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Geographically patterned variation in diapause and its relationship to other climate-associated phenotypes and genotypes of Drosophila americana

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GEOGRAPHICALLY PATTERNED VARIATION IN DIAPAUSE AND ITS RELATIONSHIP TO OTHER CLIMATE-ASSOCIATED PHENOTYPES AND GENOTYPES OF DROSOPHILA AMERICANA

by

Rebecca Anne Hart-Schmidt

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Biology
in the Graduate College of
The University of Iowa

December 2012

Thesis Supervisor: Associate Professor Bryant F. McAllister

ABSTRACT

To declare a trait adaptive, it is necessary to show heritability and beneficial fitness effects of a phenotype. Association between phenotypic and environmental variance is insufficient to identify adaptive traits because non-selective forces can build phenotype-environment associations without beneficial fitness effects. However, if the same phenotype-environment associations occur in independent lineages subject to similar environmental stresses, the traits shared among lineages may be more likely to have beneficial fitness effects and are likely targets for further study of adaptation.

I tested the hypothesis that, among species sharing similar climate and habitat distributions, shared traits are candidates for adaptations to shared selective pressures. I used *Drosophila americana* as a model because it is endemic to temperate environmental conditions similar to those of other well-studied populations and species of *Drosophila* and has other qualities that make it amenable to studies of putative adaptations. These include an ancient association with cold climates and previously defined regions of clinally distributed genetic variation maintained by selection.

I demonstrated an association between reproductive diapause and clinally distributed genetic variation in *D. americana*. The fused X-4 chromosome is sufficient to increase the probability of diapause in *D. americana*, establishing heritability of the trait and linking genetic variation under selection to a phenotype.

I also investigated geographically patterned variation in putatively adaptive traits to test whether adaptation to cold climate involves the same traits in related, but independently adapted lineages of *Drosophila*. I found that between *D. americana* and temperate populations of *D. melanogaster* some traits putatively adaptive to cold climate exhibit shared associations with climate variables.

To test the fitness effects of diapause in cold environments, I assessed the response of diapause in *D. americana* to temporal changes in the environment. In comparing diapause incidence between isofemale lines of *D. americana* derived from flies collected either prior to 2009 or following the record cold winter of 2009-2010, I found that southern populations previously accustomed to warmer climate significantly increased in diapause incidence.

My results demonstrate the interaction between variation in genotype, phenotype, and fitness for reproductive diapause in *D. americana*. I verified that identifying traits shared among species living in similar environments is an effective approach to target putative adaptations. While traits shared among species living in similar environments are good candidates for adaptations, evidence of selective advantage is necessary before a trait can be truly considered adaptive.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To my husband, Dustin E. Schmidt,
my son, Malcolm E. Schmidt,
and my parents, Dean H. and Joan M. Hart

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My results demonstrate the interaction between variation in genotype, phenotype, and fitness for reproductive diapause in *D. americana*. I verified that identifying traits shared among species living in similar environments is an effective approach to target putative adaptations. While traits shared among species living in similar environments are good candidates for adaptations, evidence of selective advantage is necessary before a trait can be truly considered adaptive.

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INTRODUCTION

Darwin's (1859) four postulates define the conditions under which evolution by natural selection occurs. First, there must be phenotypic variation among individuals. Second, phenotypic variation must be at least partially heritable. Third, there must be variation in reproductive success among individuals. Finally, the probability of reproductive success must be affected by phenotype. The simplicity and seeming inevitability of Darwin's four postulates can give the impression that natural selection should be rampant throughout life and the dominant source of biological diversity.

To the casual observer, the action of natural selection seems obvious, with most organisms appearing well-suited to their environments. This view was reinforced through the first half of the twentieth century, with many researchers working under the assumption that the majority of organisms' phenotypic features are the direct products of natural selection, conferring optimal fitness. Gould and Lewontin (1979) strongly criticized this "adaptationist programme," which they believed overemphasized the action of natural selection in shaping phenotypes and led to assertions of traits being adaptations based largely on plausibility rather than on evidence of fitness value and response to selection. They argued that observed correlations among traits and between traits and environment were not necessarily the result of natural selection, but could

be caused by such factors as allometry, pleiotropy and linkage between the loci underlying traits. This critique coincided with growing awareness of Neutral Theory and increased understanding that much biological diversity does not evolve by natural selection (Kimura and Crow, 1964).

Evolutionary biology has advanced with a stronger recognition that demonstrating heritability and the action of selection are necessary to support claims that particular traits are adaptive to environments. Much progress has been made over the past several decades in the development of methods to test both fitness in natural populations (reviewed in Kingsolver *et al* 2001) and to identify targets of past selection in the genome (*e.g.* McDonald and Kreitman 1991, Otto 2000). These techniques have allowed evolutionary biologists to posit the adaptive significance of observed phenotypes with greater rigor, requiring evidence of selection acting on traits and/or molecular signatures of selection surrounding causative loci before a trait is labeled as more than putatively adaptive (Barrett and Hoekstra 2011). Although they are relatively simple to identify, molecular signatures of selection alone are insufficient to demonstrate fitness effects at an organismal level because the effects of biased transmission of selfish genetic elements, such as meiotic drivers, can resemble selective sweeps of beneficial alleles (*e.g.* Babcock and Anderson 1996, Derome *et al* 2008, Dyer *et al* 2007, Fishman and Saunders 2008).

Barrett and Hoekstra (2011) argue that to truly demonstrate adaptation, it is necessary to fully explain the three-way interaction between fitness, genotype, and phenotype for a given trait (Figure 1.1). They identify a means to test this three-way interaction, performing selection experiments and measuring the response of gene variants known to produce phenotypic effects under natural conditions to demonstrate the fitness consequences of traits produced by those alleles. Because natural selection acts through the differential fitness among individuals, it is necessary to show that heritable differences in a phenotypic trait affect fitness to declare a phenotypic trait adaptive.

Examples of traits for which this three-way interaction has been demonstrated are few and well-known. In the classic case of industrial melanism in the peppered moth, *Biston betularia*, field experiments have shown that pigmentation matching tree color increases fitness by reducing predation pressure from birds (Kettlewell 1956, Grant *et al* 1998, Majerus 1998). Further, the locus determining moth color has been genetically mapped to a narrow chromosomal region showing a signature of recent and strong selection (van't Hof *et al* 2011). In a species of Darwin's finches, *Geospiza fortis*, beak size fluctuates in response to changes in the size of available edible seeds, a consequence of yearly variation in rainfall (Boag and Grant 1981, Price *et al* 1984). Beak size is regulated by the expression of Bmp4 and Calmodulin (Abzhanov *et al* 2004). Polymorphisms in the *FRIGIDA* gene cause flowering time in *Arabidopsis*

thaliana to vary with climate, maximizing seed production under local environmental conditions (LeCorre *et al* 2002, Korves *et al* 2007). Matching between substrate and coat color in the beach mouse, *Peromyscus polionotus*, reduces vulnerability to owl predation (Kaufman 1974). Coat color variation in *P. polionotus* is caused by sequence variation in *mc1r* and *agouti* (Hoekstra *et al* 2006, Steiner *et al* 2007).

These examples of genotype-phenotype-fitness interactions are limited in the respect that all are traits controlled by few loci. Traits affected by many genes may be more subject to constraints on other traits imposed by such factors as pleiotropy and linkage. Also, natural environments are complex, simultaneously exposing organisms to numerous potential sources of selective pressure that vary over space and time. Fitness is the result of multiple traits interacting with the full range of environmental conditions experienced by organisms. Thus, while available examples provide much information about the process and effects of natural selection, they do not address all factors influencing evolution by natural selection in real populations.

Examples of genotype-phenotype-fitness interactions are relatively rare and limited for multiple reasons. Measuring fitness is technically difficult. Tracking individual reproductive success in natural environments and is at minimum costly and challenging for most species. Fitness is context-dependant and laboratory measurements of fitness are often not reflective of natural conditions. Many natural environments cannot be

experimentally manipulated, which severely limits study of the effects of particular sources of selection. There is little overlap among species used as models in genetic and evolutionary studies, with the possible exceptions of *A. thaliana* and *Drosophila melanogaster*, so it is rare that both phenotype-genotype and phenotype-fitness interactions are well-understood for a given species. Further, in species for which genotype-phenotype relationships and/or ecology and life-history are poorly understood, it is difficult to predict which traits have heritable variation and important fitness effects.

Models used to demonstrate the genotype-phenotype-fitness interaction require several qualities. The capability to investigate the heritability and genetic control of phenotypic traits is necessary. Phenotypic traits under investigation must be defined and measurable. To investigate fitness effects, it is first necessary to have sufficient knowledge of the environmental stresses of species' habitats to reasonably predict important sources of selection, and it must also be possible to either manipulate natural environments or to follow populations through environmental changes to test interactions between environmental variables and genotype/phenotype.

An approach to identify candidate traits affecting fitness is to identify convergent traits among unrelated lineages living in similar environments (Endler, 1986). This requires the availability of lineages having similar ecological niches which have independently established

populations in similar environments, and are therefore subject to similar putative selective pressures. The general hypothesis tested in this study is that shared traits among species sharing similar climate and habitat distributions are candidates for adaptations to shared selective pressures. Adaptive traits must have a heritable basis, are expected to have an association with the environmental condition(s) under which they enhance fitness, and adaptive traits that are variable in a population are expected to respond to selection.

Members of the genus *Drosophila* supply all qualities necessary in a model to study natural selection, and also exhibit replication of environmental variables among the major species groups. Multiple lineages of *Drosophila* have independently established populations in cold climates (Gilbert *et al* 2001, Kellermann *et al* 2012). Insect species in cold climates experience similar environmental stresses, including direct cold stresses and reduced availability of food and water resources during the winter season (Danks 1996). Under these similar environments, populations of cold-climate *Drosophila* commonly exhibit parallel clines in putatively adaptive phenotypes. Because these species have independently colonized cold climates, it is likely that similar patterns of variation represent responses to the same selective pressures experienced by these populations.

Reproductive diapause is a trait that is very common among cold-climate insect species, including numerous *Drosophila* (Danks 2002,

Chapter 2). This trait arrests ovarian development at an immature stage, slows metabolism, and increases resistance to cold and starvation stresses in response to seasonal cues, typically cold temperature and shortening daylength. The capacity for reproductive diapause plausibly offers a fitness advantage, allowing flies to delay reproduction until a season when conditions are favorable for offspring survival and growth (Danks 2002). However, there is evidence that diapausing insects may experience reduced fertility, producing fewer eggs from mature ovaries than non-diapausing individuals of the same species (Hahn and Denlinger 2007, Schmidt *et al* 2005).

The genetic control of diapause varies among *Drosophila*, with differences between species and between populations within species, as well as some shared elements between species. In *D. melanogaster*, diapause has been associated with the insulin signaling genes *couch potato* and *dp110* in studies of North American populations, and with *timeless* in European populations (Schmidt *et al* 2008 Williams *et al* 2006, Tauber *et al* 2007, Sandrelli *et al* 2007). In *D. triauraria,* diapausing flies show up-regulation of *drosomycin* and *drosomycin-like*, and allelic differences in *timeless* and *cryptochrome* are associated with variation in the incidence of diapause between strains (Daibo *et al* 2001, Yamada and Yamamoto 2011). Analysis of gene expression differences between diapausing and non-diapausing *D. montana* showed that at least 6 genes,

including *couch potato,* are up or down-regulated in diapausing flies (Kankere *et al* 2010).

Climate-associated variation is evident in several additional phenotypes among multiple cold climate *Drosophila* species. Clines in body size, cold resistance, and starvation resistance are well characterized in the cold climate populations of *D. melanogaster* (Karan *et al* 1998, Robinson *et al* 2000, Trotta *et al* 2006, Imasheva *et al*. 1994, Coyne and Beecham 1987, James *et al*. 1995, Noach *et al* 1996). Australian *D. simulans* vary clinally in body size, cold resistance, and starvation resistance (Arthur *et al* 2008). *D. serrata* exhibits clines in body size and cold resistance (Hallas *et al* 2002). Independent populations of *D. subobscura* are clinally variable in body size and cold resistance, but not starvation resistance (Gilchrist *et al* 2008, Huey *et al* 2000, David *et al* 2003). *D. robusta* varies clinally in body size (Stalker and Carson 1947).

There is empirical evidence of a fitness advantage to increasing body size with increasing latitude (Bergmann's Rule) in *Drosophila*, as laboratory selection experiments show that body size increases when generations of flies are subjected to cold temperature (Partridge *et al* 1994, Anderson 1973), and body size clines have rapidly evolved following colonization of cold climates (Huey *et al* 2000, Calboli *et al* 2003). However, whether cold and starvation resistance confer overall fitness advantages is less clear. There is evidence that increased starvation resistance may be associated with decreased dessication

resistance, cold resistance, and fertility in *D. melanogaster* (Wayne *et al* 2006, Karan *et al* 1998, Hoffmann *et al* 2005).

Numerous genetic variants have been associated with cold climate populations of *D. melanogaster.* Genes involved in thermotolerance in Australian *D. melanogaster* populations have been identified (Anderson *et al* 2003, Rako *et al* 2007). Variation in body size is associated with the same genomic regions in the cold climate *D. melanogaster* populations on multiple continents (Calboli *et al* 2003, deJong and Bochdanovits 2003). Insulin signaling genes underlying growth and metabolism exhibit climate-associated variation (deJong and Bochdanovits 2003, Sezgin *et al* 2004). Genome-wide studies of latitudinally patterned genetic variation in North American and Australian populations have found that sequence and regulatory differentiation between warm and cold climate populations is overrepresented among particular classes of genes, including those functioning in steroid metabolism and insulin signaling, circadian rhythm, and immunity (Turner *et al* 2008, Kolaczkowski *et al* 2011, Fabian *et al* 2012).

1.1 Model System

The hypothesis that shared traits among species sharing similar climate and habitat distributions are candidates for adaptations to shared selective pressures can be addressed using cold-climate *Drosophila*, specifically testing whether putatively adaptive traits in cold climate populations of *D. melanogaster* and other *Drosophila* species associated

with cold-climates are also evident in *Drosophila americana*. *D. americana* is endemic to North America, with a species range between 30° and 45°N (Patterson and Stone 1952, Throckmorton 1982, McAllister 2002, McAllister *et al* 2008). This latitudinal range is comparable to that of the North American *D. melanogaster* population, so it is expected that *D. melanogaster* and *D. americana* are subject to at least some of the same selective pressures. However, these species differ in the age of their associations with cold climates. *D. americana* is a member of the *Drosophila virilis* species group, which has an ancient (>10 mya) association with cold climate (Throckmorton 1982), and is thought to have colonized North America about 3 million years ago (Caletka and McAllister 2004, Morales-Hojas *et al* 2011). *D. melanogaster* has only inhabited cold climates in Europe for less than15,000 years, and less than 500 years in North America and Australia (David and Capy 1988). These species may therefore differ in the extent to which they are adapted to the environmental stresses of cold climates.

D. americana is known to exhibit clinal genetic variation. Frequencies of a derived fusion between the X and 4th (Muller element B) chromosomes, inversions of large regions of both X and 4, and sequence variation associated with these structural rearrangements are highly correlated with latitude (McAllister 2002, McAllister 2003, Evans *et al* 2007, McAllister *et al* 2008). The cline spans all sampled populations of *D. americana*, between 44.1°N and 30.76°N, with the frequency of the

fused X-4 chromosome approaching 100% in northernmost populations and 0% in southernmost populations, with most populations including both the fused X-4 chromosome as well as unfused X and 4th chromosomes (McAllister *et al* 2008).

Polymorphic microsatellites located on the autosomes and unlinked to the X-4 centromere do not show latitudinally structured variation, so it is likely that locally adaptive alleles linked to the chromosomal rearrangement are maintained by natural selection in the face of gene flow among populations (McAllister *et al* 2008). It is therefore predicted that fused X-4 chromosomes harboring alleles for traits conferring high fitness in cold-climate conditions whereas its unfused homologs contain alleles adapted for a warmer climate.

1.2 Objectives

The main objective of this study is to investigate the evolution of similar adaptive traits in independent lineages exposed to similar environmental stresses. The general hypothesis that shared traits among species sharing similar climate and habitat distributions are candidates for adaptations to shared selective pressures is addressed by this investigation. I determined whether a genotype-phenotype-fitness interaction could be demonstrated in *D. americana* for reproductive diapause, a trait shared by numerous cold climate *Drosophila*, and also whether additional traits shared among cold climate *Drosophila* species

exhibited geographical patterns of variation consistent with adaptation to cold climate.

