

THE EVOLUTION AND GENETICS OF THERMAL TRAITS IN *DROSOPHILA*
MELANOGASTER

by

LINDSEY CAROLINE FALLIS

B.S., Texas Christian University, 2005
M.S., Texas Christian University, 2007

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

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Abstract

Temperature is a critical environmental parameter and thermal variation has significant effects on local adaptation and species distributions in nature. This is especially true for organisms that are isothermal with their environment. Variation in temperature imposes stress and directly influences physiology, behavior, and fitness. Thus, to thrive across a range of thermal environments populations must contain sufficient genetic variation, the capacity to respond plastically, or some combination of both genetic and plastic responses. In this work I first quantified patterns of phenotypic and genetic variation in nature and then dissected the genetic basis of variation in thermal traits. In the first aim I used natural populations of *Drosophila melanogaster* collected from a latitudinal transect in Argentina to investigate variation in heat stress resistance and cold plasticity within and among populations. I found heat stress resistance was highly variable within populations, but was strongly associated with the monthly maximum average temperature of each site. For cold plasticity I was able to demonstrate significant variation in plasticity within and among populations, however the among population variation was best explained by the altitude of each site. I hypothesized that this was caused by a difference in temperature fluctuations at high altitude sites relative to low altitude sites. To evaluate this hypothesis I paired our study with existing laboratory data that demonstrated significant fitness differences between high and low plasticity (and altitude) sites when these populations were reared in variable thermal environments. Thus, cold plasticity is an adaptive response to environmental variation. The final project focused on understanding the genetic basis of thermal variation. I fine-mapped a single co-localized heat and cold tolerance QTL via deficiency and mutant complementation mapping to identify four novel thermal candidate genes. There was no overlap of the deficiencies or genes associated with cold or heat stress resistance. Sequence analysis of each gene identified the polymorphisms that differentiate the lines. To test for independent associations between these polymorphisms and variation in nature the *Drosophila* Genome Reference Panel was used to confirm associations between allelic variation and cold tolerance in nature.

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Approved by:

Major Professor
Dr. Theodore Morgan

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Abstract

Temperature is a critical environmental parameter and thermal variation has significant effects on local adaptation and species distributions in nature. This is especially true for organisms that are isothermal with their environment. Variation in temperature imposes stress and directly influences physiology, behavior, and fitness. Thus, to thrive across a range of thermal environments populations must contain sufficient genetic variation, the capacity to respond plastically, or some combination of both genetic and plastic responses. In this work I first quantified patterns of phenotypic and genetic variation in nature and then dissected the genetic basis of variation in thermal traits. In the first aim I used natural populations of *Drosophila melanogaster* collected from a latitudinal transect in Argentina to investigate variation in heat stress resistance and cold plasticity within and among populations. I found heat stress resistance was highly variable within populations, but was strongly associated with the monthly maximum average temperature of each site. For cold plasticity I was able to demonstrate significant variation in plasticity within and among populations, however the among population variation was best explained by the altitude of each site. I hypothesized that this was caused by a difference in temperature fluctuations at high altitude sites relative to low altitude sites. To evaluate this hypothesis I paired our study with existing laboratory data that demonstrated significant fitness differences between high and low plasticity (and altitude) sites when these populations were reared in variable thermal environments. Thus, cold plasticity is an adaptive response to environmental variation. The final project focused on understanding the genetic basis of thermal variation. I fine-mapped a single co-localized heat and cold tolerance QTL via deficiency and mutant complementation mapping to identify four novel thermal candidate genes. There was no overlap of the deficiencies or genes associated with cold or heat stress resistance. Sequence analysis of each gene identified the polymorphisms that differentiate the lines. To test for independent associations between these polymorphisms and variation in nature the *Drosophila* Genome Reference Panel was used to confirm associations between allelic variation and cold tolerance in nature.

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Dedication

I dedicate this work to my mother for facilitating my initial interest in biology, always believing in me, and showing unwavering support throughout my education.

Chapter 1 - Thermal Adaptation in *Drosophila*

Introduction

Identifying the genes and physiological processes that underlie variation in ecologically important traits is a critical question in biology. Many organisms exhibit local adaptation in nature (Hoekstra et al., 2001, Hoffmann et al., 2001, Jenkins & Hoffmann, 1994, Winterhalter & Mousseau, 2007), an observation that has motivated evolutionary and population genetic studies for a century. Evolutionary biologists have long been interested in adaptive patterns of phenotypic divergence among populations (Mitchell-Olds et al., 2007, Zhen & Ungerer, 2008, Wittkopp et al., 2011, Kirkpatrick & Barton, 2006, Dudley, 1996, McKechnie et al., 2010), however the genes and the causal allelic variants have largely escaped description [for a few exceptions, see (Hoekstra & Coyne, 2007)].

Temperature is an environmental parameter that changes on a daily, seasonal, and spatial scale (Gibbs et al., 2003) and is a constant form of stress with which all individuals must cope. Insects are particularly vulnerable to variable temperature, as they are isothermal with their environment. Variation in temperature is one of the most important environmental factors for insects, as it drives species distributions (Clarke, 1996), influences fitness (Umina et al., 2005, Rashkovetsky et al., 2006), affects behavior (Gilchrist & Partridge, 1999), as well as whole-organism physiological performance (Denny & Helmuth, 2009). Differences in temperature across a geographical range generally follow the pattern where colder climates are associated with temperate latitudes or high altitude and warmer climates at tropical latitudes or low altitude. Such environmental gradients have led to the formation of clines in many species (Cheviron et

al., 2008, Trussell, 2000, Zhen & Ungerer, 2008), including the repeated evolution of thermal clines in insects (Gilchrist & Partridge, 1999, Hoffmann et al., 2002, Dahlhoff et al., 2008). Phenotypic clines are strong evidence for the role of selection and are usually the result of local adaptation (Duvernell et al., 2003, Endler, 1977). Research to understand temperature tolerance clines and the underlying allelic variation will expand our knowledge on how insects have adapted to their local environment and will allow predictions to be made involving insect distributions in response to a rapidly changing climate.

A powerful insect system for studying thermal adaptation is *Drosophila melanogaster*. *D. melanogaster* is a cosmopolitan species that has been extremely successful in adapting to a wide range of thermal environments in nature. This insect is found globally including both tropical and temperate regions (Ayrinhac et al., 2004, David & Capy, 1988). *Drosophila*'s success in colonizing such diverse areas can be attributed to its ability to adapt to diverse environmental conditions across generations and respond plastically to environmental variation within a generation, thus making it an interesting species to study the genetic and environmental influences on phenotypic adaptation. Furthermore, experimental lines can be and have been created from natural populations. *Drosophila* is easily bred in the lab to achieve very high sample sizes, thus making ecological and evolutionary genomic questions attainable in a laboratory setting. In addition to exhibiting abundant and interesting genetic variation in nature, *D. melanogaster* has one of the best molecular toolboxes of any model system (Drysdale & FlyBase, 2008). This presents opportunity for the mechanistic dissection of ecologically important phenotypic variation and to begin constructing predictive models of evolutionary processes via an understanding of the causal genes underlying such phenotypic variation.

Cold and heat tolerance phenotypes exhibit phenotypic clines among natural populations of *Drosophila* (Hoffmann et al., 2002, Ayirinhac et al., 2004, Karan & David, 2000, Gibert et al., 2001, Gibert & Huey, 2001, Fallis et al., 2012) and have been shown to exhibit clines that oppose each other with tolerance to heat increasing as you move toward tropical climates and tolerance to cold decreasing (Hoffmann et al., 2002). Although thermotolerance appears to represent an important adaptation for *Drosophila* species, the genetic mechanisms that mediate this response are largely unknown. Our knowledge about the genetic control of cold tolerance comes from lab artificial selection studies (Tucic 1979; Chen and Walker 1993; Anderson et al. 2005); quantifying genetic variation along cold stress clines in the field (Gibert and Huey 2001; Hoffmann et al. 2002; Ayirinhac et al. 2004; Kimura 2004; Collinge et al. 2006); and whole genome scans, such as quantitative trait loci (QTL) mapping (Morgan and Mackay, 2006; Norry et al., 2004; Norry et al., 2008). These studies have associated temperature with several candidate genes including *hsp68*, *Starvin*, *Smp-30*, *Frost*, *HSP70*, *denaturase 2*, and *CG16700* (Goto, 2000, Goto, 2001, Daibo et al., 2001, Anderson et al., 2003, Greenberg et al., 2003, Qin et al., 2005, Clowers et al., 2010, Svetec et al., 2011, Rako et al., 2007, Colinet et al., 2010, McColl et al., 1996, Colinet & Hoffmann, 2010). Anderson et al. (2003) demonstrated *Hsr-omega* exhibits an allele frequency cline, where one form of the allele gives increased cold tolerance while the other form aids in heat tolerance (pleiotropy). While others have found independent genes responding to cold or heat stress (Goto, 2000, Goto, 2001, Daibo et al., 2001, Greenberg et al., 2003, Qin et al., 2005, Clowers et al., 2010, Svetec et al., 2011, Colinet et al., 2010, McColl et al., 1996, Colinet & Hoffmann, 2010). Taken together these data demonstrate that thermotolerance phenotypes are very complex, with many genes and many interactions between genes.

While these studies have been helpful in identifying evidence for local adaptation and selecting candidate genes, still little is known about the genetics of *thermal variation* in nature. In the following chapters, I quantify patterns of phenotypic and genetic variation for heat and cold stress traits in nature, contribute to the dissection of the genetic basis of thermal phenotypes, identify genes involved in the control of natural thermal variation and link molecular variation to natural variation in cold tolerance. Together, all of these studies have been instrumental in the advancement of our understanding of the evolutionary and genetic mechanisms that mediate whole organism thermal variation in nature.

Chapter 2 - † Genetic Variation in Heat-Stress Tolerance Among South American *Drosophila* Populations

Abstract

Spatial or temporal differences in environmental variables, such as temperature, are ubiquitous in nature and impose stress on organisms. This is especially true for organisms that are isothermal with the environment, such as insects. Understanding the means by which insects respond to temperature and how they will react to novel changes in environmental temperature is important for understanding the adaptive capacity of populations and to predict future trajectories of evolutionary change. The organismal response to heat has been identified as an important environmental variable for insects that can dramatically influence life history characters and geographic range. In the current study we surveyed the amount of variation in heat tolerance among *D. melanogaster* populations collected at diverse sites along a latitudinal gradient in Argentina (24° to 38° S). This is the first study to quantify heat tolerance in South American populations and our work demonstrates that most of the populations surveyed have abundant within-population phenotypic variation, while still exhibiting significant variation among populations. The one exception was the most heat tolerant population that comes from a climate exhibiting the warmest annual mean temperature. All together our results suggest there is abundant genetic variation for heat-tolerance phenotypes within and among natural populations of *Drosophila* and this variation has likely been shaped by environmental temperature.

† Reprinted with permission from “Genetic Variation in Heat-Stress Tolerance Among South American *Drosophila* Populations” by Fallis, L. C., Fanara, J. J., Morgan, T. J., 2012, Springer, New York. 2012

Introduction

Nearly all organisms live in heterogeneous environments, which vary in biotic and abiotic factors on both spatial and temporal scales. One environmental factor that dramatically influences phenotypic evolution is the whole-organism response to temperature (Reusch & Wood, 2007, Umina et al., 2005, Rashkovetsky et al., 2006, Zhen & Ungerer, 2008).

Temperature is a critical environmental parameter and thermal variation has significant effects on local adaptation (Anderson et al., 2003) and can limit species distributions (Clarke, 1996) in nature. This is especially true for organisms that are isothermal with their environment, such as insects. Variation in temperature (Coussins & Bowler, 1987, Leather et al., 1993, Clarke, 1996) imposes stress and directly influences physiology, behavior, and fitness (Hoffmann & Parsons, 1991, Gilchrist & Huey, 1999, Gibert et al., 2001, David et al., 2003, Hoffmann et al., 2003b, Rohmer et al., 2004). Thus, for species to thrive across a range of thermal environments populations must contain either sufficient genetic variation to allow phenotypic adaptation across generations, the capacity to respond plastically to environmental variation, or some combination of both genetic and plastic responses (Hoffmann & Parsons, 1991, Ayrinhac et al., 2004, Hoffmann et al., 2005, Hoffmann & Willi, 2008).

A comprehensive understanding of genetic variation that underlies differences in thermotolerance phenotypes is critically important in light of a rapidly changing global climate. The future climate is projected to have higher global average temperatures, but also an uneven distribution of temperature changes and a greater frequency of extreme thermal events (IPCC 2007). Thus, organisms will have to cope with an increased probability of extreme weather

events including novel high temperatures across seasons (Jentsch et al., 2007). The ability of animal populations to survive these thermal shifts in the long term rests on how much genetic variation they currently harbor (Hoffmann & Willi, 2008, Potvin & Tousignant, 1996).

Quantifying how genetic variation partitioned itself within and among populations in response to natural temperature gradients will help predict the evolutionary responses to a changing global climate.

D. melanogaster is a cosmopolitan species that has been extremely successful in adapting to a wide range of thermal environments in nature (Ayrinhac et al., 2004, David & Capy, 1988). *Drosophila* has been widely used in studies of thermotolerance (Davidson, 1990, Hoffmann et al., 2002) revealing in some cases patterns of thermal variation consistent with clinal variation (Davidson 1990; Karan and David 2000; Gibert and Huey 2001; Gibert et al. 2001; Hoffmann et al. 2002; Ayrinhac et al. 2004; Rashkovetsky et al. 2006) and in other cases simply population differentiation (Parsons 1977; Stanley and Parsons 1981; Hoffmann and Watson 1993; Bublly et al. 2002; Hoffmann et al. 2005; Rako et al. 2007). Each of these studies has documented clinal and/or population variation and attempted to link this variation with changes in environmental parameters, which co-vary with latitude. Together these studies demonstrate how natural variation has been shaped by adaptation to local environments. Although these studies provide a compelling description of thermal variation, all of these studies have been performed on North American, European, Australian or African populations. To date no study has quantified thermal variation among populations in South America.

In this study we quantified phenotypic variation in heat-tolerance phenotypes within and among six natural populations of *D. melanogaster* sampled from an environmental gradient in Argentina (24° to 38° S). We used an isofemale line approach that allows us to accurately estimate the standing variation within and among populations (David et al. 2005). We find that most of the populations surveyed have abundant within-population phenotypic variation, while still exhibiting significant variation among populations.

Materials and Methods

Drosophila Stocks

Gravid females were collected from six populations in central Argentina described previously (Lavagnino et al. 2008; Folguera et al. 2008). Flies were collected by net sweeping over fermented banana baits at six locations along a north to south latitudinal gradient ranging from approximately 24° to 38° south latitude in Argentina (Figure 2.1). Populations were named for the nearby city or the providence where the sampling location was positioned (i.e., Guemes, Jachal, Chilecito, Lavalle, Uspallata, and Neuquén). Geographical locations, latitude, longitude, altitude and climatological data (<http://www.smn.gov.ar/>) for each population are presented in Table 2.1. Ten isofemale lines were created from single wild-caught females from each population and inbred via full-sib mating for 10 generations. All lines have been maintained in the laboratory since February 2004 on standard cornmeal-agar-molasses medium sprinkled with live yeast to stimulate oviposition. Flies were maintained from egg to adult at 25°C and on a light/dark cycle of 12 hours.

Heat Survivorship Profiles

Heat tolerance profiles were measured for each line within each population using a percent survival after heat-stress assay (Morgan & Mackay, 2006). Heat tolerance was measured on mated, adult flies (5-to-7 day old). Flies were anesthetized using light CO₂ and were sorted in single-sex groups of 20 individuals in standard vials containing 5 ml of cornmeal-agar-molasses medium. The experimental assay was performed at least 24 h later to allow flies to recover from the effect of CO₂. On the day of the heat stress exposure, flies from each replicate vial were transferred without anesthesia into vials without food and placed at 38° C (±0.5° C) for 60, 90, 120, 150, 180, and 210 minutes. After heat-stress exposure, flies were immediately transferred to fresh vials containing 5 ml of standard cornmeal-agar-molasses medium and returned to 25° C and 60% humidity for 24 h. After 24 h, the percentage of surviving flies per vial was recorded for each sex, line, and exposure time, generating a heat tolerance profile for each line and sex (Figure 2.2). A fly was considered a survivor if it could move when the vial was gently tapped. Four replicate assays were performed per line, sex, and exposure time resulting in a slightly unbalanced design consisting of 48,000 flies in total.

Statistical Analysis

Analysis of Phenotypic Variation

We used a series of mixed model analyses of variance (ANOVA) to determine the sources of variation in heat tolerance within and among populations. The initial full model was:

$$y = \mu + P + L(P) + S + T + S \times P + S \times L(P) + T \times P + T \times L(P) + S \times T \times P + S \times T \times L(P) + \epsilon .$$

Where y is exposure-time-, population-, line-, and sex-specific heat survivorship percentages, μ is the overall mean, while P , S , and T are the fixed effects of population, sex and heat exposure

time, respectively. $L(P)$ is the random effects of line nested within population and vial nested within the population, line, sex and exposure time, and ε is error. In this study, the terms of primary interest are population, line nested within population, and the interaction of these terms with sex and exposure time as these terms test for local phenotypic differentiation among populations, significant genetic variation within populations, and sex or treatment specific effects of populations or lines nested within populations. To further dissect the population and line-nested-within-population terms, reduced models were used, which separated the data by population and/or exposure time. The reduced analyses separated by population tested for significant genetic differences among lines within each population (i.e. significant within population genetic variation). While the reduced analyses separated by exposure time simplified the analyses, the significant differences among populations were generally consistent across exposure times (Figure 2.2; Table 2.2). For the reduced analyses at a single exposure, we used the survival data from the 180-minute exposure time. We used this time point because the mean survival across all six populations is closest to 50%, thus giving us maximum power to detect variation on the percent survivorship scale. ANOVAs and variance component calculations were performed using the PROC GLM implemented in SAS 9.2 (SAS Institute 2009). Population specific broad-sense heritabilities (H^2) were calculated as in Morgan and Mackay (2006) where $H^2 = \sigma_G^2 / \sigma_P^2$, where $\sigma_G^2 = \sigma_L^2 + \sigma_{L \times S}^2$ and $\sigma_P^2 = \sigma_L^2 + \sigma_{L \times S}^2 + \sigma_\varepsilon^2$.

Associations between Environmental and Heat Survival Variation

To test if variation in environmental or geographic factors is associated with variation in survival after heat stress, we used a stepwise forward-backward selection model implemented in PROC REG in SAS 9.2 (SAS Institute 2009). This approach tests for associations between the

line-specific mean survivorships and the geographic and/or climatological data, by evaluating the significance of each geographic or climatological factor (Table 2.1) on survival after heat stress.

Results

Phenotypic Variation within and among Populations

We observed significant variation among populations (Figure 2.2; $F_{5,56} = 2.99$, $P = 0.0184$) and among lines within populations (Figure 2.3; $F_{53,77} = 3.74$, $P < 0.0001$) across all exposure times. The largest amount of variation in survival after heat stress among populations was observed at the 180-minute exposure time. At this time point populations were the most divergent in their ability to survive heat exposure, with highly significant differences among populations ($F_{5,54} = 3.70$, $P = 0.0060$) and differences among lines within populations ($F_{53,53} = 4.01$, $P < 0.0001$). This significant population effect was driven primarily by the Chilecito population which had an elevated mean survival after heat stress score ($68.67\% \pm 2.98\%$) relative to the five other populations that were not significantly different from one another (Figure 2.2).

In addition to significant phenotypic differentiation in heat survival among populations, there was also significant genetic variation among replicate lines within each population (Figure 2.3) for five of the six populations assayed in the study (Table 2.2). Population specific broad-sense heritabilities ranged from 0.150 for Chilecito to 0.567 for Lavalley (Table 2.2). The single population that did not have significant variation among lines ($F_{9,78} = 1.79$, $P = 0.0838$) and had the smallest broad sense heritability was Chilecito. Chilecito was also responsible for the significant population effect in the global analysis across all populations (Figure 2.2, 2.4).

Associations between Environmental and Heat Survival Variation

A single environmental factor, the maximum monthly average high temperature in each population, was positively associated with variation in survival after heat stress among the six populations (Figure 2.4; $\beta = 4.064$; $P = 0.0046$). This significant association was driven primarily by the Chilecito population, which had both an extreme monthly average high temperature (31.6° C) and high average survival after heat stress (68.69% \pm 2.98%).

Discussion

In this paper we quantified genetic variation in heat tolerance within and among six populations of *D. melanogaster* collected along a latitudinal transect in Argentina. The goal of this study was to quantify the standing levels of genetic variation and thus the general ability of populations to adaptively respond to changes in their climate. We found highly significant variation in mean heat tolerance levels within population and significant variation among populations. The majority of populations exhibited significant variation among lines within each population, suggesting that although five of the six populations are not significantly different at the level of the mean survival after heat stress, each population still contains significant genetic variation for heat tolerance. We identified a predicted association between maximum monthly average high temperature and the level of heat tolerance, where populations that encounter the warmest temperatures have the greatest amount of heat tolerance (Figure 2.4). That said the Chilecito population drives this association. The combination of reduced variation within Chilecito and significant variation between Chilecito and the other populations provided a compelling pattern that may suggest a response to the extreme monthly average high temperature at this site.

Most surveyed populations of *Drosophila* have variation both within and between them for many traits. Documentation of clinal variation has previously been shown in body size (Gilchrist & Partridge, 1999), egg size (Azevedo et al., 1996), cold tolerance (Ayrinhac et al., 2004, Hoffmann et al., 2002, Gibert & Huey, 2001, Gibert et al., 2001, Karan & David, 2000), and heat tolerance (Rashkovetsky et al., 2006). Populations from many different locations and from several *Drosophila* species have been surveyed and in general, populations often harbor high levels of phenotypic and genetic variation in heat tolerance phenotypes with heritabilities ranging from 0.03-0.5 (Loeschcke & Krebs, 1996, Loeschcke et al., 1997, Jenkins & Hoffmann, 1994). In this study, our heritability estimates (Table 2.2) were all at the high end of this range (0.150 in Chilecito to 0.567 in Lavalle). These estimates should be treated with some caution because each population contains a maximum of ten lines, which is a small sample size for precise estimates of quantitative genetic parameters. As expected the broad-sense heritability estimates are high in five of the six populations due to the large amounts of within population (among line) phenotypic variation (Table 2.2).

The lack of phenotypic variation in population Chilecito is curious and can be explained by several possibilities. These include, a recent bottleneck event has reduced variation, the effective population size of Chilecito is small, thus allowing drift to eliminate allelic variation, or genetic variation could have been reduced during adaptation to the environment. The latter explanation may be most reasonable because *Drosophila* populations are continuous over the gradient sampled. In addition, a recent study has found reduced measures of narrow-sense heritability for heat shock in tropical Australian *Drosophila* species (Mitchell & Hoffmann,

2010), most likely due to previous local adaptation to constant, high thermal environments. Low narrow-sense heritability is often used as a measure of potential evolvability or potential future local adaptation. Our data suggest, a similar occurrence in the Chilecito population where local adaptation may have reduced genetic variation within this particular population and may limit response to future thermal changes.

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Figures and Tables

Figure 2.1 Sample locations.

Geographic locations of the Argentinean populations used in this study: A = Guemes, B = Chilecito, C = Jachal, D = Uspallata, E = Lavalle, and F = Neuquén.



Figure 2.2 Population-specific heat survival curves for the six natural populations.

The x-axis denotes the length of the heat exposure (0 to 210) in minutes, while the y-axis is the mean percent survival of each population averaged across lines and sexes. Error bars denote plus or minus one standard error.

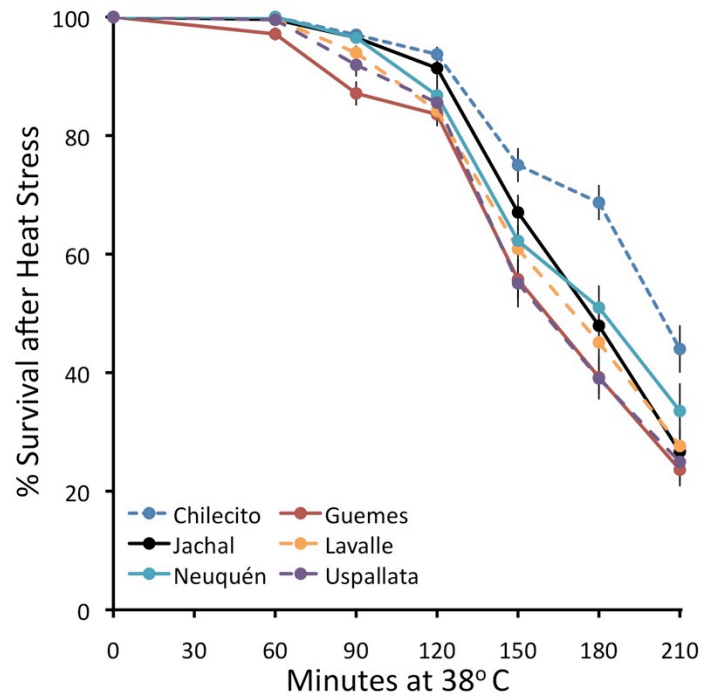


Figure 2.3 Line-specific heat survival curves for the six natural populations.

