at 18°C and 25°C overlapped significantly, and QTL for thermal plasticity shared the similar regions as QTL for CCR, but also exhibited two non-overlapping QTL on the left arm of the third chromosome. This study demonstrated the tremendous amount of variation present in cold tolerance phenotypes and identified candidate regions of the genome that contribute to thermal plasticity and require further investigation.

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Dedication

I dedicate this work to my mother and father for the unwavering support, patience, and love they demonstrated through the course of my education.

Chapter 1 - Thermal Plasticity and Adaptation in *Drosophila*

Introduction

Understanding how organisms respond to changing environments is a long standing question in evolutionary biology; however, our ability to determine the mechanisms underlying such responses has been advanced recently by increased computational power and sequencing technologies. Evolutionary biologists have long strived to identify mechanisms underlying phenotypic divergence and local adaptation (Mayr, 1963; Slatkin, 1973, 1987; Endler, 1977; West-Eberhard, 1983; Kingsolver et al., 2001), but the scale of possible investigations was limited due to technological deficits, a problem that no longer exists as investigations now investigate the molecular underpinnings of adaptive environmental responses (Reviewed in Mackay et al., 2009; Nadeau & Jiggins, 2010; Stapley et al., 2010). This change in scale has led to the field of evolutionary/ecological genomics, a discipline that seeks to identify regions of the genome that influence organismal responses to ecologically relevant environments (Feder & Mitchell-Olds, 2003; Reviewed in Ungerer et al., 2008). It is now possible to identify the molecular basis of ecologically relevant traits to the individual gene, and even polymorphism, although this challenge has been met with limited success (Hoekstra et al. 2006; Mitchell-Olds et al., 2012).

The ultimate goal of evolutionary/ecological genetics is to identify segregating natural variation in ecologically relevant traits (Orr, 2005; Hoekstra & Coyne, 2007). Genes, polygenic interactions, and interactions between an organism's genotype and their internal and external environment all contribute to phenotypic variation. This phenotypic variation can be the product of adaptive processes, phenotypic plasticity, or truly stochastic events (Falconer & Mackay, 1996; Lynch & Walsh, 1998). Gould and Lewontin (1979) argued that phenotypes observed in natural populations are not always the results of adaptation, but rather could be the result of genetic drift or selection on correlated traits. Decades later arguments still exist regarding the genetic basis and nature of adaptations: do few mutations of large effect (Reviewed in Johnson & Barton, 2005) or numerous mutations of small effect (Reviewed in Barton & Keightley, 2002) drive adaptation? Orr (1998) proposed that the effect size of mutations influencing quantitative

traits and resulting in adaptation are exponentially distributed, lending credence to the idea that many genes of small effect contribute to adaptive phenotypes. In support of this view, a large scale genome-wide association study examining common human diseases uncovered many variants of modest effect and no variants of large effect, suggesting many loci of small effect underlie complex phenotypes (The Wellcome Trust Case Control Consortium, 2007). Similar results have been reported for yeast (Brem & Kruglyak, 2005), mice (Valdar et al., 2006), and *Drosophila* (Huang et al., 2012; reviewed in Mackay, 2009). Therefore, a likely scenario for the evolution of complex traits involves many genes, of which a small number have large effects and a large number have small effects.

In addition to the controversy surrounding the effect size of mutations, uncertainty exists as to where mutations leading to adaptation actually occur in the genome: do adaptive mutations occur in the structural or regulatory regions of the genome? Hoekstra and Coyne (2007) suggest that both regulatory and structural mutations drive evolution, but structural mutations play a much greater role. However, Carroll (2008) proposed that changes in expression of proteins and cis-regulatory mutations are most likely the greatest contributors to morphological evolution. In reality, the genetic basis of complex adaptations do not fit into simple bins (e.g., structural vs. regulatory or small vs. large effect mutations), but rather the majority of these mutations are likely highly context dependent and thus likely depend on interactions between the genotype and the environment or the genetic background to determine their effects on phenotypic variation. Such interactions may explain the unique evolutionary trajectories observed in only one population, in one environment, at one specific time (Thornton-Wells et al., 2004; Mackay, 2009).

The traits that make excellent candidates for examining the above questions must be ecologically relevant, have easy laboratory assays, and exhibit diverse organismal responses. One class of such phenotypes is thermotolerance traits. Thermotolerance is an organism's ability to tolerate stressful temperatures and is a complex quantitative trait with a rich empirical history (see reviews by Hoffmann et al., 2003; Chown & Terblanche, 2007; Clark & Worland, 2008; Hoffmann & Willi, 2008; Dillon et al., 2009). Temperature has a significant influence on morphological, physiological, and fitness traits in *D. melanogaster*, ectotherms, and all

organisms. Therefore, understanding how organismal performance shifts across thermal environments is a critical field of study, especially as numerous models predict extreme temperature change as a result of global climate change (IPCC, 2007). Climate change may already be influencing the timing of life history events and range occupation of organisms (Parmesan & Yohe, 2003), decreasing chromosomal diversity of *Drosophila* species (Rodriguez-Trelles & Rodriguez, 1998), and is predicted to either increase or decrease the mean fitness of populations depending on their latitude (Deutsch et al., 2008).

Widespread ectothermic species are powerful models to study the effect of temperature, as they experience a spectrum of temperatures and must physiologically or behaviorally regulate body temperature (Gibbs et al., 2003; Chown & Terblanche, 2007; Clark & Worland, 2008). *D. melanogaster* is a cosmopolitan species with sub-Sahara African origins (David & Capy, 1988). The expansion of this ectothermic species into novel cold environments makes *D. melanogaster* a suitable organism for investigating questions related to the genetic basis and physiology of cold tolerance. To address these questions, diverse methodologies have been developed to assay thermotolerance phenotypes. The cold tolerance metrics: chill-coma recovery time , critical thermal minimum, rapid cold hardening, knockdown temperature, temperature preference, lower lethal temperature, and survivorship have been used frequently to describe the susceptibility of populations to cold temperatures (Czajka & Lee, 1989; Huey et al., 1992; Hoffmann & Watson, 1993; David et al., 1998; Kelty & Lee, 1999; Addo-Bediako et al., 2000; Gibert et al., 2001; Shreve et al., 2004; Terblanche et al., 2011). The depth of experimental procedures to assay thermotolerance, global distribution, and rich genetic tool kit of *Drosophila* confer great advantages for the mechanistic understanding of thermal biology, but contribute little to the identification of the mechanisms underlying adaptive response to cold temperatures. To identify the adaptive mechanisms it is essential to survey and dissect the natural genetic variation underlying phenotypic variation within and among populations.

Latitudinal clines in chill-coma recovery times (CCR) demonstrate that genetic variation exists for cold tolerance in *D. melanogaster*, with flies from temperate populations exhibiting increased cold tolerance as they recovery more quickly from chill coma relative to flies from tropical locations. Such phenotypic clines suggest the observed genetic differences among

populations are adaptive (Gibert et al., 2001, Hoffmann et al., 2002; Ayrinhac et al., 2004; Fallis et al., 2012). Additionally, Ayrinhac et al. (2004) demonstrated that flies from a single population, when reared at different developmental temperatures, had altered cold tolerances, indicating the population was plastic and flies reared at lower temperatures are more cold tolerant. This combination of adaptive and plastic influences on chill-coma recovery time suggests both genetic and environmental effects determine the phenotypic variation in this adaptive trait. In addition to the within species variation in chill-coma recovery speed, significant among species variation has been characterized among tropical and temperate *Drosophila* species, with species of tropical origin taking longer to recover from chill coma treatments than species of temperate origin (Gibert et al., 2001; Ayrinhac et al., 2004; Hoffmann et al., 2005). Furthermore, other thermotolerance measures are known to exhibit variation among populations and in thermal developmental environment, for example the duration of time spent at a specific temperature (i.e. acclimation or hardening) before exposure to a stressful temperature has a significant influence on mortality (Kelty & Lee, 1999; Rako & Hoffmann, 2006; Colinet and Hoffmann, 2012). These examples demonstrate that *D. melanogaster* populations differ in their basal thermotolerance and plastic acclimation abilities, suggesting genetic variation exists for both cold tolerance and thermal plasticity.

Phenotypic descriptions of thermotolerance phenotypes from *D. melanogaster* populations around the world are abundant, but examples detailing the underlying genetic architecture and physiology are poorly described. A mechanistic description of how populations respond to changing temperatures will become increasingly important if and when global climate change predictions (IPCC, 2007) are realized. Organisms can respond to changing environmental conditions through adaptation (natural selection), migration, or phenotypic plasticity. Phenotypic plasticity is a within generation response to the environment of a single genotype, where different environments elicit different phenotypes. The accuracy with which an organism responds to environmental cues and the frequency of specific environments experienced are critical determinants of plasticity prevalence (Van Tienderen, 1991; Moran, 1992; Sultan & Spencer, 2002; Pigliucci, 2001; DeWitt & Scheiner, 2004). Therefore, questions regarding the effect size and location of mutations contributing to phenotypic plasticity must be addressed to help conceptualize how organisms cope with environmental change via phenotypic plasticity.

Also, a distinction must be made between environment specific phenotypes, and plastic phenotypes, to determine if "plasticity genes" exist (Schlichting, 1986; Scheiner & Lyman, 1991), or if genes responsible for the phenotype in one environment are also responsible for the plastic response (Via & Lande, 1985).

The contributions made thus far to the fields of phenotypic plasticity and thermotolerance have aided in describing abundant genetic variation, candidate genes, and the adaptive value of both traits, but much remains to be discerned regarding the genetics of ecologically relevant trait variation. To address these questions and to identify genes involved in plastic chill coma responses to temperature, a phenotypic screen of approximately 400 recombinant inbred lines (RILs) was used to map the genetic basis of chill-coma recovery time in two thermal environments and the magnitude of phenotypic plasticity between environments. Additionally, genotyping of allelic variation in the regulatory region of a candidate cold tolerance gene, *Smp-30*, was performed in populations along the eastern seaboard of the United States to identify spatial variation associated with climatic adaptations. The following work mapped the genetic basis of chill-coma recovery time at 18°C and 25°C. Many of these regions spanned the same regions as several studies previously reported, validating the automated phenotyping method used and the mapping power of this RIL set. Also, an allele frequency cline was described for *Smp-30* regulatory polymorphisms, with alleles associated with cold tolerance increasing in frequency with latitude, and cold susceptible alleles showing the opposite pattern. This study demonstrates the tremendous amount of variation present in cold tolerance phenotypes and identified candidate regions of the genome that contribute to thermal plasticity and require further investigation. Together, these studies posit several experimentally identified regions of the genome contributing to thermotolerance and thermal plasticity variation. These findings will help further our understanding of how populations may cope with novel temperatures on both temporal and spatial scales, a question central to evolutionary biology.