1.2.1 Association Between Clinal Genetic Variation and a Putatively Adaptive Trait

Because the clinally distributed genetic variation so far identified in *D. americana* is linked to the X chromosome (Evans *et al* 2007, McAllister *et al* 2008), and because the putatively adaptive diapause trait has been linked to the X chromosome in *D. lummei* (Lumme and Karanen 1978), the closest Eurasian relative of *D. americana*, I tested the hypothesis that variation in diapause incidence is X-linked in *D. americana*. I determined whether inbred *D. americana* lines carrying the fused X-4 chromosomes that are more common in northern populations exhibit higher diapause incidence than those carrying the unfused X-4 chromosomes more common in southern populations. I also performed crosses, comparing diapause incidence among progeny known to carry different combinations of parental X and 4th chromosomes. Finally, I introgressed each X chromosome variant into an alternate genetic background and measured diapause incidence in the resulting progeny. Overall results were consistent with the presence of a dominant, high-diapause allele present on the fused X-4 chromosome of *D. americana.*

1.2.2 Geographically Patterned Variation in Putatively Adaptive Traits

To test the hypothesis that adaptation to cold climate involves the same suite of traits, indicating similar underlying function among lineages

of *Drosophila* that have independently adapted to cold climate, I determined whether variation in diapause, wing length, starvation resistance, body mass, and whole-body lipid content are clinally patterned in *D. americana.* These traits were chosen both based on simplicity of measurement as well as plausible associations with both cold climate fitness and the insulin signaling pathway, which regulates growth and metabolism and is associated with cold climate fitness in insects (Johnston and Gallant 2002, Hahn and Denlinger 2011). Diapause has been associated with the insulin signaling genes *cpo* in *D. melanogaster* and *D. montana* and *dp110* in *D. melanogaster* (Schmidt *et al* 2008 Williams *et al* 2006 Kankare *et al* 2010). Insulin signaling genes functioning in growth and metabolism show clinally distributed variation in multiple populations of *D. melanogaster* (DeJong and Bochdanovits 2003, Sezgin *et al* 2004).

I measured these phenotypes in isofemale lines derived from individuals collected from sites distributed over the latitudinal range of *D. americana*. I found that diapause incidence, wing length, and starvation resistance are significantly associated with latitude in *D. americana*, while body mass and lipid content are not. *D. americana* and cold climate populations of *D. melanogaster* share some, but not all, traits putatively adaptive to cold climate.

1.2.3 Response of Diapause to Temporal Changes in the Environment

This study took advantage of the record cold winter of 2009-2010 to test whether diapause would respond to the increased cold stress imposed on southern populations of *D. americana*. I compared diapause incidence between isofemale lines of *D. americana* derived from flies collected either prior to 2010 or during the summer of 2010. Southern populations previously accustomed to warmer climate, significantly increased in diapause incidence between samples. Northern populations, already accustomed to cold winters showed no change in diapause incidence between samples.

1.3 Overview of Conclusions

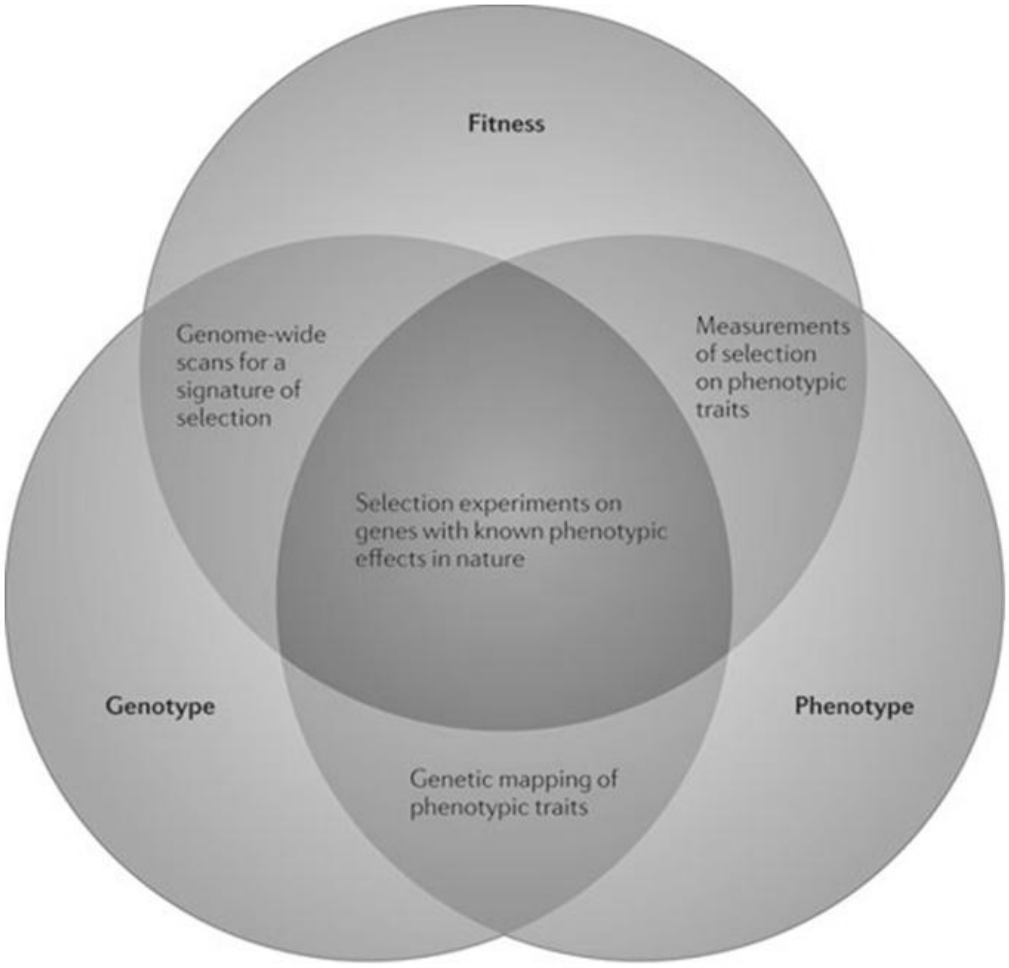
Taken together, the results obtained demonstrate the three-way interaction between variation in genotype, phenotype, and fitness for the reproductive diapause trait for *D. americana* in cold climate. Probability of diapause is at least partially controlled by at least one locus linked to a clinally distributed rearrangement of the X-4 chromosome. Variation in diapause incidence is latitudinally patterned over the *D. americana* range. Additionally, diapause incidence increased in southern populations of *D. americana* following the record-cold winter of 2009-2010, showing a response by the trait to a putative change in selective pressure.

Besides reproductive diapause, wing length and starvation resistance showed latitudinally patterned variation in *D. americana*, while body mass and lipid content did not. Thus, three of the five traits

predicted to be adaptive to cold climate in *D. americana* based on observations in other cold climate *Drosophila* did show climate-associated variation. These results are supportive of the hypothesis that shared traits among species sharing similar climate and habitat distributions are candidates for adaptations to shared selective pressures, and demonstrate that the approach employed is effective for making reasonable predictions of adaptive traits and for investigating whether traits are adaptive to a species' environment.

Cold climate *Drosophila* species are effective models in which to study the process and patterns of adaptation to complex environments. They offer well-developed tools for investigating genetic variation and function, techniques for measuring variation in numerous phenotypic traits, and replication of environmental conditions among lineages. Because studies of genotype, phenotype, and fitness can be conducted in a broad range of species that have independently colonized similar environments, analyses of genetic and phenotypic features shared among and differing between cold climate *Drosophila* species will allow researchers to address major questions in evolutionary biology through new means. Conducting these analyses will allow improved understanding of the influence of various factors in adaptation to environment through comparisons of sets of species differing in such variables as the age of the association with cold climate, and in the linkage and functional relationships among genes.

Figure 1.1 Experimental approaches used to demonstrate interactions between genotype, phenotype, and fitness (from Barrett and Hoekstra 2011).



CHAPTER 2

ASSOCIATION BETWEEN CLINAL GENETIC VARIATION AND A PUTATIVELY ADAPTIVE TRAIT IN *DROSOPHILA AMERICANA*

2.1 Introduction

Ectothermic organisms, including insects, lack internal means of regulating body temperature. Because of this, winters of temperate and cold climates in particular pose considerable physiological stress to insects; they must avoid cell damage due to freezing, endure the metabolic slow-down imposed by cold temperature, and reduce dependence on limited or unavailable resources such as plant-based food and liquid water (Danks 2002). Fitness can be maximized by avoiding reproduction and growth stages while winter environment stressors are present. Since these selective pressures are present in the habitats of all temperate-living insects, it is unsurprising that numerous lineages have evolved similar strategies to survive and successfully reproduce in these environments. Evolutionarily independent instances of facultative or obligate overwintering dormancy or diapause, spanning the range of life history phases from egg to adult, have been described in a great variety of insect species (reviewed in Danks, 2002).

There are important similarities among diapause phenotypes across insect species. Diapause phenotypes at egg, larval, pupal, and adult stages commonly enhance resistance to cold, desiccation, and starvation stresses, and most occur in response to similar environmental

triggers, a combination of decreasing photoperiod and low temperature (Tauber and Tauber 1976, Denlinger 2002). Evolution of the environmental triggers of diapause has allowed species of mosquitoes and herbivorous insects to invade novel habitats (*e.g* Hawley *et al* 1987, Feder *et al* 1997, Snodgrass 2003, Ward and Masters 2007, Mahdjoub and Menu 2008). Additionally, diapause phenotypes are regulated by photoperiod and insulin signaling pathways in the mosquitoes *Wyeomyia smithii* and *Culex pipiens* (Mathias *et al* 2005, Mathias *et al* 2007, Sim and Denlinger 2009a, 2009b Emerson *et al* 2010) and in *Drosophila melanogaster*, *D. triauraria*, and *D. montana* (Williams *et al* 2006, Tauber *et al* 2007, Sandrelli *et al* 2007, Schmidt *et al* 2008, Kankare *et al* 2010, Yamada and Yamamoto 2011). However, there are also lineage-specific features in diapause phenotypes, environmental triggers, genetic controls. These have been well-characterized in several dipteran species associated with cold-climate, including the mosquitoes, *Aedes albopictus*, *Wyeomyia smithii*, and *Culex pipiens*, the fruit fly, *Rhagoletis pomonella*, and numerous *Drosophila* (Table 2.1).

Egg diapause has been documented in *Aedes albopictus*, wherein maternal exposure to short daylength triggers a cold and desiccation-resistant dormancy in her developing offspring (Wang 1966, Mori *et al* 1981). This facultative response to environmental conditions has potentially facilitated the invasion of this species to novel habitats (Hawley *et al* 1987, Juliano and Lounibos 2005).

Bradshaw and colleagues have extensively investigated the environmental triggers and genetics underlying larval winter diapause of the pitcher plant mosquito, *Wyeomyia smithii*. Their studies have defined variation in the timing of diapause expression among populations, showing optimization to environmental signals of local seasonal change, which maximizes fitness in each population (Bradshaw 1976, Bradshaw *et al* 2000). The critical photoperiods to trigger and exit from diapause as well as the distributions of variable loci associated with critical photoperiod have changed in natural populations of *W. smithii* as a corollary of climate change (Bradshaw and Holzapafel 2001, Bradshaw and Holzapafel 2008). Differences in the expression of specific genes between diapausing and non-diapausing individuals, including the annotated genes, *Wstimeless* and *WsPpdrg1* have been identified (Mathias *et al* 2005). Further, nine QTLs are involved in the critical photoperiod for entering diapause and four QTLs are associated with the state of diapause (Mathias *et al* 2007, Emerson *et al* 2010).

Culex pipiens undergoes adult diapause through arrest of ovarian development, and shows increased cold and desiccation resistance under cool temperature and short daylength conditions (Eldridge 1968, Sanburg and Larsen 1973). In this species, diapause and associated winter-adaptive traits are controlled by multiple, pleiotropic loci (Mori *et al* 2007, Robich *et al* 2007). Gene regulation studies have shown that actins, fat metabolism genes and insulin signaling are involved in controlling

diapause in *C. pipiens* (Kim *et al* 2006, Sim and Denlinger 2008, Sim and Denlinger 2009a, 2009b).

The sympatric speciation model taxon, *Rhagoletis pomonella,* synchronizes the end of its pupal diapause with host fruiting time, producing a temporal barrier to gene flow between races (Bush 1966, Feder *et al* 1993). The allozymes associated with host differentiation among *R. pomonella* races are also markers indicative of the diapause trait (Feder *et al* 1988, Filchak *et al* 2000). Variable and heritable sensitivity to a given temperature during early pupal development triggers flies to either directly develop into adults or to overwinter in diapause. Changes in that sensitivity are likely to allow invasion of new habitats, including host shifts (Feder *et al* 1997).

Considerable diversity in the genetics of adult diapause is evident even within the *Drosophila* genus. Studies of overwintering diapause in *D. melanogaster* allow unusual power in illuminating the genetics underlying the trait due to the wealth of genetic tools developed for the system, along with its well-annotated genome. *D. melanogaster* populations across Europe and North America have independently evolved photoperiodic triggers of diapause, with diapause incidence increasing clinally with latitude (Tauber *et al* 2007, Emerson *et al* 2009). Populations on the two continents differ in the genetic variation underlying diapause clines. The diapause cline in European *D. melanogaster* is associated with variation in *timeless*, with two alleles differentially interacting with photoperiod and

genetic background to produce seasonally appropriate diapause (Tauber *et al* 2007, Sandrelli *et al* 2007).

In populations of *D. melanogaster* in North America, clinal variation in diapause incidence is due to variation in *couch potato*, wherein an additive high-diapause allele increases with latitude, and the low-diapause allele increases as latitude decreases (Schmidt *et al* 2008). Studies of laboratory strains of *D. melanogaster* have shown an association between diapause and alleles of the insulin-regulated phosphotidylinositol 3 kinase, *dp110* (Williams *et al* 2006). *dp110* is a homolog of *age-1*, a *C. elegans* gene involved in the development of the dauer state (Williams *et al* 2006). However, *dp110* variation associated with diapause is not apparent in wild North American populations of *D. melanogaster* (Williams *et al* 2006).

Adaptation of *D. melanogaster* to cold climate is recent. It has inhabited cold climate in Europe for less than15,000 years, and less than 500 years in North America and Australia (David and Capy 1988). Recent adaptation of *D. melanogaster* to temperate climates is supported by phenotypic and population genetic data (Turner *et al* 2008, deJong and Bochdanovits 2003, Schmidt *et al* 2005). For this reason, studies involving the genetic and evolutionary basis of overwintering phenotypes in *D. melanogaster* are limited to the short-term evolutionary response to these new environments, and it is unknown whether the patterns of genetic and phenotypic variation it displays over climate gradients are

evolutionarily stable and representative of long-term patterns of adaptation.

Kimura and colleagues have conducted extensive work investigating the physiology and inheritance of diapause in Japanese endemic relatives of *D. melanogaster*, the *D. auraria* species complex. Physiologically, diapause in this group entails a change in the fatty acid composition of triacylglycerols (Ohtsu *et al* 1993), increased storage of triacylglycerols (Ohtsu *et al* 1992), reduced locomotor activity, reduced feeding activity in females, and increased body weight in males (Matsunaga *et al* 1995). A study of the inheritance of diapause and its environmental triggers in *D. triauraria* has shown that variation in photoperiodic response and variation in diapause duration are associated with the same loci (Kimura and Yoshida 1995). More recent genetic studies of *D. triauraria* diapause have shown upregulation of *drosomycin* and a *drosomycin-like* gene during diapause (Daibo *et al* 2001), and allelic differences in *timeless* and *cryptochrome* are associated with variation in the incidence of diapause between strains (Yamada and Yamamoto 2011).

Diapause has also been studied in several members of the *D. virilis* species group that have ancient (>10 mya) associations with cold climate (Throckmorton 1982). In *D. littoralis,* a Northern European species, populations show clinal variation in the critical photoperiod needed to trigger diapause, with southern populations requiring shorter daylength to

enter diapause (Lumme and Oikarinen 1977). Flies derived from southern populations also show a lower propensity to diapause than flies derived from more northern populations (Lumme and Oikarinen 1977). Critical photoperiod for entering diapause and the diapause switch itself each appear to be governed by a single locus. The loci for the two traits are autosomal and closely linked, but the genes have not been specifically identified (Lumme 1981, Lankinen and Forsman 2006). Longer critical photoperiod alleles are incompletely dominant over shorter critical photoperiod alleles, and diapause promoting alleles are dominant over non-diapause alleles (Lumme *et al* 1975, Lumme and Oikarinen 1977).