Each subpanel (A - F) is for each of the six populations: A = Guemes, B = Chilecito, C = Jachal, D = Uspallata, E = Lavalle, and F = Neuquén. The x-axis denotes the length of the heat exposure (0 to 210) in minutes, while the y-axis is the mean percent survival of each population averaged across lines and sexes. *F* statistics and *P* values are for population specific analyses testing for significant variation among lines at the 180-minute exposure time.

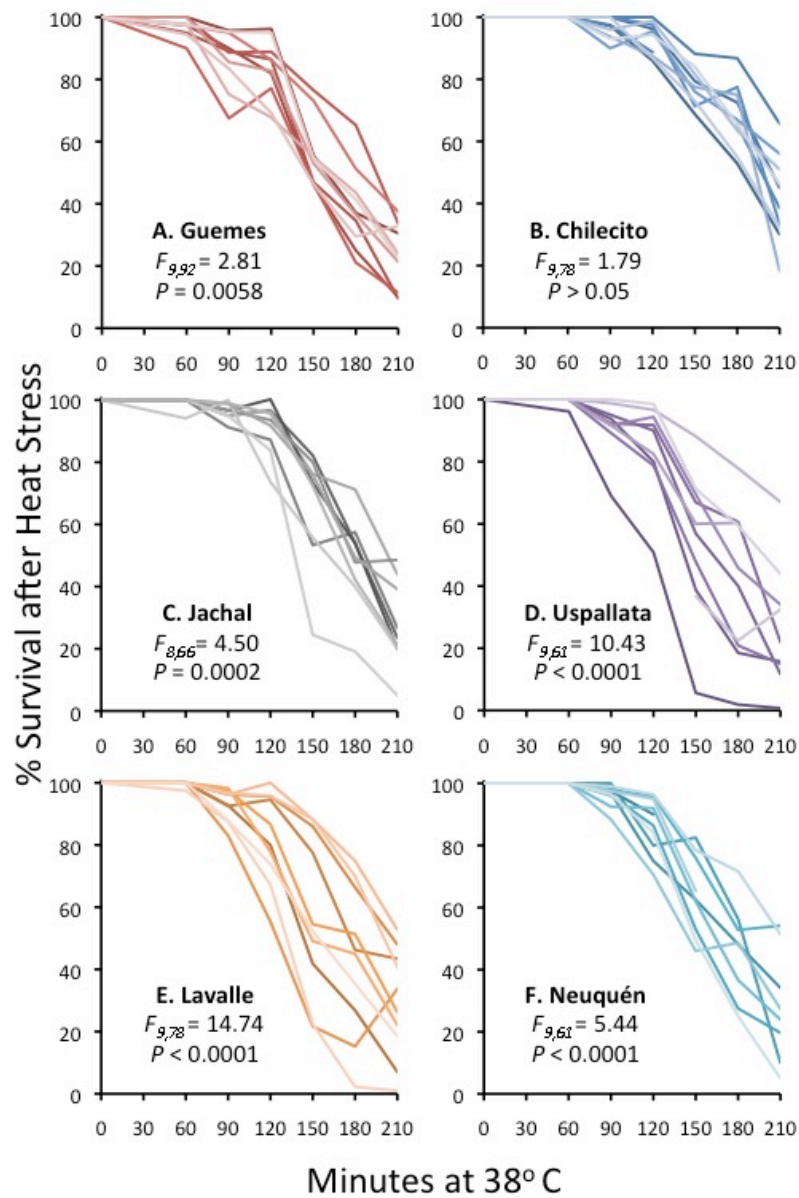


Figure 2.4 Association between maximum monthly average high temperature and mean percent survival after heat stress.

The x-axis is the maximum monthly average high temperature for each of the six populations in degrees Centigrade. While the y-axis is the percent survival after 180-minute exposure to 38° C ($\pm 0.5^\circ$ C). The small open circles are line means and the large closed circles are population means.

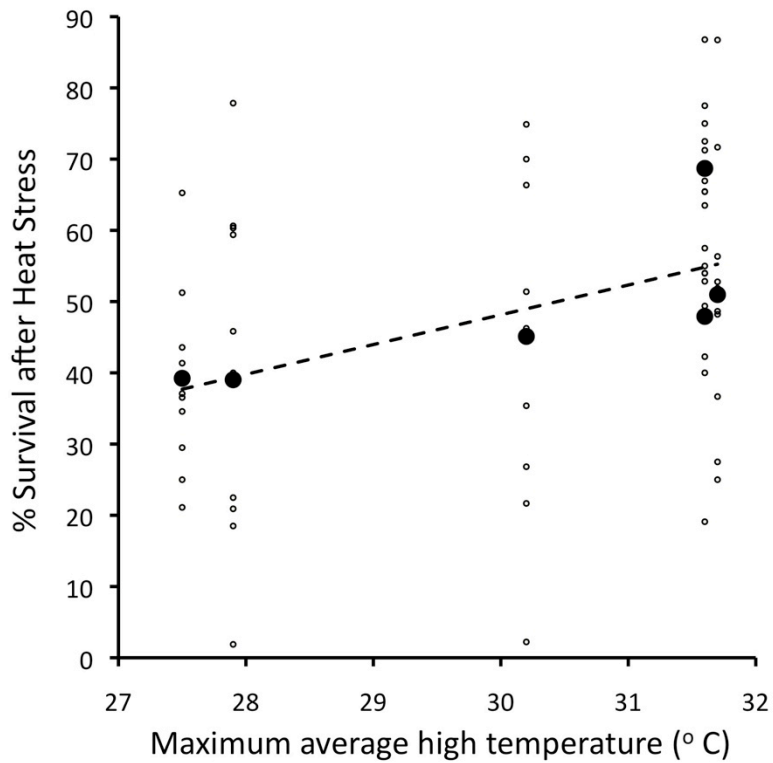


Table 2.1 Collection sites and selected climatological data for the six populations of *Drosophila melanogaster* in Argentina (<http://www.smn.gov.ar/>)

‘Maximum/minimum monthly high/low mean’ refers to an average highest/lowest temperature across all months.

Population	Latitude	Altitude (m)	Temperature (°C)			Mean rainfall (mm)	Mean humidity (%)	Isofemale lines (n)
			Mean annual	Max. monthly high mean	Min. monthly low mean			
Guemes	24°41’S	695	16.58	27.5	3.4	69.73	73.83	10
Chilecito	29°10’S	1043	17.25	31.6	2.1	15.75	59.66	10
Jachal	30°12’S	1238	16.45	31.6	0.9	11.84	54.25	9
Uspallata	32°35’S	1915	11.61	27.9	-3.7	12.75	51.45	10
Lavalle	32°50’S	647	15.93	30.2	3.2	22.53	58.75	10
Neuquén	38°57’S	260	14.74	31.7	-0.1	15.23	52.08	10

Table 2.2 Percentage of the total phenotypic variance within populations explained by among line differences, sex-specific line differences, and residual error.

Broad-sense heritabilities (H^2) were calculated as $H^2 = \frac{\sigma_G^2}{\sigma_P^2}$, where $\sigma_G^2 = \sigma_L^2 + \sigma_{L \times S}^2$ and $\sigma_P^2 = \sigma_L^2 + \sigma_{L \times S}^2 + \sigma_\epsilon^2$.

	Population:					
	Guemes	Chilecito	Jachal	Lavalle	Uspallata	Neuquén
Line	8.92*	14.97 ^{NS}	27.38***	56.72****	49.28****	24.28****
Line x Sex	10.80 ^{NS}	0.00 ^{NS}	11.27 ^{NS}	0.00 ^{NS}	0.34 ^{NS}	25.42 ^{NS}
Error	80.28	85.03	61.36	43.28	50.38	50.30
H^2	0.197	0.150	0.386	0.567	0.496	0.497

^{NS} $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

Chapter 3 - Adaptive Thermal Plasticity Among *Drosophila* populations

Abstract

Many biotic and abiotic variables influence the dispersal and distribution of organisms. Temperature has a major role in determining these patterns because it changes daily, seasonally, and spatially, and these fluctuations have a significant impact on an organism's behavior and fitness. Most ecologically relevant phenotypes that are adaptive are also complex and thus they are influenced by many underlying loci that interact with the environment. In this study we quantified the degree of thermal phenotypic plasticity within and among populations by measuring chill-coma recovery times of lines reared from egg to adult at two different environmental temperatures. We used sixty genotypes from six natural populations of *Drosophila melanogaster* sampled along a latitudinal gradient in South America. We found significant variation in thermal plasticity both within and among populations. All populations exhibit a cold acclimation response, with flies reared at lower temperatures having increased resistance to cold. We tested a series of environmental parameters against the variation in population mean thermal plasticity and discovered the mean thermal plasticity was significantly associated with altitude of origin of the population. Pairing our data with previous experiments on viability fitness assays in the same populations in fixed and variable environments reveals an adaptive role of this thermal plasticity in variable laboratory environments. Altogether, these data demonstrate abundant variation in adaptive thermal plasticity within and among populations.

Introduction

Natural environments vary in many biotic and abiotic factors on both spatial and temporal scales. Environmental variation in temperature is a critical parameter that influences many components of fitness (Reusch & Wood, 2007, Umina et al., 2005, Rashkovetsky et al., 2006), drives patterns of local adaptation (Hoffmann et al., 2003b), and affects species distributions (Clark 1996) in nature. Variation in temperature occurs on a daily, spatial, and seasonal scale (Gibbs et al. 2003) and thus for a population to persist in the long term it must harbor sufficient genetic variation to adapt across generations, the capacity of individuals to respond plastically within a generation, or some combination of both genetic and plastic responses (Hoffmann & Parsons, 1991, Ayrinhac et al., 2004, Hoffmann et al., 2005, Hoffmann & Willi, 2008).

There is abundant evidence that the thermal response phenotypes that mediate the adaptive or plastic responses are in fact under both genetic (Fallis et al., 2012, Hoffmann et al., 2003a, Zhen & Ungerer, 2008, Morgan & Mackay, 2006) and environmental control (Gibert & Huey, 2001, Gibert et al., 2001) in many species. Many studies have documented significant gene-by-thermal environment effects on thermal response phenotypes *within* populations (Deere et al., 2006, Levine et al., 2011, Swindell et al., 2007, Winterhalter & Mousseau, 2007, Ayrinhac et al., 2004), however few studies have examined genetic variation in thermal plasticity across broad geographic ranges (Trotta et al., 2006, Winterhalter & Mousseau, 2007). Studies of genetic variation in thermal plasticity from species with broad geographic ranges allow fundamental questions to be addressed including: is there genetic variation in plastic traits among populations

with distinct environments? And what is the evolutionary significance of this variation in plasticity across these diverse natural environments?

Phenotypic plasticity is a within-generation effect, where a single genotype produces distinct phenotypes in response to specific environmental conditions (Ghalambor et al., 2007). This response is quantified using a reaction norm, which is the function characterizing the response of a single genotype across multiple environments. The simplest reaction norm is a linear function quantifying the effect of two distinct environments on the expression of the phenotype. A two-environment reaction norm is beneficial because the slope of the regression between environment and phenotype estimates the degree of phenotypic plasticity for a single genotype (Scheiner, 1993, Nussey et al., 2007). Although a reaction norm measures the degree of plasticity for a single genotype, comparing variation between multiple reaction norms among genotypes, either within or among populations, can reveal evidence for genetic variation in environmental responsiveness.

Genetic variation in plasticity, within or among populations, is important as it confirms plasticity has a genetic basis and can evolve as a complex trait (Scheiner, 1993). Specifically, natural selection should favor plasticity if environmental change is frequent and environmental cues for such changes are reliable (Mitchell-Olds & Rutledge, 1986, Schlichting & Smith, 2002). The corresponding reaction norm should maintain plasticity across environments (i.e. reaction norm slopes $\neq 0$). Conversely, natural selection should limit plasticity when environmental fluctuations are rare or when cues for change are not predictable (DeWitt et al., 1998). For example, in such environments, fluctuations may be faster than the organismal response time,

making a single phenotype the best option in all environments. A single phenotype may also be favored when an organism can actively select the most suitable habitat (Hoffmann & Parsons, 1991, Hoffmann & Parsons, 1997, Schlichting & Smith, 2002). Thus, the degree of plasticity for such populations should be low (i.e. reaction norm slopes = 0) and genotypes should be robust across environments.

Drosophila melanogaster is an excellent model system to investigate genetic variation in thermal traits across temperature environments. *D. melanogaster* is a broadly distributed species that has been extremely successful in adapting to a wide range of thermal environments (Ayrinhac et al., 2004, David & Capy, 1988), thus *D. melanogaster* harbors ample amounts of genetic and phenotypic variation in thermotolerance phenotypes (Ayrinhac et al., 2004, David & Capy, 1988). Many studies have documented robust thermal responses on cold and/or heat survival/tolerance phenotypes across multiple populations (Overgaard et al., 2008, Lee et al., 1987, Sgro et al., 2010, Fallis et al., 2012), however few have measured genetic variation in thermal plasticity across multiple populations. Here we quantify the amount of thermal phenotypic plasticity variation within and among six natural populations of *Drosophila melanogaster* from a latitudinal gradient in South America (Figure 3.1). The six collection sites are diverse in many geographic, climate, and environmental parameters, including yearly thermal profiles and seasonal thermal variation (Table 3.1; Figure 3.1). We quantitatively measured plasticity in cold tolerance using a chill-coma recovery time assay (Morgan & Mackay, 2006) on ten genotypes from each population after rearing individuals from egg-to-adult at two different temperatures (18°C or 25°C). We find significant variation in thermal plasticity within populations and adaptive variation in mean thermal plasticity among populations. The among

population variation in mean thermal plasticity strongly associated with the population of origin of each population and by pairing our data with work from Folguera et al. (2008) we are able to conclude that this variation in thermal plasticity is beneficial (i.e., increases fitness) in variable laboratory environments.

Methods

Drosophila Stocks

Gravid females were collected from six populations in central Argentina, described previously (Lavagnino et al., 2008). Flies were collected by net sweeping over fermented banana baits at six locations along a north to south latitudinal gradient ranging from approximately 24° to 38° south latitude in Argentina (Figure 3.1). Populations were named for the nearby city or providence where sampling took place (i.e., Guemes, Jachal, Chilecito, Lavalle, Uspallata, and Neuquén). Collection locations, latitude, longitude, altitude and climatological data (<http://www.smn.gov.ar/>) for each population are presented in Table 3.1. Ten isofemale lines were created from single wild-caught females from each population and inbred via full-sib mating for 10 generations. All lines have been maintained in the laboratory since February 2004 on standard cornmeal-agar-molasses medium sprinkled with live yeast to stimulate oviposition. Flies were maintained from egg to adult at either 25°C or 18°C and on a light/dark cycle for 12 hours. All phenotypic assays used 5-to-7 day old flies, separated by sex to account for sex specific differences in phenotype.

Phenotypic Assays

To measure thermal plasticity, we measured chill-coma recovery time on flies reared from egg-to-adult at 18°C and 25°C. Chill-coma recovery time was measured as in Morgan and Mackay (2006). Briefly, assays were conducted by transferring 25 same-sex individuals, without the use of anesthesia, to empty shell vials immediately before cold stress. Each line was subjected to a 0°C cold stress for a 3-hour period. Upon removal from the cold, flies were placed at room temperature and allowed to recover from chill coma (i.e. able to stand on their legs) for up to 30 minutes. Chill-coma recovery times were quantified as the time (in minutes) required for a fly to recover from cold exposure within a 30-minute period. Individuals that did not recover during the observational period were given a score of 30 minutes. The mortality rate during this assay was 0%. We performed three replicates containing 25 individuals per line, sex, and developmental temperature (18° or 25°).

Statistical Analysis

We tested for the presence of variation in reaction norm slope among genotypes within each population by assessing the degree of genotype-by-environment interaction using the following mixed model: $y = \mu + G + S + E + G \times S + G \times E + S \times E + G \times S \times E + \varepsilon$, where y is the sex, line, and environment specific chill-coma recovery times. G , S , and E are the fixed effects of genotype, sex, and developmental environment (18° or 25 °C). $G \times S$, $G \times E$, $S \times E$, and $G \times S \times E$ are the interaction effects between genotype and sex, genotype and environment, sex and environment, and genotype and sex and environment, respectively and ε is the residual error. The terms of primary interest in the within population analysis are G and $G \times E$ as they

represent significant genetic variation and genotype-by-environment interaction within populations.

Variation in mean plasticity among populations was calculated by first quantifying the line-specific reaction norm slope. The line-specific regression coefficient was estimated from a simple linear regression between chill-coma recovery time and developmental environment. Specifically, for each line a simple linear regression was made using the following model: $y = \beta_0 + \beta_1 E + \varepsilon$, where y is again the sex, line, and environment specific chill-coma recovery time and E is the developmental environment (18° or 25 °C). The slopes of the regression coefficients (i.e. the β_1 s) were retained as they represent the line-specific reaction norm slope. We tested for variation in thermal plasticity (i.e., the reaction norm slopes) by performing a two-way analysis of variance with fixed effects of population and sex.

To test if variation in environmental or geographic factors associated with variation in survival after heat stress, we used a stepwise forward-backward selection model implemented in PROC REG in SAS 9.2 (SAS Institute 2009). This approach tests for associations between the line-specific mean plasticity and the geographic and/or climatological data, by evaluating the significance of each geographic or climatological factor (Table 3.1) on thermal plasticity.

Results

The developmental environment (18° C vs. 25° C) had a dramatic effect on chill-coma recovery time (Figure 3.2). The majority of the thermal reaction norms had positive slopes,

because flies reared at 18° C generally have more rapid chill-coma recovery times [\bar{x}_{18} = 12 minutes 40 seconds (\pm 3 seconds)] than flies reared at 25° C [\bar{x}_{25} = 15 minutes 5 seconds (\pm 3 seconds)]. The effect of developmental environment was highly significant in five of six populations (Table 3.2). Although there is a general pattern that decreased developmental temperature results in more rapid chill-coma recovery, there is significant variation among the genotypes within each population (Figure 3.2).

There was significant within population genetic variation in the chill-coma recovery times in all six populations (Table 3.2; Figure 3.2). All six of the populations had highly significant variation among the ten genotypes within each population, while three of the six populations (Uspallata, Lavalle, Jachal) had significant genotype-by-environment interaction (Table 3.2; Figure 3.2). To compare the population-specific thermal plasticity among the six populations, we analyzed the variation among populations in the reaction norm shapes (Figure 3.2). The thick dashed lines, superimposed on each population's set of reaction norms, represents the population mean thermal plasticity (Figure 3.2). There was significant variation in thermal plasticity among the six populations (Figure 3.3A; Table 3.3; $P=0.0113$). The Lavalle population had the lowest thermal plasticity ($\bar{\beta} = 0.246 \pm 0.08$) and thus the least dramatic shift in the chill-coma recovery time between 18° C and 25° C, while the greatest thermal plasticity occurred in the populations from Chilecito ($\bar{\beta} = 0.472 \pm 0.08$) and Uspallata ($\bar{\beta} = 0.453 \pm 0.08$).

The single environmental factor that was positively associated with variation in thermal plasticity among the six populations was population altitude (Figure 3.3B;

$\beta_1 = 0.00015 \pm 0.00006$; $P = 0.0229$). The populations from low altitude (Guemes, Neuquén, and Lavalle) had the lowest mean thermal plasticity, while populations from high altitude (Chilecito and Uspallata) had increased mean thermal plasticity (Figure 3.3B). The population from Jachal is a high altitude population (1,238 m), but exhibits a mean thermal plasticity ($\bar{\beta} = .2250 \pm 0.09$) that is similar to low altitude populations (Figure 3.3A).

To determine if variation in thermal plasticity among populations was adaptive, we complement our results with the findings of Folguera et al. (2008). Briefly, in Folguera et al. (2008) viability fitness (i.e., larval to adult viability) was measured in stable and fluctuating thermal environments on the two populations, Uspallata (that has high mean thermal plasticity and occurs at high altitude) and Lavalle (which has low mean thermal plasticity and occurs at low altitude) (Figure 3.3). There were two fixed temperature treatments (constant 17°C or 25°C) and three variable temperature treatments [day temperature: night temperature (25°C: 17°C, 30°C: 9°C, and 25°C: 9°C)]. Larval to adult viability was measured as the percent emergence of each population in each fixed or variable temperature treatment from first instar larvae to adult. Folguera et al. (2008) found no significant differences between the two populations under the fixed temperature treatments, however under two of the variable temperature treatments (25°C: 17°C, and 25°C: 9°C) the high plasticity (high altitude) population, Uspallata, had higher viability than the low plasticity (low altitude) population, Lavalle.

Discussion

The role of phenotypic plasticity in adaptation has been controversial, with some studies suggesting plasticity aids in creating new phenotypes on which evolution can act (Robinson & Dukas, 1999, Pigliucci & Murren, 2003, Price et al., 2003), while others suggest plasticity inhibits evolution because genotypes may become hidden from natural selection (Grant, 1977, Falconer, 1981, Levin, 1988, Ghalambor et al., 2007). Although a great deal of debate exists about the role plasticity plays in adaptation, the role of environmental variation on the expression of natural phenotypic variation is widely accepted and is ubiquitous for most traits (Falconer & MacKay, 1996). To link the pervasive nature of phenotypic plasticity with long standing questions about its role in adaptation it is essential to analyze many populations spanning climatically variable regions, where different degrees of phenotypic plasticity may vary in response to different evolutionary processes. Here we examined the level of phenotypic plasticity in chill-coma recovery time, an adaptive cold response phenotype (Gibert et al., 2001), within and among six *D. melanogaster* populations collected along a latitudinal and altitudinal transect in Argentina. We found very high levels of genetic variation within all populations (Table 3.2, Figure 3.2). We found that the thermal plasticity significantly varied among populations (Figure 3.3A) and that mean thermal plasticity was best explained by altitude of each population (Figure 3.3B). Populations from higher altitudes exhibited a higher level of plasticity than populations at low altitudes. Finally, we were also able to demonstrate the adaptive significance of this among population variation in thermal plasticity, by pairing our results with the results of Folguera et al. (2008), which found significant variation in larval to adult viability between the high plasticity (Uspallata) and low plasticity (Neuquén) populations when reared in a variable but not constant environments. This combination of results suggests that populations from a more variable, high altitude, environment exhibit higher levels of thermal phenotypic plasticity and this thermal

plasticity is associated with increased fitness in variable thermal environments (Folguera et al. 2008)

The overall patterns of thermal plasticity observed in each of these six populations is consistent with previous studies (Gibert & Huey, 2001, Ayrinhac et al., 2004), which have shown chill-coma recovery time to be significantly decreased when flies are developmentally acclimated in low temperature rearing environments. Across all our populations this trend is confirmed by the population mean reaction norms (Figure 3.2), where flies reared at 18°C recover more rapidly on average than flies reared at 25°C. Although the mean reaction norms are consistent with expectations, the significant variability in reaction norm slope and position is different from previous studies of chill-coma recovery time. Both Ayrinhac et al. (2004) and Gibert and Huey (2001) have previously shown that both genetic variation and developmental temperature have strong effect on chill-coma recovery time, but their effects are largely independent. In our study, we find effects that are consistent with previous studies for three populations, Guemes, Chilecito, and Neuquén, however we identified significant variation in the degree of thermal plasticity (i.e., genotype-by-environment interaction) within the Jachal, Lavelle, and Uspallata populations (Table 3.2; Figure 3.2). This variation in the degree of thermal plasticity represents genetically based differences in how genotypes within a population respond to thermal rearing environment. The finding that there was a significant effect of genetic, environmental and genotype-by-environment interactions on the expression of within population variation in chill-coma recovery time is not unexpected given the complex genetic architecture (Morgan & Mackay, 2006, Norry et al., 2004, Norry et al., 2008) that has been shown to underlie chill-coma recovery time and other thermal phenotypes.

An extremely interesting finding from the current study, was the *among* population differences in how geographically distinct populations respond to thermal rearing environment. This is the first study to our knowledge that has quantified the *among* population differences in mean thermal plasticity, based on the analysis of multiple genotypes and not overall population samples. Our finding that there is significant variation in mean thermal plasticity among populations, suggests that the evolutionary history of each population has shaped the patterns of variation in thermal plasticity among populations. However, because this variation in mean plasticity is also associated with the altitude of the population of origin, it is likely that these among-population changes in thermal plasticity were driven by biotic or abiotic differences among the sites. Our findings that high altitude populations have increased thermal plasticity relative to low altitude populations is largely consistent with previous studies that have shown increased phenotypic plasticity in populations from variable environments, relative to robust plasticity in stable environments (Cheviron et al., 2008, Ishihara, 1999, Crispo & Chapman, 2010, Trussell, 2000, Winterhalter & Mousseau, 2007, Karl et al., 2009). For example, it has been shown several times that the incident of diapause is directly related to the variation between seasonal temperatures vs. the lack there of (i.e. biboltine vs. univoltine), where populations exposed to many seasons, thus more changes in temperature, have a higher degree of phenotypic plasticity (Winterhalter & Mousseau, 2007, Ishihara, 1999). Another example can be given in the context of invasive species expanding their range into novel (i.e. more variable) environments, because invaders have a higher degree of plasticity compared to native species (Daehler, 2003, Chown et al., 2007). A common theme emerges to explain these repeated associations between phenotypic plasticity and variable environments, which is variable abiotic

environments are likely the environments where natural selection should favor plasticity (Mitchell-Olds & Rutledge, 1986, Schlichting & Smith, 2002).