Chapter 2 - The Genetic Basis of Natural Variation in Thermal Plasticity in *Drosophila melanogaster*

Introduction

The spatial and temporal heterogeneity within and among natural habitats places severe stresses on organisms, resulting in environment-specific adaptive responses (Van Tienderen, 1991; Moran, 1992). The impact of the external (and internal) environment on the expression of phenotypic variance has received increased attention as the field of phenotypic plasticity, which encompasses a wide range of organismal responses to the environment (i.e. behavioral and developmental plasticity), has grown tremendously in the last half-century (Bradshaw, 1965; Stearns, 1989; West-Eberhard, 1989; Gabriel & Lynch, 1992; Moran, 1992; de Jong, 1995, 2005; Gilchrist, 1995; Via et al., 1995; Pigliucci, 1996; Sultan & Spencer, 2002; Ghalambor et al., 2007; Auld et al., 2010; Fusco & Minelli, 2010). Phenotypic plasticity is defined as the unequal response(s) of a genotype to different environments. Multiple theoretical models have attempted to identify: the conditions that are most conducive to evolution of plastic responses, the adaptive value of plasticity, and the role plasticity plays in determining evolutionary trajectories of populations (Via & Lande, 1985; Gabriel & Lynch, 1992; Moran, 1992; Sultan & Spencer, 2002). This rich history of phenotypic plasticity theory has largely outpaced the empirical investigations, providing tremendous opportunity for the empirical dissection and validation of plasticity theory.

A complete understanding of variation in organismal responses to the environment (i.e. sensing, responding, and behaviorally/physiologically changing through time) is also important as predicted changes in the global climate will be accompanied by increases in temperature variation and thermal extremes. Such increased temperature variation and novel extremes will stress organisms outside the thermal range experienced in their native environments, and consequently force populations to tolerate novel thermal environments. This is especially true for organisms with geographical restrictions or limited mobility, long generation times, small population sizes, or narrow performance breadths, where plastic responses may be the only means of survival and reproduction (Pigliucci, 2001; DeWitt & Scheiner, 2004). The presence

of plastic responses is predicted when environmental variation exists, the cues preceding environmental change are accurate, no single genotype has the highest fitness across all environments, and the costs of plasticity are low (Van Tienderen, 1991; Moran, 1992; Sultan & Spencer, 2002; Price et al., 2003).

Genetic variation for plasticity and selection on this variation for increased/decreased plasticity have been reported in multiple systems (Brumpton et al., 1977; Scheiner & Lyman, 1991, Reboud & Bell, 1997; Pigliucci, 2001), but simply identifying plasticity as a quantitative trait with substantial genetic variation for selection to act on is no longer satisfactory. Experimental designs need to expand beyond identifying individual plastic responses, to instead examine population and species-level differences in plasticity to identify the genetic basis of plasticity, to determine how this variation is maintained, and how plasticity evolves within and among populations (Agrawal, 2001). Several theoretical models attempt to address these goals: (1) the overdominance model, (2) the pleiotropy model, and (3) the epistasis model (Reviewed in Scheiner, 1993; Piglicucci, 2005). These models differ in the causal genetic mechanisms underlying plasticity and thus result in different predictions of how plasticity should evolve. The overdominance model states that plasticity is controlled by the inverse of heterozygosity, thus as heterozygosity decreases within a genotype, plasticity will increase. This model proposes that heterozygosity functions as a buffer against environmental influences and thus as heterozygosity declines so will this buffering, resulting in increased plasticity (Marshall & Jain, 1968; Gillespie & Turelli, 1989). The pleiotropy model states that plasticity is the result of some genes having environmentally specific pleiotropic effects on a certain trait. This shift in pleiotropic genetic control among environments shifts the magnitude of plasticity (Pigliucci, 2005; Scheiner & Lyman, 1989). While, the epistasis model predicts that plasticity is the result of genetic interactions among sets of genes influencing a genotype's plasticity. These epistatic interactions mediate the properties of a genotype's plasticity (e.g., height or slope) (Scheiner & Lyman, 1989; Kassen, 2002). All of these models rely on assumptions about the genetic control and genetic interactions influencing plasticity.

Although there are a few notable examples, where we are beginning to understand the genetic basis and genetic interactions that produce morphological plasticity (e.g., morphological

polyphenisms in insects [for a review see Simpson et al., 2011]), for most phenotypically plastic and ecologically relevant traits we know little about the mechanisms underlying this variation in plasticity. This deficit in our mechanistic understanding is likely hindered by the fact that most plastic responses are likely to be mediated as part of a much larger network involving processes related to environmental sensing, relay, expression, and physiological, morphological, and behavioral responses to novel environments. Therefore, a comprehensive description of such complex phenotypic plasticity will require an understanding of genetic networks influencing traits in a single environment and the ability to predict how this network structure will shift as the environment changes (Shao et al., 2008; Mackay, 2009; Lehner, 2011; Huang et al., 2012). This complete description is beyond the scope of this work, however in this study we will demonstrate how the genetic basis of thermotolerance traits changes as the developmental environment is shifted from warm to cool.

For this work we will use the widespread model species, *Drosophila melanogaster*, and its continentally replicated thermal adaptation (David & Capy, 1988; James et al., 1995; Gibert et al., 2001; Ayrinhac et al., 2004; Sezgin et al., 2004; Schmidt et al., 2008; Hoffmann et al., 2002; Paaby et al., 2010). In *Drosophila* there is enormous amounts of variation in heat and cold stress responses within and among populations, suggesting thermotolerance has a significant heritable component (Parsons, 1977; Stanley et al., 1980; Hoffmann & Watson, 1993; Sorenson et al., 2005; Rako et al., 2007), that is also adaptive (Gibert et al., 2001; Hoffmann et al., 2002; Ayrinhac et al., 2004; Sezgin et al., 2004; Schmidt et al., 2008; Paaby et al., 2010), but is also highly plastic (Mitchell et al., 2011; Bubliy et al., 2012; Colinet & Hoffmann, 2012). The use of *Drosophila* as a model of thermal adaptation and plasticity is powerful as it is possible to leverage multiple fully sequenced reference lines and genotyped mapping populations (Macdonald & Long, 2007; Mackay, 2009, Huang et al., 2012; King et al., 2012a).

The power of genome-wide association studies (GWASs) and quantitative trait locus (QTL) mapping have been realized historically (Morgan & Mackay 2006; Carbone et al., 2006), however with the recent advances in sequencing technologies it is now possible to associate individual SNPs and candidate genes involved with complex phenotypes, like thermotolerance. Such panels permit high-resolution mapping of quantitative traits (King et al., 2012b). Although

both mapping approaches have great power, traditional QTL studies often map traits to broad intervals, while association mapping can determine the precise location and frequency, but lack the ability to estimate the allelic effect (Slate, 2005). The recent creation of a set of lines generated from an eight line intercross design, overcomes the weaknesses of both approaches, by allowing one to map the genomic location, allelic effect, and allele frequency of quantitative traits (Darvasi & Soller, 1995; Macdonald & Long, 2007).

In this study we used a synthetic mapping population, a resource for mapping quantitative traits developed via crosses between multiple parental genotypes (Darvasi & Soller, 1995). To identify potential genes involved with chill-coma recovery at 18°C and 25°C, as well as thermal plasticity, we phenotyped recombinant inbred lines from the *Drosophila* Synthetic Population Resource (DSPR). The few heterozygous founder genotypes present (1%), great map expansion, and high founder representation present in this synthetic population contribute to the precise mapping power of the DSPR (King et al. 2012a). The mapping power and resolution of the DSPR was validated by mapping QTL for overall activity of ADH enzyme that explained 57% of the genetic variance, confirming loci previously identified for ADH activity , and mapping QTL with modest-effects (King et al. 2012b). Besides the power to detect QTL of modest effect, the DSPR is able to determine allele frequencies because eight founding populations were used to develop the recombinant inbred lines (RILs), as opposed to the two used in standard QTL studies. These features give us the utmost confidence that the DSPR is a great resource for detecting and identifying candidate plasticity genes, the first step to a greater understanding of phenotypic plasticity variation, penetrance, and evolution.

Plasticity in response to temperature is a widely observed phenomenon in *D. melanogaster*. Effects of temperature have been documented for numerous morphological, physiological, and behavioral traits (Pétavy et al., 1997; David et al., 1990; Delpuech et al., 1995; Peng et al., 2007; Cooper et al., 2012), but we are most interested in the effect of temperature on cold tolerance phenotypes (Hoffmann & Watson, 1993; Gibert & Huey, 2001; Overgaard et al., 2011; Nyamukondiwa et al., 2011). The vast differences in response to temperature suggest that temperature dictates numerous changes in morphology, physiology, and behavioral responses. Thus, understanding the evolution of thermal plasticity will require an

integrative approach, involving the investigation of numerous traits and identification of genetic influences shaping phenotypic plasticity. To help achieve this goal, we focus on the physiological response to an acute cold stress, chill-coma recovery, across two developmental temperatures, with the ultimate goal of identifying genomic regions influencing thermal plasticity and contributing insight into phenotypic plasticity evolution.

In the absence of a within generation response to extreme temperatures, populations will migrate or perish. Alternatively, a within generation response (phenotypic plasticity) can serve as a buffer, allotting organisms the necessary time to adapt or fix an environmentally induced response (Waddington, 1942, 1952; West-Eberhard, 2003, 2005). Such responses may facilitate the persistence of populations inhabiting environments predicted to be affected adversely by global climate change, which could otherwise reach extinction over short periods of time. Large scale questions regarding the evolvability of adaptive traits remain unanswered, and an in-depth knowledge of the evolution of phenotypic plasticity may help elucidate the role plasticity plays in fostering or constraining evolution. Here we use the DSPR to estimate the locations, effects, and frequencies of QTL contributing to CCR and thermal plasticity in an effort to contribute knowledge to long-standing questions regarding the evolution of plasticity. Specifically, do genes responsible for the focal trait (cold tolerance) co-localize with those responsible for plasticity, or do plasticity-specific loci exist? What regions of the genome and what types of genes contribute to the propensity of plasticity? On smaller scales, do the sexes respond similarly when mapped for thermal plasticity QTL, and how many loci are potentially involved in thermal plasticity phenotypes? An understanding of the underlying mechanistic basis driving phenotypic plasticity can help shape novel models for phenotypic plasticity and formulate hypotheses regarding the evolution of phenotypic plasticity.

Materials & Methods

Founding Stocks and Variation in Plasticity (pA and pB lines)

The Drosophila Synthetic Reference Panel founding lines consist of 15 founder strains collected from around the world, which were used to create two synthetic populations, pA and pB. A round robin intercross scheme was used to construct the two subpopulations, which would

later lead to the recombinant inbred lines derived from the pA and pB synthetic populations. Both subpopulations contain seven unique founders, each sharing one common founder. Following 50 generations of random mating and free recombination, the recombinant inbred lines were started using 576 pairs from each subpopulation followed by 20 generations of inbreeding (King et al. 2012a). Thus, pA and pB constitute independent units containing mostly unique alleles, affording the researcher the opportunity to select a mapping population that best suits their questions and traits of interest. The 15 founding lines of the DSPR were screened using the phenotyping assays described in Morgan & Mackay (2006). Briefly, flies were reared at either 18°C or 25°C for all of development and 5-7 day old flies were placed at 0°C for 3 hours, returned to room temperature (23°C), and observed until a chill-coma recovery score was derived. Chill-coma recovery time (CCR), was the score (in minutes) generated when flies were able to stand upright on their legs upon removal from a 0°C incubator and placement at room temperature (23°C). As a result of the increased variation for thermal plasticity among the founding lines of the pA population, we focused the mapping analyses described below on the pA population.