More recent work has identified numerous genes involved in the diapause of *D. montana*, a member of the *D. virilis* species group that is widespread over Asia, Northern Europe, and North America. Microarray analysis showed that at least six genes are up or down-regulated in diapausing flies, including genes annotated as having functions in circadian rhythm, temperature stress, and insulin signaling. In the same study, qRT-PCR confirmed that *couch potato* is differently expressed in diapausing and non-diapausing *D. montana* (Kankare *et al* 2010).

Diapause in *D. lummei,* native to Northern Europe, was found to be controlled by a single, X-linked locus (Lumme and Karanen 1978). However, this result is based upon inheritance of the trait in a cross between *D. lummei* and a non-diapausing strain of *D. virilis,* and so may not accurately reflect all sources of variation within *D. lummei*. The

genetics of diapause in *D. lummei* have not been further analyzed using more recently developed techniques. It is therefore possible and perhaps likely that additional loci also function in the control of diapause.

Drosophila americana is a member of the *D. virilis* species group that is endemic to North America. Its species range spans 30° to 45°N (Patterson and Stone 1952, Throckmorton 1982, McAllister 2002, McAllister *et al* 2008). This latitudinal range is comparable to that of the North American *D. melanogaster* population, although the *D. americana* lineage is thought to have colonized North America about 3 million years ago (Hilton and Hey 1996, Hilton and Hey 1997, Spicer and Bell 2002, Caletka and McAllister 2004). Because *D. americana* is closely related to *D. lummei* and is thought to have colonized North America from Northern Eurasia, it is also likely to be ancestrally adapted to cold climate.

D. americana is known to exhibit clinal genetic variation; frequencies of a fusion between the X and 4th chromosomes, inversions of large regions of both X and 4, and sequence variation associated with these structural rearrangements are highly correlated with latitude (McAllister 2003, Evans *et al* 2007, McAllister *et al* 2008). Because other markers across the genome do not show geographically structured variation, it is likely that locally adapted X-linked alleles are maintained by natural selection in the face of gene flow among populations (McAllister *et al* 2008).

This study examines X-linked phenotypic variation in *D. americana* in relation to local winter severity, focusing on the inheritance of reproductive diapause. Because the clinally distributed genetic variation so far identified in *D. americana* is linked to the X chromosome (Evans *et al* 2007, McAllister *et al* 2008), and because the diapause trait has been linked to the X chromosome in *D. lummei* (Lumme and Karanen 1978), the closest Eurasian relative of *D. americana*, this study tests the hypothesis that variation in diapause incidence is X-linked in *D. americana*.

To identify an association between X chromosome variation and diapause incidence, we compared diapause incidence among inbred fly lines carrying X chromosome variants known to be clinally distributed across *D. americana* populations. We tested whether inbred fly lines carrying X chromosome variants more common in northern populations exhibit higher diapause incidence than those carrying X chromosome variants more common in southern populations. To test whether inheritance of diapause is consistent with X-linkage of the trait in *D. americana,* we performed both between and within species crosses, comparing diapause incidence among progeny known to carry different combinations of parental X chromosome variants. To further test whether diapause is associated with the presence of particular X chromosome variants, we introgressed each X chromosome variant into a genetic background associated with the alternative chromosomal arrangement and measured diapause incidence in the resulting progeny.

2.2 Materials and Methods

2.2.1 Measuring Diapause Incidence

Diapause incidence of 16 inbred lines of *D. americana* was measured in a standard laboratory assay. Inbred lines were originally derived from single, wild-caught female flies collected between 1996 and 2001 from four sites (Table 2.2). Lines have been inbred by mating full siblings over at least 11 generations and maintained under standard laboratory conditions. All lines have previously been assessed for presence or absence of the X-4 chromosomal fusion (Mena, 2009) (Table 2.2). Twelve lines are fixed for the fused X-4 chromosome and four lines are fixed for unfused X and 4^{th} chromosomes.

Flies used in the diapause assay were reared at low density on standard cornmeal media at 22°C under a 12:12 light:dark cycle. Virgin adults were collected at 0-48 hours following eclosion, placed in mixed-sex food vials containing a maximum of 20 individuals. Mixed-sex vials were maintained in an incubator at 11°C under a 10h:14h light:dark cycle for a 4-5 week period (Saunders *et al* 1989, Williams and Sokolowski 1993). Following this cool, short day length treatment, female flies were isolated and frozen at -20°C. Females were thawed, and the ovaries dissected and photographed. The developmental stage of each individual's ovaries was determined by applying the criteria defined by Lumme and Lakovaara (1983). Stage one ovaries, which correspond to King's stages 1-7, have round or slightly elongate cells, and no visible yolk

(King 1970, Lumme *et al* 1974) (Figure 2.1A). Stage two ovaries correspond to King's stages 8-13, and have more elongated cells with visible yolk (King 1970, Lumme *et al* 1974) (Figure 2.1B). Stage three ovaries, King's stage 14, contain at least one mature egg which is elongate, fully yolked, and has dorsal appendages (King 1970, Lumme *et al* 1974) (Figure 2.1C). Individuals having only ovaries in stage one upon dissection were classed as diapausing, while those having at least one ovary in stages two or three were classed as not diapausing.

Diapause incidence of each line was defined as the number of females in diapause divided by the total number of females dissected. Diapause incidence was compared between X-4 fused and unfused lines with a Chi-square test. One-way ANOVA was used to test for differences in diapause incidence between latitudes of origin among inbred lines. Measurements of diapause incidence of individual inbred lines were used to inform choice of lines for subsequent experiments.

2.2.2 Patterns of Diapause Inheritance

Crosses were performed between high and low diapausing lines of *D. virilis*-group species to determine whether diapause is inherited in a pattern consistent with X-linkage in *D. americana* and its close relatives. Because a previously published result suggests that diapause is an X-linked trait in *D. lummei* (Lumme and Karanen 1978), a close relative of *D. americana,* crosses were performed between the lines 1051.46 of *D. virilis* and 1011.01 of *D. lummei* (UCSD stock center) to replicate this result and

to validate the method for further studies of diapause inheritance in *D. americana*. Crosses between the *D. americana* lines, G96.10, OR01.50, and HI99.34 (Table 2.2), and the 1051.46 line of *D. virilis* were performed to determine whether the pattern of diapause inheritance is consistent with X-linkage of the trait in *D. americana*.

Additionally, because patterns of diapause inheritance in the between-species crosses could reflect common effects of *D. virilis* alleles or cross-species epistatic interactions in the hybrid genomes, crosses were performed between pairs of *D. americana* inbred lines (Table 2.2); the X-4 fused line G96.10 was crossed to the unfused line FP99.2, the X-4 fused line HI99.18 was crossed to the unfused line FP99.46, G96.10 was crossed to HI99.18, and FP99.2 was crossed to FP99.46.

In each case, reciprocal crosses were performed between the two parental lines (Figure 2.5A). Female representatives of the parental lines and F1 offspring were collected and subjected to the diapause-inducing treatment as described above. F1 males were backcrossed to the parental lines, and backcrossed offspring also subjected to the diapause-inducing treatment. Diapause incidence was measured for parental lines, the F1 generation and the backcross generation of each cross. Diapause incidence was compared between each parental line and respectively, F1 females heterozygous for X-linked loci, backcrossed heterozygotes, and backcrossed homozygotes using Chi-square tests. The probability of diapause of the F1 females produced by reciprocal crosses was also

compared using Chi-square tests, in order to identify possible major maternal effects on diapause inheritance.

2.2.3 Introgression of X-Chromosome Variants

In order to test the effects of X-linked loci on diapause more independently of genetic background effects, the fused X-4 chromosome was introgressed into a genetic background associated with the unfused X chromosome using crosses between the fused inbred line, OR01.92 and the unfused inbred line, FP99.2 (Table 2.2). In contrast, the unfused X-chromosome was introgressed into genetic backgrounds associated with fused X-4 chromosomes using crosses between the fused inbred lines, G96.10 and OR01.92 and the unfused inbred line, FP99.2 (Table 2.2).

Crosses were performed as shown in Figure 1. In each case, the X-chromosome associated with the maternal line was introgressed into the genetic background associated with the paternal line. In the initial cross, two parental lines differing in X-chromosome status (X-4 fused or unfused) were mated to produce female offspring heterozygous for fused and unfused X-chromosomes and male offspring carrying the X-chromosome associated with the maternal line. To decouple the maternally derived X-chromosome from the maternal line's cytoplasm, F1 male offspring were mated to females of the paternal line (F1 in Figure 2.2). This cross produced females heterozygous for fused and unfused X-chromosomes and carrying the cytoplasm associated with the paternal line, as well as males carrying the maternally derived X-chromosome. These females

were then backcrossed to males derived from the initial paternal line (BC1 in Figure 2.2).

Four offspring genotypes were produced by this cross; females heterozygous for fused and unfused X-chromosomes, females homozygous for the paternally derived X-chromosome, and two male types differing in the X-chromosome carried. Female offspring were mated to males of the paternal line, allowed to oviposit, and then frozen for genotyping. DNA was extracted from each individual and the X-linked locus *fu1* amplified as described in Vieira et al, 2001. Amplified *fu1* products were subjected to RFLP analysis, using *ClaI* to distinguish a single nucleotide polymorphism fully associated with the respective centromeric regions of X-4 fused and unfused X-chromosomes (Vieira et al 2001, Mena 2009). Previous studies have shown that much recombination along the length of fused and unfused X and 4th chromosomes is blocked by paracentric inversions observed only in fused chromsomes (Vieira et al 2006, McAllister 2003, McAllister and Evans, 2006). Consequently, although recombination between chromosomal forms is possible in heterokaryotypic females, most loci along the X and 4th chromosomes were expected to remain linked to their original centromeres throughout these crosses. Those regions which freely recombine between fused and unfused forms of the chromosomes do not exhibit sequence differentiation between the two forms. Female progeny of those females carrying the maternally derived X-chromosome form

were collected for continued backcrossing to the paternal line (BC2-8 in Figure 2.2). This process was repeated until eight generations of backcrossing to the paternal line were complete.

Progeny of the eighth backcross were composed of the four X-chromosome genotypes described above, and estimated to carry a genetic background that was 99.7% paternally derived, based on the breeder's equation. Full sib-matings were performed for two generations in order to produce female offspring homozygous for the maternally derived X-chromosome. Each mated pair was genotyped as described above, and offspring were saved from those pairs able to produce the desired X-chromosome composition.

Female progeny of sib-matings were subjected to the diapause-inducing treatment, dissected and assessed for diapause, and individually genotyped for *fu1* as described above. For each cross, the probability of diapause was compared between females homozygous for the introgressed X-chromosome and that of each parental line using Cochran-Mantel-Haenszel statistics. The probability of diapause for heterozygous females produced by each cross was also compared that of each parental line in order to test for dominance effects of X-linked loci.

2.3 Results

2.3.1 Diapause Incidence of Inbred Lines

Sixteen inbred *D. americana* lines of known X-chromosome type were subjected to the diapause induction treatment and their diapause

incidence measured (Table 2.2). Among the 12 lines carrying the fused X-4 chromosome, mean diapause incidence was measured as 0.73 (se = 0.087). Among the four lines carrying the unfused X-chromosome, mean diapause incidence was measured as 0.19 (se = 0.089). Individuals whose genome includes the fused X-4 chromosome have a significantly higher probability of diapause than flies carrying only the unfused X-chromosome (Chi-square = 113.89, $p < 0.0001$). One-way ANOVA revealed no significant relationship between latitude of origin as a continuous variable and diapause incidence for the inbred lines ($F = 0.6748$, $p = 0.584$).

2.3.2 Patterns of Inheritance of Diapause Among *D. virilis*-Group Species

Among crosses between the non-diapausing *D. virilis* line, V46, and highly diapausing lines of its close relatives, *D. lummei* and *D. americana*, diapause incidence was measured for parental lines and hybrid offspring carrying an X-chromosome derived from each parent or homozygous for X-chromosomes derived from the *D. virilis* parent (Table 2.3).

The results of the *D. virilis* by *D. lummei* cross were strongly consistent with a major, dominant X-linked factor determining diapause (Figure 2.3). F1 and backcrossed hybrid offspring carrying an X-chromosome derived from each parental line exhibited a probability of diapause that was significantly higher than that of the *D. virilis* parental line and not significantly different from the *D. lummei* parental line. Backcrossed hybrid offspring carrying two X-chromosomes derived from

D. virilis were significantly less likely to be in diapause than flies of the parental *D. lummei* line, and did not differ significantly from the parental *D. virilis* line.

For the *D. virilis* by *D. americana* crosses, results were largely consistent with X-linkage of the diapause phenotype (Figure 2.4). The probability of diapause of F1 hybrid offspring did not differ between reciprocal crosses for any lines used. For all crosses, F1 hybrid offspring showed significantly higher probability of diapausing than the *D. virilis* V46 parental line. In crosses using the lines G96.10 and OR01.50, F1 hybrid offspring showed significantly lower probability of diapausing than the *D. americana* parental line. Backcrossed hybrid offspring carrying an X-chromosome derived from both the V46 and a *D. americana* line showed a significantly higher probability of diapausing than the V46 parental line in all crosses, and a significantly lower probability of diapausing than the *D. americana* parental line in crosses using the lines G96.10. Backcrossed hybrid offspring carrying two X-chromosome derived from V46 showed no difference in probability of diapausing from the V46 parental line in crosses involving the *D. americana* lines, G96.10 and OR01.50, and a significantly higher probability of diapause than V46 in the cross using the *D. americana* line, HI99.34. In all crosses, backcrossed hybrid offspring carrying two X-chromosome derived from V46 showed a significantly lower probability of diapause than the *D. americana* parental line.

2.3.3 Inheritance of Diapause Among *D. americana* Lines

Among crosses between inbred lines of *D. americana*, diapause incidence was measured for parental lines and hybrid offspring known to be heterozygous or homozygous for each parental X-chromosome (Table 2.4) (Figure 2.5). F1 offspring produced by reciprocal crosses did not differ in probability of diapause for any combination of parental lines. Backcrossed offspring produced by crosses differing in direction and carrying one X-chromosome derived from each parent did not differ in probability of diapause for any combination of parental lines. Results were consistent with X-linkage of a major, dominant high-diapause allele.

When the low diapausing line FP99.2 was crossed to the highly diapausing line G96.10, F1 and backcrossed hybrid offspring carrying one X-chromosome derived from each parental line showed significantly higher probability of diapause than the FP99.2 parental line (Figure 2.5B). Backcrossed hybrid offspring homozygous for X-chromosomes derived from FP99.2 showed significantly lower probability of diapause than the G96.10 parental line. Backcrossed hybrid offspring homozygous for X-chromosomes derived from G96.10 showed significantly higher probability of diapause than FP99.2.

Interestingly, when the low diapausing line FP99.2 was crossed to the low diapausing line FP99.46, F1 and backcrossed hybrid offspring carrying one X-chromosome derived from each parental line showed significantly higher probability of diapause than both the FP99.2 and

FP99.46 parental lines (Figure 2.5C). Backcrossed hybrid offspring homozygous for X-chromosomes derived from FP99.46 showed significantly higher probability of diapause than both FP99.2 and FP99.46.

When the low diapausing line FP99.46 was crossed to the moderately diapausing line HI99.18, F1 hybrid offspring showed significantly higher probability of diapause than both FP99.46 and HI99.18(Figure 2.5D). Backcrossed hybrid offspring carrying one X-chromosome derived from each parental line showed significantly higher probability of diapause than FP99.46. Backcrossed hybrid offspring homozygous for X-chromosomes derived from FP99.46 showed significantly higher probability of diapause than FP99.46.

When the highly diapausing line G96.10 was crossed to the moderately diapausing line HI99.18, F1 and backcrossed hybrid offspring carrying one X-chromosome derived from each parental line showed significantly higher probability of diapause than HI99.18 (Figure 2.5E). Backcrossed hybrid offspring homozygous for X-chromosomes derived from HI99.18 showed significantly lower probability of diapause than the G96.10 parental line.

2.3.4 Introgression of X-Chromosomes Variants

Flies carrying X-chromosomes on genetic backgrounds normally associated with the alternative form of the X-chromosome were assessed for diapause tendency and compared to that of both parental classifications (Table 2.5). Flies homozygous for fused X-4 chromosomes

on a genetic background associated with unfused X-chromosomes were significantly more likely to be in diapause than flies of the unfused parental line. Flies carrying only one copy of the fused X-4 chromosome on a genetic background associated with unfused X-chromosomes were significantly more likely to be in diapause than flies of the unfused parental line. Flies homozygous for unfused X-chromosomes on a genetic background associated with fused X-4 chromosomes were significantly more likely to diapause than flies of the unfused parental line and were significantly less likely to diapause than flies of the fused parental line.