To determine if populations with high thermal plasticity actually do better than populations with low thermal plasticity, it is essential to measure fitness or a component of fitness for each of these populations in multiple fixed and variable environments. To this end our discovery of abundant variation in thermal plasticity within and among population, as well as the population mean thermal plasticity and altitude association result is further supported by the work of Folguera et al. (2008). Folguera et al. (2008) measured larval to adult viability to assay fitness in a low altitude, low thermal plasticity population (Lavallo) and in a high altitude, high thermal plasticity population (Uspallata) in both fixed and variable temperature environments. When reared in the fixed environments (constant 17°C or 25°C) the populations showed no significant differences in larval to adult viability, however when reared in variable environments (daytime: nighttime temperatures of 25°C: 17°C, 30°C: 9°C, and 25°C: 9°C) the high altitude, high plasticity population had higher larval to adult viability in two of the variable environments relative to the low altitude, low plasticity population. Although first instar to adult viability is not a complete estimate of fitness, Folguera et al. (2008) show there are significant differences between Uspallata (high altitude and plasticity) and Lavallo (low altitude and plasticity) that occur in fluctuating environments in the predicted direction based on the mean thermal plasticity phenotypes presented in this current work. Altogether, our results and the results of Folguera et al (2008) clearly demonstrate there is abundant variation in thermal plasticity within and among populations and the significant among population variation in thermal plasticity was likely shaped by local adaption to different degrees of local environmental heterogeneity.

Acknowledgments

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Figures and Tables

Figure 3.1 Population sites.

Geographic locations of the Argentinean populations used in this study: A = Guemes, B = Chilecito, C = Jachal, D = Uspallata, E = Lavalle, and F = Neuquén. Insets show mean highest and lowest monthly temperatures (filled and open circles, respectively) from collection locations. Meteorological data from <http://www.smn.gov.ar/>.

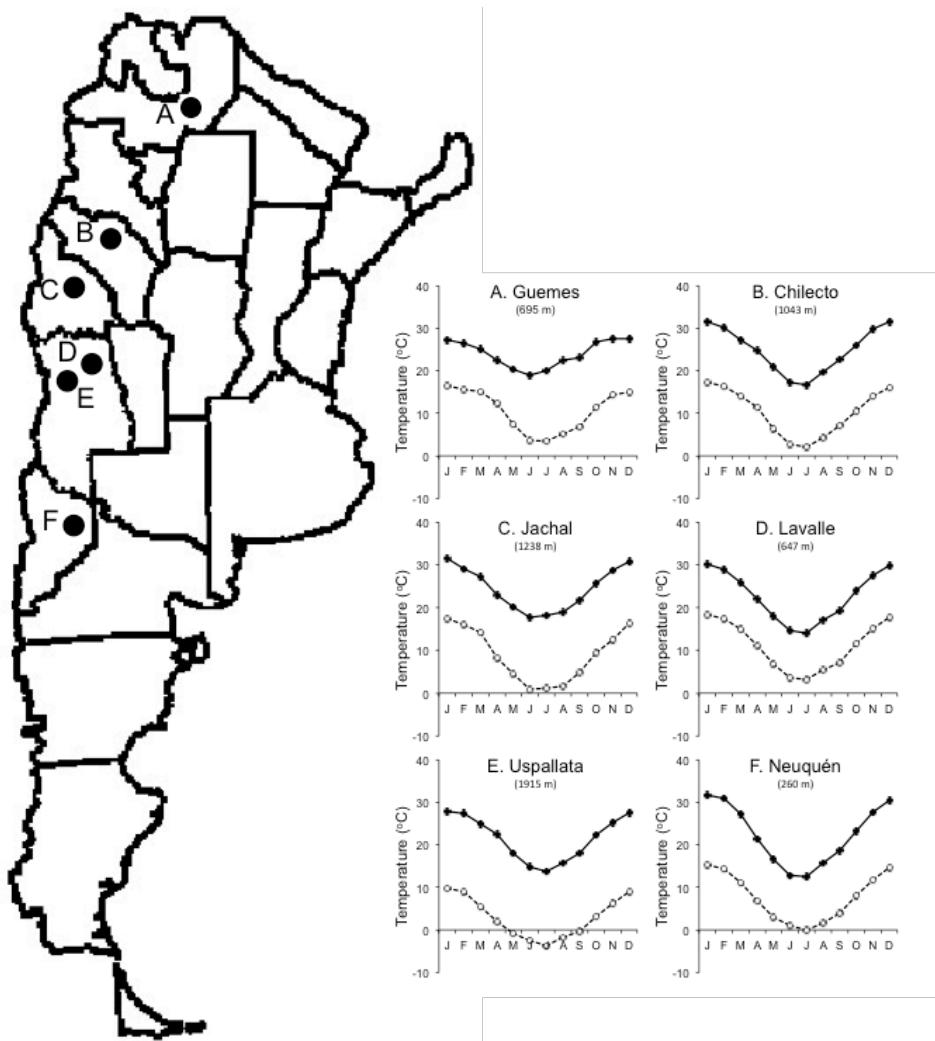


Figure 3.2 Thermal reaction norms.

Line-specific chill-coma recovery reaction norms are presented for the each line grouped within the six populations (A-F; See Fig.1). The reaction norm with the open diamond and bold dashed line is the mean population chill-coma recovery reaction norm. The x-axis is the developmental temperature, 18° C or 25° C, while the y-axis is the chill-coma recovery time in minutes.

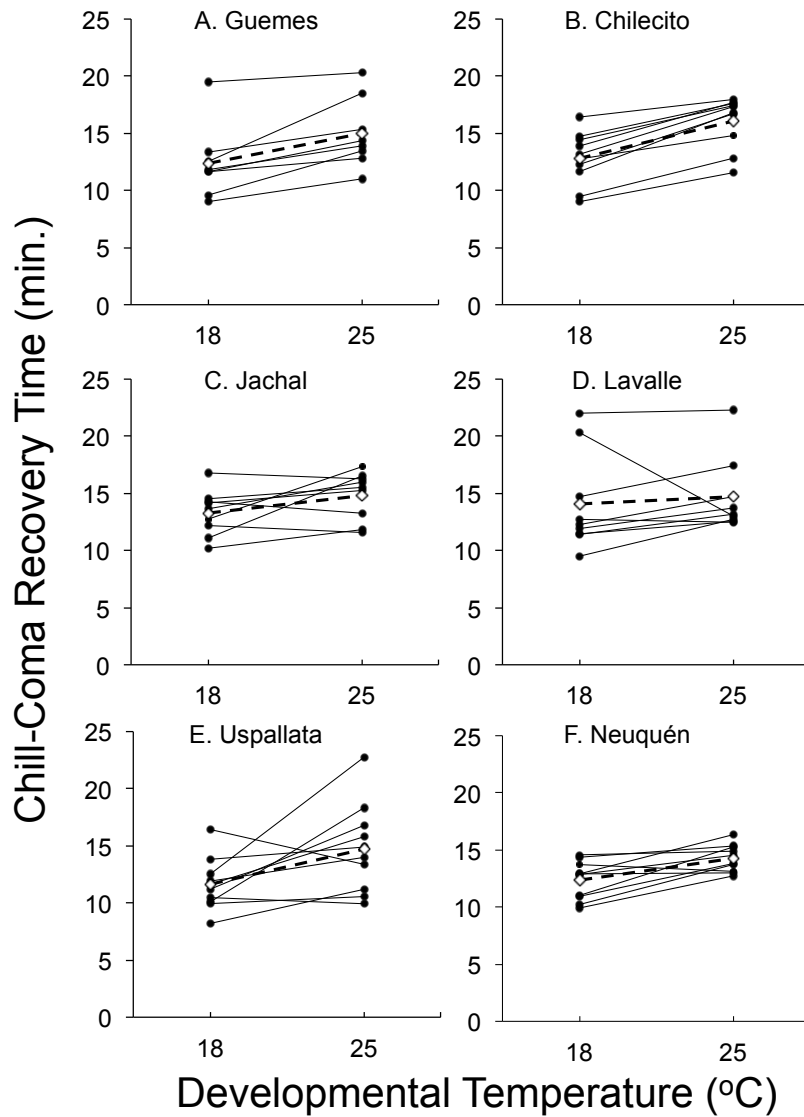


Figure 3.3 Among population variation in thermal reaction norms.

A. Variation in population mean plasticity among the six populations. The y-axis is the population mean plasticity, while the x-axis is the population of origin (ordered by latitude from north to south). Lower case letters denote the Duncan's post hoc means groupings. B. Relationship between population mean plasticity and altitude of the population. Error bars denote plus or minus one standard error.

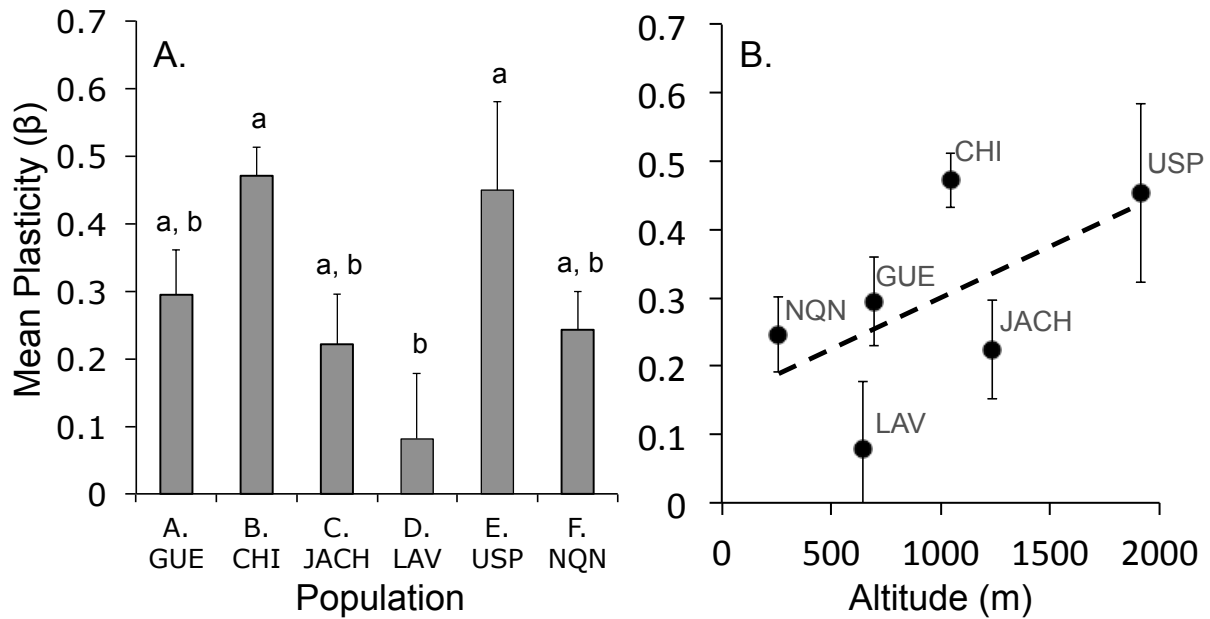


Table 3.1 Collection sites and selected climatological data for the six populations of *Drosophila melanogaster* in Argentina

Population	Latitude	Altitude (m)	Temperature (°C)			Mean rainfall (mm)	Mean humidity (%)	Isofemale lines (n)
			Mean annual	Max. monthly high mean	Min. monthly low mean			
A. Guemes	24°41'S	695	16.58	27.5	3.4	69.73	73.83	10
B. Chilecito	29°10'S	1043	17.25	31.6	2.1	15.75	59.66	10
C. Jachal	30°12'S	1238	16.45	31.6	0.9	11.84	54.25	10
D. Lavalle	32°50'S	647	15.93	30.2	3.2	22.53	58.75	10
E. Uspallata	32°35'S	1915	11.61	27.9	-3.7	12.75	51.45	10
F. Neuquén	38°57'S	260	14.74	31.7	-0.1	15.23	52.08	10

Table 3.2 Genetic and Genotype-by-environment variation within each of the six populations.

F values and significance are presented for each population.

	Population:					
	Guemes	Chilecito	Jachal	Lavalle	Uspallata	Neuquén
<i>Environment (E)</i>	18.28 ^{****}	50.65 ^{****}	11.10 ^{**}	1.91	73.39 ^{****}	19.38 ^{****}
<i>Genotype (G)</i>	10.93 ^{****}	9.23 ^{****}	6.42 ^{****}	26.52 ^{****}	19.12 ^{****}	4.24 ^{****}
<i>G ! E</i>	0.96 ^{NS}	0.51 ^{NS}	2.11 [*]	4.92 ^{****}	13.19 ^{****}	1.52 ^{NS}

^{NS} *P* > 0.05; ^{*} *P* < 0.05; ^{**} *P* < 0.01; ^{***} *P* < 0.001; ^{****} *P* < 0.0001

Table 3.3 Variation in thermal plasticity (reaction norm slope) among populations

Source	df	MS	F	P
Population	5	0.4404	3.13	0.0113
Sex	1	0.0030	0.02	0.8836
Population x Sex	5	0.0434	0.31	0.9068
Error	108			

Chapter 4 - Genetic Basis of Variation in Thermotolerance Phenotypes in *Drosophila melanogaster*: Tight Linkage or Pleiotropy?

Abstract

Temperature is an important abiotic factor that affects all organisms in nature. Fluctuations in temperature can initiate organismal responses from the cellular level up to complex behaviors. Thus, temperature will directly and indirectly impact the survival and fitness of most organisms. Insects are especially susceptible to changes in temperature because they are isothermal with their environment. *Drosophila melanogaster* is a widespread species that has adapted to many novel thermal environments. As a result, there is abundant within and among population adaptive genetic variation in thermotolerance phenotypes. To dissect this important trait variation many recent studies have mapped multiple quantitative trait loci (QTL). From this work, several studies have independently identified a major effect QTL in the middle of the second chromosome influencing both recovery time from cold and heat stress resistance. The co-localization of these QTL could either be the result of the same gene controlling both cold and heat stress resistance (i.e., pleiotropy) or different genes affecting cold and heat stress resistance in close proximity to each other (i.e., tight linkage). We found three small regions affecting cold stress resistance and two different regions affecting heat stress resistance. These results suggest that the co-localization of these cold and heat stress QTL is caused by *different* causal loci in tight linkage.

Introduction

Understanding the genetic architecture underlying adaptive trait variation is a central question in biology. One environmental parameter that is crucial to organismal fitness and behavior is temperature (Gilchrist & Partridge, 1999). Temperature changes on a daily, seasonal, and spatial scale (Gibbs et al., 2003) and therefore, is a constant form of stress with which all individuals must cope. The organismal response to temperature results in varying effects ranging from the cellular level to complex behavior. Thus, it is important to dissect the genetics of thermal variation in natural populations. It is also timely as the environment is currently changing rapidly; temperatures are expected not only to increase on a global average, but also have less predictability with more dramatic fluctuations on a local scale (Jentsch et al., 2007).

Drosophila melanogaster is a cosmopolitan species that has been extremely successful in adapting to a wide range of thermal environments in nature. *D. melanogaster* is found in a variety of tropical and temperate regions (Ayrinhac et al., 2004, David & Capy, 1988) and the role of thermal adaptation in *Drosophila* has been demonstrated in many studies from regions across the globe. For example Hoffmann et al. (2001), showed Australian *D. melanogaster* populations exhibit significant clinal variation in heat and cold tolerance phenotypes. In these populations, those closer to the equator are more heat tolerant and cold susceptible than populations found at more extreme southern latitudes. Similarly, Ayrinhac et al. (Ayrinhac et al., 2004), Schmidt and Paaby (2008), and Fallis et al. (2012) have shown evidence for phenotypic clines in Europe, North America, and South America suggesting that resistance to thermal stress is a climatic adaptation on a global scale (Gibert & Huey, 2001, Hoffmann et al., 2002, Kimura, 2004). Although the adaptive significance of temperature stress phenotypes has been

established, one of the primary objectives, when studying such variation, is to link clinal phenotypic variation to the underlying genes contributing to local adaptation in nature.

Several studies have demonstrated cases where allelic variation in candidate thermotolerance loci exhibit allele frequency clines in candidate genes across latitude. For example, *Hsr-omega* and *hsp68* have been associated with variation in heat stress and cold tolerance (Anderson et al., 2003, McColl et al., 1996). Segregating genetic variation and associations have also been identified between cold tolerance and many candidate genes; including *Smp-30*, *Frost*, *HSP70*, *denaturase 2*, and *CG16700* (Goto, 2000, Goto, 2001, Daibo et al., 2001, Anderson et al., 2003, Greenberg et al., 2003, Qin et al., 2005, Clowers et al., 2010, Svetec et al., 2011, Rako et al., 2007, Colinet et al., 2010). At the genomic level, mapping studies have localized variation in most of these candidate genes to the right arm of chromosome III, however, several quantitative trait loci (QTL) studies have identified many different thermal QTL spanning all chromosomes in *Drosophila* (Svetec et al., 2011, Norry et al., 2008, Norry et al., 2004, Morgan & Mackay, 2006). Three of these studies (Norry et al., 2004, Norry et al., 2008, Morgan & Mackay, 2006) have independently identified a large effect QTL influencing both heat and cold tolerance on the second chromosome, at cytological position 37C5-48D5. This replicated co-localization is interesting because the recombinant inbred lines (RILs) used in each study represent unique samples of natural genetic variation. That is, founder stocks used for each segregating cross were originally collected from Denmark, Australia, Russia, and the United States. This suggests alleles underlying this heat- and cold-stress resistance QTL are important for variation in thermotolerance phenotypes and this variation segregates on a global scale.

In the current study, we build on the work of Morgan and Mackay (2006), Norry et al. (2004), and Norry et al. (2008) by fine-mapping the co-localized thermotolerance region on chromosome II via a series of deficiency lines. We ask two primary questions: first, what is the genetic architecture underlying variation in each thermotolerance phenotype? And second, is the co-localization of this heat and cold stress QTL caused by pleiotropy or tight linkage?

Materials and Methods

Phenotypic Assays

All phenotypic assays were conducted in an identical manner to Morgan and Mackay (2006). Briefly, all parental and deficiency stocks used in this study were maintained on cornmeal-molasses fly food at 25°C and on a 12-hr light/dark cycle. Flies used in screens were 5-7 days old and reared at low density by placing five males and five females in each vial for 3 days. Phenotypic assays were conducted by transferring 10 same-sex individuals of each genotype, without the use of anesthesia, to empty shell vials immediately before subjecting individuals to heat or cold stress. For chill-coma recovery, individuals were placed at 0°C for a 3-hr period. Upon removal from the cold, flies were placed at room temperature (23°C ± 0.5°C) and allowed to recover from chill coma (i.e. able to stand on their legs). The chill-coma recovery score was quantified as the number of flies recovered from cold exposure within an 11-min time period. The 11-min time interval represents the 50% chill-coma recovery time point for the parental lines (Oregon-R and 2B-3; Morgan and Mackay 2006). For survival after heat stress, flies were subjected to the 50% heat survival time-point of 110 minutes, at 38°C (± 0.5°C)

(Morgan and Mackay 2006). Upon removal from the heat stress, flies were immediately tipped into new shell vials containing approximately 5 mL of fresh food. The survival after heat-stress score was the number of survivors out of 10 after a 24-hr recovery period at room temperature ($23^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$).

Stocks

The parental lines used to fine map the region on chromosome II are the same recombinant inbred lines used in Morgan and Mackay (2006) to identify thermotolerance QTL. Oregon-R and 2B-3 were created from naturally derived flies lines collected from Oregon and Russia, respectively (Lindsley & Zimm, 1992, Pasyukova & Nuzhdin, 1993). Neither Oregon-R nor 2B-3 has been selected for thermotolerance phenotypes. Individual lines are homozygous at all loci with Oregon-R and 2B-3 having significantly different percent survival after heat stress and percent recovery from chill coma (Morgan and Mackay 2006). Oregon-R is tolerant to cold and susceptible to heat, while 2B-3 is tolerant to heat and susceptible to cold.

Deficiency stocks were ordered from Bloomington Stock Center (Bloomington, IN) that span the QTL region from cytological marker 37A-49A on chromosome II (Figure 4.2). All deficiency stocks were created as part of the larger Drosdel collection and are thus in a common *w¹¹¹⁸iso;2iso;3iso* strain, chosen for its lack of P elements and its use in some behavioral studies (Dura et al., 1993). Deficiencies are maintained over balancer chromosomes (*FM7*) marked with a visible curly wing phenotype (*CyO*) (Ryder et al., 2007, Ryder et al., 2004). A total of twenty-six overlapping deficiencies (Table A.1, Figure 4.2) were used that tile the 11MB thermal QTL region between 37A-49A on chromosome II.

Crosses

The crossing scheme for each of the 26 deficiencies paired virgin females of each parental line, Oregon-R and 2B-3, with males from each deficiency stock (Table A.1). Crosses were performed using five virgin females and five mated males per cross to control for progeny density. Because each deficiency is maintained over a balancer chromosome, each cross resulted in four distinct genotypes containing either the deficiency or balancer chromosome over each parental background (i.e., Oregon-R/deficiency, Oregon-R/balancer, 2B-3/deficiency, 2B-3/balancer). All resulting progeny were sorted by sex and genotype and screened for heat and cold tolerance phenotypes. For heat and cold assays, data was collected in an initial screen made of four blocks, consisting of four replicates of each genotype. A second analysis was used to identify final significant deficiencies. The significant deficiencies were re-tested using two more blocks of four replicates, to be confident in the significance of each region. The final analyses, reports the result of the pooled data from both the initial screen and the retests for each individual deficiency.

Complementation tests and analysis

To test the phenotypic contribution of each deficiency region, quantitative complementation tests were used (Pasyukova et al., 2000). Quantitative complementation tests are used to detect small differences in allelic effects between parental genotypes. In our design, parental lines, Oregon-R and 2B-3 were crossed to each deficiency line (Oregon-R/Oregon-R x deficiency/balancer and 2B-3/2B-3 x deficiency/balancer). This resulted in four different

genotypic classes (Oregon-R/deficiency, Oregon-R/balancer, 2B-3/deficiency, 2B-3/balancer). The mutant progeny (Oregon-R/deficiency, 2B-3/deficiency), in each of the wildtype backgrounds, was compared to the corresponding wildtype line with the functional allele (Oregon-R/balancer, 2B-3/balancer). This comparison of four genotypes allows differences in segregation of parental QTL located at other regions of the genome, outside the deficiency, to be eliminated and thus only reveal differences uncovered by the deficiency that are the focus of the comparisons. Quantitative failure to complement was indicated when the phenotypic difference between the two wildtype backgrounds carrying deficiency alleles is greater than the phenotypic difference between the wildtype over a functional allele. That is, the expression $(\text{Oregon-R/balancer} - 2\text{B-3/balancer}) < (\text{Oregon-R/deficiency} - 2\text{B-3/deficiency})$ must be satisfied and statistically significant. This criterion eliminates epistatic interactions between genes on the balancer chromosome and Oregon-R and 2B-3 thermotolerant phenotypes. To identify deficiencies that exhibit failure to complement statistically, we used the following three-way ANOVA model: $y = \mu + L + S + G + L \times S + L \times G + G \times S + L \times G \times S + R(L \times G \times S) + \epsilon$, where y is the phenotype, L , G , and S are the fixed effects of parental lines, genotype, and sex, while R is the random effect of replication nested within the line, genotype, and sex and ϵ is error. Quantitative failure to complement was indicated when the $L \times G$ term was significant ($p < 0.05$) and the relationship $(\text{Oregon-R/balancer} - 2\text{B-3/balancer}) < (\text{Oregon-R/deficiency} - 2\text{B-3/deficiency})$ was satisfied. The final list of significant deficiencies was made based on both the statistical calculation and the complementation test plot. Deficiencies were only termed significant when they met the requirements for both tests. For instance, in some complementation plots the lines crossed giving a significant p -value, but did not satisfy the expression $(\text{Oregon-R/balancer} - 2\text{B-3/balancer}) < (\text{Oregon-R/deficiency} - 2\text{B-3/deficiency})$.

These deficiencies were no longer considered, as these cases represent epistasis between the parental line genotype and the background genotype, not significance of the deficiency region. In several cases failure to complement was sex specific and indicated by a significant LxGxS term.

Results

Significant Deficiencies

The experiment was conducted in two phases, an initial screen that consisted of four replicates per genotype to identify the possible regions that were significant. The initial screen was followed by a secondary screen, containing at least eight additional replicates of all significant deficiencies. This design allows strong confidence in the identified regions because of the replicated screen approach.

In the initial screen, six deficiencies had significant LxG terms for percent recovery from chill coma (Df(2L)ED1303, Df(2L)ED1315, Df(2L)ED1317, Df(2L)ED1454, Df(2R)ED1673, and Df(2R)ED1725) and four deficiencies had significant LxGxS terms for percent recovery from chill coma (Df(2L)ED1315, Df(2L)ED1473, Df(2R)ED1791, and Df(2R)ED2076). For percent survival after heat stress we found two deficiencies that had significant LxG terms (Df(2L)ED1305 and Df(2L)ED1454) and three deficiencies that had significant LxGxS terms (Df(2L)ED1305, Df(2L)ED1317, and Df(2L)ED1454). Final deficiencies were selected based on significant LxG and LxGxS terms, as well as visually through the quantitative complementation test plot. We did this to eliminate significant terms based on epistasis [plots

where lines crossed, and (Oregon-R/balancer – 2B-3/balancer) < (Oregon-R/deficiency – 2B-3/deficiency) was not satisfied] and to not exclude deficiencies where the expression was true, but the interaction was not significant statistically. This eliminated many of the originally significant deficiencies, leaving three significant deficiencies for cold (Df(2R)ED1725, Df(2L)ED1473, Df(2R)ED2076) and two significant deficiencies for heat (Df(2L)ED1317, Df(2R)ED1770).