Drosophila Stocks

Based upon the results of a power analysis performed by King et al. (2012a), in which the power to detect QTL of large and small effect increases greatly from 200 to 400 lines and remains relatively constant above 400 lines, we randomly chose 431 recombinant inbred lines (RILs) from the DSPR to map the genetic basis of thermotolerance traits (King et al. 2012a). RILs were used from the pA synthetic population only, which was developed from eight founding lines, harboring genetic variation from around the globe, as described in King et al. (2012a). Lines were maintained under standard conditions (25°C, 12:12 light/dark cycle) on standard cornmeal, molasses, and agar for three generations after flies were received in the Morgan Lab.

Thermal Environments

All of the RILs were reared in warm and cool environments. The “warm” environment was 25°C (12:12 L:D cycle), while the “cool” environment was 18°C (12:12 L:D cycle).

Experimental flies were generated at low density, 5 males and 5 females, in vials containing ~20 ml of standard cornmeal:molasses:agar. Vials were cleared of adults after 3 days at 25°C and 5 days at 18°C to ensure flies present in the vials at time of collection were of the appropriate age and to maintain a low larval density for the developing flies.

Chill-coma Recovery

Experimental flies from each treatment were reared from egg to adult at the "warm" or "cool" treatment temperature (12:12 L:D cycle). Upon the first day of eclosion, all flies were cleared from their vial to ensure the flies present on the following day eclosed on that day. Two days following the presence of eclosed flies, all flies in each respective vial were transferred to a collection vial under light CO_2 anesthesia. Same sex groups containing 10 flies were placed into collection vials (~10 mLs of standard food). Four replicates of 10 flies per line, per sex, were measured in a randomized design. Four days after placement in collection vials, flies were screened for CCR, ensuring that all experimental flies were 5-7 days old and any residual effects of CO_2 exposure were removed. To measure chill-coma recovery times, flies were transferred to empty vials without anesthesia immediately before exposure to 0°C for 3 h. Upon removal from the cold, flies were placed in 1 of 20 randomly selected compartments for chill-coma measurement in a 23°C incubator. Approximately 4 minutes post removal from the cold exposure, a time-lapse photography session was initiated. Photographs were captured using a digital camera (Canon EOS Rebel T3 Digital SLR Camera) running off the time-lapse function of a computer program (DSLR Remote Pro for Windows). Photos were saved to an external hard drive, transferred to an iMac, and pairwise comparisons were made between the first image and all subsequent images via a custom script executed in ImageJ. This script tests for minimal movements that are defined as chill-coma recovery time. Movements were defined based on the area (number of pixels) and circularity (4π (area/perimeter2)) of individual flies as defined by script written for ImageJ. Output files from ImageJ were deposited into Excel and a chill-coma recovery time (CCR) value was derived for each individual to determine mean recovery times in each treatment, and ultimately a plasticity value (the difference between recovery times in the 18°C treatment and 25°C treatment). The automated phenotyping process was validated by

regressing automated phenotyping results against historical phenotyping results collected in the manner described in Morgan & Mackay (2006).

Quantitative Genetic Analyses

Mixed model analyses of variance (ANOVAs) and variance component calculations were used to determine sources of variation between lines, sexes, treatments, and interactions using PROC GLM implemented in SAS 9.2 (SAS Institute 2009). The initial full model was:
$y = \mu + L + S + T + L \times S + L \times T + T \times S + L \times S \times T + \varepsilon$, where y is CCR time at each treatment, μ is the overall mean, ε is the residual error, while L, S, and T are fixed effects of line, sex, and treatment respectively. The terms of primary interest are L, $L \times S$, $L \times T$, and $L \times S \times T$, as they represent genetic variation, genotype-by-sex, genotype-by-environment, and genotype-by-sex-by-environment interactions, respectively. Temperature specific analyses were performed using a model identical to the one above but with the fixed effect of temperature removed.

Broad-sense heritabilities (H^2) for CCR times were calculated as $H^2 = \sigma_L^2/(\sigma_L^2 + \sigma_E^2)$, where σ_L^2 is the among-line variance component and σ_E^2 is the within-line variance component. The coefficient of genetic variance was calculated as $CV_G = \sqrt{\sigma_G^2}/\bar{x}$, where $\bar{x}$ is the average CCR and σ_G^2 is the genetic variance.

QTL Mapping

Standard interval mapping quantitative trait locus mapping was used to identify QTL for chill-coma recovery time (at 18°C and 25°C) and thermal plasticity (calculated as the difference in CCR between flies reared at 18°C and 25°C) using the R package DSPRqtl (flyrils.org). Briefly, the R package performed a multiple regression of our measure of cold tolerance, CCR, on the eight additive probabilities of founder genotypes from the pA population with no covariates. The *F*-statistic was converted to a LOD score and trait data was randomly permuted 1,000 times to determine if LOD scores exceeded the threshold determined by randomly assigning trait values to random markers (Churchill and Doerge, 1994). Standard interval mapping (Lander & Botstein, 1989; Broman & Sen, 2009) was also performed around each

mapped QTL to precisely map peaks and determine confidence intervals (as described in King et al. 2012a).

Results

Thermal Plasticity among the Parental Stocks

Chill-coma-recovery times at 18^{o} C, 25^{o} C, and thermal plasticity were measured on the parental stocks, which founded the pA and pB populations (Fig. 2.1). Both sets of parental lines exhibit highly significant genotype-by-environment interactions for chill-coma recovery time, however the founders of the pA population have more significant thermal plasticity ($F = 20.85$; $P < 0.0001$) relative to the founders of the pB population ($F = 11.22$; $P < 0.0001$). Thus, as a result of experimental limitations we focused on the screening of the pA population as its founders exhibited a greater degree of thermal plasticity relative to the pB population. Focusing on a single set of RILs does not decrease the impact of the current study as the pA and pB founding populations and resulting RIL sets, represent unique allelic combinations and thus if we were to repeat this analysis on the pB population we would expect overlapping results but not identical results as the pools of genetic variation segregating within each RIL set are independent and unique (King et al. 2012a).

Quantitative Genetic Variation

Mean chill-coma recovery times ranged from 8.63 min (cold tolerant lines) to 28.52 min (cold susceptible lines), with normally distributed intermediate cold tolerance phenotypes (Fig. 2.2a). Cold tolerance responses between treatments were not correlated, meaning a cold tolerant fly when reared at 18^{o} C was not necessarily cold tolerant when reared at 25^{o} C (Fig. 2.2b) There was significant genetic variation among the 431 RILs for chill-coma recovery at 18^{o} C, 25^{o} C, and thermal plasticity ($F_{18} = 14.15$ $P< 0.0001$; $F_{25} = 8.00$ $P< 0.0001$; $F_{Plasticity} = 8.01$ $P< 0.0001$; Fig. 2.3a). However the magnitude of the genetic variation (σ^2_L) differed among environments and traits, with the chill-coma recovery at 18^{o} C having the highest genetic variation ($\sigma^2_L = 10.7$; $CV_G = 0.189$; $H^2 = 0.238$). Chill-coma recovery at 25^{o}C had decreased genetic variation ($\sigma^2_L = 8.47$; $CV_G = 0.146$; $H^2 = 0.150$) relative to chill-coma recovery at 18^{o}C. Thermal plasticity

exhibited genetic variation intermediate between the two single environment measures (σ^2_{LxT} = 9.34; H^2 = 0.179).

Mean thermal plasticity scores ranged from -12.47 (recovery at 18°C takes 12.47 minutes longer than recovery at 25°C) to 12.61 (recovery at 18°C is 12.61 minutes quicker than recovery at 25°C), with normally distributed intermediate plasticity scores (Fig. 2.3c). As expected, flies reared at 18° C recovered significantly faster from chill coma, with an average recovery time of 17.40 minutes compared to an average recovery time of 19.55 minutes for flies reared at 25°C (F_{Temp} = 930.90; P<0.0001; Fig. 2.2a). The overall average plasticity had a positive value of 2.11 (flies reared at 18°C recovered, on average, 2.11 minutes quicker than flies reared at 25°C). Sex specific effects were identified, as females were more cold tolerant than males, recovering more quickly from chill coma when reared at 18° C and 25° C than males, 17.04 min compared to 17.70 min and 19.21 min (F_{18} = 59.71; P < 0.0001) compared to 19.82 min, respectively (F_{25} = 24.31; P<0.0001).

Developmental temperature had a significant effect on cold tolerance phenotypes. The influence of developmental temperature was evidenced by a majority of thermal reaction norms having positive slopes, with chill-coma recovery time being quicker, on average, in flies reared at 18°C (Fig. 2.2a). Interestingly, most of the thermal reaction norm space was occupied, indicating that some lines responded in an unexpected fashion, performing better when reared at a higher developmental temperature (Fig. 2.3c). Crossing of reaction norms, indicated by a highly significant *line-by-temperature treatment* interaction term (F = 8.01; P < 0.0001), confirmed substantial genetic variation exists for thermal plasticity.

QTL Mapping

We mapped 5 QTL for chill-coma recovery time at 25°C, 4 QTL for chill-coma recovery time at 18°C, and 4 QTL for thermal plasticity (Fig. 2.4, Table 2.1). All QTL mapped to the autosomes and explained a modest portion of the phenotypic variation (6.88% to 9.85%) in the pA population. The majority of the chill-coma recovery QTL identified on the second and third chromosomes were previously shown to be involved with both cold and heat tolerance using different mapping populations (Morgan & Mackay, 2006; Norry et al., 2007, 2008).

Interestingly, differences between the main effect QTL for CCR at 18°C and 25°C (i.e. peaks present at 25°C, but absent at 18°C) appear to describe half of the thermal plasticity QTL, with the exception of the two thermal plasticity QTL on the left arm of the third chromosome.

For the chill-coma recovery QTL at 25^{o}C, four QTL were mapped to the second chromosome, with one QTL on the left arm and three QTL on the right arm. The QTL on the left arm was located at 2L: 19,520,000 (confidence interval: 19,290,000 – 19,670,000). The most significant QTL was on the right arm of the second chromosome, and explained 8.85% of the genetic variation, and a 2-LOD support interval includes a 690-kb region encompassing 119 genes, located at 2R: 4,470,000 (confidence interval: 4,220,000 – 4,910,000). Only one QTL was present on the third chromosome (3R: 7,470,000; confidence interval: 7,360,000 – 7,490,000), explaining 7.19% of the variance in chill-coma recovery time at a developmental temperature of 25° C. The QTL identified above explain 39.80% of the variation in chill coma recovery time at 25°C and encompass 859 genes.

The largest chill-coma recovery time QTL for development at 18° C was located on the right arm of the third chromosome (3R: 4,140,000; confidence interval: 4,060,000 – 4,820,000) and explained 7.50% of the variation associated with chill coma recovery time. Three other QTL were identified, one on the right arm of the second chromosome (2R: 1,910,000; confidence interval: 1,830,000 – 1,960,000), and two on the right arm of the third chromosome (3R:5,350,000 and 3R:22,91,000; confidence intervals: 5,220,000 – 5,390,000 and 22,860,000 – 23,050,000, respectively). Together, these QTL explain 29.28 % of the variation in chill coma recovery time when flies are reared at 18° C and only 223 genes are present below the significant peaks.