2.4 Discussion

Among the inbred lines of *D. americana* assessed for diapause incidence, flies belonging to lines fixed for the X-4 fused chromosome are significantly more likely to diapause than flies belonging to lines fixed for unfused X-chromosomes. These data did not reveal a significant relationship between latitude of origin and diapause incidence of the inbred lines. However, this sample included only four inbred lines fixed for the unfused X-chromosome, and three of these lines were derived from a single population, so geographical effects on diapause incidence are unlikely to be evident among these lines.

Crosses between *D. virilis* group species showed inheritance patterns of diapause consistent with X-linkage of the trait. The cross between *D. virilis* and *D. lummei* replicated the findings of a similar cross performed by Lumme and Karenen (1978), indicating at least one major,

dominant X-linked locus conferring high diapause incidence. Crosses between *D. virilis* and high-diapausing *D. americana* produced similar results, except that, in most cases, flies carrying only a single X-chromosome derived from *D. americana* showed diapause incidence intermediate between the two parental lines. This effect could indicate the influence of additional loci on the trait or additive effects of *D. americana*-derived high-diapause alleles. However, between-species crosses are vulnerable to the effects of interactions between heterospecific loci that would not occur in natural conspecific matings (Landry *et al* 2005a, Landry *et al* 2005b). For example, the observed effects in these crosses could be due to a recently derived low-diapause allele present in the *D. virilis* line which would make the homologous alleles of the other species appear as high-diapausing alleles in the interspecific hybrids.

Within *D. americana*, crosses between high and low-diapausing inbred lines show patterns of diapause inheritance consistent with X-linkage of at least one dominant major-effect allele conferring a high probability of diapause on carriers. Crosses between two lower diapausing lines exhibit complementation, with offspring carrying an X-chromosome derived from each parental line showing higher diapause incidence than both parental lines. This result could be due to the action of multiple loci, not necessarily X-linked, additively contributing to diapause, so that as more diapause-promoting alleles are present in an individual's genome, probability of diapause is higher. This would allow

for considerable variation in probability of diapause among individuals in populations where multiple diapause promoting alleles segregate. Alternatively, multiple, complementing low-diapause alleles at a single, not necessarily X-linked locus could produce highly diapausing offspring in crosses between low-diapausing lines. This would also allow variation in probability of diapause among individuals in populations where multiple low-diapause alleles segregate, with homozygotes having low probability of diapause and heterozygotes having high probability of diapause.
It is likely that low-diapause alleles are a derived trait in *D. americana*, and may have risen in frequency as *D. americana* invaded warmer climates at southern latitudes.

Introgression of each X-chromosome variant of *D. americana* into a genetic background associated with the alternate variant allowed X-linked effects to be identified in isolation. In agreement with the other crossing experiments, a fused X-chromosome derived from a high-diapausing line conferred a high probability of diapause as a dominant effect on flies with a genetic background derived from a low-diapausing line. An unfused X-chromosome derived from a low diapausing line conferred a low probability of diapause as a recessive effect on flies with a genetic background derived from a high-diapausing line. However, the diapause probability of these flies was higher than that of the unfused parental line, suggesting that additional autosomal loci could also contribute to the diapause phenotype.

The high-diapause phenotype observed in some lines of *D. americana* are shared by Northern European members of the *D. virilis* species group. Because the ancestors of *D. americana* colonized North America from Northern Europe, the high-diapause trait is likely ancestral. Additionally, because diapause is similarly inherited in *D. americana* and its close relative, *D. lummei*, the same loci may control diapause in both species. In *D. americana*, the high-diapause allele is associated with the derived fusion between X and 4^{th} chromosomes. This fusion is associated with multiple nested inversions on both chromosomes, which restrict recombination between chromosomal forms, and could thus serve as a mechanism for maintaining linkage between the high-diapause allele and other loci also conferring cold climate adaptations in the face of gene flow from populations at warmer latitudes. It is also possible that multiple loci functioning together to produce diapause phenotypes remain linked within inversions (Dobzhansky 1970).

These inversions do prevent the identification of individual genes involved in the diapause phenotype of *D. americana* by traditional genetics approaches. It is important to note, however, that these regions contain excellent candidate genes to investigate for diapause function as expression-based approaches become available for the *D. americana* genome. Diapause in *D. montana,* a close relative of *D. americana,* is associated with downregulation of both *nonA* and *cacophony* (Kancere *et al* 2010). Variation in both of these genes has been linked to the *Xc*

inversion of *D. americana*, which is fully associated with the fused X-4 chromosome (Mena 2009). *Timeless* does not show sequence variation associated with the chromosome rearrangement of *D. americana* (McAllister 2003).

Chromosomal inversions are associated with diapause in cold climate *Drosophila* species besides *D. americana*. *Drosophila triauraria*, of the *D. melanogaster* species group has several diapause-associated and winter adaptation loci within clinally distributed inversions (Kimura and Yoshida 1995). The major diapause locus of *D. littoralis* of the *D. virilis* species group is associated with a clinally distributed inversion (Lumme 1981, Lankinen and Forsman 2006). However, diapause loci have not been associated with inversions in *D. melanogaster*, or in the *D. virilis* group species, *D. lummei* and *D. montana*. So, while chromosomal inversions may be one mechanism of building or maintaining associations between loci with related functions in winter adaptation, they are certainly not a universal mechanism, even among closely related lineages of *Drosophila*.

Among *Drosophila* species that have been well studied for diapause, there are important similarities in the genetic regulation of the trait. The same genes, including *timeless* and *couch potato*, are known to be involved in the control of diapause in at least some independent lineages (Tauber *et al* 2007, Sandrelli *et al* 2007, Schmidt *et al* 2008, Kankare *et al* 2010, Yamada and Yamamoto 2011). Additionally,

pathways involved in circadian rhythm and insulin signaling are known to play a role in diapause phenotypes not only in *Drosophila* species, but in other cold climate adapted insects as well. This is not particularly surprising, given that the environmental triggers of diapause and the physiological changes involved in diapause, including delayed maturation, increased energy storage, and slowed metabolism are shared among lineages.

However, this work stands as evidence that, given similar selective pressures, evolutionary events are to some degree repeatable across lineages. To better understand the conditions and limits of this repeatability within the *Drosophila* diapause model, it will be necessary to increase the sampling of species for which the genetics, history, and selection pressures surrounding diapause have been studied. This will allow robust comparisons of these factors to be made between lineages which have recently gained diapause and those which have maintained the trait over many generations, as well as to identify commonly shared features of independently derived diapause.

Table 2.1 Reproductive diapause among temperate species of *Drosophila.*

Species	*Diapause*	*References*	*Species Group / Subgroup*
D. lummei	Present	Lumme 1978, Watabe 1983	Virilis / Virilis
D. virilis	Absent	Watabe 1983, Kimura 1984	Virilis / Virilis
D. virilis	Present	Hart-Schmidt unpublished data (Table A.2)	Virilis / Virilis
D. americana	Present	Hart-Schmidt (this work)	Virilis / Virilis
D. montana	Present	Watabe 1983	Virilis / Montana
D. ezoana	Present	Watabe 1983	Virilis / Montana
D. kanekoi	Present	Watabe 1983	Virilis / Montana
D. borealis	Present	Hart-Schmidt unpublished data (Table A.2)	Virilis / Montana
D. littoralis	Present	Lumme *et al* 1974	Virilis / Montana
D. robusta	Present	Carson and Stalker 1948	Robusta
D. lacertosa	Present	Ichijo *et al* 1980	Robusta
D. sordidula	Present	Ichijo *et al* 1980	Robusta
D. phalerata	Present	Geyspits and Simonenko 1970	Quinaria
D. transversa	Present	Geyspits and Simonenko 1970	Quinaria
D. tristis	Present	Basden 1954	Obscura / Obscura
D. alpina	Absent	Goto *et al* 1999	Obscura / Obscura
D. subsilvestris	Absent	Goto *et al* 1999	Obscura / Obscura
D. bifasciata	Present	Lakovaara *et al* 1972, Watabe 1979, Goto *et al* 1999	Obscura / Obscura
D. subobscura	Absent	Basden 1954, Goto *et al* 1999	Obscura / Obscura
D. guanche	Absent	Goto *et al* 1999	Obscura / Obscura
D. obscura	Present	Basden 1954, Begon 1976, Goto *et al* 1999	Obscura / Obscura
D. pseudoobscura	Present	Collett and Jarman 2001	Obscura / Pseudoobscura

Table 2.1 Continued

D. lowei	Present	Heed *et al* 1969	Obscura / Pseudoobscura
D. lutescens	Present	Ohtsu *et al* 1995	Melanogaster / Takahashii
D. takahashii	Absent	Ohtsu *et al* 1992	Melanogaster / Takahashii
D. auraria	Present	Kimura 1984	Melanogaster / Montium
D. biauraria	Present	Kimura 1984	Melanogaster / Montium
D. triauraria	Present	Kimura 1983	Melanogaster / Montium
D. quadraria	Present	Kimura 1983	Melanogaster / Montium
D. subauraria	Present	Ohtsu *et al* 1995	Melanogaster / Montium
D. rufa	Present	Ohtsu *et al* 1995	Melanogaster / Montium
D. serrata	Absent	Magiafoglou *et al* 2002	Melanogaster / Montium
D. melanogaster	Present	Saunders *et al* 1989, Williams and Sokolowski 1993	Melanogaster / Melanogaster
D. simulans	Absent	Bouletreau-Merle *et al* 2003	Melanogaster / Melanogaster

Table 2.2 X-4 fusion status of inbred lines and coordinates of sites from which flies founding each line were collected (Mena, 2009).

Line	X-4 Fusion	Latitude	Longitude	N	# in Diapause	Diapause Incidence
FP99.2	Unfused	34.19	-91.08	40	1	0.03
FP99.34	Fused	34.19	-91.08	17	17	1.00
FP99.46	Unfused	34.19	-91.08	25	11	0.44
FP99.52	Unfused	34.19	-91.08	40	5	0.13
HI99.4	Fused	38.66	-90.68	30	22	0.73
HI99.18	Fused	38.66	-90.68	31	14	0.45
HI99.34	Fused	38.66	-90.68	13	13	1.00
HI99.48	Fused	38.66	-90.68	13	11	0.85
HI99.50	Fused	38.66	-90.68	39	1	0.03
G96.10	Fused	41.55	-87.37	34	33	0.97
G96.13	Fused	41.55	-87.37	46	40	0.87
G96.21	Fused	41.55	-87.37	13	5	0.38
G96.45	Fused	41.55	-87.37	19	13	0.68
OR01.46	Unfused	41.61	-83.20	23	4	0.17
OR01.50	Fused	41.61	-83.20	29	26	0.90
OR01.92	Fused	41.61	-83.20	48	44	0.92

Table 2.3 Results of between-species crosses.

Species/Line or Cross Composition	Gen.	X-Chromosome Composition	N	# in Diapause	D.I.	Chi-square vs. V46	Chi-square vs. other parent
D. virilis/V46	P	X_{V46} X_{V46}	40	0	0.00	-	-
D. lummei/1011.01	P	$X_{1011.01}$ $X_{1011.01}$	40	39	0.98	-	-
V46x1011.01	F1	X_{V46} $X_{1011.01}$	20	19	0.95	72.25**	0
1011.01xV46	F1	X_{V46} $X_{1011.01}$	20	20	1.00		
V46x(1011.01xV46)	BC1	X_{V46} $X_{1011.01}$	8	8	1.00	41.07**	0.02
V46x(V46x1011.01)	BC1	X_{V46} X_{V46}	44	3	0.07	1.20	65.34**
D. americana/G96.10	P	$X_{G96.10}$ $X_{G96.10}$	40	39	0.98	-	-
V46xG96.10	F1	X_{V46} $X_{G96.10}$	4	3	0.75	21.89**	8.68**
G96.10xV46	F1	X_{V46} $X_{G96.10}$	6	3	0.50		
V46x(G96.10xV46)	BC1	X_{V46} $X_{G96.10}$	22	15	0.68	32.36**	8.40**
V46x(V46xG96.10)	BC1	X_{V46} X_{V46}	45	0	0.00	0	77.19**
D. americana/OR01.50	P	$X_{OR01.50}$ $X_{OR01.50}$	40	34	0.85	-	-
V46xOR01.50	F1	X_{V46} $X_{OR01.50}$	5	1	0.20	16.48**	3.80
OR01.50xV46	F1	X_{V46} $X_{OR01.50}$	8	5	0.63		
V46x(OR01.50xV46)	BC1	X_{V46} $X_{OR01.50}$	48	39	0.81	55.12**	0.03
V46x(V46xOR01.50)	BC1	X_{V46} X_{V46}	27	0	0.00	0	43.26**

Table 2.3 Continued

D. americana/HI99.34	P	$X_{HI99.34}$ $X_{HI99.34}$	40	39	0.98	-	-
V46xHI99.34	F1	$X_{V46}X_{HI99.34}$	2	2	1.00	29.29**	0.26
HI99.34xV46	F1	$X_{V46}X_{HI99.34}$	4	3	0.75		
V46x(HI99.34xV46)	BC1	$X_{V46}X_{HI99.34}$	42	35	0.83	54.80**	3.20
V46x(V46xHI99.34)	BC1	X_{V46} X_{V46}	6	2	0.33	7.08**	16.05**

D.I. represents diapause incidence. Significantly different diapause incidences between parents and crossed offspring are shown. ** denotes p-value <0.01, * denotes p-value <0.05.

Table 2.4 Results of crosses between *D. americana* lines.

Line or Cross Composition	Gen.	X-Chromosome Composition	N	# in Diapause	D.I.	Chi square vs. Parent [a]	Chi square vs. Parent [b]
G96.10 (X-4 fused)	P	$X_{G96.10}$ $X_{G96.10}$	20	20	1.00	-	-
FP99.2 (X and 4 unfused)	P	$X_{FP99.2}$ $X_{FP99.2}$	20	1	0.05	-	-
HI99.18 (X-4 fused)	P	$X_{HI99.18}$ $X_{HI99.18}$	10	5	0.50	-	-
FP99.46 (X and 4 unfused)	P	$X_{FP99.46}$ $X_{FP99.46}$	20	2	0.10	-	-
G96.10 [a] xFP99.2 [b]	F1	$X_{G96.10}$ $X_{FP99.2}$	20	20	1.00	0.40	40.27**
FP99.2 [b] xG96.10 [a]	F1	$X_{G96.10}$ $X_{FP99.2}$	20	17	0.85		
FP99.2x(G96.10xFP99.2)	BC1	$X_{G96.10}$ $X_{FP99.2}$	22	21	0.96	1.06	39.08**
G96.10x(FP99.2xG96.10)	BC1	$X_{G96.10}$ $X_{FP99.2}$	24	20	0.83		
G96.10x(G96.10xFP99.2)	BC1	$X_{G96.10}$ $X_{G96.10}$	16	16	1.00	0	28.49**
FP99.2x(FP99.2xG96.10)	BC1	$X_{FP99.2}$ $X_{FP99.2}$	22	3	0.14	28.15**	0.18
FP99.2 [a] xFP99.46 [b]	F1	$X_{FP99.2}$ $X_{FP99.46}$	20	14	0.70	25.32**	21.89**
FP99.46 [b] xFP99.2 [a]	F1	$X_{FP99.2}$ $X_{FP99.46}$	20	17	0.85		
FP99.46x(FP99.2xFP99.46)	BC1	$X_{FP99.2}$ $X_{FP99.46}$	21	8	0.38	6.86**	4.76*
FP99.2x(FP99.46xFP99.2)	BC1	$X_{FP99.2}$ $X_{FP99.46}$	23	10	0.44		
FP99.2x(FP99.2xFP99.46)	BC1	$X_{FP99.2}$ $X_{FP99.2}$	25	7	0.28	2.60	1.27
FP99.46x(FP99.46xFP99.2)	BC1	$X_{FP99.46}$ $X_{FP99.46}$	21	14	0.67	14.24**	11.55**

Table 2.4 Continued

FP99.46 [a] xHI99.18 [b]	F1	$X_{FP99.46}$ $X_{HI99.18}$	20	19	0.95	25.93**	3.02
HI99.18 [b] xFP99.46 [a]	F1	$X_{FP99.46}$ $X_{HI99.18}$	20	14	0.70		
HI99.18x(FP99.46xHI99.18)	BC1	$X_{FP99.46}$ $X_{HI99.18}$	19	8	0.42	8.92**	0.05
FP99.46x(HI99.18xFP99.46)	BC1	$X_{FP99.46}$ $X_{HI99.18}$	20	13	0.65		
FP99.46x(FP99.46xHI99.18)	BC1	$X_{FP99.46}$ $X_{FP99.46}$	21	14	0.67	11.55**	0.25
HI99.18x(HI99.18xFP99.46)	BC1	$X_{HI99.18}$ $X_{HI99.18}$	13	2	0.15	0.21	1.77
G96.10 [a] xHI99.18 [b]	F1	$X_{G96.10}$ $X_{HI99.18}$	20	20	1.00	0.51	12.89**
HI99.18 [b] xG96.10 [a]	F1	$X_{G96.10}$ $X_{HI99.18}$	20	19	0.95		
HI99.18x(G96.10xHI99.18)	BC1	$X_{G96.10}$ $X_{HI99.18}$	20	14	0.70	2.20	3.52
G96.10x(HI99.18xG96.10)	BC1	$X_{G96.10}$ $X_{HI99.18}$	23	22	0.96		
G96.10x(G96.10xHI99.18)	BC1	$X_{G96.10}$ $X_{G96.10}$	12	10	0.83	1.28	1.47
HI99.18x(HI99.18xG96.10)	BC1	$X_{HI99.18}$ $X_{HI99.18}$	23	8	0.35	17.26**	0.19

D.I. represents diapause incidence. Significantly different diapause incidences between parents and crossed offspring are shown. ** denotes p-value <0.01, * denotes p-value <0.05.