From Deficiency to QTL

From the pattern of significant and non-significant deficiencies, the QTL can be further refined to regions that are smaller than the original significant deficiency. For example if a significant deficiency is partially overlapped by two non-significant deficiencies then the QTL region can be narrowed to the region unique to the significant deficiency. However if there are no partially overlapping deficiencies or there are multiple significant overlapping deficiencies, narrowing the QTL region within the deficiency breakpoints is impossible. We applied this multiple deficiency framework to the cold and heat deficiency sets and identified three regions for cold and two regions for heat.

Cold tolerance QTL

For percent recovery from chill coma there were three significant deficiencies, one common to both sexes and two that were sex specific (Table 4.1, Figure 4.1A-C). Deficiency Df(2R)ED1725 at cytological marker 43E-44B is significant for both sexes and is overlapped by two overlapping non-significant deficiencies (Figure 4.2), Df(2R)ED1715 and Df(2R)ED1735. Thus, the significant region can be narrowed to a 25kb area near cytological marker 43F. The

significant male-specific deficiency, Df(2R)ED2076 (Figure 4.1B), spans cytological markers 46F-47B corresponding to a 330kb region, there were no overlapping deficiencies in this region. The significant female-specific region, Df(2L)ED1473, is located at cytological regions 39B-40A (Table 4.1A, Figure 4.1C) and this region contains two non-significant deficiencies (Df(2L)ED1466 and Df(2L)ED1378). Thus the significant region can be narrowed to a 232kb area near cytological marker 39E. Together the three regions span a total of 587 kb, which is substantially smaller than the original 11MB region.

Heat survival QTL

For heat survival, two deficiencies of interest were identified in our final screen (Table 4.1B and Figure 4.1D-E). Deficiency, Df(2L)ED1317 has a nearly significant LxG term ($p=0.09$) and a quantitative complementation plot suggesting it to be a region of interest (Figure 4.1D). This region is between cytological markers 38D1-38D5. The second interesting deficiency is female-specific (Df(2R)ED1770, Figure 4.1E), with a LxGxS term of $p=0.0593$ (Table 4.1B). This deficiency is overlapped by two other non-significant deficiencies, (Df(2R)ED1791 and Df(2R)ED1742), which narrows the region to about 300kb.

Pleiotropic thermal QTL or Tightly linked heat and cold QTL?

When the locations of the three significant cold and the two significant heat deficiencies are compared (Figure 4.2), the results support the hypothesis that the co-localization of this QTL is the result of tight linkage of different genes influencing heat and cold stress resistance. We reject the pleiotropy hypothesis because none of the deficiencies are the same or overlapping between heat and cold stress resistance.

Discussion

Variation in thermal stress response in *Drosophila* represents an important phenotype that contributes to patterns of local adaptation on a global scale (Hoffmann et al., 2003b). Despite the clear importance of thermal stress phenotypes to the success of populations in variable local and regional environments, many questions persist about the genetic basis of thermal traits in *Drosophila*. Multiple studies have dissected the genetic basis of heat- and cold-stress resistance and identified thermal QTL on all major chromosomes of the *Drosophila* genome (Svetec et al., 2011, Norry et al., 2008, Norry et al., 2004, Morgan & Mackay, 2006). Three of these studies (Morgan & Mackay, 2006, Norry et al., 2004, Norry et al., 2007, Norry et al., 2008) identified a single replicated region on chromosome II (cytological position 37C5-48D5) that is associated with both heat survival and chill-coma recovery. This chromosome II QTL is exciting, as it is known to influence variation in thermal stress in many populations sampled from around the world (Norry et al., 2004, Norry et al., 2008, Morgan & Mackay, 2006). In this study, we answer two questions that focus on this co-localized QTL region. First we fine-mapped this 11Mb region to three small regions for cold tolerance and two small regions for heat tolerance. This reduced the candidate regions for cold tolerance to a total of 587kb and 500kb for heat tolerance. Second, we are able to reject the hypothesis that the co-localization of this heat and cold tolerance QTL is the result of pleiotropic effects and have strong evidence that the co-localization is the result of tight linkage.

This study reduced the number of candidate genes under this QTL, from nearly 2,000 genes to 108 candidates for cold tolerance and 73 candidates for heat tolerance. For cold tolerance these 108 candidates are contained within three significant regions, while for heat tolerance these 73 candidate genes are grouped into two significant regions (Figure 4.2). The primary advantage of QTL analysis followed by fine-scale mapping over candidate gene approaches is that there is reduced bias, as a result of candidate gene list selection. Using fine mapping via deficiency stock analyses coupled with further analysis of mutations we are able to test deficiencies and eventual mutations in an unbiased manner. This is essential, because focusing exclusively on candidate genes will miss unannotated genes, which are likely involved in environmental responses (Coolon et al., 2009). Thus, deficiency mapping is an efficient and unbiased method to identify genomic regions influencing variation in a quantitative trait. Our fine-mapping has identified five candidate genomic regions via this unbiased approach. Although we have not localized the causal loci within each of these genomic regions, we can discuss the classes of genes that are contained within these five candidate genomic regions. Even though we are discussing the classes of genes within the five genomic regions without functional tests, we argue that combination of the unbiased deficiency screen coupled with a survey of the classes of genes is a significant improvement. We are also in the process of testing each of the candidate genes within the five genomic regions using mutant complementation tests.

Within the three cold tolerance genomic regions, there are two unannotated candidate genes, *CG11165* and *CG30378* underlie deficiency Df(2R)ED1725, at cytological marker 43F, that have suggested function associated with calcium ion binding. Movement of calcium across a membrane has been shown to be part of the rapid cold hardening mechanism in the arctic midge

(Teets et al., 2008). Additionally, Ca⁺ is needed for electrochemical potential across the cell membrane. Misregulation of Ca⁺ can lead to cold induced immobilization (Hochachka, 1986, Pullin & Bale, 1988, Kelty et al., 1996, Kostal et al., 2007, Kostal et al., 2004, Takeuchi et al., 2009). *TATA binding protein associated factor 5 (Taf5)* and *Lsm10* underlie deficiency Df(2R)ED2076 at cytological markers 47C5 and 47C1, respectively. *Taf5* is involved in preinitiation complex assembly for the start of transcription (Dynlacht et al., 1991). While, *Lsm10* is part of a protein complex that has an important role in histone pre-mRNA processing (Pillai et al., 2001, Godfrey et al., 2009). These genes have seemingly general functions, but may be important in the regulation of genes in response to cold exposure. In the third significant cold region near cytological marker 39B4-40A5 we found no previously studied cold tolerance candidate gene or one with intriguing putative function, thus highlighting the value of the unbiased fine-mapping approach, as well as the need for additional functional tests. For heat tolerance, the gene *Phosphoglucose isomerase (Pgi)*, is located under deficiency Df(2R)ED1770 at cytological marker 44F6. *Pgi* has been shown important in heat survival in other organisms, such as butterflies and leaf beetles (Wheat et al., 2011, Wheat et al., 2009, Nearing et al., 2003). In order to mechanistically implicate allelic variation in these candidate genes to natural variation in thermotolerance, additional ongoing work is required to test all genes in the five identified regions.

In addition to fine-mapping the genetic basis of variation in heat- and cold-stress resistance, we have also determined that the co-localization of this QTL (Morgan & Mackay, 2006) is the result of tight linkage and not pleiotropy. This finding is significant as the physical linkage of genes along a chromosome has major consequences on the trajectories of correlated

evolution for multivariate phenotypes in response to selection. For example when a pleiotropic gene controls two phenotypes, this will impose a functional genetic constraint on the independent evolution of each phenotype. That is, a response of one phenotype will result in a direct correlated response of the other phenotype (Kelly, 2009, Arnold, 1992, Schwenk, 1994). This functional constraint cannot be decoupled via recombination and thus will limit the possible evolutionary trajectories for this multivariate phenotypic combination (Lande & Arnold, 1983, Jones et al., 2003, Weinreich et al., 2005). Alternatively, if the genetic basis of two phenotypes is controlled by independent genetic architectures (i.e., different genes affecting each phenotype), then the multivariate trait combination will be free to evolve in response to selection without a functional genetic constraint and thus, without a limited set of evolutionary trajectories. These two scenarios, complete constraint (pleiotropy) and genetic independence, represent the extremes of the spectrum for the genetic architecture of multivariate quantitative trait evolution. However understanding where thermal trait combinations (e.g., heat- and cold-stress resistance) are in this spectrum of genetic integration/independence is important if we want to understand the adaptive capacity of populations in a warming but increasingly fluctuating thermal environment.

In the current study, the significant regions for cold and heat tolerance are located on different albeit tightly linked segments of chromosome II (Figure 4.2). There is no overlap between the cold and heat tolerance regions, thus the loci are in tight linkage with one another. The fact that these loci are in tight linkage means that there is no absolute functional constraint as a result of a pleiotropic gene, however it does not mean that cold and heat tolerance will evolve completely independently of one another. The physical linkage between these regions

suggests that there is likely limited recombination among regions harboring cold and heat genes, this will likely result in haplotypes that segregate together. So although our results do not identify a true genetic constraint, it is likely that this region of chromosome II is limiting the genetic independence of this multivariate trait combination. It should be noted that this chromosome II QTL is not the only genomic region influencing variation in cold- and heat-stress resistance. A global analysis of all genomic regions associated with heat and cold tolerance clearly demonstrates a complex genetic architecture underlying both of these phenotypes with associated candidate genes and QTL mapping to all chromosomes of the *Drosophila* genome (Anderson et al., 2003, Clowers et al., 2010, Colinet et al., 2010, Daibo et al., 2001, Goto, 2000, Goto, 2001, Greenberg et al., 2003, Morgan & Mackay, 2006, Norry et al., 2004, Norry et al., 2008, Qin et al., 2005, Rako et al., 2007, Svetec et al., 2011). Thus, at a genomic level there appears to be a significant amount of genetic independence between heat- and cold-resistance in *Drosophila*, however our results and previous studies on the right arm of chromosome III suggest some degree of genetic integration, via tight linkage in our work, and via a pleiotropic gene, *hsr-omega* (Anderson et al., 2003). This dynamic set of unique and overlapping genetic architectures place the heat- and cold-stress resistance multivariate trait combination at an intermediate level of genetic integration between full genetic constraint and genetic independence.

Acknowledgements

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Figures and Tables

Figure 4.1 Quantitative complementation test plots for deficiencies of interest.

Failure to complement is indicated by the exacerbated difference between the phenotype of the parental lines when over the deficiency line. Red solid lines represent parental line Oregon-R and dashed blue lines represent parental line 2B-3. A. Df(2R)ED1725 B. Df(2R)ED2076 C. Df(2L)ED1473 D. Df(2L)ED1317 E. Df(2R)ED1770.

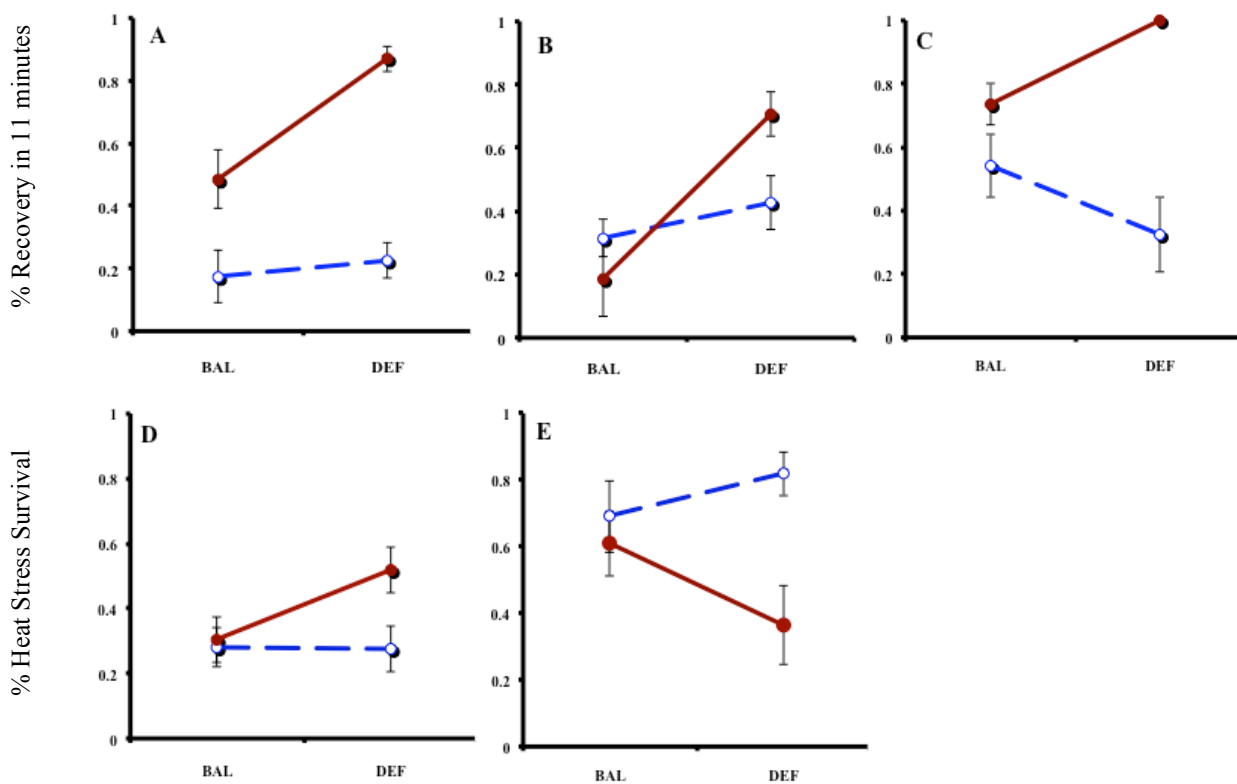


Figure 4.2 Each grey box represents individual deficiencies tested in our study.

Numbers correspond to list of deficiencies in Appendix A1. Colored boxes show the significant cold (blue) and heat (red) deficiencies identified. Note that the significant deficiencies are not the same or overlapping between cold and heat suggesting underlying loci are tightly linked, not pleiotropic for thermal tolerance phenotypes.

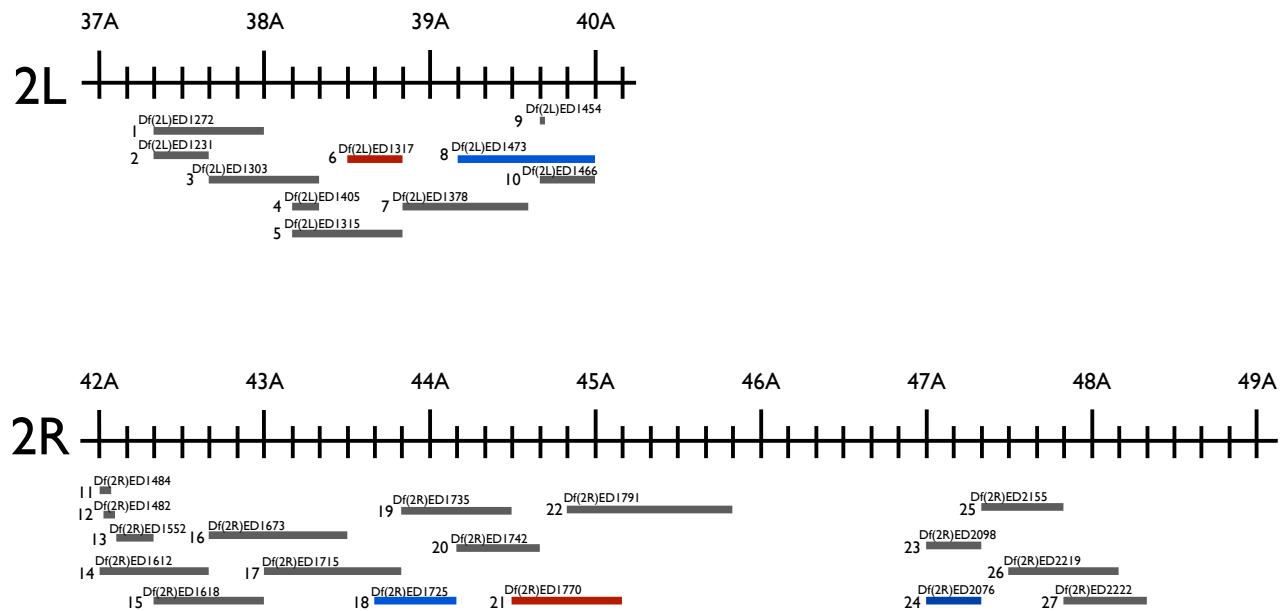


Table 4.1 Analysis of Variance for cold deficiencies of interest.

* indicates $p < 0.05$

Cold	<u>Both sexes</u>		<u>Female</u>	<u>Male</u>
<i>Deficiency</i>	<i>LxG</i>	<i>LxGxS</i>	<i>LxG</i>	<i>LxG</i>
Df(2R)ED1725	0.0281*	0.6628	0.0543	0.2346
Df(2R)ED2076	0.3800	0.0225	0.3101	0.0284*
Df(2L)ED1473	0.1148	0.2820	0.0144*	0.7606

Table 4.2 Analysis of Variance for heat deficiencies of interest.

* indicates $p < 0.05$

Heat	<u>Both sexes</u>		<u>Female</u>	<u>Male</u>
<i>Deficiency</i>	<i>LxG</i>	<i>LxGxS</i>	<i>LxG</i>	<i>LxG</i>
Df(2L)ED1317	0.2368	0.2347	0.0593*	0.2506
Df(2R)ED1770	0.09230	0.9230	0.2150	0.2506

Chapter 5 - The Identification of Novel Cold Tolerance Genes in *Drosophila*

Abstract

Temperature is an important factor that drives the dispersal and distribution of organisms. Temperature changes on a spatial and temporal scale and directly affects organismal fitness and survival. Many studies have characterized the adaptive significance of thermal stress phenotypes, but relatively little is known about the genetics of thermotolerance. In this study, *Drosophila melanogaster* was used to gain insight into the genetics of thermal phenotypes by taking advantage of its ubiquitous range across continents and abundant genetic tools. We continue work from our previous study focused on fine-mapping a cold tolerance QTL on the second chromosome of *Drosophila melanogaster*. We have now reduced the QTL to four novel candidate genes using an unbiased mutant complementation approach. We surveyed molecular variation in each of the candidate genes between the parental lines and assigned functional significance to a structural, nonsynonymous polymorphism via an association analysis between variation in cold tolerance and this focal polymorphism.

Introduction

Thermotolerance is a complex phenotype that is controlled by many genes that interact with the environment (Kristensen et al., 2008, Overgaard et al., 2008, Rako & Hoffmann, 2006, Mackay, 2001). Many studies have demonstrated the genetic complexity of thermal regulation

in nature, however our current understanding of the *individual* genes controlling natural variation in temperature-stress resistance is limited. This is an oversight given the central role environmental temperature has on organismal fitness, survival, and distribution of organisms across a landscape (Hoffmann & Parsons, 1991, Gilchrist & Huey, 1999, Gibert & Huey, 2001). Temperature has been shown to drive local adaptation among populations as evidenced by thermal clines across latitude or altitude (Cheviron et al., 2008, Ayres & Scriber, 1994, Zhen & Ungerer, 2008). Thermal phenotypes show a general trend across species; populations are generally more heat tolerant and cold susceptible in the tropics and at low altitudes, while temperate and high altitude populations are generally more cold tolerant and heat susceptible (Cheviron et al., 2008, Demont & Blanckenhorn, 2008, Gilchrist & Partridge, 1999, Hoffmann et al., 2002, Trussell, 2000). Although the adaptive significance of temperature stress phenotypes has been established, one of the primary objectives when studying such natural variation is to link phenotypic clines with the underlying genes contributing to local adaptation in nature.

Drosophila melanogaster is a powerful system for the dissection of the genetic basis of thermal adaptations in nature. *D. melanogaster* originated in Sub-Saharan Africa, but has subsequently colonized the majority of the globe and adapted to many novel local environments. Multiple studies have documented evidence of phenotypic clines in thermal phenotypes in Australia (Hoffmann et al., 2001), Europe (Ayrinhac et al., 2004), North America (Schmidt & Paaby, 2008) and South America (Fallis et al., 2012). Thus, resistance to thermal stress is a climatic adaptation on a global scale (Gibert & Huey, 2001, Hoffmann et al., 2002, Kimura, 2004).

Prior work on the genetics of thermal adaptation in *Drosophila* has included QTL mapping and candidate gene association studies (Clowers et al., 2010, Hoffmann et al., 2003b, Anderson et al., 2003, Morgan & Mackay, 2006). Candidate gene studies have yielded many interesting findings but the dissection of thermal *variation* via QTL analysis is essential, as these analyses focus directly on the genetic variation influencing phenotypic variation in nature. From these QTL studies many different thermal QTL have been mapped, which span all of the major chromosomes in the *Drosophila* genome (Svetec et al., 2011, Norry et al., 2008, Norry et al., 2004, Morgan & Mackay, 2006). Three of these studies (Norry et al., 2004, Norry et al., 2008, Morgan & Mackay, 2006) have independently identified a large effect QTL influencing cold tolerance on the second chromosome, at cytological position 37C5-48D5. This replicated QTL is interesting because the recombinant inbred lines (RILs) used in each study represent unique samples of natural genetic variation, with founder stocks from Denmark, Australia, Russia, and the United States. Thus, this replicated QTL suggests that alleles underlying this cold-stress resistance QTL are important for variation in cold-stress resistance and this variation segregates on a global scale.

In a previous study, we fine mapped this QTL (11 MB; at cytological position 37C5-48D5) on the second chromosome to three small candidate regions associated with chill-coma recovery (Fallis et al., *submitted*). In the current study we follow up on the deficiency mapping study to move from candidate regions to candidate genes. The power of this design is that we have isolated each candidate region (Morgan & Mackay, 2006, Fallis et al., *submitted*) in an unbiased manner. We identify four novel cold tolerance candidate genes from the three regions isolated in Fallis et al. (*submitted*). We sequenced all candidate genes to identify polymorphisms

that differentiate the two parental lines from one another. The gene *lsm10* emerged as an interesting candidate as it contained a non-synonymous polymorphism that differentiated the parental genotypes and was associated with natural phenotypic variation in cold tolerance.

Materials and Methods

Phenotypic assays

All stocks used in assays were maintained on common cornmeal-molasses fly food at 25°C and on a 12-hr light/dark cycle. Flies used in screens were 5-7 days old and kept at equal densities until use. Assays were conducted by transferring 10 same-sex individuals of each genotype, without the use of anesthesia, to empty shell vials immediately before they were subjected to cold stress. To measure chill-coma recovery, individuals were placed at 0°C for a 3-hr period. Upon removal from the cold, flies were placed at room temperature (23°C ± 0.5°C) and allowed to recover from chill coma (i.e. able to stand on their legs). The chill-coma recovery score was quantified as the number of flies recovered from cold exposure within an 11-min time period. The 11-min time interval represents the 50% chill-coma recovery time point for the parental lines (Oregon-R and 2B-3; Morgan and Mackay 2006).

Stocks

The parental lines used to test the candidate genes in the three candidate cold regions on chromosome II are the same recombinant inbred lines used to identify the thermotolerance QTL (Morgan & Mackay, 2006) and fine-map the QTL to three candidate cold regions (Fallis et al.,

submitted). Oregon-R and 2B-3 were created from naturally derived flies lines collected from Oregon and Russia, respectively (Lindsley & Zimm, 1992, Pasyukova & Nuzhdin, 1993). Neither Oregon-R nor 2B-3 has been selected for thermotolerance phenotypes, however 2B-3 has been selected for reduced male mating activity. Individual lines are homozygous at all loci with Oregon-R and 2B-3 having significantly different percent recovery from chill coma (Morgan and Mackay 2006). Oregon-R is tolerant to cold, while 2B-3 is susceptible to cold. Although, the parental lines represent a very limited sample of the naturally occurring allelic variation in nature they are useful in determining loci that may be important on a cosmopolitan level.

All available single-gene mutants (35 in total) underlying the three significant deficiency regions (Fallis et al. *submitted*) were obtained from Bloomington Stock Center (Bloomington, IN). All mutant lines have null mutations in the gene of interest and are part of the larger Exelixis collection (Harvard University; Table 5.1).

Crosses

The crossing scheme paired virgin females of each parental line, Oregon-R and 2B-3, with males from each of the 35 mutant stocks (Table 5.1). Crosses were assembled using five females and five males per cross to control for progeny density. Nearly all of the mutations were homozygous viable stocks and thus resulted in a single, heterozygous genotype (Oregon/mutant or 2B-3/mutant), when crossed to the two parental lines. A second cross was used to evaluate the effect of the genetic background on the mutant by parental line cross. For this set, each parental line was crossed to the isogenic background within which the mutant stock was created.

This yields a second set of genotypes (Oregon/wildtype and 2B-3/wildtype) and can be directly compared to the mutant cross. All four genotypes, Oregon/mutant, Oregon/background, 2B-3/mutant, 2B-3/background, were contemporaneously phenotyped for cold tolerance.