Thermal plasticity QTL were generally located near chill coma recovery QTL at 25° C and 18° C, except for the plasticity QTL located on the left arm of the third chromosome. The largest plasticity QTL was on the right arm of the second chromosome (2R:8,010,000; confidence interval: 8,000,000 – 8,080,000) and explained 9.26% of the variation in thermal plasticity. Two QTL on the left arm of the third chromosome (3L:5,530,000 and 3L:14,990,000; confidence interval: 5,120,000 – 5,570,000 and 13,390,000 – 15,320,000, respectively) were

specific to thermal plasticity and explained 16.06 % of thermal plasticity variation, with a possible 239 genes residing in the region. The remaining thermal plasticity QTL was on the right arm of the third chromosome (3R:23,400,000; confidence interval: 23,380,000 – 24,340,000). Together, all identified thermal plasticity QTL explain 33.51% of the variation in thermal plasticity and encompass 354 genes.

Discussion

A comprehensive understanding of thermotolerance phenotypes and phenotypic plasticity is becoming increasingly important as organismal habitats experience novel extreme temperatures and thermal fluctuations as a result of global climate change. A large amount of information has been generated investigating organismal performance across multiple temperatures (Rako & Hoffmann, 2006; Chown & Terblanche, 2007; Clark & Worland, 2008; Nyamukondiwa et al., 2011; Colinet & Hoffman, 2012), but a void exists as to the underlying genetic architecture and physiology driving the organismal response to environmental variation. A near complete description of the genetic architecture of thermal tolerance will aid in the identification of candidate genes and proposed physiological mechanisms controlling thermotolerance at both high and low temperatures. This is especially true for populations predisposed to the greatest temperature swings in the immediate future. The strategies an organism can use to cope with changing environments include migration, evolution of tolerance (adaptation), and/or phenotypic plasticity. With regards to environmental changes involving temperature, we are especially interested in thermal plasticity, as populations with different geographical, and associated climatic origins are hypothesized to have different plastic responses (van Heerwaarden & Sgrò, 2010). Here we characterize the genetic architecture of cold tolerance and thermal plasticity phenotypes in *D. melanogaster* after development at two temperatures, 18°C and 25°C.

Chill-coma recovery time is a standard metric of cold tolerance (Gibert et al., 2001), but debate exists regarding which phenotypes are the best for assessing thermotolerance in ectotherms (Hazell & Bale, 2011; Ransberry et al., 2011). To our knowledge, no study to date has demonstrated a clear link between greater cold tolerance and fitness, using any metric of cold

tolerance, in a convincing manner, which contributes to the uncertainty of a superior cold tolerance metric. Two cold tolerance assays, critical thermal minimum (CT_{min}) and chill-coma recovery (CCR), are used commonly, and it has been suggested that CT_{min} may be the most ecologically relevant as this is the temperature at which organisms lose locomotor ability, exposing them to possible predation, further injury due to cold, and the inability to forage for food and mates, all resulting in reduced fitness (Hazell & Bale, 2011), however the speed with which an insect recovers from chill coma may also reduce the amount of time spent in these adverse conditions, affecting fitness as well. Ransberry et al. (2011) examined the relationship between CCR and CT_{min} across a wide range of temperatures and demonstrated that both exhibit plasticity at opposite temperature extremes, suggesting that the underlying physiological mechanisms are different, but the correlation between both over all non-extreme temperatures suggests both are useful ecologically relevant measures for assessing cold tolerance (Ransberry et al., 2011).

Studies of thermal tolerance must incorporate the standing natural genetic variation upon which selection acts to identify truly adaptive processes, as opposed to artificially selected. To our knowledge, this study is the first to assess genetic variation in globally distributed *D. melanogaster* genotypes and map the genetic basis of thermal plasticity. Using the DSPR has allowed us to determine the effect, frequency, and genomic locations of the QTL (King et al., 2012b) influencing thermal tolerance and thermal plasticity. As expected, significant Genotype-by-Environment interaction was observed, however a surprising number of RILs responded in the unexpected direction, the non-developmental acclimation direction (i.e., genotypes that recover more quickly when reared at warmer temperatures). Although the presence of these seemingly "maladaptive" genotypes seems anomalous, not all responses to the environment need be adaptive (Ghalambor et al., 2007; Valladares et al., 2007), especially in populations from geographic regions infrequently experiencing chill-coma inducing temperatures. The majority of lines (70.91%) had plasticity values in the expected positive direction, and the mean thermal plasticity value was positive (2.11), that is on average flies reared at a warmer developmental temperature were more cold susceptible than flies reared at a colder developmental temperature, a pattern consistent with developmental acclimation (Kristensen et al., 2008). As expected, females recovered more quickly than males and flies reared at 18°C recovered more quickly than

flies reared at 25°C (Fig. 2.2). Also, thermal plasticity scores were normally distributed (Fig. 2.3b); suggesting plasticity is a quantitative trait with a genetic basis, which has the ability to evolve.

The QTL we identified are from a global source of alleles, harboring variation from temperate and tropical regions, and many of the chill-coma recovery QTL at 25°C overlap with QTL identified using Australian (Norry et al., 2007), Australian and Danish (Norry et al., 2008) and American and Russian parental lines (Morgan & Mackay, 2006). These findings validate the accuracy and validity of the DSPR for mapping thermotolerance phenotypes. The high level of recombination in the DSPR allowed for precise mapping of QTL, with some regions encompassing only 80kb or containing 19 genes; precision not usually afforded by traditional QTL mapping studies (Mackay, 2001, 2009). Thermal plasticity QTL did not overlap with any chill-coma recovery time QTL on the left arm of the third chromosome, suggesting these may be regions contributing to thermal plasticity variation. Interestingly, the other 2 plasticity QTL were located in close proximity of chill-coma recovery time at 18°C and 25°C QTL. Therefore, it seems plausible that plasticity evolution does not have to fall into one of three models: overdominance, epistasis, or pleiotropy. Instead, genes contributing to thermal plasticity could be independent of the environment (i.e. plasticity QTL not overlapping with CCR QTL) or could be environment-dependent (i.e. plasticity QTL located in the same vicinity as CCR QTL), suggesting components of the epistasis and pleiotropy models could both be accurate (Scheiner & Lyman, 1989).

A description of the physiological processes related to cold tolerance will help describe possible suites of genes that influence cold tolerance based on their function. Suites of genes involved in ion transport and ATPase activity were located under significant QTL in multiple traits. Based on their molecular and physiological function, these genes may be involved in cold tolerance. This is supported by findings (MacMillan et al., 2011b; Reviewed in MacMillan et al., 2011a) that led to the development of a model for the effects of decreasing temperatures on muscle resting potential in insects (MacMillan et al., 2012). The model describes the shift of water and Na^+ from the hemolymph to the gut upon prolonged exposure to cold temperatures, increasing the volume of the gut and increasing hemolymph K^+ concentration. These concurrent

processes depolarize muscle K^+ equilibrium potentials (E_K), resulting in chill-coma due to a loss of ion homeostasis, which when K^+ homeostasis is restored, chill-coma recovery begins (MacMillan et al. 2012). This model was not tested on Drosophilids, but it is likely that similar responses to prolonged freezing temperatures occur in Drosophilids.

Based on the QTL positions and the mechanistic model detailed by MacMillan et al., (2012), plausible candidate genes identified by our QTL analysis are those involved with ion transport and ATPase activity. One such gene contributing to CCR at 25°C, *Trap1*, located under a QTL peak at 2.83 Mb on the right arm of the second chromosome (2R: 2.83 Mb), influences mitochondrial function, and exhibits ATPase activity (Felts et al., 2000), as mutants had decreased respiratory functions compared to controls (Costa et al., 2013). *Trap1* mutants also had decreased lifespans and decreased coordination as evidenced by lower climbing index scores (Costa et al., 2013), this is compelling evidence that *Trap1* may contribute to chill-coma recovery as temperature influences both life span and how quickly coordination is regained after a thermal stress. Lastly, *Trap1* has been suggested as a candidate cold tolerance gene in several other studies as well, providing a compelling argument that *Trap1* contributes to phenotypic variation in cold tolerance (Norry et al., 2008; Morgan & Mackay, 2006; Vermeulen et al., 2013). Changes in Na^+ and K^+ concentrations beyond a certain point induce chill coma, therefore genes involved in the transport of these ions are good cold tolerance candidate genes. *Task7,* a two-pore domain potassium (K_{2P}) channel, does not function properly by itself, but forms a functional heteromeric channel with *Task6* that is most likely involved in setting membrane potential, and this specific channel might exhibit different functional and pharmacological properties such as pH sensitivities (Döring et al., 2006). *Task7* was located under a peak for CCR at 18°C at 3R: 5.35 Mb. Two other plausible candidate genes located in regions with statistically significant LOD scores included *Best 3* and *Best4*, which are involved in anion and chloride transport (Sun et al., 2002) and cell volume homeostasis due to similarity to *Best1* (Chien & Hartzell, 2008). The extent to which these genes interact or contribute to cold tolerance at all is uncertain, but based on the known chill-coma recovery time physiology (MacMillan et al., 2012) and their association with a thermal plasticity QTL on the left arm of the third chromosome, they represent promising candidates.

The final and most recognizable step of chill-coma recovery involves the ability to stand up straight, a process that involves fine motor coordination and therefore genes involved in locomotion and flight may contribute to the chill-coma recovery process. We identified one such gene in our QTL analysis for thermal plasticity, *starvin*, underlying a peak at 3L:14.99 Mb. Adult flies with *starvin* mutations experience impaired climbing and flight abilities that progress with age and larvae with *starvin* overexpressed suffer reduced mobility and increased death (Arndt et al., 2010). Also, *starvin* forms a chaperone complex that degrades damaged products to help maintain muscle integrity and proper function (Arndt et al., 2010). In addition to these possible functional roles involved in conferring cold tolerance, *starvin* has also been identified as a possible cold tolerance gene through expression studies (Colinet & Hoffmann, 2010, 2012).

Alterations to the chordotonal organs may affect gravitaxis behavior, as previous studies have suggested chordotonal organs are involved in gravitaxis behavior (Armstrong et al., 2006; Kamikouchi et al., 2009; Desroches et al., 2010). Therefore, a particular gene of interest we identified is *nan,* and interestingly it also contributes to thermal plasticity variation and was under the same peak as *starvin. nan* is an ion channel protein similar to the transient receptor potential (TRP) superfamily of channels, which expression studies demonstrated it is located in chordotonal organs (Kim et al., 2003) . Based on sequence similarity to the TRP family, *nan* is believed to be involved in calcium ion transport (Littleton & Ganetzky, 2000) and hearing (Kim et al., 2003). Also, *nan* mutants displayed defective negative geotaxis, uncoordinated movements, and reduced locomotion. Another candidate involved in hearing that we identified as contributing to cold tolerance at 25°C, is *nompA*, located under a peak at 2R: 6.61 Mb. *nompA* is expressed in Johnston's organ and involved in the perception of mechanical stimuli, a process that is interrupted in *nompA* mutants (Chung et al., 2001; Gopfert & Robert, 2003). Lastly, the gene *Eb1* was associated with CCR at 25°C (located at 2R:2.83 Mb) and mutants in *D. melanogaster* had severe coordination problems, so much so, flies could not fly and took longer to right themselves after being placed on their back compared to wildtype flies (Elliot et al., 2005). The intricate relationship between mechanoreception and chordotonal organs may also be intertwined with responses to cold as indicated by significant regions of the genome we identified.