Table 2.5 Results of introgression crosses.

Classification	X-Chromosome Composition	Genetic Background	N	# in Diapause	D. I.	cmh vs. fused parent (logit)	cmh vs. unfused parent (logit)
Unfused into Fused	$X_{unfused}\,X_{unfused}$	Fused X	9	5	0.56	8.0 (0.1136)**	18.9 (48.8)**
Fused into Unfused	$X_{unfused}\,X_{fused}$	Unfused X	88	70	0.80	ns	65.6 (151.7)**
Fused into Unfused	$X_{fused}\,X_{fused}$	Unfused X	32	27	0.84	ns	49.4 (210.6)**
Fused Parental	$X_{fused}\,X_{fused}$	Fused X	48	44	0.92	-	-
Unfused Parental	$X_{unfused}\,X_{unfused}$	Unfused X	40	1	0.03	-	-

D.I. represents diapause incidence. Significantly different diapause incidences between parents and crossed offspring are shown. ** denotes p-value <0.01, * denotes p-value <0.05.

Figure 2.1 Example images of stages of ovarian development in *Drosophila*. A) Stage 1, B) Stage 2, C) Stage 3 (Lumme 1983)

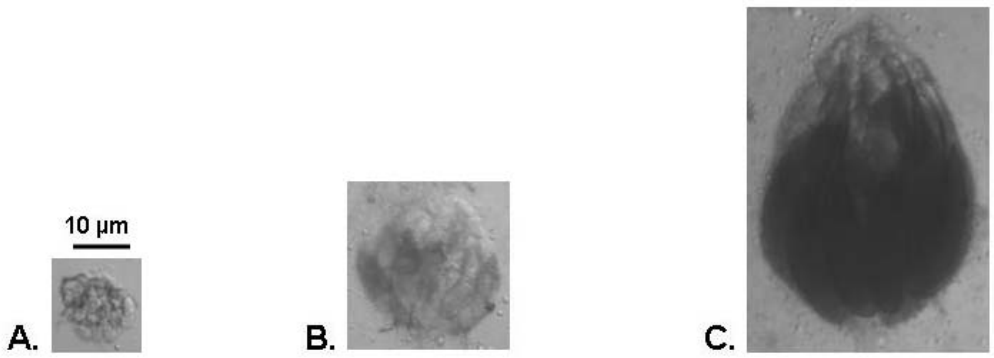


Figure 2.2 X-4 chromosome inheritance throughout introgression crossing design for (A.) unfused X and 4 into fused genetic background and (B.) fused X-4 into unfused genetic background. Boxed chromosome representations represent hybrid progeny each generation.

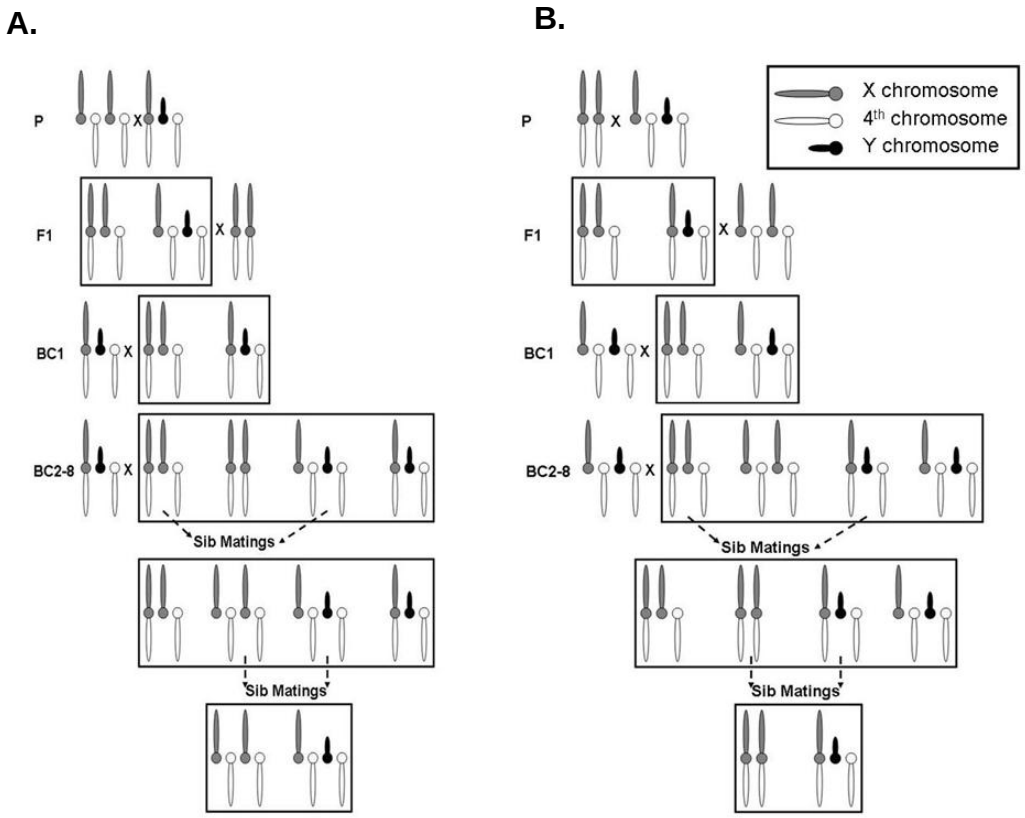


Figure 2.3 Measured proportions of females in diapause among parental lines and hybrid offspring in a cross between *D. virilis* and *D. lummei*. Among hybrid offspring, the maternal species of the cross producing the hybrid class is listed first. V indicates *D. virilis* and L represents *D. lummei.*

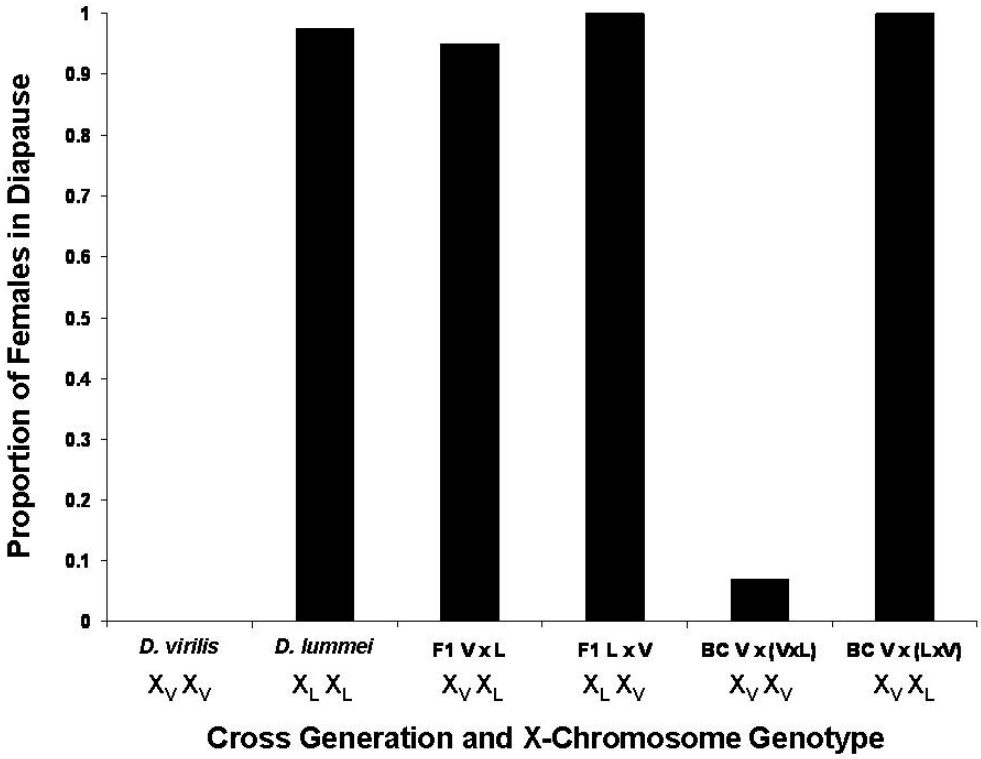


Figure 2.4 Measured proportions of females in diapause among parental lines and hybrid offspring in a cross between *D. virilis* and *D. americana*. Among hybrid offspring, the maternal species of the cross producing the hybrid class is listed first. V indicates *D. virilis* and A represents *D. americana.*

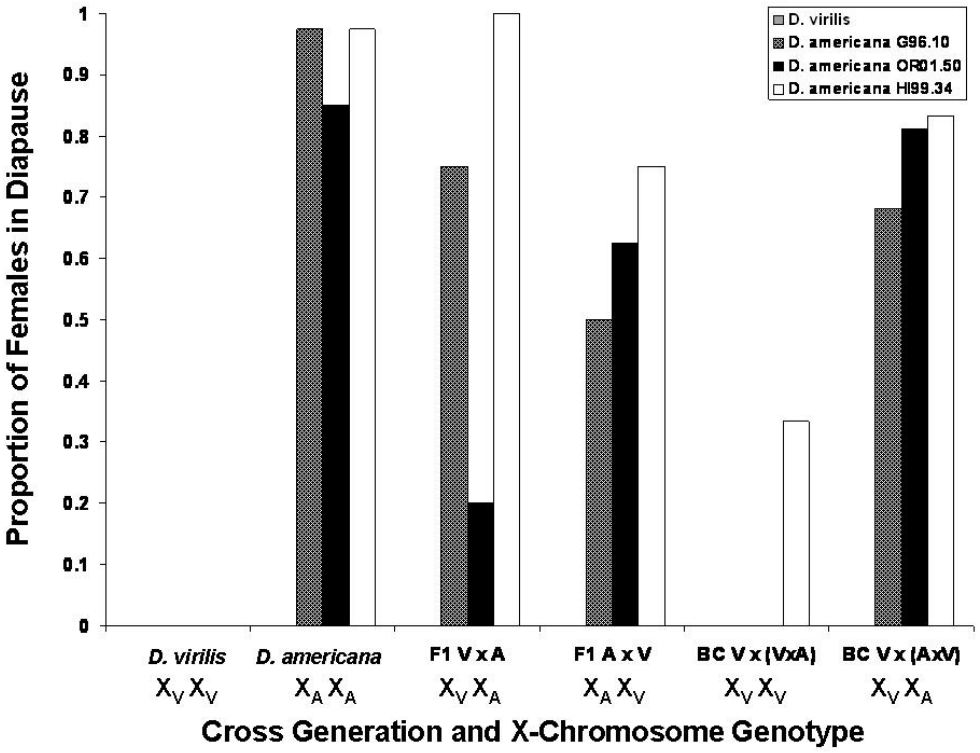
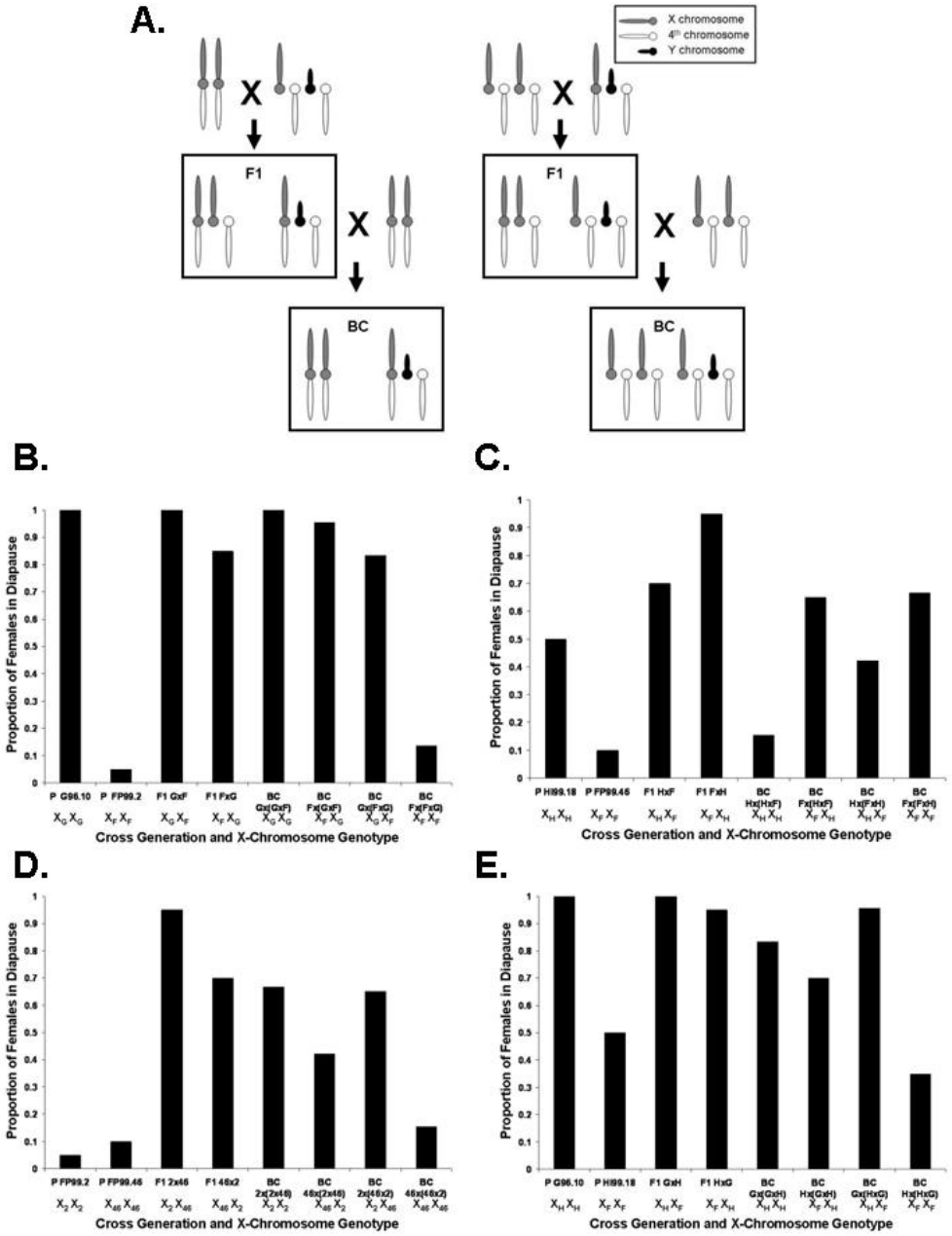


Figure 2.5 Diagram of crossing design and measured proportions of females in diapause among parental lines and hybrid offspring in crosses between inbred lines of *D. americana*. A) Inheritance of X and 4th chromosomes in crosses between X-4 fused and unfused lines. B-E) Results of crosses between four pairs of inbred lines. Among hybrid offspring, the maternal line of the cross producing the hybrid class is listed first.



CHAPTER 3

GEOGRAPHICALLY PATTERNED VARIATION IN PUTATIVELY ADAPTIVE TRAITS IN *DROSOPHILA AMERICANA*

3.1 Introduction

Complex environments impose numerous sources of selective pressure on organisms, including temperature stresses, resource limitations, parasite and disease loads, as well as predation and competition. High fitness individuals are those able to simultaneously avoid or resist multiple stresses and obtain multiple resources in order to survive and reproduce (Lande and Arnold 1983, Phillips and Arnold 1989). This requires the expression of multiple traits, and it is expected that the suite of traits that enhance fitness in a given environment will covary with the relevant environmental variable(s), and that species having similar ecology and environments are predicted to share some, but not all traits covarying with environmental variables (Endler 1995).