Mutant tests and analysis

To test the phenotypic contribution of each mutation, mutant quantitative complementation tests were used (Jordan et al., 2006, De Luca et al., 2006). The approach tests for quantitative failure to complement, and thus shows evidence that the gene disrupted by a known mutation contains allelic variation affecting the trait between lines. This method assumes that there are different QTL alleles at a locus affecting the trait in each parental line, Oregon-R and 2B-3. These lines (*Oregon/Oregon* & *2B-3/2B-3*) are crossed to a non-functional mutant in the gene of interest (Table 5.1). This cross of the parental line to the mutant and the parent line and the co-isogenic background genotype yields four genotypes: *Oregon/Mutant*, *Oregon/Background*, *2B-3/Mutant*, *2B-3/Background*. Complementation mapping is simply testing whether there is an interaction between the genotype (*Mutant* or *Background*) and the line (*Oregon* or *2B-3*), that is, $(Oregon/Mutant - 2B-3/Mutant) - (Oregon/Background - 2B-3/Background)$ must be significantly different from zero. The QTL allele in question must perform differently when combined with the *Mutant* allele than when combined with the functional *Background* allele. This comparison of four genotypes allows differences in segregation of other parental line QTL to be eliminated, because these are the differences that persist between the lines in either genetic background (*Mutant* or *Background*). Thus, allelic differences uncovered by the mutation result in quantitative failure to complement, which is indicated when the phenotypic difference between the two parental lines over the mutant alleles

is greater than the parental lines over a functional (*Background*) allele. That is, the expression $(Oregon-R/Background - 2B-3/Background) < (Oregon-R/Mutant - 2B-3/Mutant)$ must be statistically significant.

To statistically demonstrate quantitative failure to complement, we used mutant-specific analysis of variance (ANOVA) via the following three-way ANOVA model: $y = \mu + L + S + G + L \times S + L \times G + G \times S + L \times G \times S + R(L \times G \times S) + \epsilon$, where y is percent recovery from chill coma, L , G , and S are the fixed effects of parental lines (Oregon-R or 2B-3), genotype (*Mutant* or *Background*), and sex, while R is the random effect of replication nested within the line, genotype, and sex and ϵ is error. Failure to complement was indicated when the $L \times G$ or $L \times G \times S$ term was significant ($p < 0.05$).

Sequencing candidate genes

To identify polymorphisms that differentiate the parental lines in the candidate mutations that exhibit quantitative failure to complement, we extracted DNA from five Oregon-R and five 2B-3 males using the Puregene DNA isolation kit (Gentra Systems). Specific candidate genes were amplified and sequenced using primers designed from the *Drosophila* reference genome (<http://www.flybase.org>) and the Primer3 website (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). All candidate genes were amplified using 20ul PCR reactions (11ul ddH₂O, 2ul Mango buffer, 1ul MgCl₂, 1ul dNTP, 1ul forward primer, 1ul reverse primer, 2ul DNA, and 1ul Mango Taq) and standard PCR profile (initial denaturation at 96°C for 15 seconds, annealing at 56.5°C for 30 seconds, and extension at 72°C for 30 seconds, cycled 30 times and a final extension step at 72°C for 5 minutes). All candidate genes

were sequenced in both directions by the AGTC Sequencing Center (University of Kentucky). Sequence reads were assembled into contigs using SeqMan Pro (DNA* Lasergene suite) and aligned using MUSCLE (<http://www.ebi.ac.uk/Tools/muscle/index.html>). Individual polymorphisms were identified and translated using Jalview (Clamp et al., 2004, Waterhouse et al., 2009).

SNP-cold tolerance associations

The identification of a pattern from quantitative failure to complement of a known mutation, coupled with a polymorphism between the parental lines in the same gene, is compelling evidence for a link between genes and phenotype. However, to expand these results to natural populations we also tested if an association exists between candidate polymorphisms and natural variation in cold tolerance within an independent population of natural genotypes. To do this the *lsm10* SNP was tested for association with percent recovery from chill coma in the community resource, the *Drosophila* Genetic Reference Panel (DGRP) (Mackay et al., 2012). The DGRP is a panel of natural inbred lines created from wild-caught females in a single Raleigh, NC population. Wild-caught females were used to initiate, isofemale lines, which were then inbred via full-sib mating for 20 generations. This resulted in 192 isogenic lines, with limited to no variation within lines, but abundant variation among lines. In addition to representing a community sample of natural phenotypic variation, the DGRP is also a fantastic genomic resource as nearly all of 192 lines have been sequenced, annotated, and has all of the segregating polymorphisms identified (Mackay et al., 2012). To test for an association between the non-synonymous SNP in *lsm10* and natural variation in cold tolerance, we downloaded the genotypes for the seven SNPs that were segregating in the DGRP within the gene *lsm10* and

paired the mean percent recovery from chill coma data for each line (Clowers et al., 2010). Each of the seven SNPs was tested for its association with natural variation in chill-coma recovery by a simple SNP specific analysis using the following model: $y = \mu + \text{SNP} + \text{Sex} + \text{SNP} \times \text{Sex} + \epsilon$, where y is the line and sex specific percent recovery from chill coma, μ is the overall mean, SNP and Sex are the fixed effects of the different alleles and sexes, respectively, while ϵ is the residual error. In this genotype-phenotype association we are testing a specific hypothesis about the focal non-synonymous SNP that differentiates the two parental lines from one another, as a result of this we do not correct for multiple tests as is commonly done in gene (Clowers et al., 2010) or genome-wide (Mackay et al., 2012) association studies.

Results

Our results further refine the three cold candidate regions identified in our previous work (Fallis et al. *submitted*), by testing all of the available mutations in these three cold tolerance candidate regions (Table 5.1). From 35 cold candidate mutant complementation tests, we identified four cold tolerance candidate genes that influence variation in percent recovery from chill coma between the parental lines.

Mutant Complementation Tests

We performed thirty-five mutant complementation tests by crossing mutations and the genetic background stock to the parental lines Oregon-R and 2B-3. Individual candidate genes were identified by the combination of percent recovery from chill coma values that are consistent

with quantitative failure to complement and a significant interaction between parental line (Oregon-R or 2B-3) and genotype (Mutant or Background) (Table 5.2; Figure B.1). Specifically, mutations were nominated if the LxG term or LxGxS were significant in the mutant specific analysis and the expression $(Oregon-R/Background - 2B-3/Background) < (Oregon-R/Mutant - 2B-3/Mutant)$ was satisfied. Four genes of the thirty-five mutations satisfied both of these criteria and thus, are thought to harbor allelic variation influencing percent recovery from chill coma between the parental lines (Table 5.2). Three of the significant mutations were located between cytological positions 47C1 and 47C5; these include *CG30016* (p=0.0402), *Taf5* (p=0.0107), and *lsm10* (p=0.0034) (Figure 5.2A-C; Table 5.2). A single candidate cold tolerance gene *CG8791* (p=0.0524) was identified at cytological position 43F2 (Table 5.2).

Little is known about these candidate mutations. *CG30016* has unknown molecular function, but has gene ontology term inferring a biological process involved in transport (FlyBase et al., 2004). *CG8791* is thought to be involved in phosphate sodium symporter activation, although this is based completely on sequence similarity and has biological function inferred to be involved in transmembrane transport (FlyBase et al., 2004). *lsm10* also has unknown molecular function, but has experimental evidence indicating it has a role in mRNA processing (Godfrey et al., 2009, Pillai et al., 2001). *Taf5* is the only gene to have both molecular and biological process information based on experimental data, and functions as part of transcription regulation in eukaryotes (Dymlacht et al., 1991).

Sequence Analysis

To identify the polymorphisms that differentiate the Oregon-R and 2B-3 genotypes from one another, we sequenced the transcribed regions (i.e., exons, introns and 5' and 3' UTRs) plus 100 bp upstream and downstream of the four candidate genes. All candidate genes have simple gene structure with *CG30016*, *Taf5*, and *lsm10* all having a single exon, ranging in size from about 500 nucleotides (*CG30016* and *lsm10*) to slightly over 2kb (*Taf5*) and are positioned in reverse orientation. *CG8791* has three small exons, but total gene size is small (about 1.5kb; 1819nt). Three of the four have synonymous changes that differentiate the parental lines. *CG8791* has five synonymous changes in exon two, *Taf5* has ten synonymous changes scattered through out its coding region, and *CG30016* has one synonymous change. The only gene with a nonsynonymous change was *lsm10*, which was an A to T change near the 5' end of coding region. Oregon-R has the T allele, corresponding to amino acid Histidine, while 2B-3 has the A allele and changes the amino acid to Leucine. Histidine is a polar molecule with a net positive charge, while Leucine, is nonpolar and has no net charge. Thus, these changes in amino acid sequence may influence the function or folding of the *lsm10* protein. Based on a recent study, *lsm10* is part of a ring of proteins involved in the initiation complex of mRNA processing (Godfrey et al., 2009). Thus, the shape of the *lsm10* protein is very specific and important to the function of the complex. Any change in amino acid may affect the resulting protein and the overall phenotype.

lsm10 SNP Association

To determine if the non-synonymous polymorphism in *lsm10* is associated with natural variation in cold tolerance, we used the *Drosophila* community resource, the DGRP, which

represents an independent set of natural genotype and phenotype data. Within the DGRP the polymorphism (A/T) that differentiates Oregon-R from 2B-3 was segregating among 162 lines. The A allele was at much lower frequency (4.938%), than the T allele (95.062%). The polymorphism was significantly associated with variation in percent recovery from chill coma ($p=0.0193$), where individuals with the A allele had a 74.198% mean percent recovery from chill coma, while the T allele had a 48.837% mean percent recovery from chill coma (Figure 5.3A). We also tested the associations between six neighboring polymorphisms in *lsm10* that were present in the DGRP but not segregating between Oregon-R and 2B-3. None of the six neighboring SNPs in *lsm10* were significantly associated with percent recovery from chill-coma (Figure 5.3B) and the strongest association with percent recovery from chill coma was at the non-synonymous change identified between Oregon-R and 2B-3.

Discussion

In this study, we have fine mapped a cold tolerance QTL on *Drosophila's* second chromosome to individual genes via a series of mutant complementation tests. This 2nd chromosome cold tolerance QTL is significant, as it has been identified in studies from multiple independent recombinant inbred line sets, each containing genetic variation sampled from around the globe (Norry et al., 2004, Norry et al., 2008, Morgan & Mackay, 2006). Thus, identifying the individual genetic changes responsible for this replicated cold tolerance QTL advances our understanding of the genetic basis of thermal adaptation on a worldwide scale. We have previously fine-mapped this cold tolerance QTL to three small regions affecting variation in percent recovery from chill coma (Fallis et al. *submitted*) via deficiency complementation tests.

In this study we tested all of the available mutations within the three regions and identify four novel thermal candidate genes affecting natural variation in chill-coma recovery time. The discovery that a single QTL fractionates into multiple causal regions and then multiple causal genes is not surprising; many other studies on *Drosophila* lifespan (De Luca et al., 2006), behavior (Jordan et al., 2006), and bristle number (Gurganus et al., 1999). Our results are consistent with these previous studies that demonstrate a large effect QTL is often the result of multiple linked genes with additive phenotypic effects in the same direction across loci.

The four genes that exhibit quantitative failure to complement and likely harbor allelic variation affecting variation in percent recovery from chill coma are *CG30016*, *CG8791*, *Taf5*, and *lsm10* (Table 5.2; Figure 5.2A-D). All four of these genes are novel cold tolerance genes in that none of them have previously been associated with the genetic control of any thermal phenotype. Although each of these candidate genes are novel, the known or inferred function of each candidate provides some insight into the link between genetic variation and natural variation in cold tolerance between Oregon-R and 2B-3. For example *CG30016* and *CG8791* are both computation predicted coding genes, which are both thought to be involved in the transport of molecules across the cell membrane based on sequence comparisons. This is significant because the movement of ions and molecules across the membrane has been found to be physiologically important in cell homeostasis, especially in regards to cold temperature stress in other species of insects (Hochachka, 1986, Kostal et al., 2007, Kostal et al., 2004). The other two candidate cold genes are involved in transcription or mRNA processing and have less clear links with the whole organism response to cold. *Taf5* functions broadly in the regulation of transcription (Dymlacht et al., 1991), while *lsm10* is a 14KDa protein previously involved in

mRNA processing (Pillai et al., 2001). *Lsm10* is part of the U7 small nuclear ribonucleoprotein complex (U7 snRNP), which plays an essential role in histone pre-mRNA processing (Godfrey et al., 2009).

Although we are the first to fine-map the 2nd chromosome of the *Drosophila* genome to individual cold tolerance genes, our results are complemented by a growing body of candidate thermotolerance loci. These include, *Hsr-omega* and *hsp68* that have been associated with variation in heat stress and cold tolerance (Anderson et al., 2003, McColl et al., 1996). As well as multiple studies that have documented segregating genetic variation and associations have also been identified between cold tolerance and many candidate genes; including *Frost*, *SMP-30*, *HSP70*, *desaturase 2* and *CG16700* (Goto, 2000, Goto, 2001, Daibo et al., 2001, Anderson et al., 2003, Greenberg et al., 2003, Qin et al., 2005, Clowers et al., 2010, Svetec et al., 2011, Rako et al., 2007, Colinet et al., 2010). These studies and our work have demonstrated a link between candidate genes and thermal phenotypes, however to identify the link between true *natural* genetic variation and *natural* phenotypic variation in cold tolerance it is essential to test for genotype-phenotype associations in nature.

We sequenced the transcriptional unit (i.e., the 5' UTR, exons, introns, and 3' UTR) plus 100 basepairs upstream and downstream for all four cold candidate genes in the parental lines. For three (*CG30016*, *CG8791*, *Taf5*) of four genes sequenced we identified multiple polymorphisms that differentiated the Oregon-R and 2B-3 parental genotypes from one another, however all of these polymorphisms were synonymous and thus do not influence the protein between Oregon-R and 2B-3. In contrast *lsm10* had a single nonsynonymous change near the 5'

end of coding region resulting in a change from a Histidine to a Leucine. Thus, this modification to the amino acid sequence likely influence the function of the *lsm10* protein. Including the role *lsm10* plays in the formation of a ring of proteins involved in the initiation complex of mRNA processing (Godfrey et al., 2009). This nonsynonymous change is very exciting as it provides a possible link between a structural genetic change and the whole organism thermal phenotype.

To test this link between the nonsynonymous change in *lsm10* and cold tolerance we leveraged the DGRP lines (Mackay et al., 2012), which is a fully sequenced community resource for association genetics. We tested the genotype-phenotype association at this focal nonsynonymous change in *lsm10*. There were seven polymorphisms in *lsm10* that were segregating within the DGRP lines, of these seven, this A/T nonsynonymous change was the only polymorphism significantly associated with natural variation in the percent recovery from chill coma.

Altogether this study demonstrates the genetic basis of this replicated cold tolerance QTL is not simple and represents the combined effects of at least four genes. The genetic mechanisms mediating the allelic effects on differences in cold tolerance are diverse and likely involve a combination of regulatory and structural changes. Finally, these results suggest that mutations identified by the comparison of ecologically important phenotypes and laboratory genotypes can reveal important polymorphisms affecting natural variation in cold tolerance.

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Figures and Tables

Figure 5.1 Significant deficiencies.

Deficiencies are represented by the grey numbered bars. Blue colored bars are significant for chill-coma recovery time.

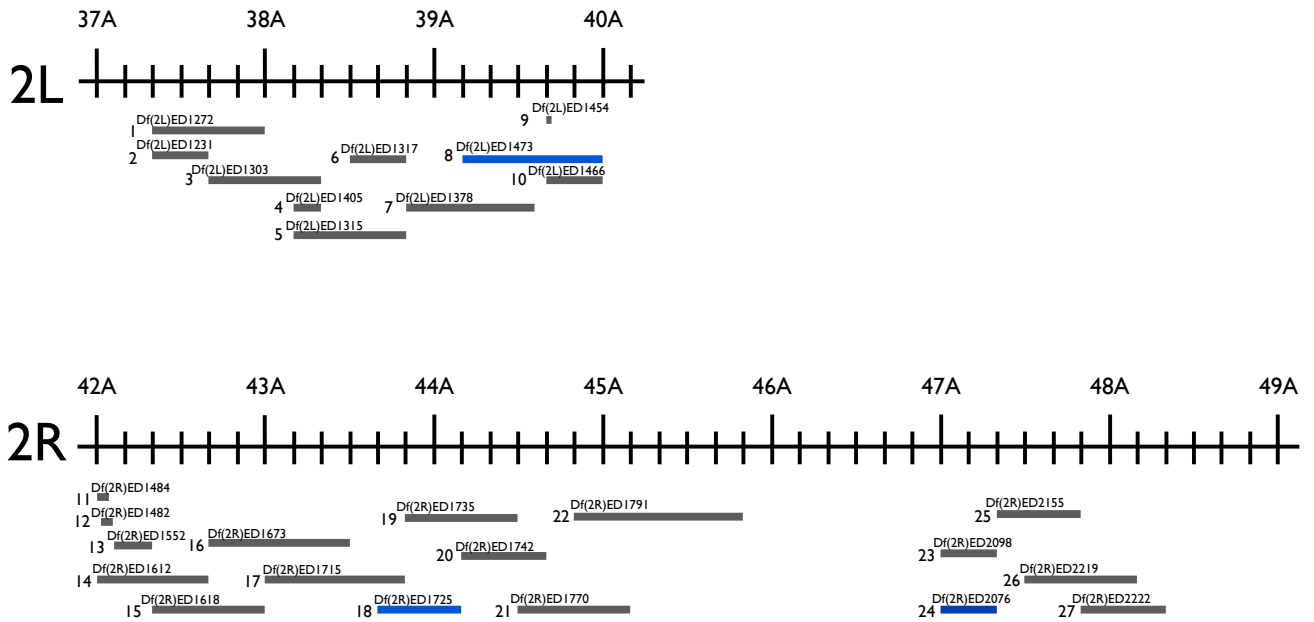


Figure 5.2 Complementation Plots.

Test plot for candidate genes A. *CG8791* (LxG p=0.0524), B. *lsm10* (LxG p=0.0034), C. *CG30016* (LxG p=0.0402), D. *Taf5* (LxG p=0.0107). Oregon-R is represented by solid red lines and 2B-3 is represented by dashed blue lines.

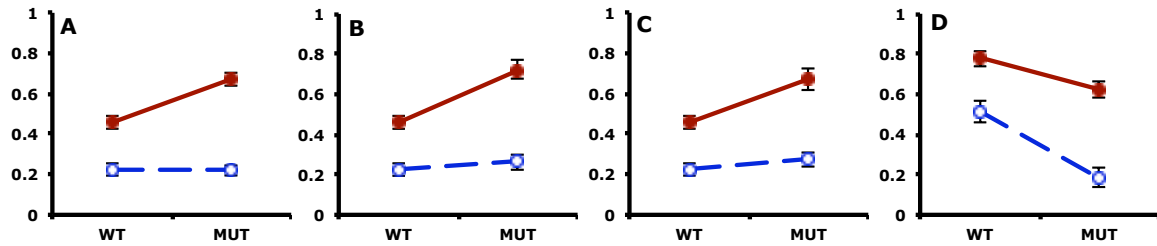
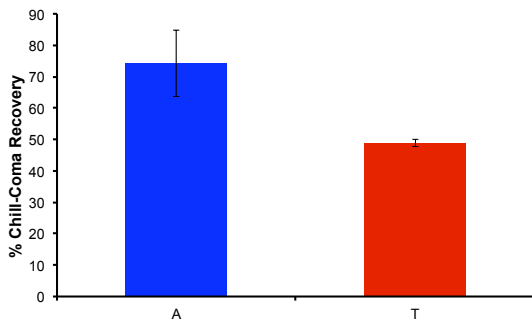


Figure 5.3 DGRP SNP associations.

A. Significant association in *lsm10* A/T SNP and the DGRP ($p=0.0193$). SNP A, found in parental line 2B-3, associated with faster recovery (74.198% in 11 min) compared to SNP T, found in Oregon-R (48.837% in 11 min). B. We tested all SNPs found within *lsm10* found in the DGRP. Only the A/T SNP we found was significant in this test.

A.



B.

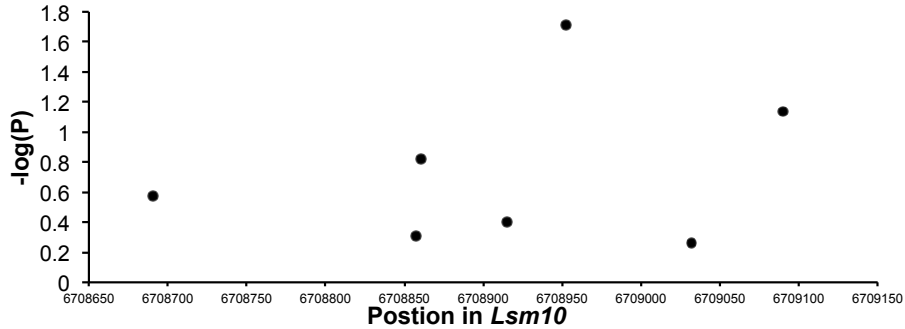


Table 5.1 List of genes underlying each significant deficiency that were tested via mutant complementation tests.

Number in the left column refers to the significant deficiency for cold tolerance in Figure 1.

Deficiency	Gene	Molecular function	Biological function			
18	Df(2R)ED1725	<i>nito</i>	mRNA binding	Nuclear mRNA slicing		
		<i>CG2915</i>	Metalloprotease activity	Proteolysis		
		<i>CG30379</i>	N-methyl-D-aspartate	Unknown		
		<i>rnh1</i>	Ribonuclease H activity	Unknown		
		<i>CG8791</i>	Phosphate sodium symporter activation	Transmembrane transport		
		<i>Kdm4a</i>	Histone demethylase	Histone H3-K36 demethylation		
		<i>CG30377</i>	Unknown	Unknown		
		<i>Lin19</i>	Ubiquitin protein ligase binding	Ubiquitin protein ligase dependent protein catabolic process		
		<i>CG30381</i>	Unknown	GPI anchor biosynthetic process		
		<i>CG14764</i>	Unknown	Unknown		
		<i>trsn</i>	Endoribonuclease activity	RNA interference; protein stabilization; jump response		
		<i>CG17765</i>	Calcium ion binding	Unknown		
		24	Df(2R)ED2076	<i>lola</i>	Protein binding	Anatomical structure development; cellular component organization or biogenesis; locomotion; multi-organism process; sensory organ development; organ morphogenesis; immune response; behavior; neuromuscular process; behavioral interaction between organisms; cell projection organization; biological regulation; cellular process involved in reproduction
				<i>psq</i>	Sequence-specific DNA binding	Olfactory behavior; embryonic pattern specification; regulation of chromatin silencing; imaginal disc-derived wing morphogenesis; anterior/posterior axis specification, embryo; chromatin silencing
<i>stan</i>	Receptor signaling protein activity			Neuron differentiation; cellular component organization or biogenesis; biological regulation; system development; neuron projection development; establishment of planar polarity; sensory organ development; regulation of developmental process; cell adhesion; embryonic pattern specification; compound eye photoreceptor development		
<i>CG12934</i>	Unknown			Unknown		
<i>Caf1-105</i>	DNA binding			Chromatin assembly or disassembly		
<i>Mat1</i>	Cyclin-dependent protein kinase activity; RNA polymerase II carboxy-terminal domain kinase activity			Transcription initiation from RNA polymerase II promoter		
<i>CG12338</i>	D-aspartate oxidase activity			Oxidation-reduction process		
<i>CG7220</i>	Ubiquitin-protein ligase activity			Proteolysis		
<i>CG33144</i>	Zinc ion binding			Unknown		
<i>Lsm10</i>	Unknown			mRNA processing; histone mRNA 3'-end processing		
<i>Taf5</i>	Sequence-specific core promoter binding RNA polymerase II transcription factor activity involved in preinitiation complex assembly			Positive regulation of transcription from RNA polymerase II promoter; neurogenesis		
<i>CG30016</i>	Unknown			Transport		
<i>CG12344</i>	Unknown			mRNA processing		
<i>CG30015</i>	RNA pol II transcription factor			Positive regulation of transcription of RNA pol II		
<i>CG30020</i>	Unknown			Transport		
<i>cag</i>	DNA binding			Unknown		
<i>CG12343</i>	Unknown			Neurogenesis		
<i>whd</i>	Carnitine O-palmitoyltransferase activity			Response to starvation; response to oxidative stress; response to metal ion		
<i>dgo</i>	Unknown			Establishment of planar polarity; establishment of imaginal disc-derived wing hair orientation; establishment of ommatidial planar polarity		
<i>cg11919</i>	Nucleoside-triphosphatase activity; ATP binding			Peroxisome organization		
<i>nclb</i>	Chromatin DNA binding			Germ-line stem cell maintenance; ovarian follicle cell development; ovarian fusome organization; male gonad development; regulation of transcription from RNA polymerase II promoter; germ cell development		
<i>wde</i>	Unknown			Unknown		
<i>CG12943</i>	Amino acid transmembrane transporter activity			Amino acid transmembrane transport		

Table 5.2 Analysis of Variance for candidate genes of interest

* indicates $p < 0.05$

Cold	<u>Both sexes</u>		<u>Female</u>	<u>Male</u>
<i>Gene</i>	<i>LxG</i>	<i>LxGxS</i>	<i>LxG</i>	<i>LxG</i>
<i>CG8791</i>	0.0524*	0.9641	0.1611	0.1736
<i>Lsm10</i>	0.0034*	0.4169	0.0119*	0.1160
<i>CG30016</i>	0.0402*	0.5631	0.3315	0.0416*
<i>Taf5</i>	0.0107*	0.0590*	0.0012*	0.6497

Chapter 6 - Conclusions

Temperature is a critical environmental parameter, which varies on many spatial and temporal scales. This environmental variation has well-defined influences on patterns of local adaptation and species distributions across natural landscapes. For organisms that are isothermal with their environment, variation in temperature influences physiology, behavior, fitness and directly impacts the whole organism responses to stress. Thus, for species to thrive across a range of variable thermal environments populations must contain sufficient genetic variation, the capacity to respond plastically, or some combination of both genetic and plastic responses. In this work I first quantified patterns of phenotypic and genetic variation for thermal traits in nature and then dissected the genetic basis of variation in thermal traits.