The mechanistic bases of certain relationships between temperature and *Drosophila* life history traits and physiology have not been described, but make intuitive sense. Flies reared at colder temperatures have longer developmental times and generally live longer (Egge et al., unpublished data; Huey et al., 1991; Norry & Loeschcke, 2002). Candidate genes with influences on longevity, *Coq2* (Liu et al., 2011), *lt* (Simonsen et al., 2007), *bmm* (Grönke et al., 2007), and *wdb* (Funakoshi et al., 2011) may share developmental processes linked to longevity and larger size from rearing or adaptation to colder temperatures. Both *bmm* and *wdb* were associated with thermal plasticity and located at 3L: 14.99 Mb and 3R: 2.4 Mb, respectively. *Lt* and *Coq2* were associated with CCR at 25°C and 18°C, respectively. Besides longevity, energetic reserves must be conserved during thermal stresses, and loci involved in glycolysis may contribute to cold tolerance. The genomic region of a QTL for CCR at 25°C harbored a gene involved with glycolysis, *Pgi*, at 2R: 4.47 Mb. Associations between *PGI* genotypes/allele frequencies and thermal traits exist in a number of insect systems (Neargarder et al., 2003; Karl et al.., 2008), although such clinal associations are not always present or clear for *Drosophila* (Eanes et al., 2005). It becomes evident how many genes and processes can contribute to ecologically relevant phenotypes, thus we must be careful to not narrow the scope with which we think about cold tolerance.

One gene underlying a significant QTL on the 2nd chromosome (2R: 6.61 Mb), influencing CCR at 25°C is *Lsm10*, which was recently reported by Fallis et al. (in review) to be a cold tolerance gene via fine mapping of a previous QTL study mapping heat and cold tolerance in *D. melanogaster*. Interestingly, out of the five significant genes identified, *Lsm10* was the only gene with a non-synonymous polymorphism that may alter the structure of the resulting protein, and ultimately phenotype. *Lsm10* mutants experience premature death and irregular histone functioning (Godfrey et al., 2009). The alteration of histone functioning in *Lsm10* mutants, and discovery of a non-synonymous polymorphism (Fallis et al. in review), may disrupt the expression of cold tolerance genes via faulty regulatory regions. This alteration of regulatory regions could also drive expression leading to plastic responses to novel environments and temperatures.

Here we described the phenotypic and genetic variation present for a thermotolerance phenotype, chill-coma recover time, at two development temperatures and for thermal plasticity in a synthetic population of *Drosophila* harboring a global set of alleles. Flies reared at colder temperatures tend to be more cold tolerant, and all three traits measured were quantitative traits with abundant genetic and phenotypic variation. A surprising number of lines had negative plasticity scores, indicating flies were more cold tolerant when reared at warmer temperatures, a result that seems counterintuitive, but flies harbored alleles from across the globe, including equatorial regions, where selection pressures to be cold tolerant are most likely weak or nonexistent. Therefore, the lack of an adaptive response to rearing temperature may not be surprising, and suggest a cost to thermal plasticity that has eroded the ability to be plastic. Alternatively, it may hint at the fact that cold tolerance is an extremely complex phenotype, and perturbations to genomes from natural populations from recombination events with genetic material from global populations disturbed genetic pathways and networks, leading to decreased cold tolerance. No clear pattern for the genetic architecture of thermotolerance or thermal plasticity was present, as half of novel thermal plasticity QTL co-localize with chill-coma recovery time QTL, suggesting plasticity QTL may be the same as environment specific QTL, and that plasticity QTL may be independent of environment specific QTL (i.e. "plasticity genes" do indeed exist). Future studies will aim to mechanistically describe regions of the genome involved in thermal plasticity and describe the adaptive ability and potential costs of plasticity. Also, local populations of Drosophila need to be examined from across the globe to describe natural variation, which can be used to link climatic patterns to variation, and to determine the evolutionary patterns and possible models for plasticity and complex trait evolution.

The diversified findings of studies from various systems and environments suggesting costs, the adaptive value of, and fixation of plasticity demonstrate the great progress made in the field, and may highlight even more, the amount of work that remains. Questions of great interest to this study are diverse and aim to contribute to the rich theoretic work surrounding phenotypic plasticity evolution. Are the genes influencing traits of interest (i.e. cold tolerance) the same as those influencing plasticity (i.e. thermal plasticity), or are there "plasticity genes"? If plasticity genes exist, are their contributions to plasticity dictated by many genes of small effect, or few genes of large effect? These questions address just a small proportion of phenotypic plasticity

knowledge, but once answered, they will help in the development of novel models that incorporate facets that were previously thought to be unimportant. The study presented here described genomic regions contributing to thermal plasticity and candidate genes. Describing basic patterns of phenotypic plasticity is the first step in describing the mechanistic basis and evolution of plasticity. As the epigenetic community continues to blossom and sequencing and computational power increases, questions addressing the adaptive value of and costs of plasticity can be addressed, an area or plasticity research we were unfortunately not able to make contributions to directly. Future directions involve deciphering the costs of plasticity and evolutionary trends associated with plasticity, long term selection experiments in controlled heterogeneous environments with lines of varying plasticity can help address these long-standing evolutionary questions.

Figure 2.1 Founding line chill-coma recovery time for (a) the pA population and (b) pB population of the DSPR. The x-axis is the developmental temperature, 18°C or 25°C, and the y-axis is chill-coma recovery time in minutes.

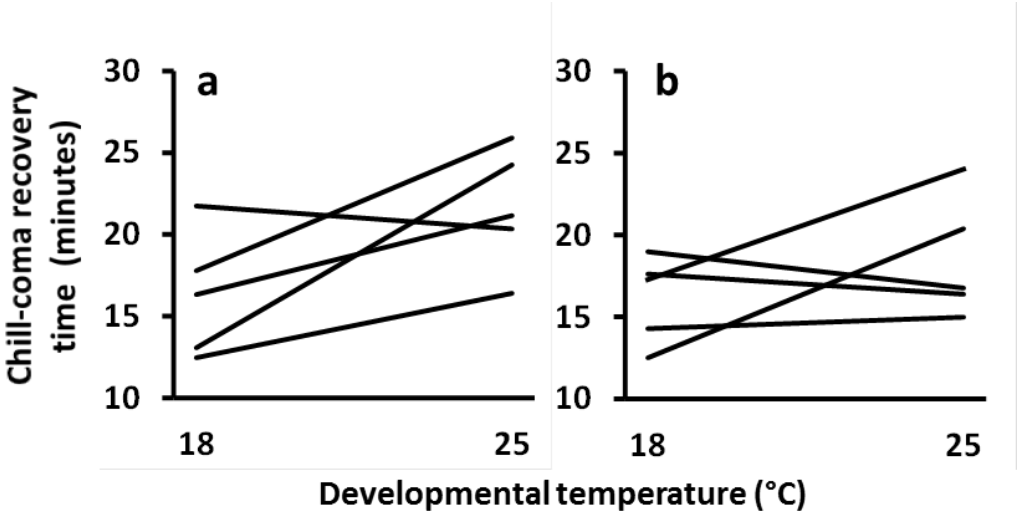

Figure 2.2 (a) Distribution of chill-coma recovery times after development at 18°C (blue) and 25°C (red). The x-axis is chill-coma recovery time in minutes and the y-axis is the frequency. (b) The relationship between chill-coma recovery times after development at 18°C (on the y-axis) and 25°C (on the x-axis).

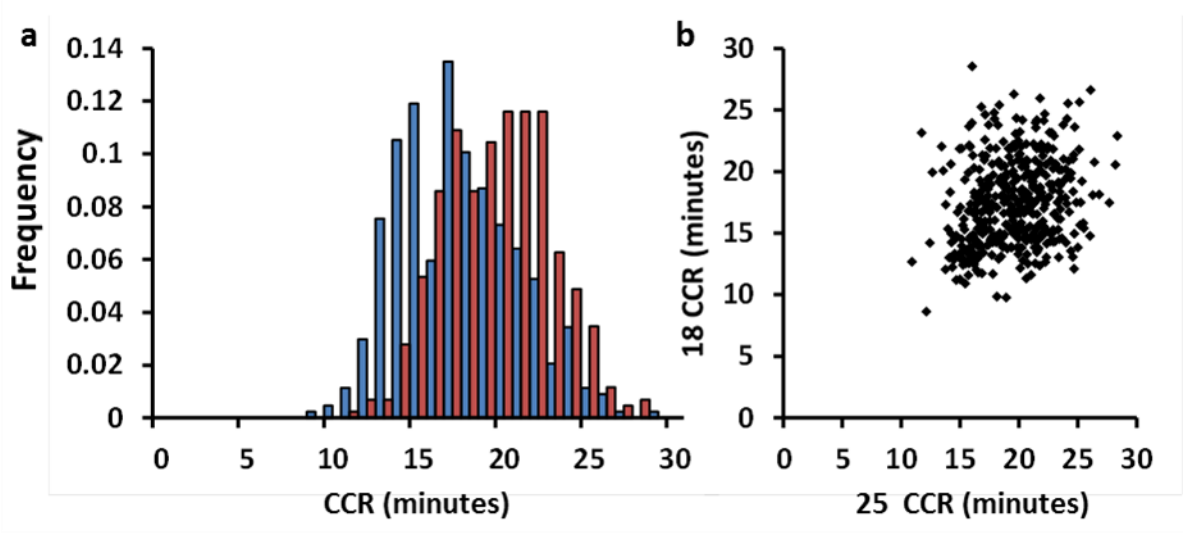

Figure 2.3 Thermal plasticity variation. (a) A representative sample of line-specific chill-coma recovery time reaction norms. Reaction norms with cool colors (blues) are plastic in the expected direction, reaction norms with warm colors (reds) are plastic in the unexpected direction, and neutral color (gray) reaction norms do not exhibit plasticity. (b) The distribution of thermal plasticity scores (the difference between CCR at 25°C and 18°C) is on the x-axis, and the frequency is on the y-axis. (c) Rank order of all RILs phenotyped. RILs in blue were more cold tolerant when reared at cooler temperatures, and RILs in red exhibited the opposite pattern. Thermal plasticity is on the y-axis and each RIL is on the x-axis.