However, selection affects not only loci controlling a trait, but also those genetically and functionally linked to target loci, some selectively neutral traits will also covary with those enhancing fitness (Gould and Lewontin 1979, Lande 1979, Lande and Arnold 1983). These traits are likely to differ between lineages due to such neutral factors as differences between lineages in the linkage relationships among loci, variation in the composition or organization of regulatory networks, or from the demographic factors determining the available genetic variation for

selection to act upon. Taken together, a consequence is that the identification of traits associated with increased fitness in a given environment requires either direct measures of the fitness value of each trait, which is likely impossible in cases of covarying traits (Barrett and Hoekstra 2011), or by identifying those traits covarying with environment in multiple independent lineages sharing ecology and sources of selection (Calboli et al 2003, Boughman et al 2005, Crozat et al 2010).

Members of the genus *Drosophila*, inhabiting cold climates can serve as an effective model to employ the second approach. European, Australian, Indian, South and North American populations of *D. melanogaster* represent independent colonizations of cooler climates from the ancestral African population (David and Capy 1988). All cold climate insects must avoid or resist the stresses of cold, reduced availability of food and water, and unfavorable conditions for reproduction that occur during winter (Danks 1996).

Clinally patterned phenotypic variation across latitude has been studied in multiple *D. melanogaster* populations. Adult diapause, a facultative reproductive quiescence in response to cold temperature and short daylength, has been documented in both European and North American *D. melanogaster*. Besides delaying reproduction, the diapause state results in decreased senescence, increased lifespan, and increased resistance to temperature and starvation stresses (Schmidt et al 2005, Tatar et al 2001, Tauber et al 2007). Further, in the North American *D.*

melanogaster population, variation at *couch potato*, the locus controlling the diapause state, has been linked to variation in stress resistance and fecundity (Schmidt et al 2005, Schmidt et al 2008). Among North American lines of *D. melanogaster*, those which diapause have higher lipid content per body mass than those which do not (Schmidt et al 2005).

There is also geographically patterned variation in *D. melanogaster* traits that have not been functionally or genetically linked to diapause. Berry and Kreitman (1993) described clinal variation in *adh* alleles which have phenotypic effects on the metabolism of ethanol in the laboratory. *D. melanogaster* populations outside of North America show clinal variation in resistance to dessication, starvation, and thermal stresses (Karan et al 1998, Robinson et al 2000, Trotta et al 2006). Body size is a clinally distributed trait in all studied *D. melanogaster* populations, with flies living at higher latitudes achieving larger size measured by either body mass or wing length (Imasheva *et al*. 1994, Coyne and Beecham 1987, James *et al*. 1995, Noach et al 1996). Cold climate *D. melanogaster* also show higher glycogen levels and glycogen storage than those from more tropical sites (Eanes 1999; Verrelli and Eanes 2001, Bochdanovits and deJong 2003).

Several studies have linked diapause and other traits associated with cold climate populations of *D. melanogaster* to the insulin signaling pathway, which regulates physiological processes including growth and metabolism (Johnston and Gallant 2002). *Couch potato* controls diapause

in wild populations of North American *D. melanogaster* (Schmidt et al 2008), while *dp110* has been shown to play a role in diapause in laboratory strains (Williams et al 2006). Both North American and Australian populations show clinally distributed variation in *chico, TOR,* and *Pten* (DeJong and Bochdanovits 2003). Alleles of additional metabolic genes such as *pgm* are commonly found to be clinally distributed in cold climate populations of *D. melanogaster* (Sezgin et al 2004).

Insulin signaling genes also appear to be important to the fitness of an independently cold-adapted lineage of *Drosophila*, and other insect species (reviewed in Hahn and Denlinger 2011). Insulin signaling genes have been associated with diapause and metabolic traits proposed to function in cold-climate fitness in *D. montana,* and in *Culex pipiens* (Kankare et al 2010, Sim and Denlinger 2008). This observation supports the prediction that multiple growth and metabolism traits could be associated with both climate and diapause across *Drosophila* lineages. However, the extent to which cold-climate associated traits in other lineages of *Drosophila* mirror those observed in *D. melanogaster* has not been clearly established. This can be tested by determining whether variation in this class of phenotypes is predicted by variation in diapause incidence and/or climate variables in lineages of *Drosophila* which have evolved under cold-climate conditions independently from *D. melanogaster.*

D. americana has several features particularly appealing for comparison to putative cold-adaptation traits in *D. melanogaster*. *D. americana* is endemic to North America, with a latitudinal range comparable to that of North American *D. melanogaster* (Patterson and Stone 1952, Throckmorton 1982, McAllister 2002, McAllister et al 2008). However, the *D. americana* lineage has inhabited North America for at least 3 million years (Caletka and McAllister 2004), and shares a recent common ancestor with Northern European members of the *D. virilis* species group, and so likely has an even more ancient association with cold climates (Throckmorton 1982). *D. americana* is known to exhibit clinally distributed genetic variation, in the form of a fusion between the X and 4^{th} chromosomes, with associated chromosomal inversions and sequence divergence between fused and unfused X and 4^{th} chromosomes (McAllister 2003, Evans et al 2007, McAllister et al 2008). Inversions along the fused X and 4^{th} chromosomes of *D. americana* contain several insulin signaling genes, and sequence variation in other genes is associated with the presence of the fusion (McAllister 2003, Evans et al 2007, McAllister et al 2008, Mena 2009). X-4 linked structural and sequence variation is associated with probability of diapause and of cold coma recovery time in inbred lines of *D. americana* (Chapter 2, Mena 2009).

Numerous isofemale lines of *D. americana* derived from wild flies collected from sites spanning the species range have been maintained in

the McAllister laboratory. These lines reflect natural geographic patterns of phenotypic and genetic variation in the species. This study sets out to test the hypothesis that adaptation to cold climate involves the same traits in related, but independently adapted, lineages of *Drosophila,* 72 isofemale lines derived from individuals collected from 18 sites distributed over the latitudinal range of *D. americana* were utilized to assess patterns of phenotypic variation with respect to latitude of origin and probability of diapause. Phenotypes assessed in this study were probability of diapause, wing length, starvation resistance, body mass, and whole-body lipid content. These were chosen both based on simplicity of measurement as well as plausible associations with both cold-climate fitness and insulin signaling.

3.2 Materials and Methods

3.2.1 Isofemale Lines

Isofemale lines are composed of the descendants of single, wild-caught female flies collected between 1997 and 2007. Lines were chosen to represent the breadth of latitudinal variation present in the species, therefore, a total of 18 populations located along the Mississippi River Valley between 30.70˚N and 41.78˚N were sampled (Table 3.1). Four isofemale lines derived from each population were used in this study for a total of 72 lines (Table 3.1). For each experiment, a sample of breeding flies was taken from laboratory stocks of isofemale lines and reared at low density for one generation before being subjected to experimental

conditions. In some cases, a subset of lines failed to produce offspring or suffered excessive deaths during experimental set up, so measurements of every phenotype were not recorded for all lines (Table A.2).

3.2.2 Diapause

Flies used in the diapause assay were reared at low density on standard cornmeal media at 22°C under a 12:12 light:dark cycle. Virgin adults were collected at 0-48 hours following eclosion, placed in mixed-sex food vials containing a maximum of 20 individuals. Mixed-sex vials were maintained in an incubator at 11°C under a 10h:14h light:dark cycle for a 4-5 week period (Saunders et al 1989, Williams and Sokolowski 1993). Following this cool, short day length treatment, female flies were isolated and frozen at -20°C. Females were thawed, and the ovaries dissected and photographed. The developmental stage of each individual's ovaries was determined by applying the criteria defined by Lumme and Lakovaara (1983), described in Chapter 2 of this work.

Diapause incidence of each line was defined as the number of females in diapause (neither ovary developed beyond stage 1) divided by the total number of females dissected. Logistic regression was performed between the number of flies in diapause divided by the number of flies dissected against the latitude of origin for each isofemale line. Mean probability of diapause was compared between lines derived from the northernmost nine populations (36.27°N and above) and the southernmost nine populations (34.71°N and below) using the F-statistic (ANOVA).

3.2.3 Wing Length

Flies used to measure wing length were reared at low density on standard cornmeal media at 22°C under a 12:12 light:dark cycle. Virgin adults were collected at 0-48 hours following eclosion, and placed 10-12 flies per vial, five vials per line and sex, in single sex vials on standard cornmeal media. They were aged for seven days at 22°C under a 12:12 light:dark cycle, and frozen at -20°C for later analysis. A single wing was dissected from each frozen fly and mounted on a glass slide. Digital photographs were taken of each wing at a standard magnification, and calibrated with photographs of a slide micrometer taken at the same magnification. Wings were measured from photographs along wing vein L3 from the anterior cross vein to tip, converting pixels to micrometers using ImageJ (http://rsbweb.nih.gov/ij/). A minimum of 15 and a maximum of 50 individuals were measured for each line and sex. Mean wing length for each line and sex was calculated. Pearson correlation coefficients (R) were calculated for each sex between mean wing length and latitude of origin of isofemale lines. Mean wing length was compared using between females and males overall, and within the nine northern and nine southern populations respectively, as well as between sexes within the northern and southernmost populations respectively using the F-statistic (ANOVA).

3.2.4 Starvation Resistance

Flies used in the starvation resistance assay were reared at low density on standard cornmeal media at 22°C under a 12:12 light:dark cycle. Virgin adults were collected at 0-48 hours following eclosion. They were then placed seven days to age on standard cornmeal media 10-12 individuals per vial at 22°C under a 12:12 light:dark cycle. Following this, each group of flies was transferred to vials containing sterilized 2% agar. Agar was considered to provide adequate water and negligible nutrition. For each line and sex, five agar vials, each containing 10-12 individuals were housed at 22°C under a 12:12 light:dark cycle. Every 24 hours, vials were visually examined, and the number of dead flies recorded until all flies were dead. Mean days survived was figured for each vial, and averaged over all five vials per sex and line to produce a mean survival time in days for each sex and line. Pearson correlation coefficients (R) were calculated for each sex between mean survival time and latitude of origin of isofemale lines. Mean survival time was compared using between females and males overall, and within the nine northern and nine southern populations respectively, as well as between sexes within the northern and southernmost populations respectively using the F-statistic (ANOVA).

3.2.5 Mass and Lipid Content

Flies used in the starvation resistance assay were reared at low density on standard cornmeal media at 22°C under a 12:12 light:dark

cycle. Virgin adults were collected at 0-48 hours following eclosion, and placed 22 flies per vial, five vials per line and sex, in single sex vials on standard cornmeal media. They were aged for seven days at 22°C under a 12:12 light:dark cycle and frozen at -80°C for later analysis. Lipid extraction was performed using a modified version of the protocol employed by Ballard et al (2008). Five batches of 20 flies per sex and line were placed in a 70°C incubator for 48 hours. Following this, the mass of each batch of desiccated flies was measured. Each batch of desiccated flies was then treated with10mL di-ethyl ether for 24 hours to extract lipids. Ether containing extracted lipids was removed, and each batch of flies was dried for an additional 24 hours at 70°C. The mass of each batch of flies following ether extraction and drying was measured. The lipid content as a proportion of each batch of flies was calculated as (dry mass – ether extracted mass) / dry mass. Mean dry mass and lipid content was calculated for each sex and line. Pearson correlation coefficients (R) were calculated for each sex between mean dry mass and latitude of origin of isofemale lines, as well as between mean lipid content and latitude of origin of isofemale lines. Further, Pearson correlation coefficients (R) were calculated for each sex between mean dry mass and mean survival time, as well as between mean lipid content and mean survival time. Mean body mass and lipid content were compared using between females and males overall, and within the 9 northern and 9 southern populations

respectively, as well as between sexes within the northern and southernmost populations respectively using the F-statistic (ANOVA).

3.3 Results

3.3.1 Diapause

Among 64 sampled isofemale lines of *D. americana* from 18 populations, mean probability of diapause is 0.69 with a standard error of 0.039. The difference between northern and southern means is non-significant (Table 3.2). However, logistic regression shows a significant association ($p<0.0001$, odds ratio 1.25), between probability of diapause and latitude of origin (Figure 3.1). The 32 lines derived from Southern populations are quite variable in probability of diapause, with eight lines having probabilities of diapause below 0.2, eight lines above 0.8, and the remaining 16 lines having intermediate probabilities (Table 3.3). Among those 32 lines derived from Northern populations, probability of diapause is almost universally high, with only one line having a probability of diapause below 0.2, 24 lines having probabilities of diapause above 0.8, and the remaining seven lines having intermediate probabilities (Table 3.3).

3.3.2 Wing Length

Wing length was measured for females of 51 isofemale lines derived from 16 populations and males of 38 isofemale lines derived from 15 populations. Comparisons of means between sexes and northern and southern localities yielded no significant differences (Table 3.2). Latitude

of origin is significantly correlated with wing length in both male (R=0.759, p<0.0001) and female *D. americana* (R=0.697, p<0.0001) (Figure 3.2). Partial regression excluding probability of diapause, reveals a significant association between wing length and latitude for both males (R=0.6701, p<0.0001) and females (R=0.5182, p=0.0001). Probability of diapause is also significantly correlated with both male and female measures of wing length; with R=0.575 (p=0.0002) for males and R=0.675 (p<0.0001) for females. Partial regression excluding latitude of origin reveals a significant association between wing length and probability of diapause for females (R=0.3484, p=0.0142), but not for males (R=0.2672, p=0.1152).

3.3.3 Starvation Resistance

Starvation resistance was measured for females and males of 71 isofemale lines derived from 18 populations. Significant differences in mean days surviving the starvation treatment were observed for the overall male and female means, as well as between the sexes derived from both southern and northern populations, with females always surviving significantly longer than males (Table 3.2). Means within sexes between southern and northern populations were not significantly different for either females or males (Table 3.2). Male starvation survival is significantly correlated with both latitude of origin (R=0.275, p=0.0201) and probability of diapause (R=0.445, p=0.0002) (Figure 3.3). Female starvation survival is also correlated with both latitude of origin (R=0.321, p=0.0064) and probability of diapause (R=0.390, p=0.0015).

Partial regression excluding probability of diapause, reveals no significant association between starvation resistance and latitude for males (R=0.0591, p=0.6457) or females (R=0.1602, p=0.2095). Partial regression excluding latitude of origin reveals a significant association between starvation resistance and probability of diapause for males (R=0.3550, p=0.0043), but not for females (R=0.1731, p=0.1748).

3.3.4 Body Mass and Lipid Content

Body mass and lipid content show no significant differences between the sexes or between localities (Table 3.2). Neither do they show significant correlation with either latitude of origin or probability of diapause. However, body mass and lipid content are significantly correlated with each other; R= 0.672 ($p<0.0001$) for males and R=0.753 ($p<0.0001$) for females.

3.4 Discussion

The probability of diapause does not significantly differ between lines derived from Northern populations and those derived from Southern populations. This is likely due to high variance in probability of diapause among Southern lines, as probability of diapause is nearly universally high among isofemale lines originating from northern latitudes. Both this and the observation that the probability of diapause is positively associated with latitude are consistent with a selective advantage to diapause under conditions present in Northern populations and relaxed selection on the trait in Southern populations. Also, high variation in probability of

diapause among southern-derived lines of *D. americana* is consistent with previous observations of the trait's inheritance. Unfused X and 4th chromosomes, at high frequency in southern latitudes, appear to harbor multiple alleles and/or loci contributing to probability of diapause (Chapter 2).

The potential for relaxed selection on diapause at Southern latitudes would suggest that *D. americana* does not experience a fitness cost to diapause in the form of reduced fertility of high-diapause lines as *D. melanogaster* does (Schmidt et al 2008). The ancient association between the *D. americana* lineage and cold climate may have allowed disassociation between diapause and fertility traits (Moran 1994). Fertility could be compared between high and low-diapausing lines of *D. americana.* However, laboratory comparisons may not accurately reflect realized fertility under natural conditions (Leroi et al 1994, Berger et al 2008, Messina and Fry 2003). Alternatively, because southern sites harboring *D.* americana do experience occasional harsh winters, periodic selection favoring diapausing flies could maintain variation for the trait in the face of unknown fitness costs (Hendrick 1974).