To address the first two sets of questions, I measured phenotypic variation in heat stress phenotypes using *Drosophila melanogaster* populations collected across a latitudinal gradient in South America. I found significant variation within and among populations and the variation among populations was best explained by the maximum mean annual temperature for each location. To further explore the environment's role in adaptation, I measured the degree of cold tolerance plasticity in the South America populations by rearing flies at two developmental temperatures (18°C and 25°C). Interestingly, populations from higher altitudes, which face greater fluctuations in temperature, had higher levels of cold tolerance plasticity than did populations from low altitudes, which encounter more stable annual temperatures. Fitness measurements (egg-to-adult viability) on the most and least plastic populations indicate populations have equal fitness when temperatures were held constant. However, the high-

plasticity population had higher fitness than the low-plasticity population in multiple fluctuating thermal environments (different day and nighttime temperatures). Thus, there is not only significant variation in the degree of cold tolerance plasticity within and among populations in nature, but cold plasticity is likely an adaptive response to environmental variation.

The final two chapters focused on understanding the genetic basis of thermal variation. The motivation for this work comes from a set of *D. melanogaster* QTL studies that found a co-localized peak for heat and cold tolerance in the middle of the 2nd chromosome. I fine-mapped this co-localized QTL via deficiency complementation mapping. There was no overlap of the deficiencies associated with cold or heat stress resistance, indicating that in this region of the genome heat and cold stress genes are independent, but arranged closely along the chromosome. I next fine-mapped the genetic basis of cold stress candidate regions using mutant complementation tests, revealing four novel cold stress genes. Sequence analysis of each gene identified the polymorphisms that differentiate the lines. Candidate gene *lsm10* has a nonsynonymous change that segregated between the two parental lines. To test for independent associations between these polymorphisms and variation in nature, the *Drosophila* Genome Reference Panel was used. Future work is needed to functionally test the specific role of *lsm10* in cold tolerance in nature.

This work has made significant contributions to the field of thermal biology. It has identified thermal phenotype clines in South America, a continent previously lacking from thermal cline studies, and has linked phenotypic clines to environmental parameters. It has demonstrated the adaptive significance of phenotypic plasticity in thermal traits in variable environments, as opposed to stable environments, and has contributed to our understanding of

the benefits and costs of phenotypic plasticity. This work has also demonstrated the complex, yet independent, genetic architectures that underlie different thermal phenotypes (i.e., heat and cold tolerance). Finally, this work has advanced the current list of cold tolerance candidate genes, as I have added four novel genes contributing to the underlying genetic architecture of this complex phenotype.

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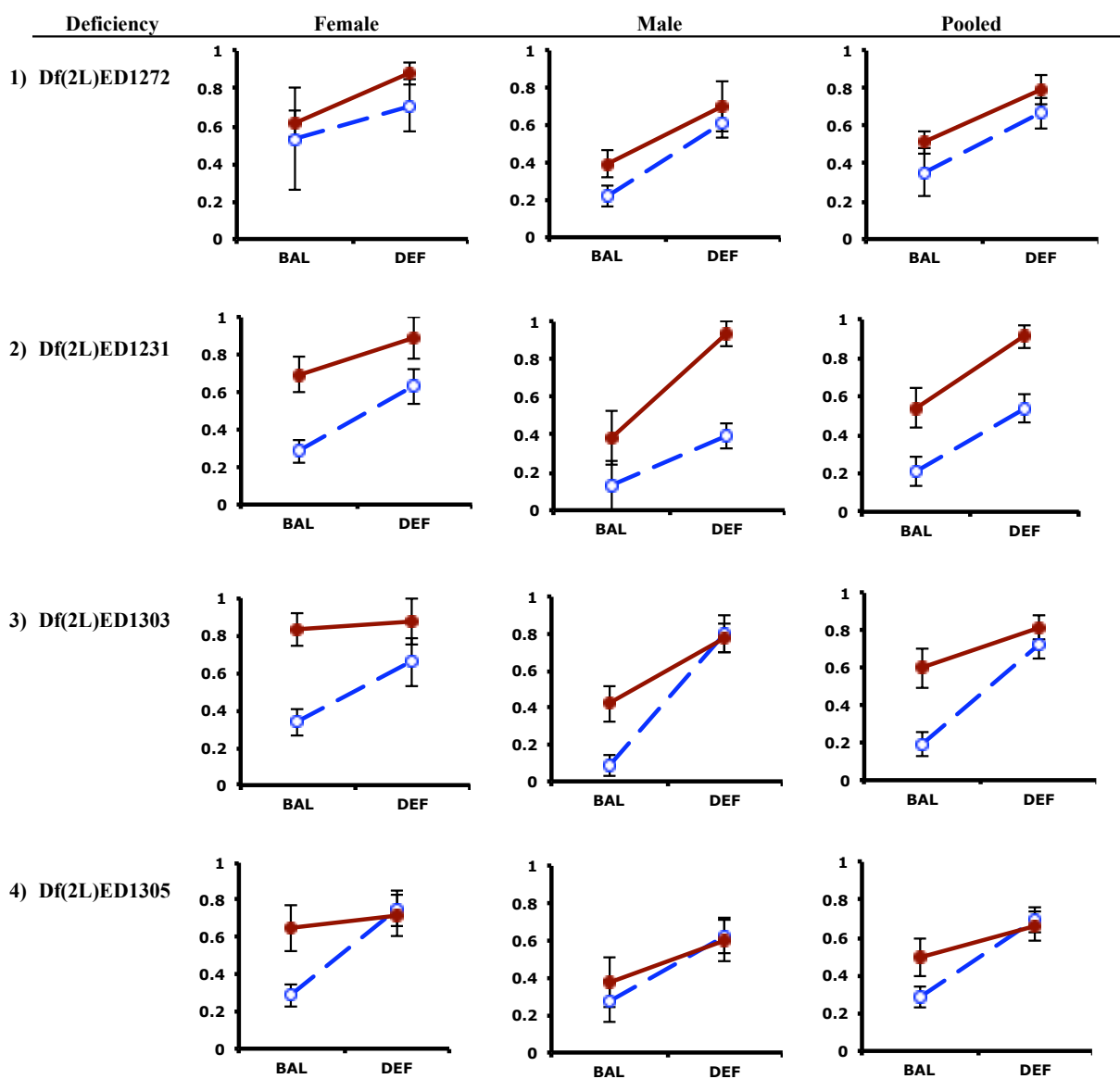
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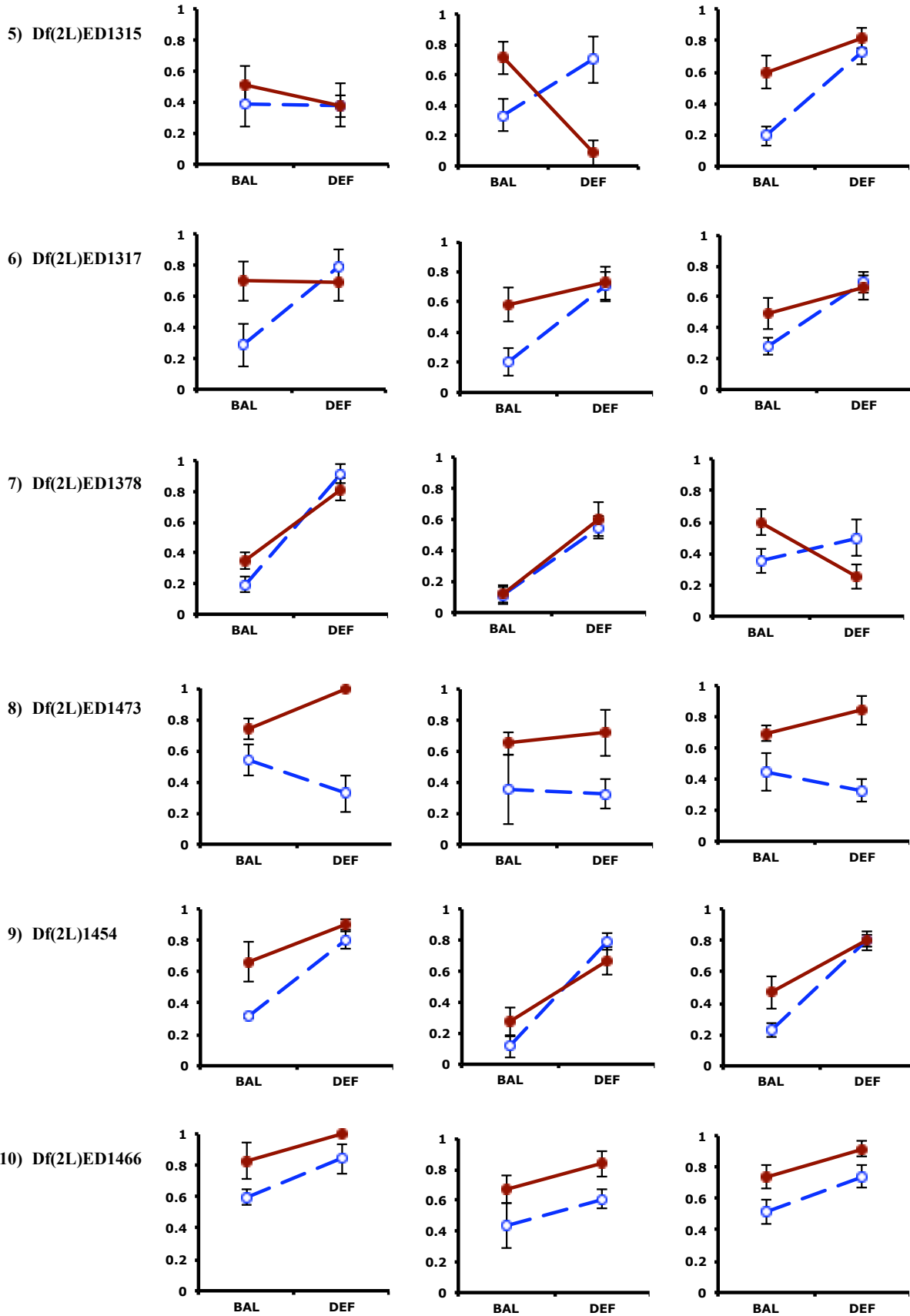
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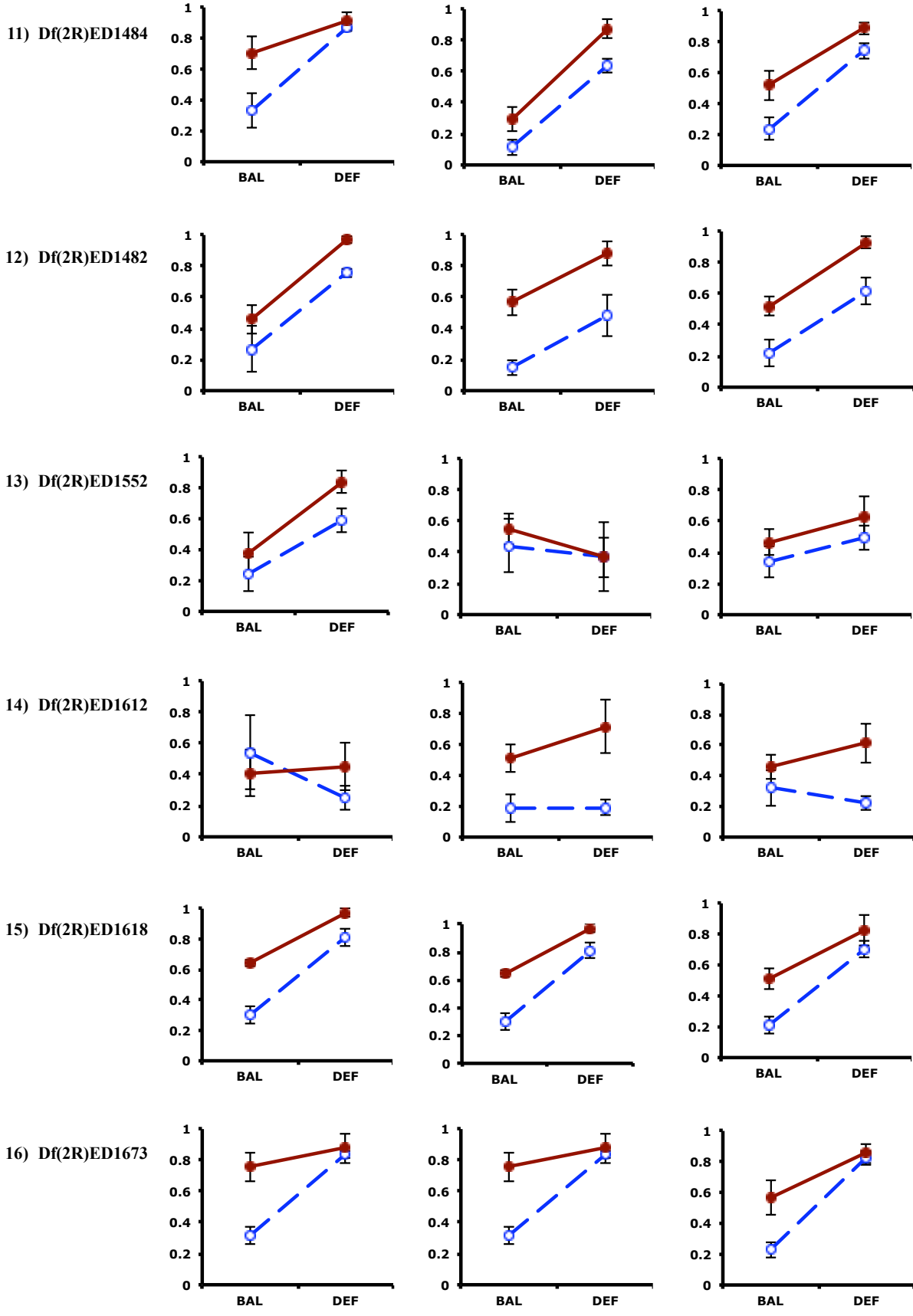
Figures and Tables

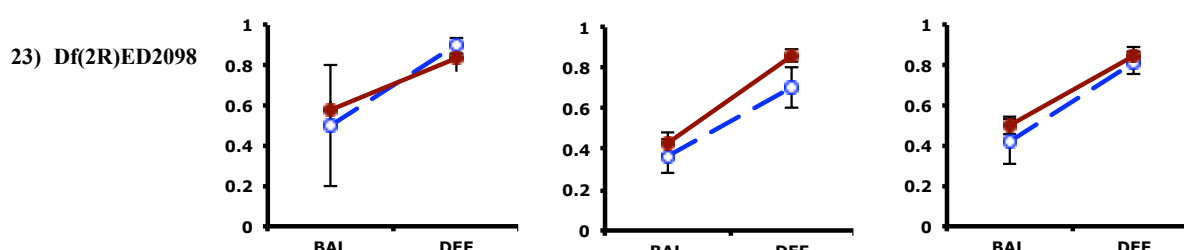
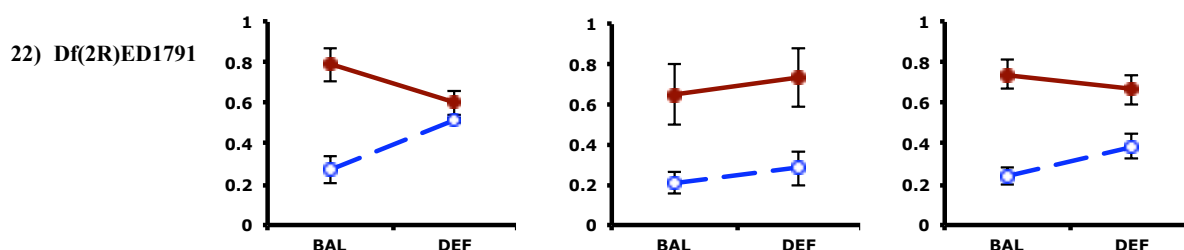
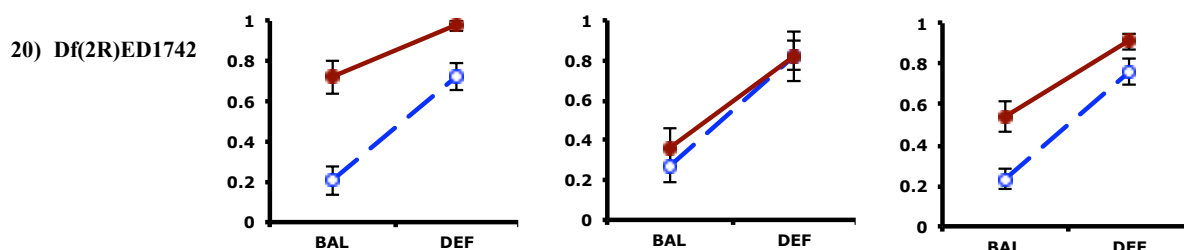
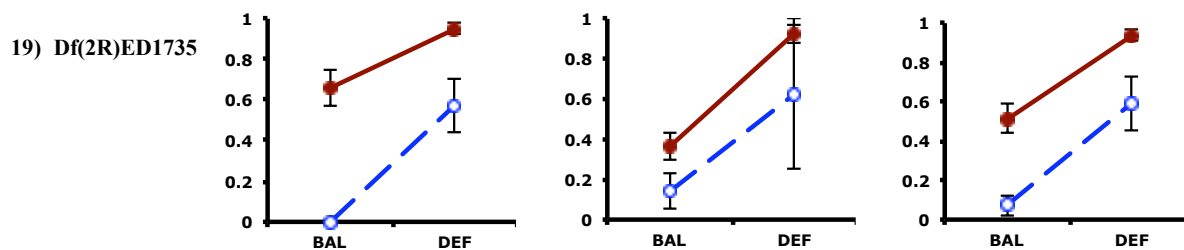
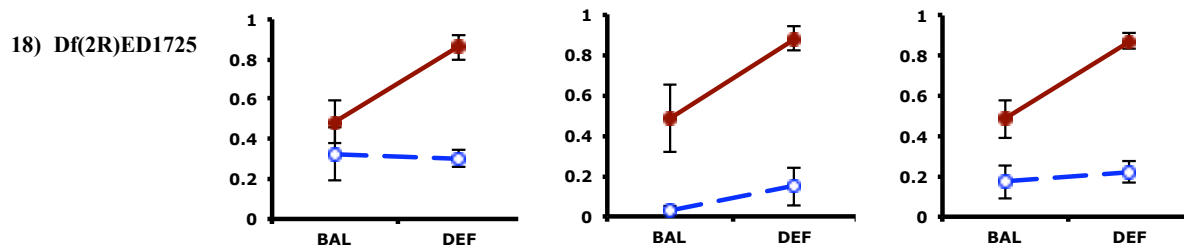
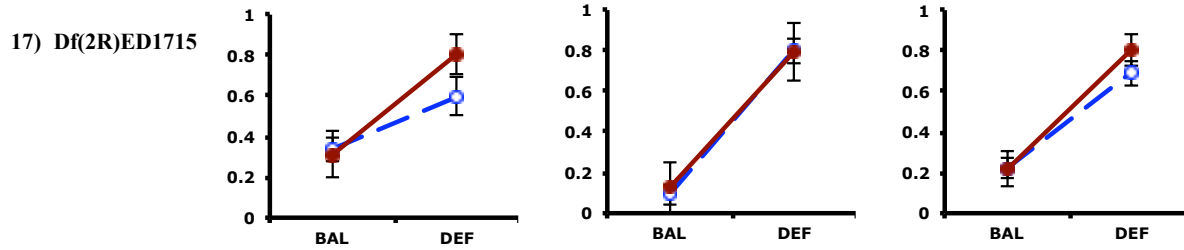
Figure A.1 Cold tolerance quantitative complementation plots for all tested deficiencies.

The number in the left column is consistent with numbers in Appendix A Table 1. Failure to complement is indicated by the exacerbated difference between the phenotype of the parental lines when over the deficiency line. Red solid lines represent parental line Oregon –R and dashed blue lines represent parental line 2B-3.