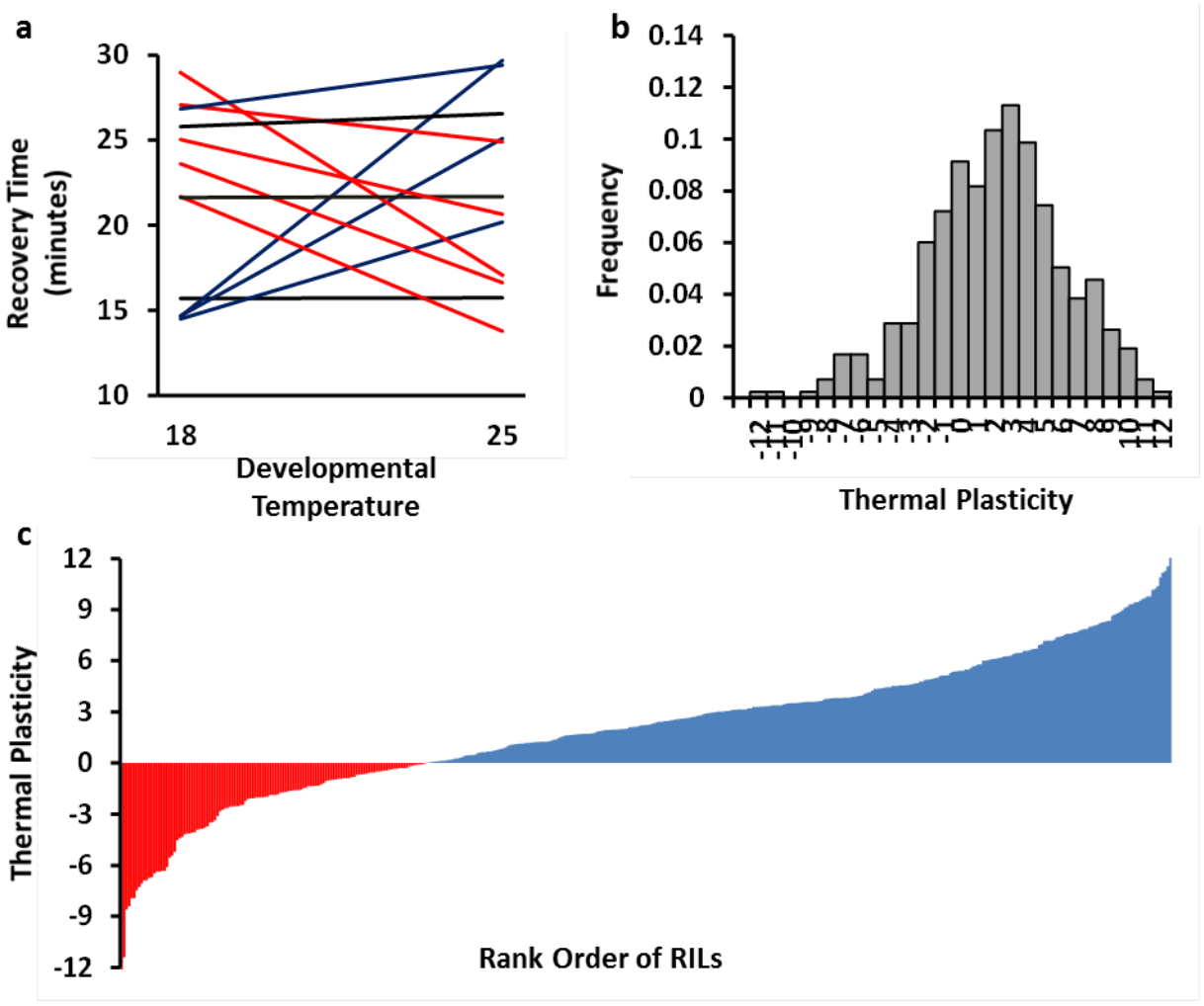

Figure 2.4 Genome scan for thermotolerance QTL. QTL plots for (a) chill-coma recovery time at 18°C, (b) chill-coma recovery time at 25°C, and (c) thermal plasticity. Genome position (in cM) is on the x-axis, and the LOD score, determined by 1,000 permutations, is on the y-axis.

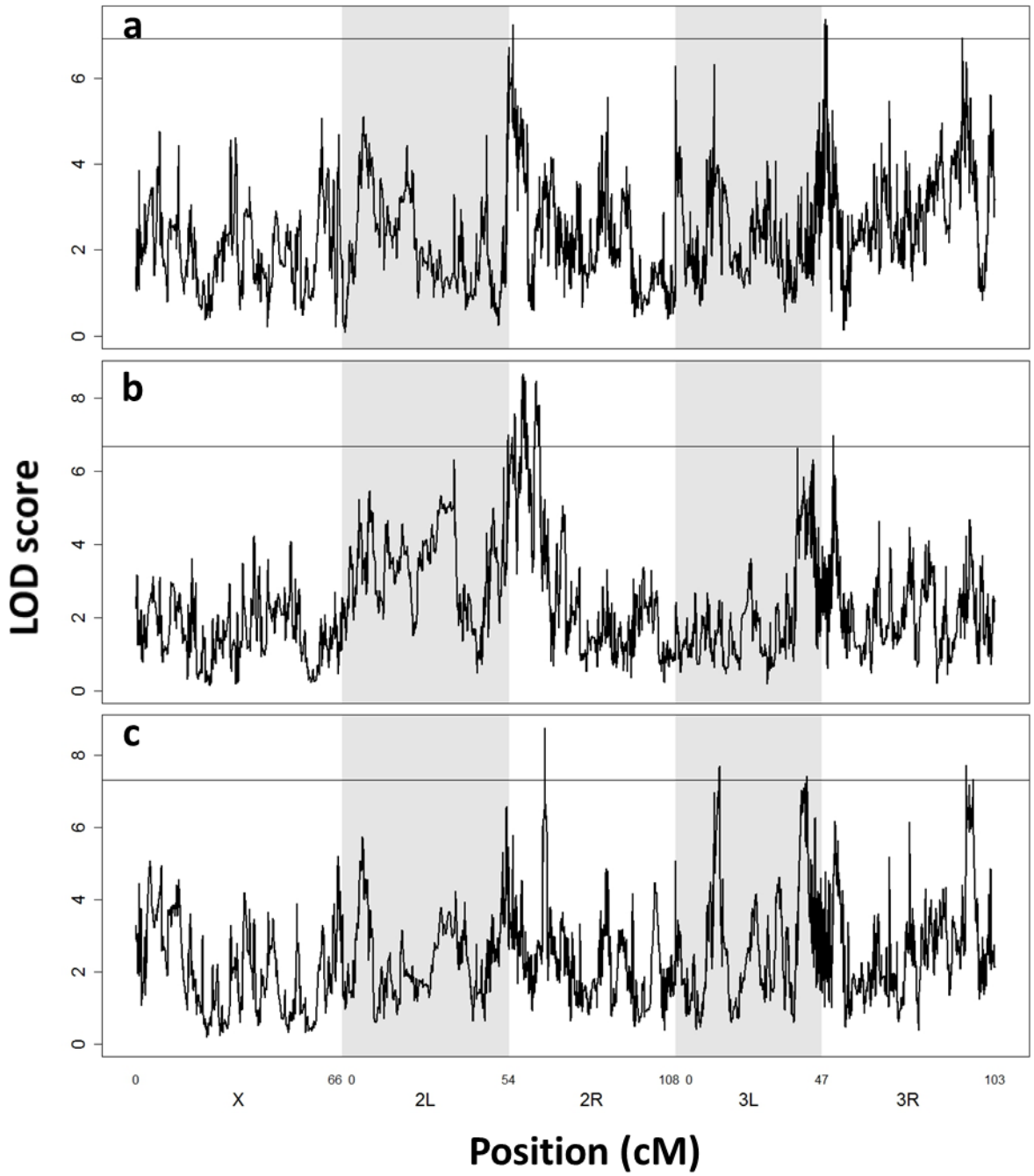

Table 2.1 Thermotolerance QTL for the pooled sexes.

Treatment	Chr	Position (Mb)	LOD score	CI (Mb)	% Var
18° C	2R	1.91	7.240	1.83 - 1.96	7.3620
18° C	3R	4.14	7.383	4.06 - 4.82	7.5018
18° C	3R	5.35	7.232	5.22 - 5.39	7.3538
18° C	3R	22.91	6.934	22.86 - 23.05	7.0620
25° C	2L	19.52	6.993	19.29 - 19.67	7.2159
25° C	2R	2.83	7.570	21.29 (2L) - 3.04 (2R)	7.8732
25° C	2R	4.47	8.657	4.22 - 4.91	8.8550
25° C	2R	6.61	8.462	6.29 - 7.24	8.6644
25° C	3R	7.47	6.971	7.36 - 7.49	7.1938
Plasticity	2R	8.01	8.752	8.00 - 8.08	9.2555
Plasticity	3L	5.53	7.681	5.12 - 5.57	8.1701
Plasticity	3L	14.99	7.402	13.39 - 15.32	7.8853
Plasticity	3R	23.4	7.712	23.38 - 24.34	8.2021

Chapter 3 - An Allele Frequency Cline in Cold Tolerance Associated Alleles in *Drosophila*

Introduction

The primary aims of evolutionary genetics are to identify traits that contribute to adaptive evolution, elucidate the genetic basis of adaptations, and ultimately determine how functional genetic variation evolves in nature (Orr, 2005; Hoekstra & Coyne, 2007). Climatic variation plays a critical role in driving patterns of adaptive evolution (Davis & Shaw, 2001), and one of the most important parameters is temperature. Temperature changes spatially and temporally and thus requires behavioral, physiological, and/or genetic responses to cope with this variation (Hoffmann & Parsons, 1991; Ayrihnac et al., 2004). Environmental temperatures are extremely heterogeneous, with tropical habitats infrequently cooling below 0°C, while temperate habitats routinely experience temperatures below freezing. These environmental gradients result in complex patterns of selection resulting in local adaptation (Endler, 1986; Kingsolver et al., 2001) and phenotypic clines (Endler, 1977).

The resistance to both hot and cold thermal environments is thought to be adaptive in *Drosophila* (Hoffmann et al., 2002). However, the ability to cope with cold temperatures has been repeatedly identified as a climatic adaptation among geographically distinct *D. melanogaster* populations (Ayrinhac et al., 2004; Hoffmann et al., 2002; Schmidt & Paaby, 2008). This has motivated a number of genetic/genomic studies to identify the loci responsible for variation in cold tolerance. These studies have used association mapping (Collinge et al., 2008; Clowers et al., 2010); QTL analyses [Morgan & Mackay, 2006; Norry et al., 2008); mutational analyses (Takeuchi et al., 2009)]; and gene expression studies (Goto, 2000; Qin et al., 2005; Sinclair et al., 2007). One gene that has been shown to influence natural variation in cold tolerance is *Senescence Marker Protein-30* [*Smp-30* (Clowers et al., 2010; Morgan & Mackay, 2006; Goto, 2000; Qin et al., 2005; Sinclair et al., 2007). *Smp-30* is a compelling candidate gene for cold tolerance as it has been shown to be responsive to cold stress (Goto, 2000; Qin et al., 2005; Sinclair et al., 2007). It has also been demonstrated to contain regulatory polymorphisms that are associated with variation in cold tolerance (Clowers et al., 2010). Finally, *Smp-30* is

involved in Ca^{2+} ion homeostasis (Fujita et al., 1998; Inoue et al., 1999; Son et al., 2008), which is significant because shifts in ion homeostasis can lead to changes in the electrochemical potential across membranes, which can lead to cold-induced immobilization or injury (Takeuchi et al., 2009; Kelty et al., 1996; Kostal et al., 2007).