Mean wing length is not significantly different between lines derived from Northern and Southern populations, nor between males and females. Mean starvation resistance is significantly higher in females than in males, and higher among Northern lines than Southern lines. Mean wing length and starvation resistance are positively associated with both latitude and

mean probability of diapause. Partial regressions reveal that wing length is more strongly associated with latitude, while starvation resistance is more strongly associated with probability of diapause. Body size is known to have a strong association with latitude but has not been directly associated with diapause in *D. melanogaster* (Imasheva et al 1994, Coyne and Beecham 1987, James et al 1995, Noach et al 1996). Growth is regulated by numerous loci in *Drosophila*, of which at least some appear unassociated with the pathway regulating diapause, and could respond to selection on traits unrelated to the potential for diapause (Oldham et al 2000). However, in *D. melanogaster*, increased starvation resistance occurs during diapause, and presence of the high-diapause allele of North American *D. melanogaster* is predictive of increased starvation resistance (Schmidt et al 2008). Further, regulation of metabolism and control of energy allocation to various physiological functions such as growth, reproduction, or storage, likely in large part through insulin signaling, are important in both the diapause state and resistance to starvation stresses (DeJong and Bochdanovits 2003).

Mean body mass and lipid content do not significantly differ between the sexes or between northern and southern lines. Neither is associated with latitude or probability of diapause in *D. americana*, but body mass and lipid content are associated with each other. Regulation of body mass and lipid storage may have been decoupled from that of diapause over the longer history of exposure to cold-climate selective

pressures in the *D. americana* lineage, as both growth and energy allocation are likely to be subject to selection on numerous traits (Moran 1994, Partridge and Sibly 1991).

In *D. melanogaster* and *D. simulans*, lipid content is associated with starvation resistance (Chippindale et al 1996, Schmidt et al 2005, Schmidt et al 2008, Ballard et al 2008). This relationship was not directly analyzed in this study, in which body mass and lipid content were measured after allowing *D. americana* to develop on abundant food resources. These conditions do not reflect natural conditions (Atkinson 1979).

The *D. americana* lineage has had greater opportunity to produce mutations affecting the regulation of these traits, as well as to optimize fitness in cold-climates with respect to the functional relationships between diapause and these traits (Throckmorton 1982, Caletka and McAllister 2004). Additionally, selection on these traits may differ between *D. melanogaster* and *D. americana*, as the life histories, habits and resource requirements almost certainly differ between the two species.

These observations show that there are important similarities among the phenotypic traits of lineages independently adapted to similar conditions. However, some traits covarying with environmental variables are not shared across all lineages living in similar environments. Though this result is not unexpected, it does serve as a further caution against making the assumption that covariance with environmental variables, or even known adaptive traits, is sufficient evidence to call a trait adaptive.

Actually determining which traits are and are not adaptive to cold climate *Drosophila* will require additional experiments.

To test which traits are adaptive within a species/population, it is necessary to make direct measures of fitness value, either by directly determining survival or fertility advantages associated with individual traits, or by identifying genetic signatures of selection at the loci regulating particular traits. To determine which traits are generally adaptive to *Drosophila* living in cold climates, it will be necessary to sample more species that have independently colonized these environments and measure the same set of putatively adaptive traits in all of them. It is expected that more closely related species would share more covarying traits due to linkage (Bhutkar et al 2008), so those traits covarying with an environmental variable among a broad range of distantly related linages are much stronger candidates for adaptive traits. Heritability and fitness measures would still be necessary to absolutely confirm adaptive value of traits, however (Barrett and Hoekstra 2011).

A better understanding the causes and limits of convergent evolution will become possible as comparative studies between lineages independently adapted to similar environments define their similarities and differences in selective constraints, patterns of phenotypic and genetic variation, and linkage relationships among loci. This study shows that cold climate *Drosophila* species offer great potential as a model to advance this end. Species from almost every major group of the genus

have been recorded as living in cold climates, and diapause is present in many of these (Chapter 2). Tools to investigate genetic variation, gene function, and genome organization more efficiently in species even distantly related to *D. melanogaster* are increasingly available. Comparative studies among *Drosophila* species differing in these respects will allow identification of factors influencing convergent evolution and adaptation to complex environments.

Table 3.1 Populations represented in this study, with locations and dates of collection.

Population	Latitude (˚N)	Longitude (˚W)	Year Collected	Isofemale Lines
FG	30.70	84.86	2006	FG06.06, FG06.18, FG06.24, FG06.30
CI	30.76	91.47	2005	CI05.08, CI05.18, CI05.20, CI05.38
BU	31.99	85.05	2005	BU06.10, BU06.12, BU06.22, BU06.48
ML	32.52	92.13	1997	ML97.03, ML97.04, ML97.05, ML97.06
DA	32.54	87.84	2006	DA06.02, DA06.10, DA06.16, DA06.24
CB	32.98	92.85	2005	CB05.06, CB05.14, CB05.22, CB05.26
FP	34.19	91.08	1999	FP99.24, FP99.32, FP99.34, FP99.52
WR	34.60	86.92	2006	WR06.16, WR06.24, WR06.40, WR06.48
LR	34.71	89.99	2005	LR05.02, LR05.24, LR05.30, LR05.32
LA	36.27	90.75	1999	LA99.02, LA99.24, LA99.48, LA99.54
PM	36.97	90.14	1999	PM99.18, PM99.24, PM99.32, PM99.44
OC	38.34	87.31	2007	OC07.02, OC07.08, OC07.12, OC07.26
HI	38.66	90.68	1999	HI99.20, HI99.38, HI99.48, HI99.58
MK	38.94	85.83	2007	MK07.18, MK07.20, MK07.32, MK07.36
DI	40.45	89.94	2005	DI05.06, DI05.54, DI05.62, DI05.82
WS	40.70	82.00	2007	WS07.14, WS07.16, WS07.18, WS07.20
SB	41.50	91.16	2002	SB02.02, SB02.06, SB02.08, SB02.10
IR	41.78	91.72	2004	IR04.10, IR04.18, IR04.32, IR04.96

Table 3.2 Comparisons between phenotypic means for sex and geographic class.

Phenotype	Sex	Locality Class	# Pop.	# Lines	Mean	SE	F-statistic	p-value
Diapause Probability	n/a	Southern	9	32	0.53	0.056	0.065	ns
		Northern	9	32	0.84	0.039		
Wing Length (µm)	Female	all	16	51	1887.1	15.382	0.0002	ns
	Male		15	38	1883.8	16.591		
	Female	Southern	9	29	1820.7	17.590	0.7510	ns
		Northern	7	22	1927.5	12.581		
	Male	Southern	8	17	1806.8	0.400	1.7240	ns
		Northern	7	21	1946.1	0.476		
	Female	Southern	9	29	1820.7	17.590	0.7510	ns
	Male		8	17	1806.8	0.400		
	Female	Northern	7	22	1927.5	12.581	0.330	ns
	Male		7	21	1946.1	0.476		
Starvation Survival (days)	Female	all	18	71	11.72	0.171	4.950	< 0.05
	Male		18	71	7.82	0.118		
	Female	Southern	9	36	11.25	0.226	0.238	ns
		Northern	9	35	12.20	0.235		
	Male	Southern	9	36	7.55	0.135	0.162	ns
		Northern	9	35	8.09	0.185		
	Female	Southern	9	36	11.25	0.226	5.478	< 0.05
	Male		9	36	7.55	0.135		
	Female	Northern	9	35	12.20	0.235	5.385	< 0.05
	Male		9	35	8.09	0.185		
	Male		9	33	0.21	0.015		

Table 3.2 Continued

Body Mass (g)	*Female*	*all*	*18*	*67*	*0.011*	*0.00059*	*0.0565*	*ns*
	Male		18	66	0.009	0.00044		
	Female	Southern	9	33	0.011	0.00080	0.0005	ns
		Northern	9	34	0.011	0.00087		
	Male	Southern	9	33	0.009	0.00058	0.0232	ns
		Northern	9	33	0.010	0.00067		
	Female	Southern	9	33	0.011	0.00080	0.1111	ns
	Male		9	33	0.009	0.00058		
	Female	Northern	9	34	0.011	0.00087	0.0232	ns
	Male		9	33	0.010	0.00067		
Lipid Content	Female	all	18	67	0.21	0.012	0.006	ns
	Male		18	66	0.20	0.010		
	Female	Southern	9	33	0.21	0.012	0.002	ns
		Northern	9	34	0.20	0.012		
	Male	Southern	9	33	0.19	0.012	0.027	ns
		Northern	9	33	0.21	0.015		
	Female	Southern	9	33	0.21	0.012	0.041	ns
	Male		9	33	0.19	0.012		
	Female	Northern	9	34	0.20	0.012	0.001	ns
	Male		9	33	0.21	0.015		

ns = non-significant.

Table 3.3 Mean phenotypic values for all isofemale lines by sex.

	Proportion in Diapause	*Wing Length (µm)*		*Starvation Survival (days)*		*Body Mass (g) (20 flies)*		*Proportion Lipids*	
Line		*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*
FG06.06	0.71	1790.1	---	11.06	7.30	0.014	0.010	0.23	0.16
FG06.18	0.18	1760.7	1726.3	11.45	7.75	0.016	0.012	0.24	0.18
FG06.24	0.51	1716.0	1714.4	12.67	7.10	0.015	0.013	0.27	0.27
FG06.30	0.12	1726.9	1707.8	10.75	8.80	0.019	0.016	0.31	0.28
CI05.08	0.88	1846.9	1827.0	9.83	7.35	0.007	0.006	0.15	0.14
CI05.18	0.04	---	1807.8	9.90	7.35	0.010	0.008	0.22	0.22
CI05.20	0.55	1799.0	---	11.40	7.60	0.009	0.008	0.17	0.19
CI05.38	0.13	---	1849.8	10.90	7.23	0.007	0.005	0.19	0.09
BU06.10	0.80	1706.9	1651.1	11.36	7.21	0.018	0.013	0.37	0.33
BU06.12	0.37	1739.8	---	11.20	7.93	0.015	0.013	0.24	0.23
BU06.22	0.83	1977.5	---	14.05	8.70	0.016	0.012	0.26	0.24
BU06.48	0.78	1755.7	1767.5	12.86	8.34	0.010	0.011	0.21	0.24
ML97.03	0.11	1631.9	---	11.64	7.16	---	---	---	---
ML97.04	0.33	1737.5	---	9.75	6.27	0.009	0.007	0.15	0.14
ML97.05	0.008	1792.7	1779.4	11.33	7.43	0.006	0.006	0.21	0.24
ML97.6	---	---	---	8.96	7.30	---	---	---	---
DA06.02	0.45	1694.9	1714.0	10.03	6.13	0.005	0.005	0.18	0.19
DA06.10	---	---	---	11.80	8.80	0.006	0.006	0.03	0.11
DA06.16	1.00	1952.1	1910.3	10.63	8.25	0.006	0.006	0.25	0.27
DA06.24	0.87	1910.5	---	10.90	7.33	0.013	0.008	0.24	0.10
CB05.06	0.52	1892.3	---	10.13	7.05	0.011	0.012	0.15	0.20
CB05.14	0.67	1949.7	---	9.43	6.20	0.014	0.011	0.22	0.15
CB05.22	0.95	1960.5	1956.4	9.80	7.20	0.007	0.006	0.17	0.18

Table 3.3 Continued

CB05.26	0.06	1813.6	1847.5	10.23	6.80	0.012	0.008	0.28	0.17
FP99.24	---	---	---	10.31	6.65	0.006	0.005	0.12	0.13
FP99.32	0.27	1735.5	---	12.44	7.83	0.007	0.006	0.21	0.17
FP99.34	0.75	1863.8	---	11.73	7.69	0.008	0.007	0.11	0.07
FP99.52	---	---	---	11.25	7.63	0.007	0.007	0.16	0.13
WR06.16	0.60	1797.5	---	12.67	7.39	0.018	0.011	0.32	0.25
WR06.24	0.80	1889.5	1883.8	13.50	9.11	0.020	0.016	0.32	0.35
WR06.40	0.43	1885.9	1859.8	11.48	7.33	0.009	0.007	0.20	0.15
WR06.48	0.90	1901.7	---	14.85	7.25	0.018	0.014	0.24	0.24
LR05.02	0.85	1953.1	---	11.90	8.60	---	---	---	---
LR05.24	0.95	1874.2	1917.3	13.00	7.28	0.009	0.008	0.22	0.19
LR05.30	0.07	---	---	10.23	7.75	0.007	0.006	0.15	0.13
LR05.32	0.39	1743.4	1795.4	9.53	6.03	0.008	0.007	0.12	0.10
LA99.02	0.94	1818.6	1833.0	11.16	9.08	0.006	0.005	0.14	0.09
LA99.24	0.88	---	1891.4	14.80	9.78	0.006	0.006	0.16	0.15
LA99.48	0.74	---	---	9.74	7.00	0.008	0.008	0.13	0.16
LA99.54	0.00	---	---	9.59	5.45	0.006	0.006	0.10	0.11
PM99.18	0.97	1882.8	---	11.80	8.38	0.007	0.006	0.15	0.10
PM99.24	0.94	---	---	13.78	9.20	---	---	---	---
PM99.32	0.95	1985.1	1955.7	12.31	6.73	0.008	0.007	0.16	0.11
PM99.44	0.97	1988.9	1942.6	12.86	8.82	0.005	0.005	0.15	0.16
OC07.02	1.00	1908.3	1894.3	10.81	7.84	0.010	0.010	0.26	0.25
OC07.08	0.64	1970.1	1932.1	12.47	7.74	0.022	0.018	0.27	0.23
OC07.12	0.93	1908.8	1852.3	11.12	8.24	0.011	0.009	0.22	0.20
OC07.26	0.71	1957.3	2027.2	12.88	8.01	0.016	0.014	0.21	0.25
HI99.20	0.90	---	---	10.98	6.88	0.006	0.007	0.11	0.11
HI99.38	0.35	---	---	12.85	7.88	---	---	---	---

Table 3.3 Continued

HI99.48	---	---	---	14.99	10.04	0.008	0.008	0.16	0.14
HI99.58	0.51	---	---	11.81	6.14	0.008	0.008	0.16	0.14
MK07.18	0.66	1931.0	1848.9	12.83	7.99	0.017	0.013	0.22	0.26
MK07.20	1.00	1916.5	1947.4	13.98	8.77	0.019	0.015	0.24	0.24
MK07.32	0.70	2033.7	2022.7	12.37	7.76	0.017	0.015	0.30	0.23
MK07.36	0.87	1978.2	1946.3	14.40	7.26	0.017	0.012	0.31	0.27
DI05.06	0.98	1949.6	1988.3	11.60	9.18	0.007	0.006	0.18	0.19
DI05.54	0.81	2080.3	2051.5	13.83	10.30	0.008	0.015	0.07	0.27
DI05.62	1.00	2065.2	2047.4	13.20	8.85	0.007	0.007	0.17	0.25
DI05.82	0.96	2013.5	1978.1	12.13	7.90	0.007	0.007	0.20	0.19
WS07.14	---	1932.9	1865.2	---	---	0.015	0.014	0.30	0.55
WS07.16	0.95	1850.7	---	12.79	7.60	0.016	0.013	0.33	0.29
WS07.18	0.89	2096.4	2020.6	10.93	7.59	0.015	0.014	0.26	0.27
WS07.20	0.82	---	1916.6	10.87	7.44	0.019	0.016	0.31	0.30
SB02.02	1.00	1959.3	1951.0	11.90	8.47	0.007	0.007	0.23	0.24
SB02.06	1.00	---	---	12.28	9.35	0.005	---	0.24	---
SB02.08	1.00	1952.3	---	14.20	9.53	0.008	0.008	.019	0.20
SB02.10	1.00	1987.3	1955.0	10.77	7.17	0.007	0.005	0.12	0.13
IR04.10	---	---	---	10.28	7.08	0.011	0.011	0.31	0.25
IR04.18	---	---	---	12.10	7.13	0.015	0.012	0.20	0.16
IR04.32	0.86	---	---	11.55	8.30	0.007	0.007	0.16	0.17
IR04.96	0.98	---	---	10.88	8.45	0.017	0.013	0.29	0.24

--- = missing value.

Figure 3.1 Logistic regression of proportion of flies diapausing (probability of diapause) to latitude of origin (˚N) of isofemale lines. Markers represent observed proportion of diapausing flies of each sampled isofemale line. The black and grey lines represent predicted probability of diapause and the 95% confidence interval, respectively.