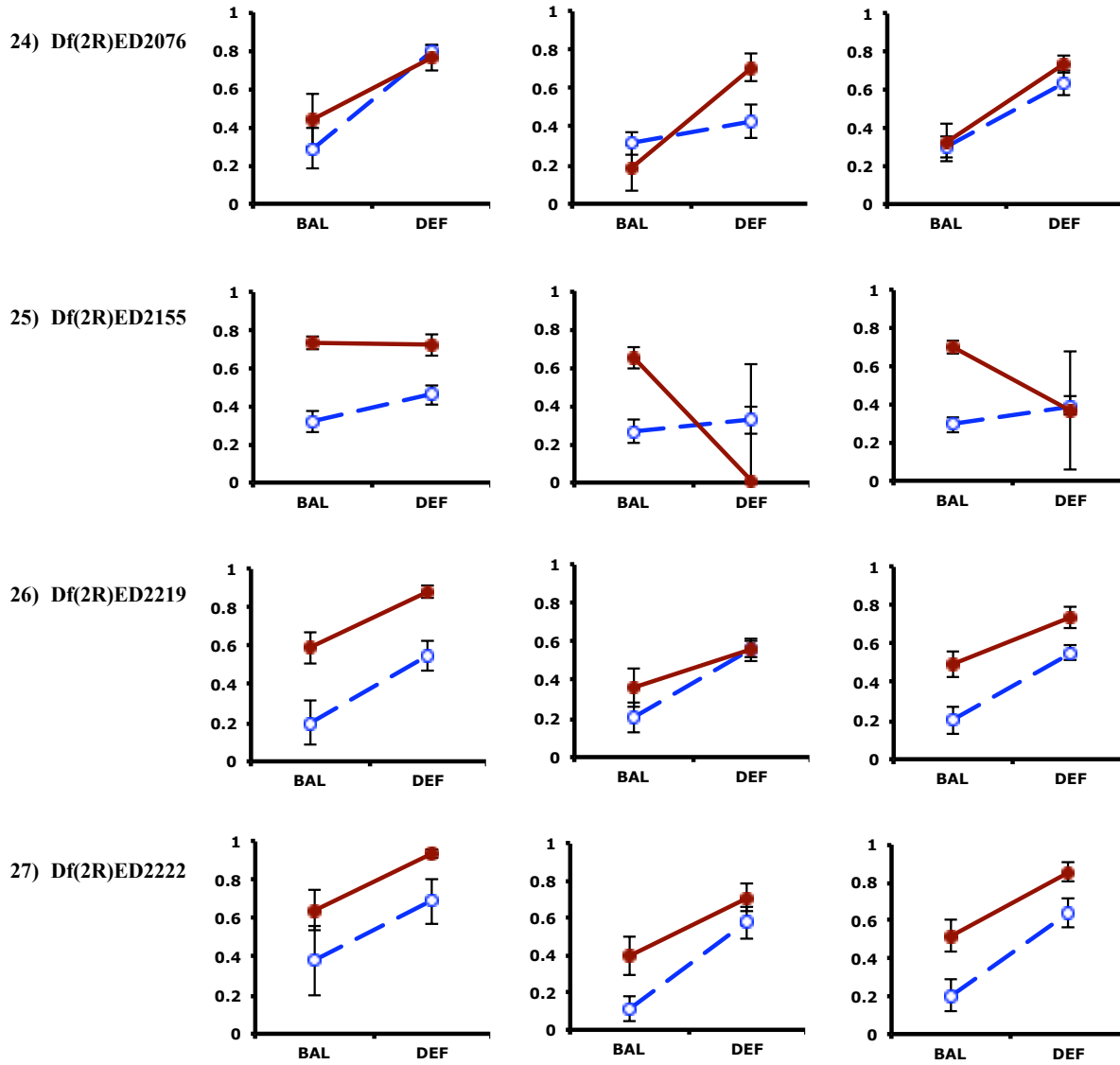
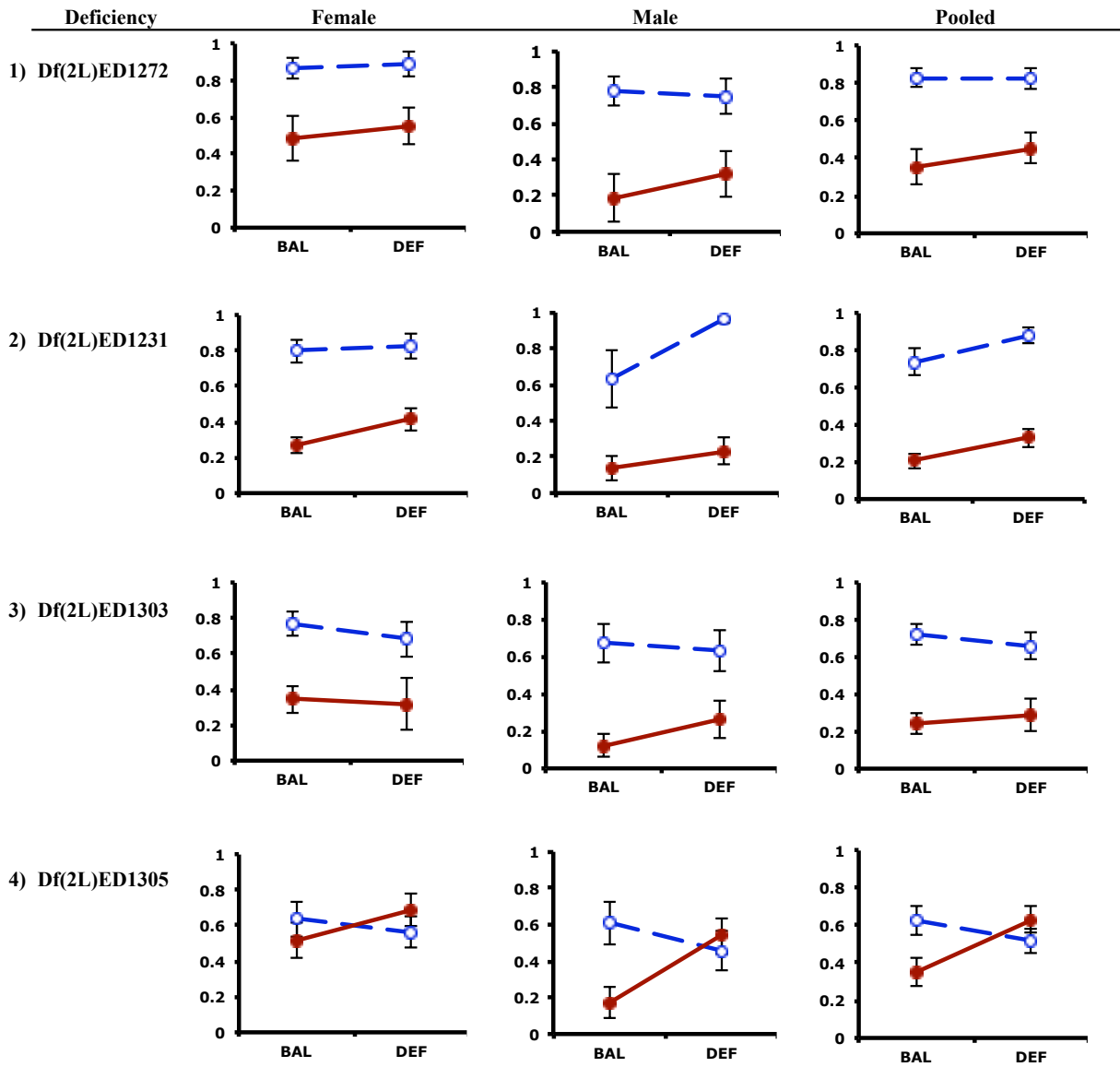
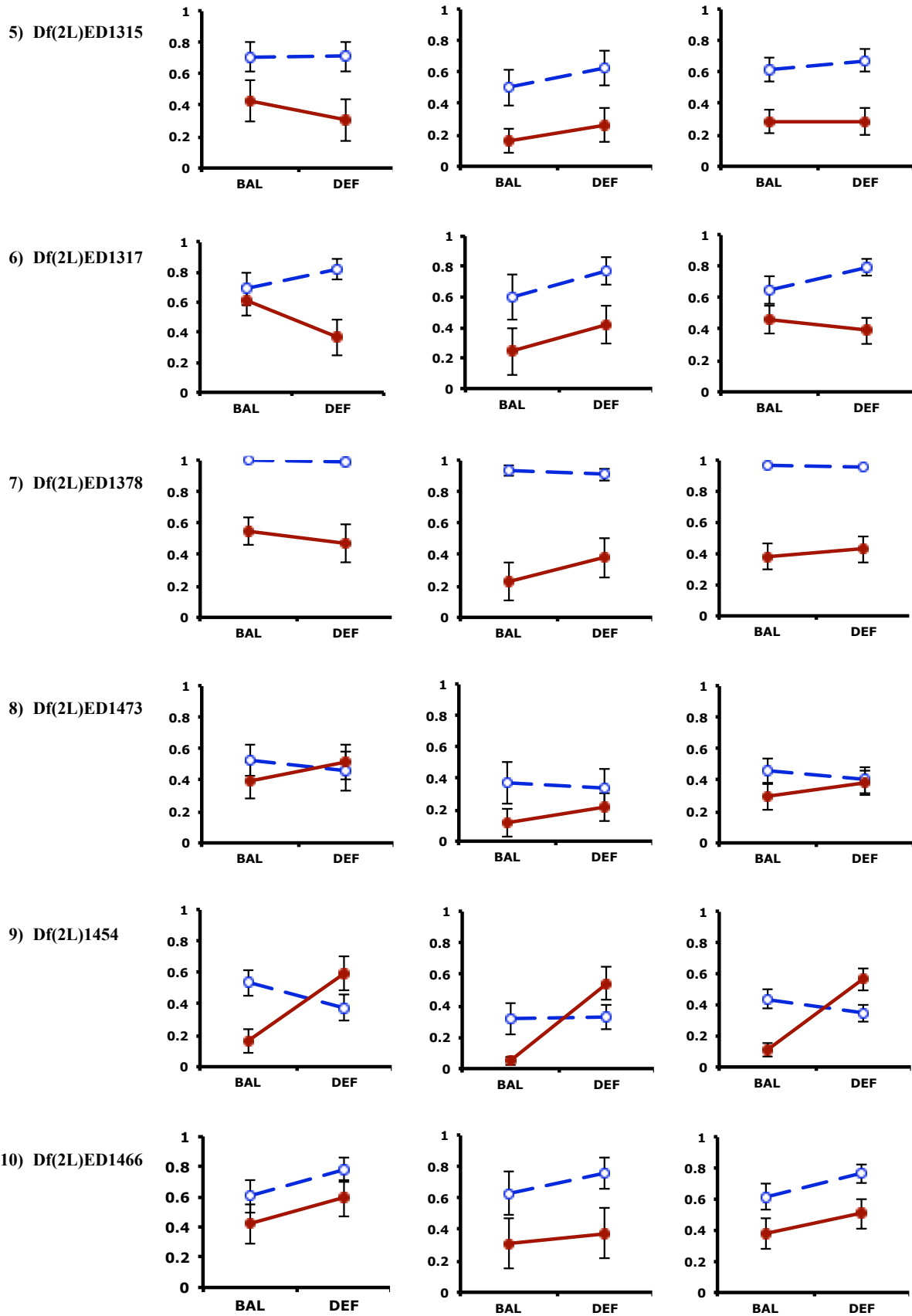
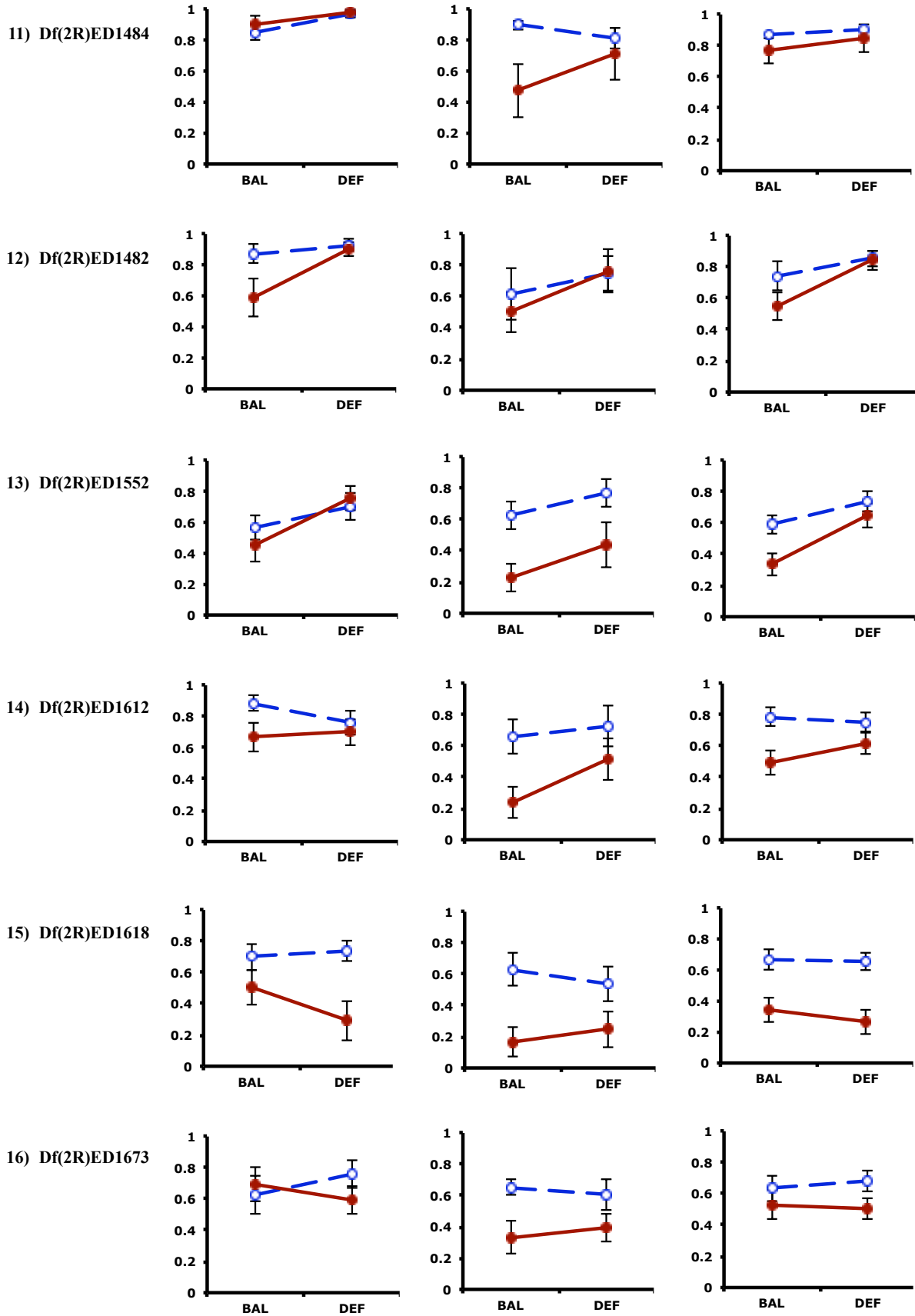


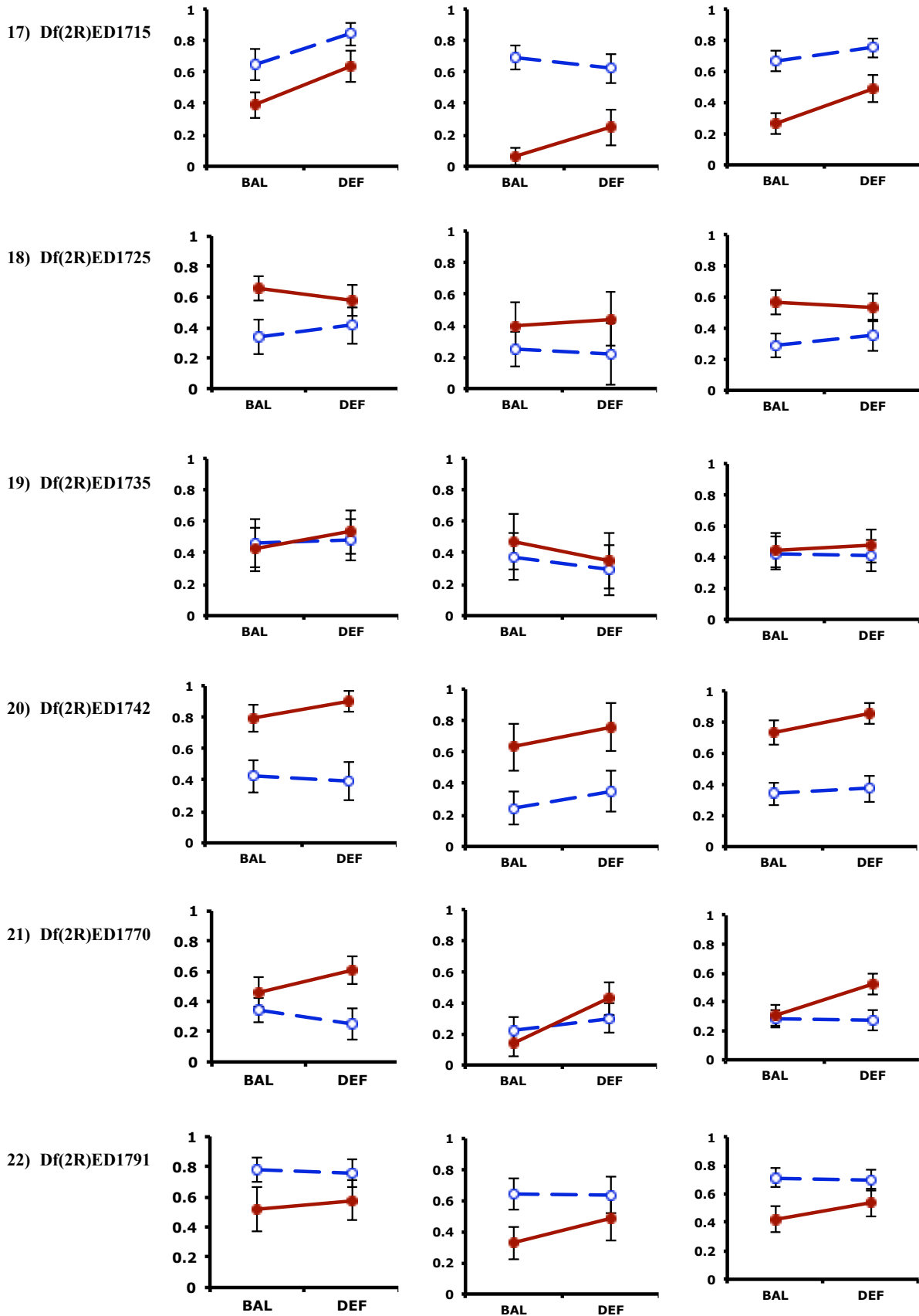
Figure A.2 Heat tolerance quantitative complementation plots for all tested deficiencies.

The number in the left column is consistent with numbers in Supplemental Table 1. Failure to complement is indicated by the exacerbated difference between the phenotype of the parental lines when over the deficiency line. Red solid lines represent parental line Oregon –R and dashed blue lines represent parental line 2B-3.

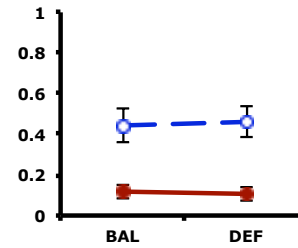
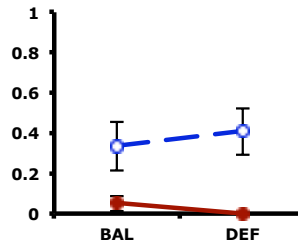
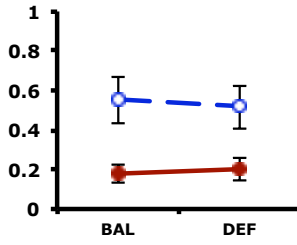




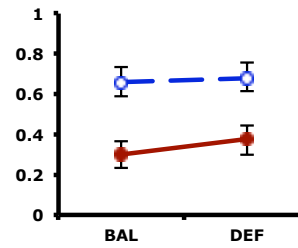
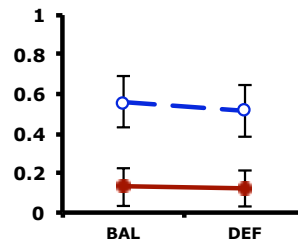
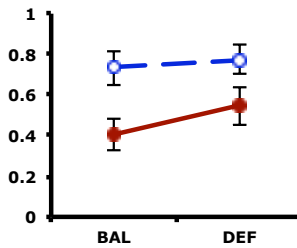




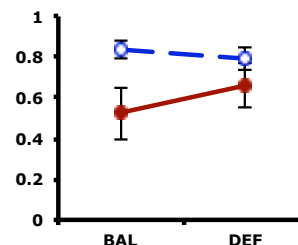
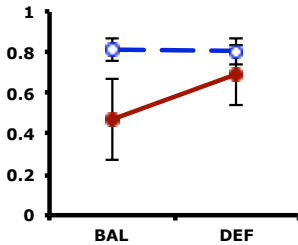
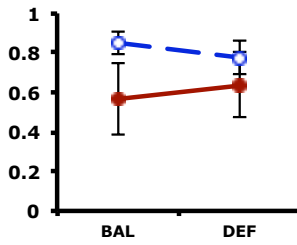
23) Df(2R)ED2098



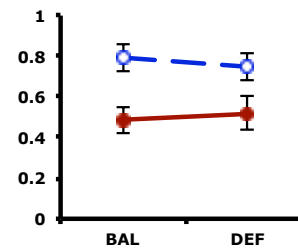
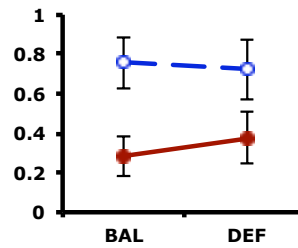
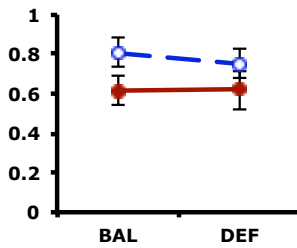
24) Df(2R)ED2076



25) Df(2R)ED2155



26) Df(2R)ED2219



27) Df(2R)ED222

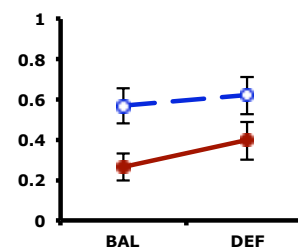
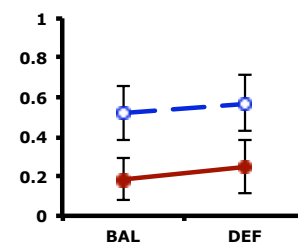
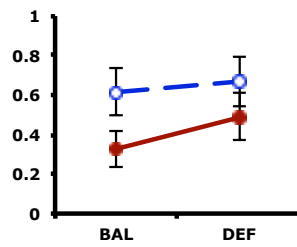


Table A.1 List of all deficiencies used in the analysis.

The numbered first column corresponds to numbers in Figure 3.2.

	Stock	Deficiency	Breakpoints	Molecular Breakpoints
1	24116	Df(2L)ED1272	37C5;38A2	2L:19158440;19753324
2	9174	Df(2L)ED1231	37C5;37E3	2L:19158440;19464056
3	8679	Df(2L)ED1303	37E5;38C6	2L:19517610;20382385
4	9222	Df(2L)ED1305	38B4;38C6	2L:20085397;20382385
5	9269	Df(2L)ED1315	38B4;38F5	2L:20085397;20917519
6	9175	Df(2L)ED1317	38D1;38F5	2L:20638580;20917519
7	9682	Df(2L)ED1378	38F1;39D2	2L:20823195;21397328
8	9266	Df(2L)ED1473	39B4;40A5	2L:21250892;21828548
9	9357	Df(2L)ED1454	39E3;39E3	2L:21629316;21657677
10	9340	Df(2L)ED1466	39E3;40A5	2L:21629316;21828548
11	9683	Df(2R)ED1484	42A2;42A14	2R:1781142;2132933
12	23228	Df(2R)ED1482	42A8;42A11	2R:1923128;2019615
13	8044	Df(2R)ED1552	42A11;42C7	2R:2019519;2628201
14	8045	Df(2R)ED1612	42A13;42E6	2R:2108037;2937177
15	8939	Df(2R)ED1618	42C4;43A1	2R:2556592;3074730
16	9062	Df(2R)ED1673	42E1;43D3	2R:2873307;3421058
17	8931	Df(2R)ED1715	43A4;43F1	2R:3214456;3804428
18	8941	Df(2R)ED1725	43E4;44B5	2R:3501429;4043550
19	9275	Df(2R)ED1735	43F8;44D4	2R:3849654;4487956
20	9276	Df(2R)ED1742	44B8;44E3	2R:4061673;4611634
21	9157	Df(2R)ED1770	44D5;45B4	2R:4543134;5095046
22	9063	Df(2R)ED1791	44F7;45F1	2R:4810235;5440757

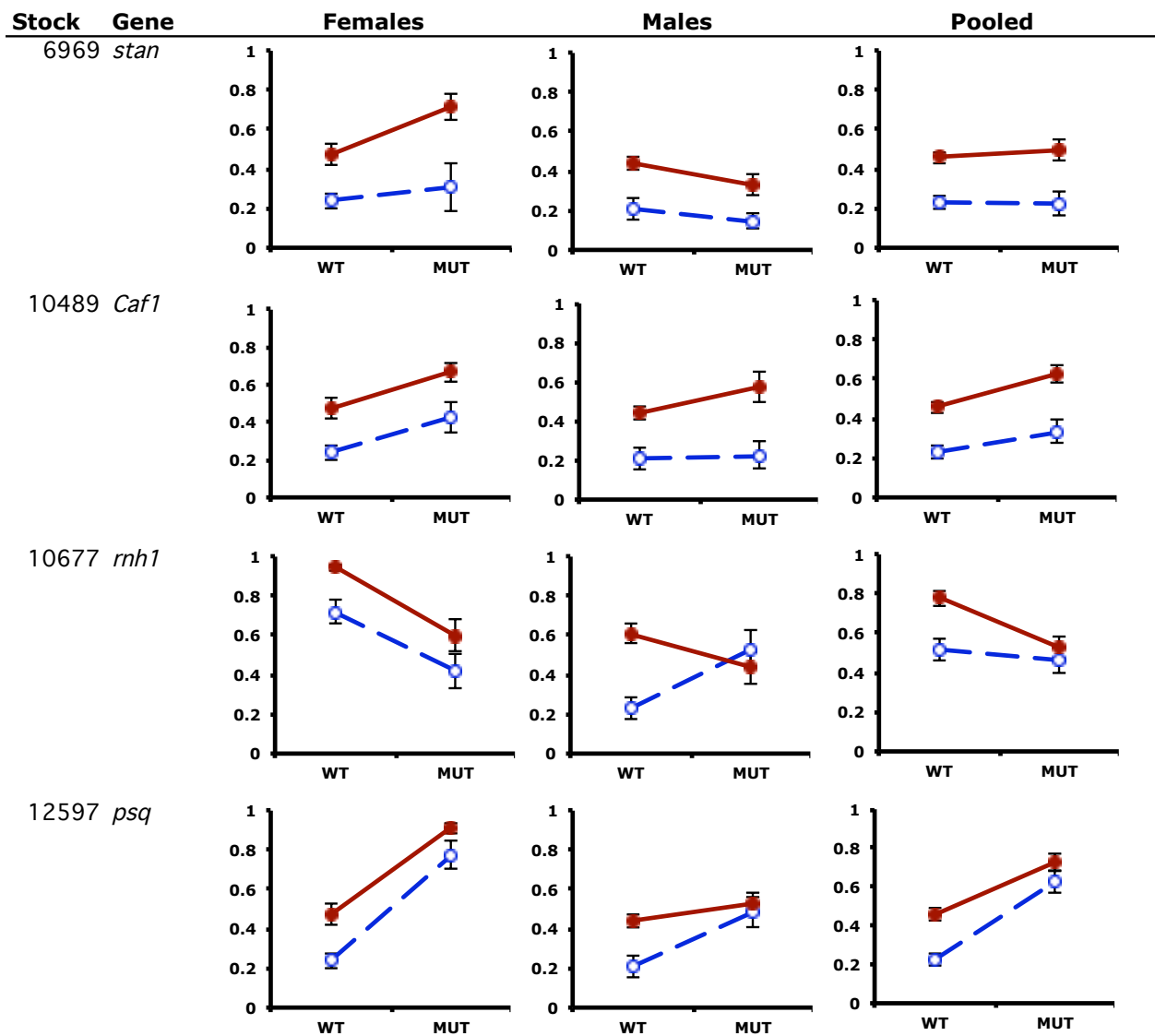
23	9277	Df(2R)ED2098	47A7;47C6	2R:6304366;6786711
24	8909	Df(2R)ED2076	47A10;47C1	2R:6364289;6707491
25	9344	Df(2R)ED2155	47C6;47F8	2R:6781601;7284947
26	8910	Df(2R)ED2219	47D6;48B6	2R:7084917;7552896
27	8911	Df(2R)ED2222	47F13;48B6	2R:7340485;7552896

Appendix B - Cold Tolerance Mutant Complementation Tests

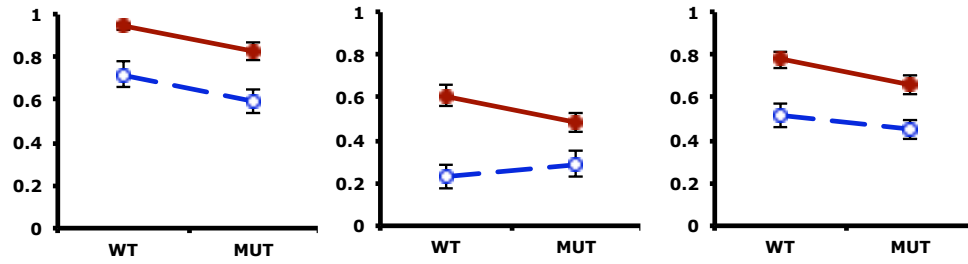
Figures and Tables

Figure B.1 Cold tolerance mutant complementation plots for all tested mutants.