In this paper we investigate the link between cold tolerance clines [Ayrinhac et al., 2004; Hoffmann et al., 2002; Schmidt & Paaby, 2008) and among population genetic variation in *Smp-30.* We specifically test if alleles associated with natural variation in cold tolerance (Clowers et al., 2010) exhibit clines among populations in the US (25.46°N to 44.046°N). We ask do allele frequencies align with their hypothesized function based on associations with variation in cold tolerance (Clowers et al., 2010)? That is, alleles associated with cold susceptibility should be at high frequency in southern populations and low frequency in northern populations, while alleles associated with cold resistance should exhibit the opposite pattern.

Materials and methods

Population samples

Nine populations were sampled from Florida (25.46°N) to Maine (44.046°N) as in Paaby *et al.* (2010). Flies were collected off fallen fruit at seven sites including Bowdoinham, ME (44.046°N latitude; *n*=26), Princeton, NJ (40.35°N latitude; *n*=45), Eutawville, SC (33.39°N latitude; *n*=42), Morven, GA (30.94°N latitude; *n*=45), Jasper, FL (30.54°N latitude; *n*=28), Ft. Pierce, FL (27.44°N latitude; *n*=30), and Homestead, FL (25.46°N latitude; *n*=36). Two additional sites were included. Raleigh, NC (35.77°N latitude; *n*=225) was the allele frequency data from 225 isogenic lines reported in Clowers et al. (2010), while the Mt. Sinai, NY (40.947°N latitude; *n*=36) population were a set of chromosome extraction lines as described in Schmidt et al. (2008). A total of 517 lines were sampled.

Genotyping of Smp-30

Each line was genotyped at a single insertion/deletion (indel) polymorphism (*D1-603In,D2*) that was previously shown to be associated with variation in cold tolerance in the Raleigh, NC farmer's market population (Clowers et al., 2010). This indel is located 603

basepairs upstream of the start of the coding sequence and has three alleles; a common insertion allele (*In*) that is associated with cold tolerance, a 9-bp deletion allele (*D1*) that is associated with cold susceptibility, and a 10-bp deletion allele (*D2*) that is also associated with susceptibility. The *D2* allele is rare (0.09) in the Raleigh population and is an independent deletion, occurring on a different haplotype background than *D1* (Clowers et al., 2010). Three different PCR primers, corresponding to the three different alleles (*In*, *D1*, and *D2*), were designed that anneal at the indel. Each primer was paired with a universal reverse primer, thus resulting in three PCR reactions per line. Using this technique we could accurately infer *Smp-30* genotype based on the presence/absence of product in the various PCRs. To ensure precision each line was genotyped twice and blindly scored. Genotyping was performed using 10 uL PCR reactions and the following primers combinations: *Smp30-D1F* (CATGGAGCCTGATAACAC) with *Smp30-R* (ACTCGAATAAGCCAGAGAG), *Smp30-D2* (CATCGAGCCTGAGCCTG) with *Smp30-R*, and *Smp-In* (CATGGAGCCACAACATTTC) with *Smp30-R*. PCR products were visualized and scored using 1% agarose gels.

Statistical Analysis

Allele frequencies were calculated by hand. We tested for allele clines using a simple linear regression of allele frequency versus latitude for each allele (*In*, *D1*, and *D2*). Regression analyses were performed in SAS v9.1 in PROC REG.

Results

We previously demonstrated this indel polymorphism (*D1-603In,D2*) is strongly associated with variation in cold tolerance (Clowers et al., 2010). The *D2* allele is associated with cold susceptibility and is common (52%) at extreme southern latitude (25.46°N), but is very rare (4%) at extreme northern latitudes in the United States (44.046°N). In contrast, the *In* allele that is associated with cold tolerance is rare (10%) in southern populations (25.46°N), but increases in frequency (48%) in northern populations (44.046°N). Finally, the *D1* allele that is also associated with cold susceptibility is common at all latitudes, ranging in frequency of 36.8% at extreme southern latitudes to 67.8% in Princeton, NJ (40.35°N).

There is a significant cline in frequency at the *Smp-30* indel alleles across latitude (Fig. 3.1; Table 3.1). The cline in the *D2* allele is highly significant ($b = -0.01852$, SE = 0.00579, *P* = 0.0151). The decrease in frequency with increasing latitude matches the expectation for a susceptibility allele (Clowers et al., 2010). The trend observed for *In* allele is not significant ($b = 0.01053$, SE = 0.00561, *P* = 0.1024), but the increase in frequency with increasing latitude matches the expectation for a tolerance allele (Clowers et al., 2010). Finally, the *D1* allele is the most common allele and the change in frequency across latitude is not significant ($b = 0.00860$, SE = 0.00492, *P* = 0.1241).

Discussion

Cold tolerance is a climatic adaptation in *Drosophila*, which exhibits phenotypic clines on multiple continents (Ayrinhac et al., 2004; Hoffman et al., 2002; Schmidt & Paaby, 2008). We have previously shown that natural variation in cold tolerance is significantly associated with regulatory variation in the cold tolerance gene, *Smp-30* (Clowers et al., 2010). Thus, an essential next step is to link cold tolerance clines with cold associated polymorphisms in *Smp-30* and test if the tolerance/susceptibility alleles exhibit clines across latitudes spanning thermal extremes. We use nine populations sampled along the east coast from southern Florida (25.46°N) to Maine (44.046°N).

We identified an adaptive regulatory polymorphism 603 basepairs upstream of the *Smp-30* coding sequence. The combined evidence of the presence of clines in allele frequency at the *D1-603In,D2* polymorphism, the highly significant negative cline for *D2* (cold-susceptible) allele, the positive relationship between the frequency of the *In* (cold-resistant) allele with latitude, and the previously reported association between variation at this (*D1-603In,D2*) polymorphism and variation in cold tolerance (Clowers et al., 2010), strongly implicate the regulatory variation in *Smp-30* with adaptation to thermal environments in North America. We hypothesize that the differences between the *In, D1*, and *D2* allele frequency clines suggest different molecular functions for each allele. Specifically, we expect the *D2* and *In* alleles in *Smp-30* will represent functional regulatory variation that will respond to thermal variation and significantly contribute to thermal adaptation among populations. In contrast, the lack of a cline

in the *D1* (cold- susceptible) allele may suggest a lack functional regulatory variation for *D1* individuals in nature. We are currently isolating the influence of each of these regulatory regions and testing the functional role of each allele by assessing their effects on whole-organism chill-coma recovery time as well as dissecting expression differences across ecologically relevant thermal environments.

Although we have shown a link between adaptive regulatory alleles and phenotypic clines in cold tolerance it is essential to note that the *D1-603In,D2* polymorphism is one of four polymorphisms in *Smp-30* associated with variation in cold tolerance in Raleigh, NC (Clowers et al., 2010). Three of these four cold-associated polymorphisms (*G-632A*, *A-630G*, *D1-603In,D2*) are within 30 basepairs and are in strong albeit not perfect linkage disequilibrium with one another (Clowers et al., 2010). Thus, it is impossible to assign causation to the *D1-603In,D2* polymorphism when there are two additional cold-associated polymorphisms in high LD with the indel polymorphism that is the focus of the current study. That said, the presence of a significant cline for this cold-associated indel polymorphism suggests that additional functional work is justified to isolate the individual effects of multiple polymorphisms upstream of *Smp-30.*

Figure 3.1

Allele frequency cline across latitude in *Smp-30*. The two rare variants *D2* and *In*, exhibit the expected shift in allele frequency across latitude based on their phenotypic associations. The cline in *D2* allele is highly significant ($b = -0.01852$, SE = 0.00579), while the shift in allele frequency at the *In* allele is not significant ($b = 0.01053$, SE = 0.00561). The *D1* allele is the most common allele, the change in frequency across latitude is not significant ($b = 0.00860$, SE = 0.00492) and the trend is opposite what is expected based on the phenotypic associations.

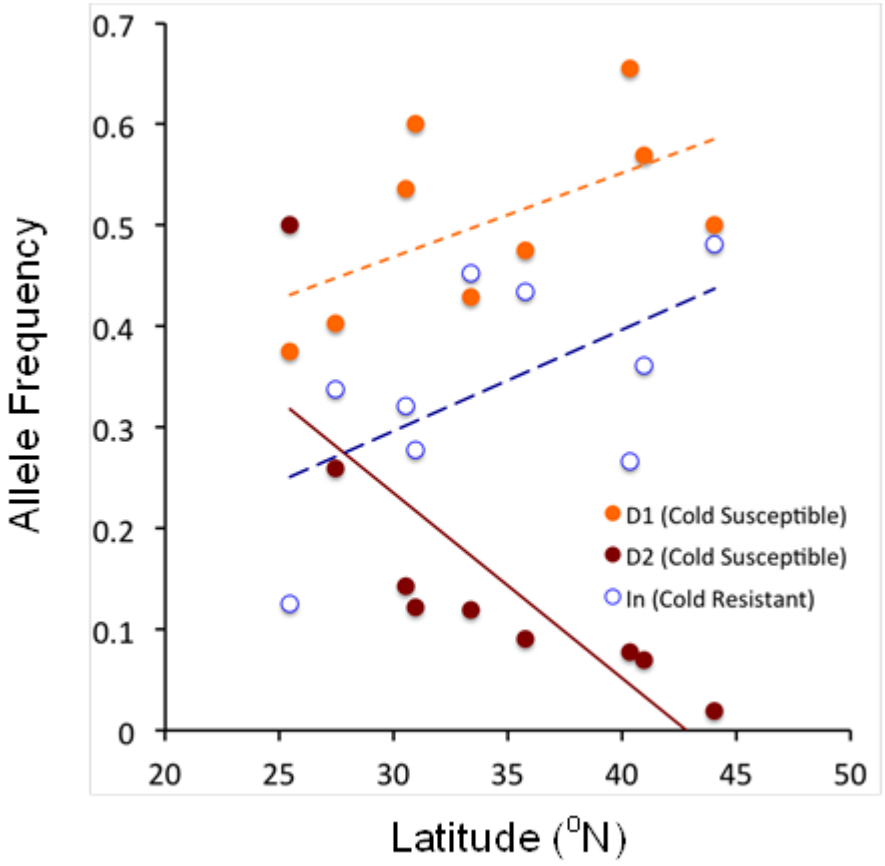

Table 3.1

Linear regression for *Smp-30* alleles analysis across latitude.

Allele	**df**	***F***	***R^2***	***P***
In-cold resistant	1,7	3.53	0.3352	0.1024
D1-cold susceptible	1,7	3.05	0.3036	0.1241
D2-cold susceptible	1,7	10.24	0.5940	**0.0151**

Chapter 4 - Conclusions

Temperature is one of the most important abiotic factors an organism experiences. Sustained temperatures outside of an organism's optimal range lead to decreased performance, and can ultimately lead to decreases in fitness. Temperatures changes with the seasons (temporally) and with altitude and latitude (spatially), which demands the appropriate organismal response to cope with changing, and often stressful, temperatures. For populations to deal with such stresses they must harbor sufficient genetic variation to respond over multiple generations (adaptation), the ability to shift their phenotypes within a generation (phenotypic plasticity), or some combination of both. Here, I described phenotypic and genetic variation in chill-coma recovery time and thermal plasticity, and identified an allele frequency cline in a cold tolerance gene in the model organism, *D. melanogaster*.