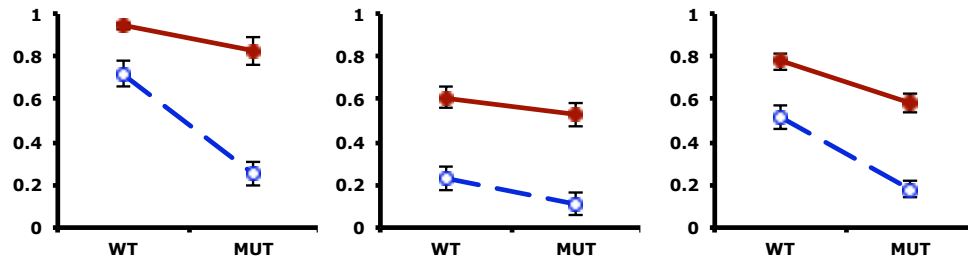
Failure to complement is indicated by the exacerbated difference between the phenotype of the parental lines when over the mutant line. Red solid lines represent parental line Oregon –R and dashed blue lines represent parental line 2B-3.



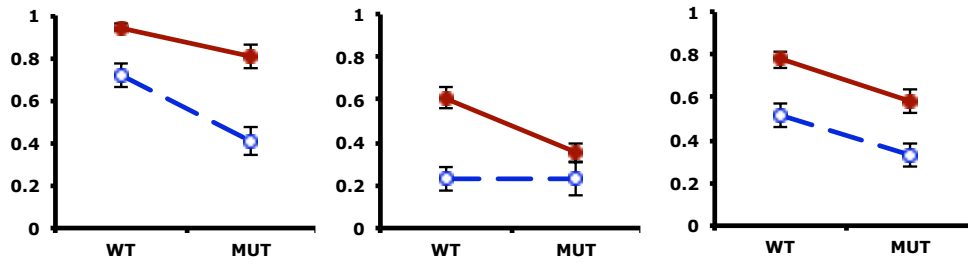
13352 *CG2915*



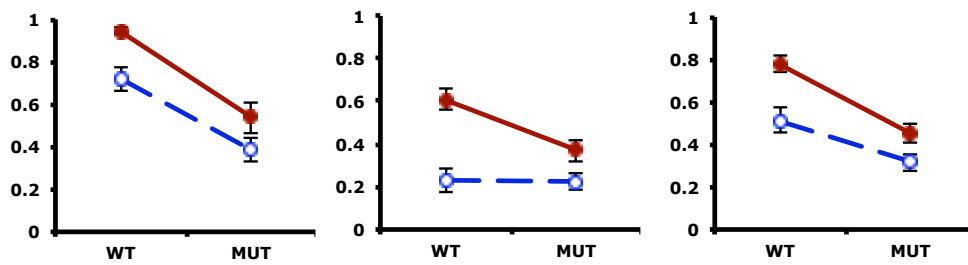
13359 *CG30379*



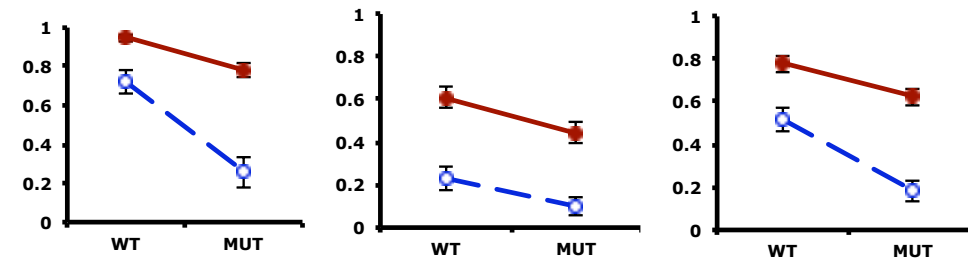
13828 *Kdm4A*



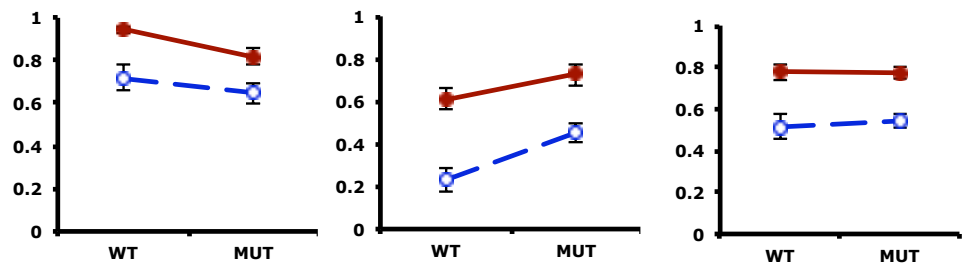
14027 *cag*

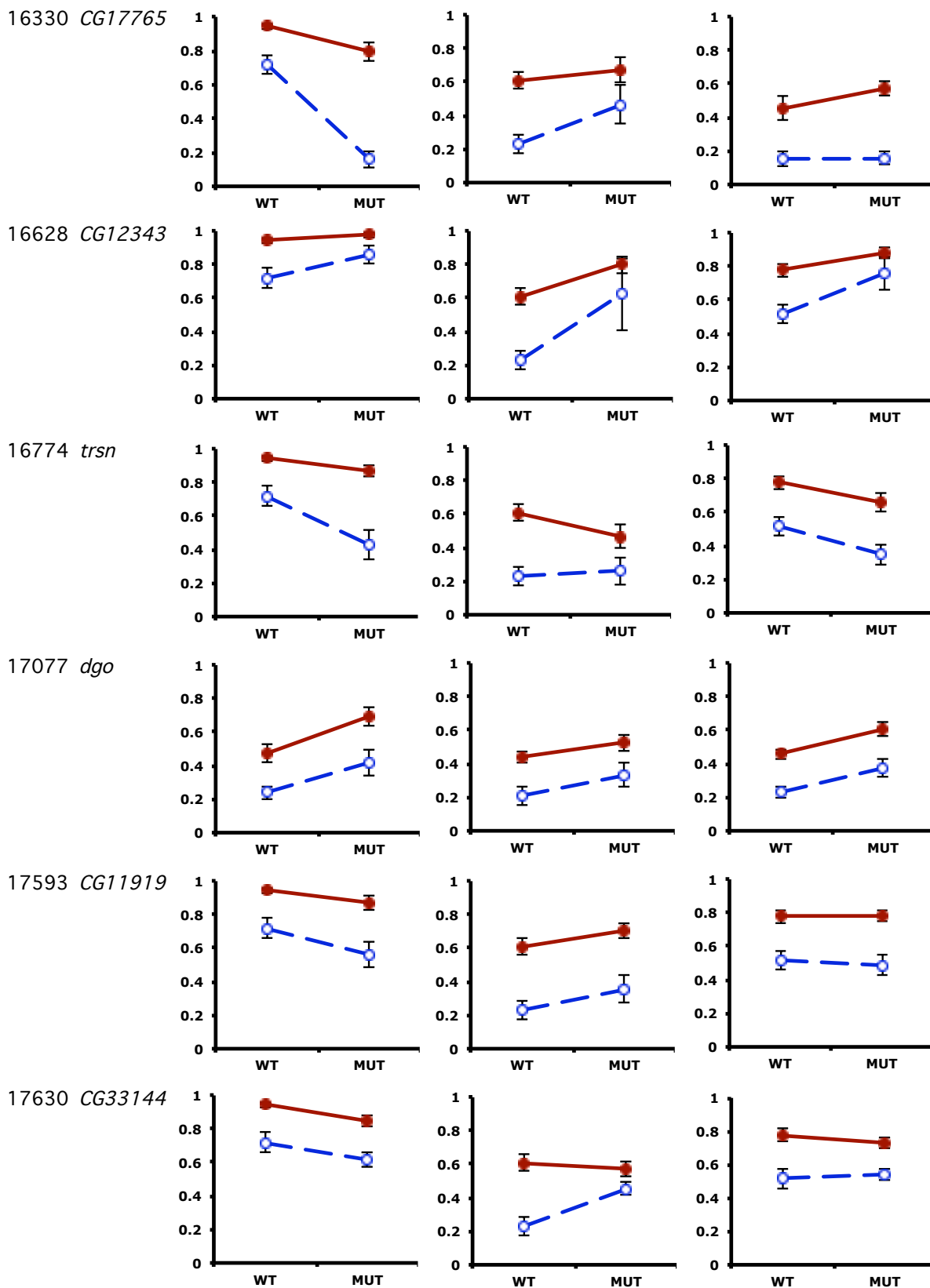


15373 *Taf5*

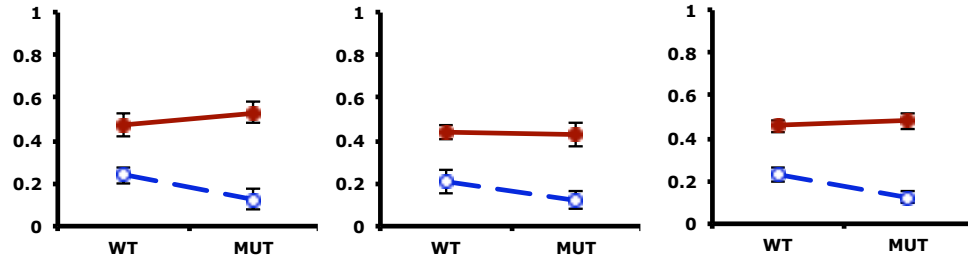


15999 *whd*

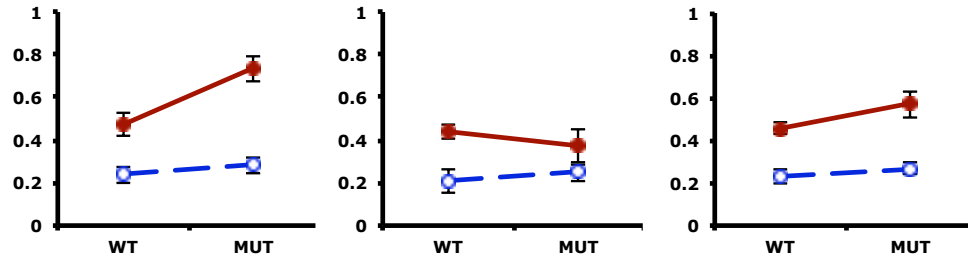




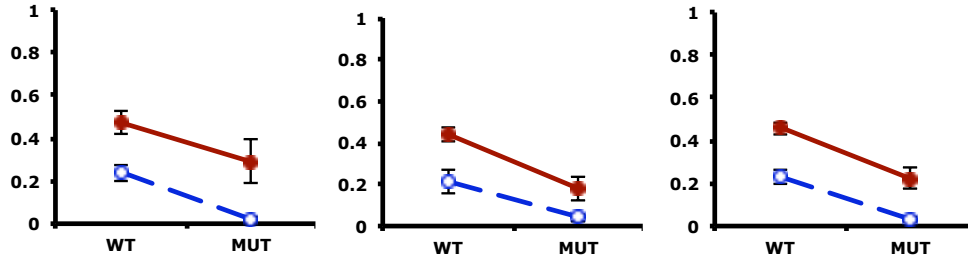
17722 CG12338



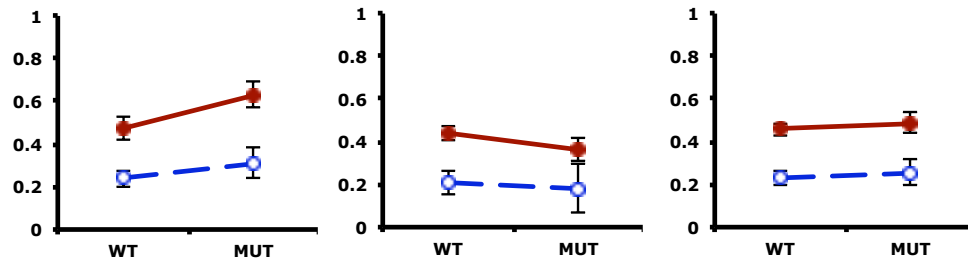
18009 CG30381



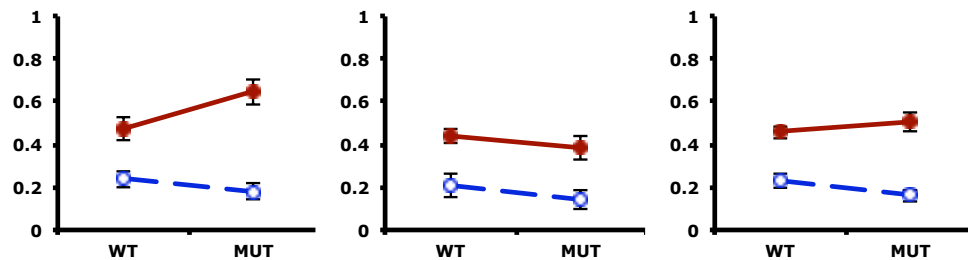
18217 CG12934



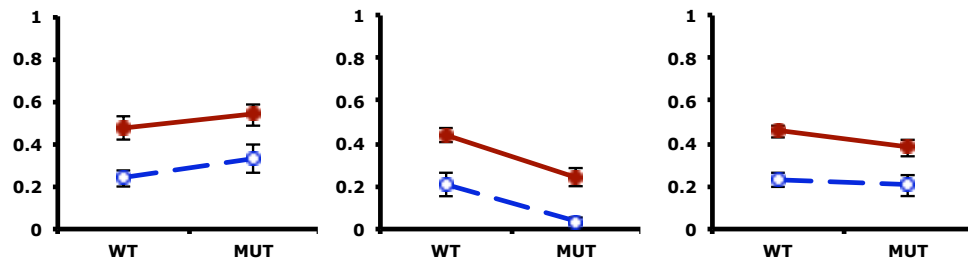
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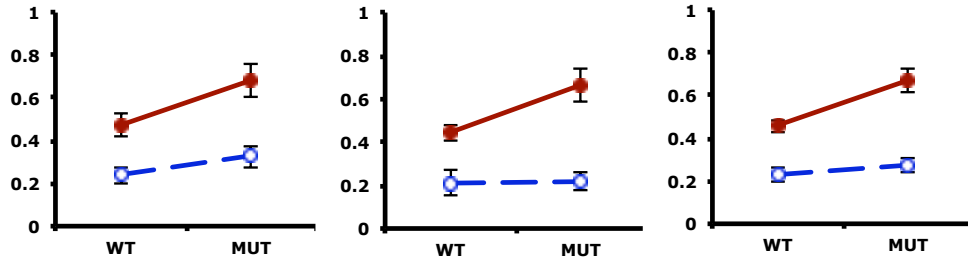
18442 CG30015



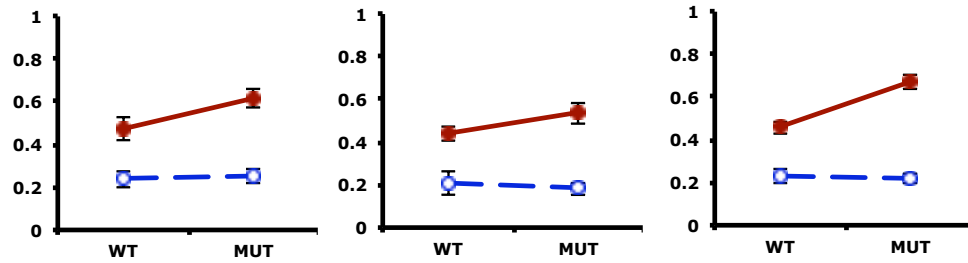
18519 CG20020



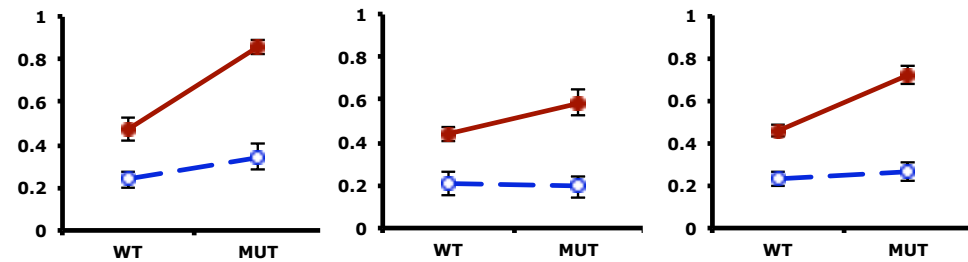
18554 *CG30016*



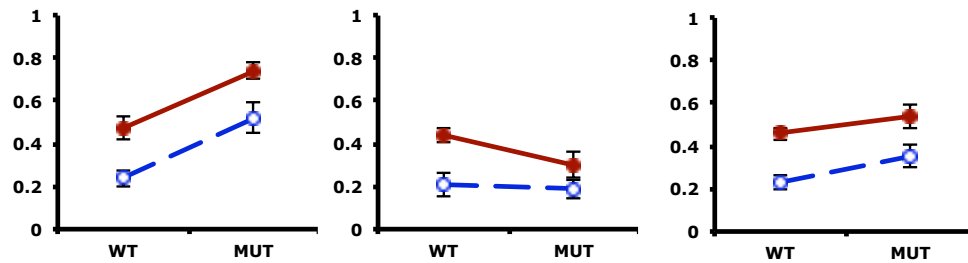
18878 *CG8791*



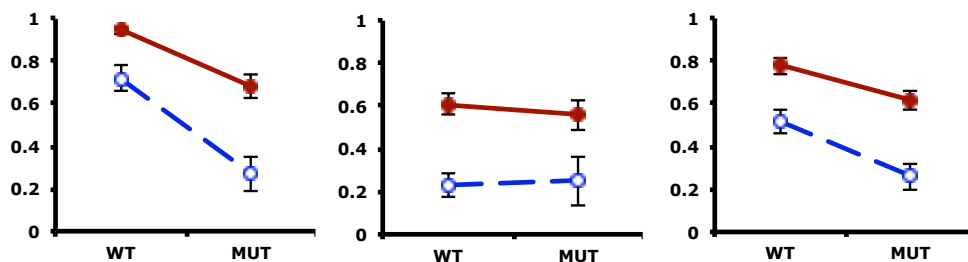
18995 *lsm10*



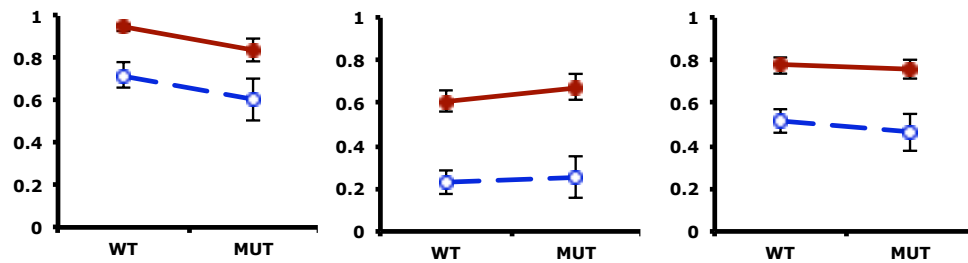
19152 *CG30377*



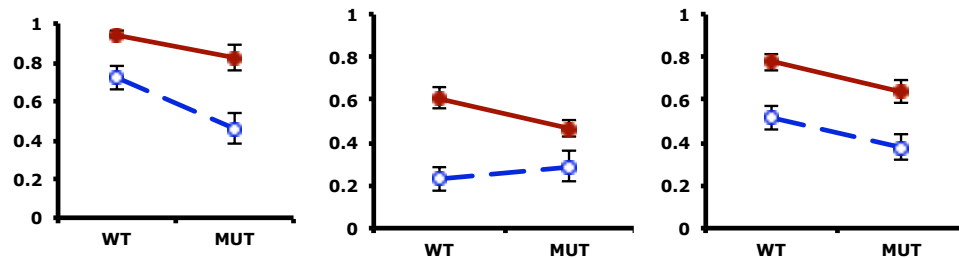
19872 *lola*



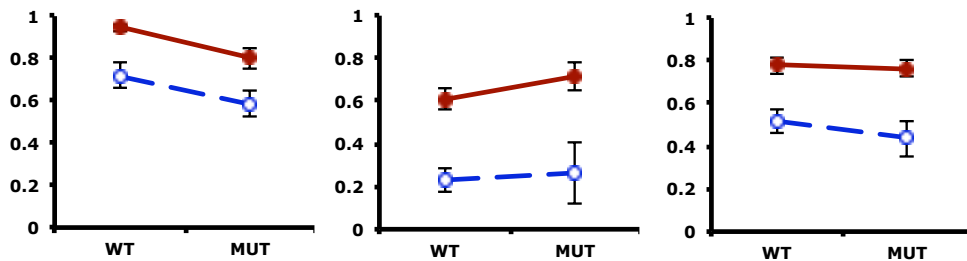
20206 *nclb*



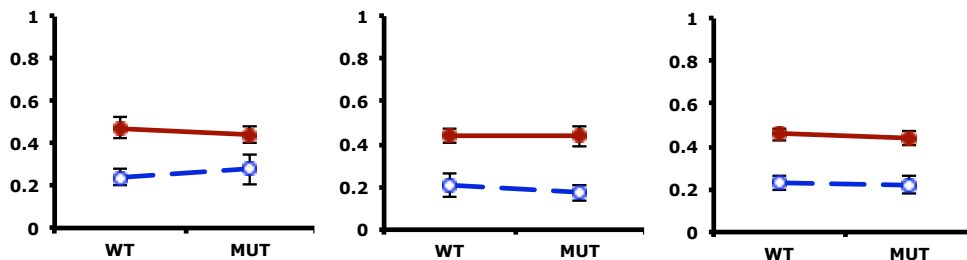
20813 *lin19*



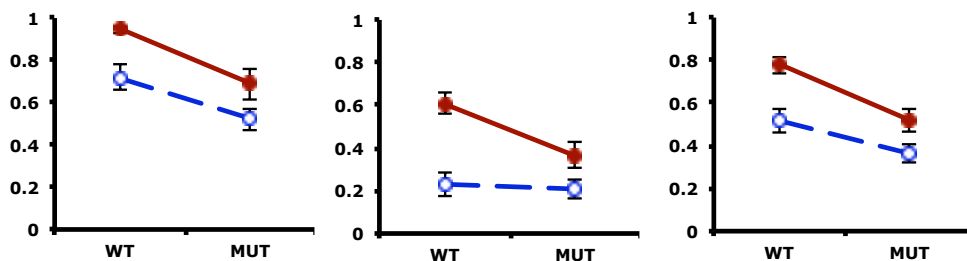
21669 *wde*



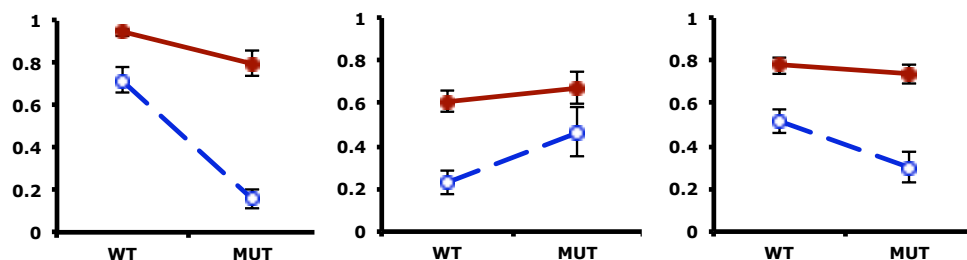
22092 *nito*



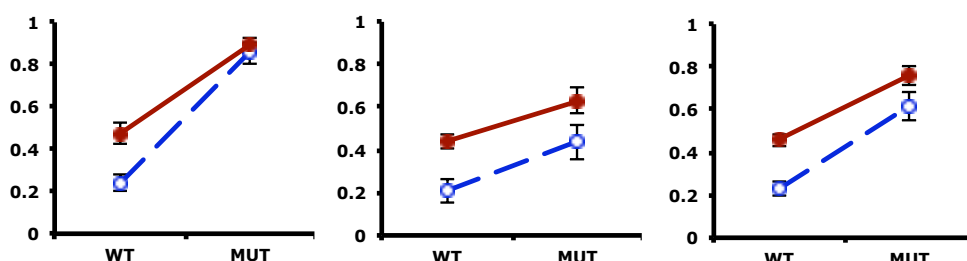
22153 *CG14764*



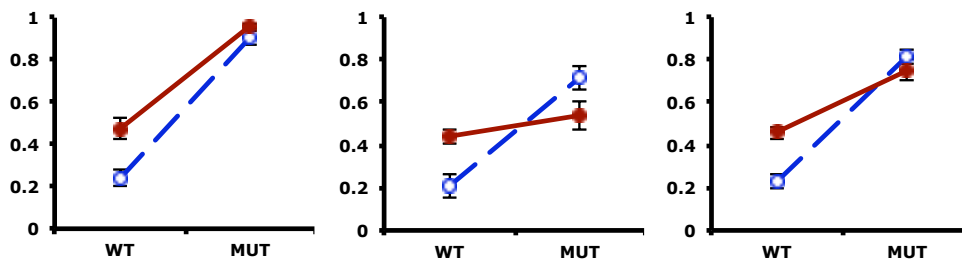
22334 *Mat1*



23427 *CG12943*



26336 CG7220



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