In the first data chapter (Chapter 2), I leverage the recent development of advanced intercrossed mapping populations that have increased the resolution with which quantitative traits can be mapped. To this end, I characterize the genetic architecture of thermotolerance phenotypes in *Drosophila* by phenotyping approximately 400 RILs from the *Drosophila* Synthetic Population Resource (DSPR), an advanced intercross mapping population. Each RIL was phenotyped for chill-coma recovery after egg-to-adult development at 18°C and 25°C, which allowed for the calculation of a third phenotype, thermal plasticity, the difference between chill-coma recovery at the two temperatures. I identified significant effects of genotype, environment, sex, and genotype-by-environment interactions for all traits measured; suggesting significant genetic variation is present for both chill-coma recovery and thermal plasticity. Through the detection of associations between genotype and phenotype via QTL mapping, I identified genomic regions contributing to chill-coma recovery time and thermal plasticity. Regions contributing to chill-coma recovery time were similar regardless of developmental temperature, and regions contributing to thermal plasticity also shared some overlap, but two QTL on the left arm of the third chromosome were specific to thermal plasticity.

The second data chapter (Chapter 3) was a narrowly focused project that investigated the contributions of polymorphisms in the regulatory region of a candidate cold tolerance gene, *Smp-*

30, to a known cold tolerance cline along the east coast of the United States. Flies were genotyped from along a latitudinal gradient on the eastern seaboard of the United States. Populations from higher latitudes, and consequently cooler temperatures, had high frequencies of the allele that conferred cold tolerance, and the opposite pattern held for another allele associated with cold susceptible flies. A third allele exhibited the opposite expected pattern, but this may simply imply that two of the alleles are involved in regulatory mechanisms, and the last allele is not. Further characterization of allele-specific constructs will be required to confirm these predictions. Regardless, these observations suggest adaptive regulatory mechanisms occur clinally in natural populations.

The research presented here has contributed to the growing knowledge of thermotolerance genetic and phenotypic variation, but more so, it has strived to address long-standing evolutionary questions regarding adaptation and the genetic basis of phenotypic plasticity. We demonstrated that several thermal plasticity QTL are independent of environment-specific QTL, a finding that could aid in answering questions regarding the evolution of phenotypic plasticity: do "plasticity" genes exist, or are environment-dependent genes responsible for plasticity? Also, the description of numerous RILs exhibiting maladaptive plasticity may illustrate just how complex thermotolerance phenotypes are, as the recombination experienced in this mapping population altered genetic networks, leading to unexpected phenotypes. Alternatively, flies exhibiting these maladaptive responses may have harbored genetic material from equatorial parental populations, which seldom experience freezing temperatures and would not be selected for cold tolerance. Overall, this suggests multiple genes of modest to small effect are most likely responsible for complex traits involved in adaptive processes. I have also contributed to the list of possible candidate cold tolerance and thermal plasticity genes, replicated QTL for cold tolerance, and validated the mapping power of the DSPR.

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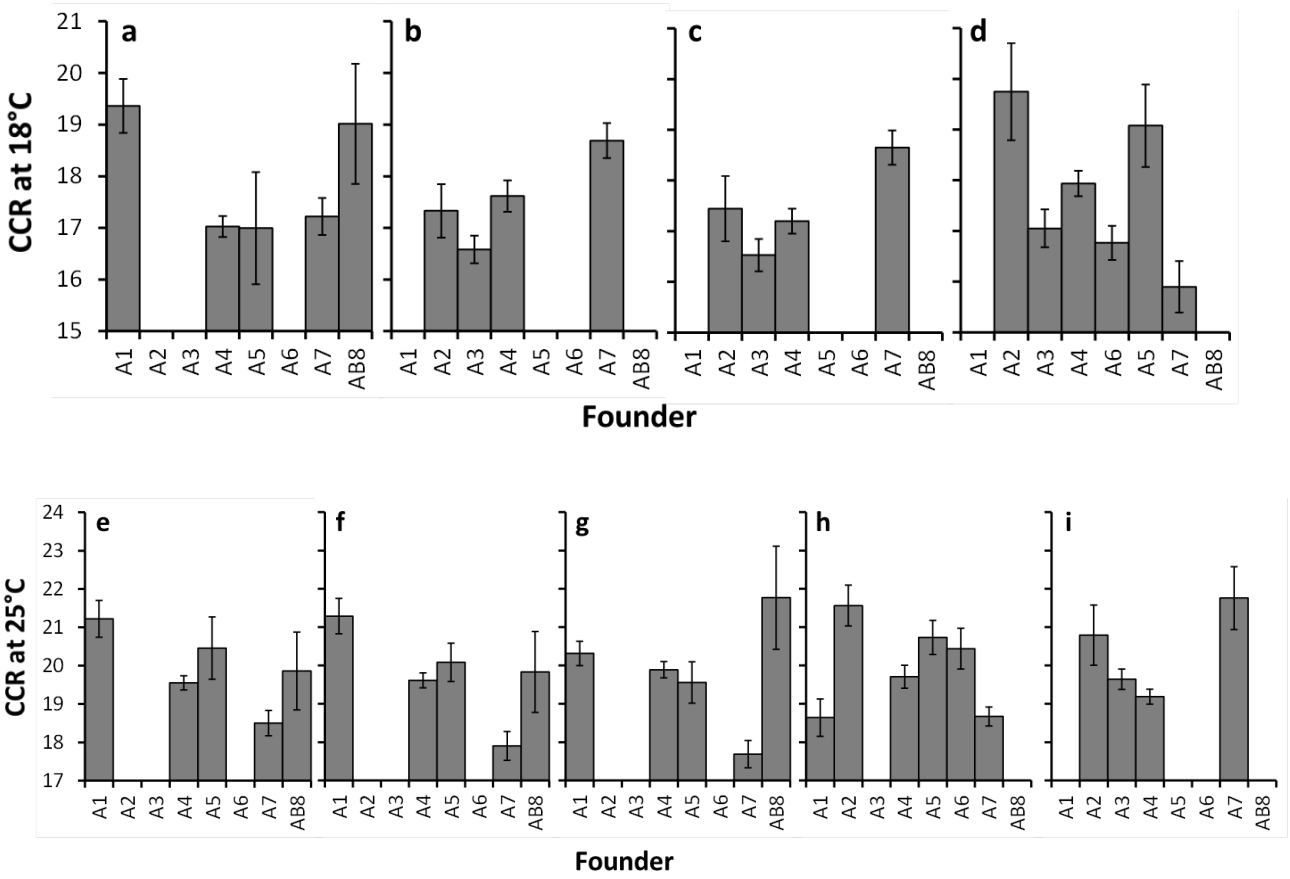
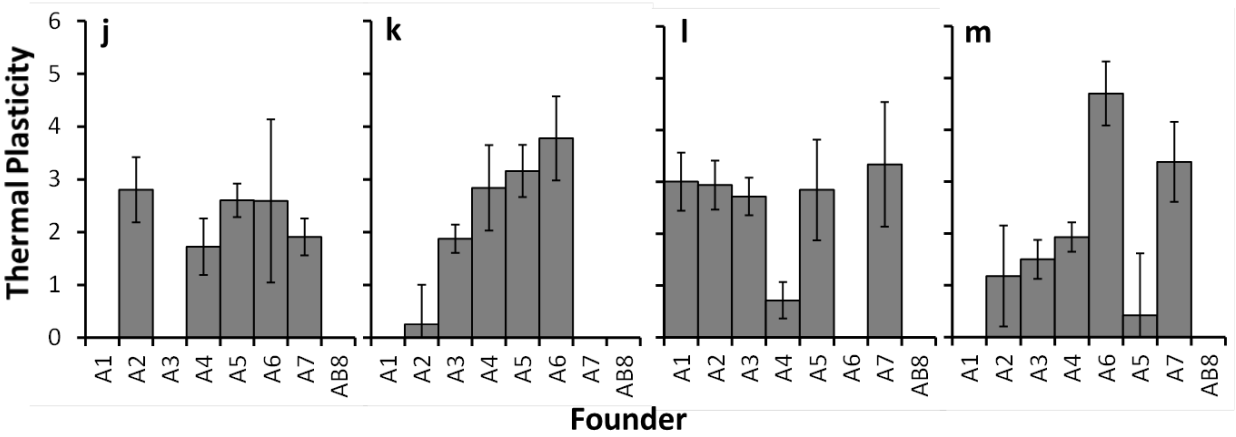
Appendix A - Additional QTL Information

Figures and Tables

Figure A.1 Founder haplotype means for all thermotolerance QTL identified (sexes pooled). Founder haplotype means for chill-coma recovery time at 18°C QTL on (a) 2R: 1.91 Mb (the right arm of the second chromosome at the physical location 1.91 Mb), (b) 3R: 4.14 Mb, (c) 3R: 5.35 Mb, and (d) 3R: 22.91 Mb. Founder haplotype means for chill-coma recovery time at 25°C QTL on (e) 2L: 19.52 Mb, (f) 2R: 2.83 Mb, (g) 2R: 4.47 Mb, (h) 2R: 6.61 Mb, and (i) 3R: 7.47 Mb. Founder haplotype means for thermal plasticity QTL on (j) 2R: 8.01 Mb, (k) 3L: 5.53 Mb, (l) 3L: 14.99 Mb, and (m) 3R: 23.4 Mb. The x-axis is the founding line of the pA population in all instances, and the y-axis is either the respective chill-coma recovery time or thermal plasticity.




Thermal Plasticity
0
1
2
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A1
A2
A3
A4
A5
A6
A7
AB8
Founder

Table A.1 Thermotolerance QTL for Females.

Treatment	Chr	Position (Mb)	LOD score	CI (Mb)	% Var
18° C	2R	1.93	7.325	1.84-1.98	7.380
18° C	3R	4.50	8.685	4.06-4.84	8.689
25° C	2L	5.03	7.360	4.80-5.25	7.398
25° C	2L	9.46	6.824	9.43-9.49	6.878
25° C	2R	7.09	8.203	6.33-7.29	8.210
Plasticity	2R	8.01	7.423	8.00-8.41	7.710
Plasticity	3R	23.39	7.230	23.36-23.46	7.519

Table A.2 Thermotolerance QTL for Males.

Treatment	Chr	Position (Mb)	LOD score	CI (Mb)	% Var
18° C	2L	20.75	6.965	20.50(2L) - 1.63(2R)	7.045
18° C	3L	4.94	7.240	4.90-4.98	7.313
18° C	3R	22.90	7.745	22.86-23.21	7.803
18° C	3R	23.36	7.342	23.30-23.53	7.412
25° C	2R	1.35	9.751	22.55(2L) - 1.93(2R)	9.851
25° C	2R	2.87	9.037	2.50-3.04	9.164
25° C	2R	4.47	8.873	4.21-5.35	9.005
25° C	2R	6.55	7.012	6.27-6.95	7.186
25° C	3L	13.01	7.284	12.99-13.06	7.454
Plasticity	2L	18.73	7.426	18.19-19.53	7.820
Plasticity	2R	19.10	7.414	18.40-19.40	7.807
Plasticity	3L	4.94	7.645	4.91-4.98	8.041