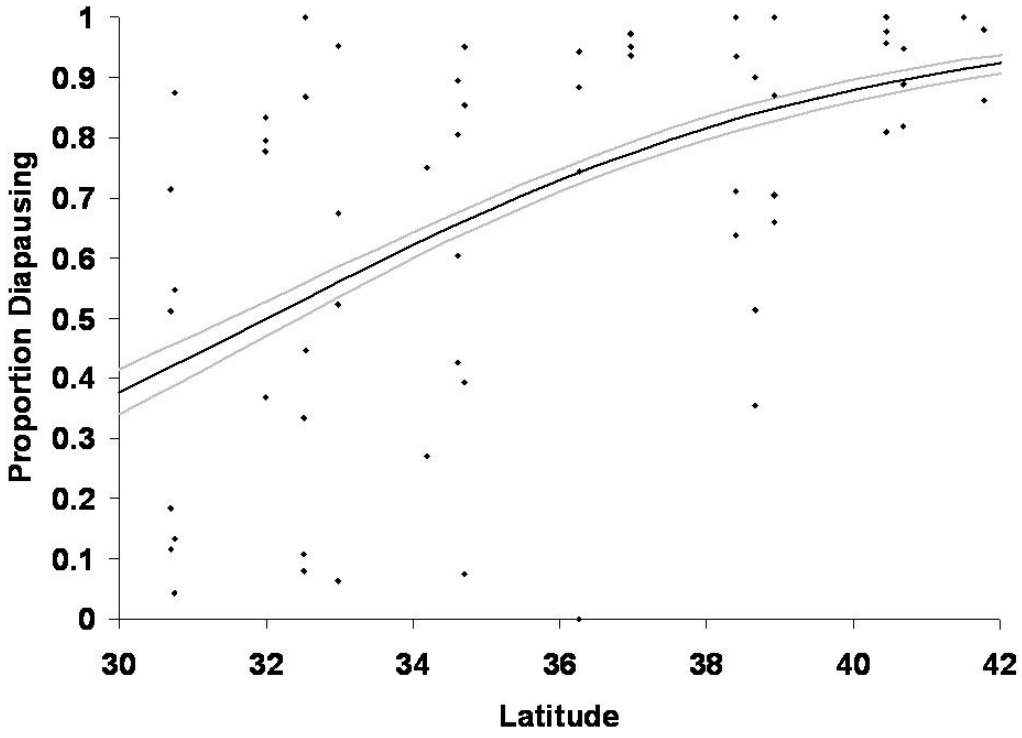


Figure 3.2 Linear regression of Female (A) and Male (B) wing length by latitude of origin (˚N) of isofemale lines. Markers represent observed mean wing length of each sampled isofemale line. The black and grey lines represent predicted wing length and the 95% confidence interval, respectively.

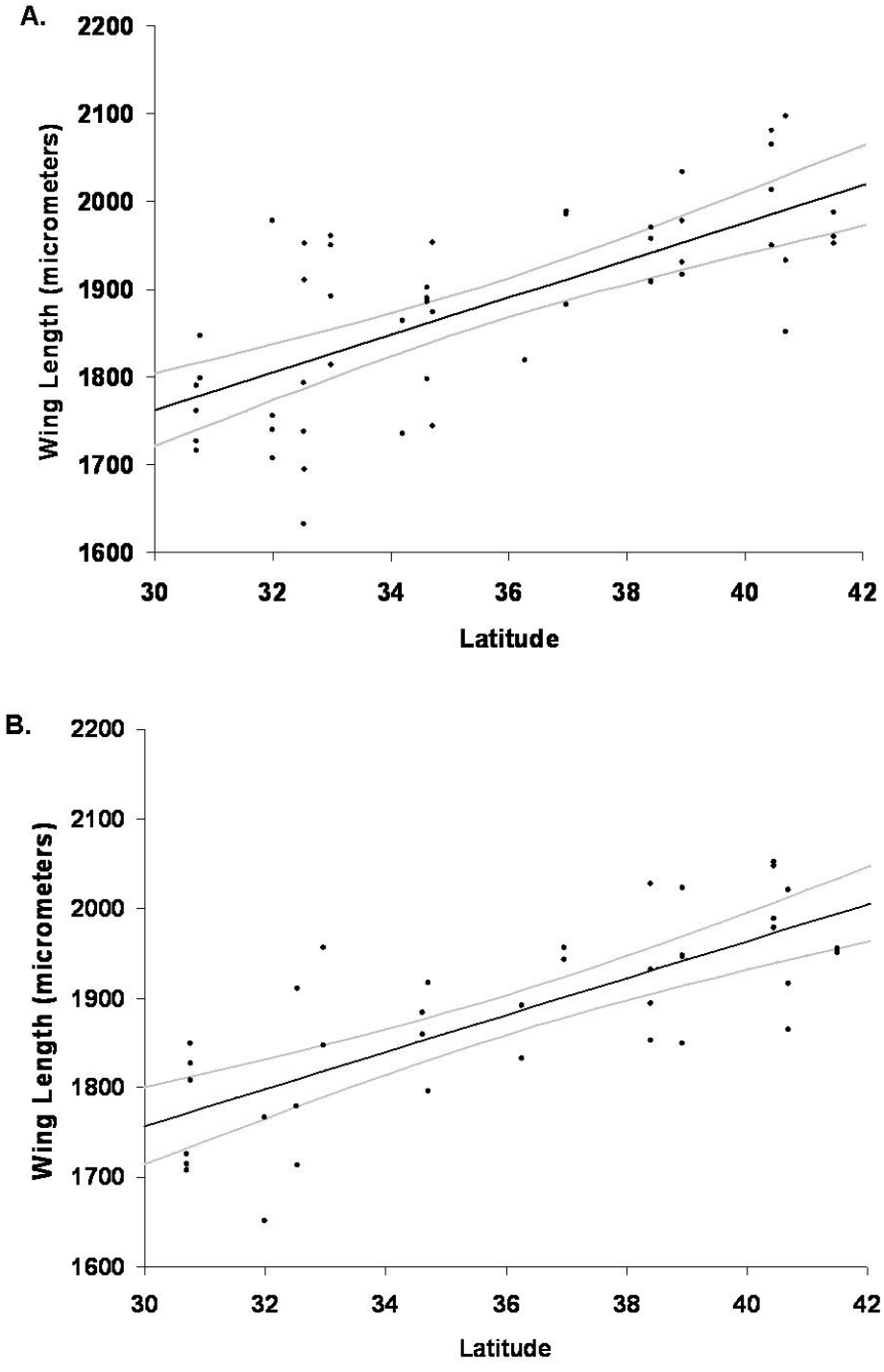
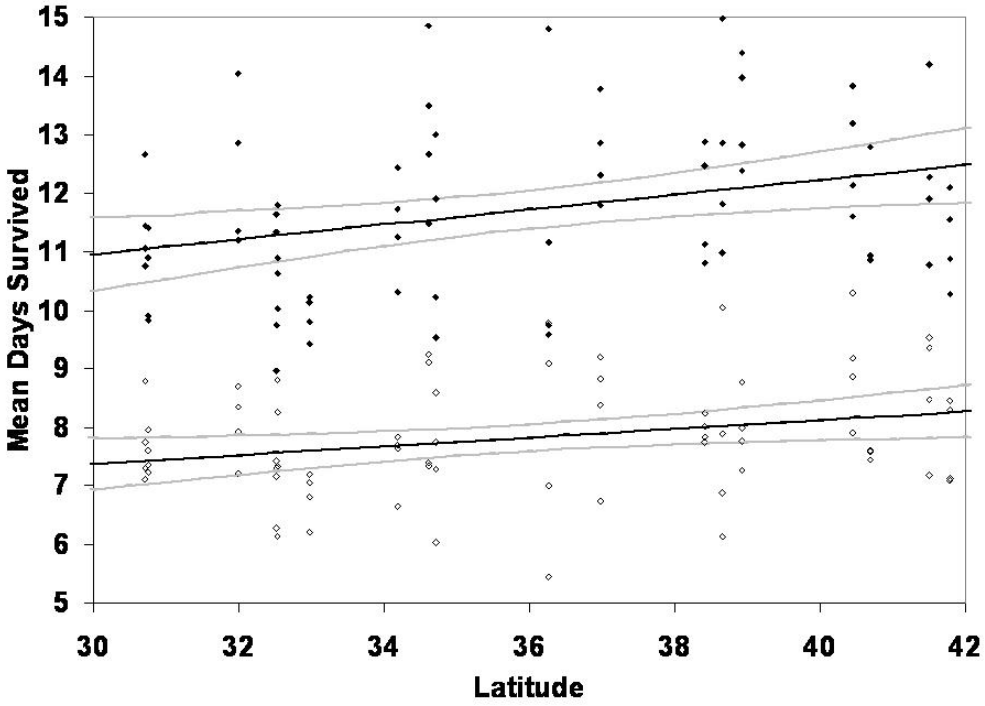


Figure 3.3 Linear regression of Female (and Male starvation survival (days) by latitude of origin (˚N) of isofemale lines. Markers represent observed mean wing length of each sampled isofemale line. Filled markers represent female values and open markers represent male values. The black and grey lines represent predicted wing length and the 95% confidence interval, respectively.



CHAPTER 4

RESPONSE OF DIAPAUSE TO TEMPORAL CHANGES IN THE ENVIRONMENT IN *DROSOPHILA AMERICANA*

4.1 Introduction

Establishing that a given trait enhances fitness is a necessary step before declaring that the trait is adaptive to a given environment or set of conditions (Gould and Lewontin, 1979). Fitness values of traits are often indirectly assessed by studying phenotypic distributions in association with environmental variables and/or population genetic signatures of selection (e.g. Karan et al 1998, Sezgin 2004 , Linnen et al 2009). Genome-wide studies to identify genetic targets of selection are powerful, especially when used with appropriate models and controls (Bustamante et al 2005, Nielsen et al 2005, Thornton and Jensen 2007). Studies at both the genotypic and phenotypic levels are valuable to understanding adaptation, as those assessing either molecular signatures of selection or individual fitness can fail to identify the effects of past or current selection on alleles and traits (Barrett 2012, Brown 2012, Domingues et al 2012, Gratten et al 2012). However, it is rarely feasible to take direct measures of the fitness value of individual traits in natural environments due to the major technical challenges of sampling, non-invasively measuring phenotypes, and accurately tracking survival and fertility of individuals (Kingsolver et al 2001).

In many cases, whether a trait enhances fitness is tested by observing individuals or populations of known genotype/phenotype in artificially manipulated environments. This may be done in a laboratory simulated environment, which allows greater control of experimental variables, but may not reflect important aspects of natural conditions because environment is necessarily simplified in the laboratory (Endler 1986). Alternatively, natural environments or populations may be experimentally manipulated using such techniques as predator exclusion or transplantation. This allows a more realistic environment than the laboratory approach, but less control over experimental variables (Reznick and Bryga, 1987, Reznick and Ghalambor 2005)

Yet another method of testing whether a trait enhances fitness under natural conditions is to assess the response of traits to temporal changes in environmental stresses or to conduct follow-up studies on previously observed natural populations soon after ecological events posing novel selection. This type of study has provided the “textbook” examples of adaptive changes in beak size in Darwin’s finches and of industrial melanism and its reversal in peppered moths, which demonstrate evolution in response to selective pressure within a human lifetime (Boag and Grant 1981, Grant et al 1998). This approach does depend on opportunity, with knowledge of a plausible change in the direction or strength of selection, as well as the availability of phenotypic data from the same populations both before and after the change in

selection being necessary to demonstrate a response to environmental change. Knowledge of the genetic variation underlying the traits in question strengthened the evidence that these phenotypes are adaptive (Abzhanov et al 2004, Abzhanov et al 2006, Mallarino et al 2011, van't Hof et al 2011).

An opportunity of this sort arose following the winter of 2009-2010 in the southern United States. This period caused record cold temperatures and freezing periods in typically warm-climate locations (National Climatic Data Center 2010). These unusual environmental conditions posed significant stress to at least some endemic species. For example, manatees and coral reef cover in Florida were strongly affected by cold-related mortality during this period (Florida Fish and Wildlife Conservation Commission 2010, Colella et al 2012).

D. americana is endemic to the Mississippi River Valley, between Wisconsin and Florida. Populations spanning the species range were sampled prior to the 2009-2010 winter, and isofemale lines derived from those samples have been maintained in the McAllister laboratory. These were available for comparison to samples of the same populations taken following the 2009-2010 winter.

Previous studies of *D. americana* have shown evidence for selective maintenance of locally adaptive genotypes. The species is polymorphic for a clinally distributed fusion between the X and 4th chromosomes, with this cline maintained by selection in the face of gene

flow between populations (McAllister 2002). There is significant genetic differentiation between fused and unfused X and 4th chromosomes, with inversions blocking recombination between chromosomal forms in regions containing multiple genes (McAllister 2003, Evans et al 2007, McAllister et al 2008). Additionally, *D. americana* varies clinally in diapause incidence, a trait associated with adaptation to cold climate in numerous insect species (Chapters 2 and 3). A high probability of diapause in *D. americana* has been associated with the fused X-4 chromosome (Chapter 2).

This study tests the hypothesis that diapause incidence would increase in populations of *D. americana* adapted to warm climate in response to the selective pressure imposed by the record cold winter of 2009-2010. Diapause incidence was compared between isofemale lines of *D. americana* derived from flies collected either prior to 2009 (pre-2010) or during the summer of 2010. While Southern populations, adapted to warmer climate, are expected to have increased in diapause incidence between samples, Northern populations, already adapted to cold climate are predicted to show no change in diapause incidence between samples. Comparison of pre-2010 and 2010 lines derived from Northern populations allows the possibility of loss of the diapause trait during laboratory maintenance to be eliminated as a cause for increased diapause incidence between samples.

4.2 Materials and Methods

Diapause incidence of 164 isofemale lines of *D. americana* was measured in a standard laboratory assay (Table 4.1). Lines were derived from single, wild-collected female flies whose descendents have been maintained under standard laboratory conditions. Pre-2010 lines were derived from wild females collected from 8 populations between 1996 and 2007. 2010 lines of the same 8 populations were derived from females collected during the summer of 2010.

Flies used in the diapause assay were reared at low density on standard cornmeal media at 22°C under a 12:12 light:dark cycle. Virgin adults were collected at 0-48 hours following eclosion, placed in mixed-sex food vials containing a maximum of 20 individuals. Mixed-sex vials were maintained in an incubator at 11°C under a 10h:14h light:dark cycle for a 4-5 week period (Saunders et al 1989, Williams and Sokolowski 1993). Following this cool, short day length treatment, female flies were isolated and frozen at -20°C. Females were thawed, and the ovaries dissected and photographed. The developmental stage of each individual's ovaries was determined by applying the criteria defined by Lumme and Lakovaara (1983).

Diapause incidence of each line was defined as the number of females in diapause (neither ovary developed beyond stage 1) divided by the total number of females dissected. For each population, diapause

incidence was compared between pre-2010 and 2010 lines using F-statistics (ANOVA)

4.3 Results

Among the four southernmost sampled populations of *D. americana*, three showed a significant increase in mean diapause incidence following the 2009-2010 winter (Table 4.2, Figure 4.1). The population RB showed a non-significant increase in diapause incidence, but power to detect an increase was limited by the availability of only two pre-2010 isofemale lines. The minimum increase in mean diapause incidence among southern populations between pre-2010 and 2010 lines was 0.24 among LR lines, and the maximum increase was 0.43 among DA lines. A subset of 2010 lines derived from each southern site exhibited diapause incidence within the range of pre-2010 lines derived from the same site (Table 4.1).

In contrast, mean diapause incidence did not significantly change following the 2009-2010 winter for any of the four northernmost sampled populations (Table 4.2, Figure 4.1). Lines derived from females collected in 2010 showed very similar mean diapause incidence to those collected from the same sites in years prior to 2010 with a minimum difference of 0.01 among IR lines and a maximum difference of 0.03 among MK lines. Before and in 2010, northern populations exhibited high mean diapause incidence, with most lines showing diapause incidence above 0.5 and few lines showing low diapause incidence (Table 4.1).

4.4 Discussion

The hypothesis that diapause incidence would increase in populations of *D. americana* adapted to warm climate in response to the selective pressure imposed by the record cold winter of 2009-2010 was supported by the data. Mean diapause incidence was significantly higher among lines derived from females collected in 2010 than from pre-2010 in three of four southern populations sampled, and no significant change was observed between pre-2010 and 2010 lines derived from northern populations.

Because several 2010 lines derived from southern populations did exhibit low diapause incidence, it is unlikely that low-diapause alleles were purged from the populations. Instead, it is possible that the observed change in diapause incidence reflects an increase in the frequency of high-diapause alleles, either through the action of selection or due to migration from high-diapause populations, shifting mean diapause incidence in southern populations upward. If this change is due to migration from northern high-diapause populations, an increase in the frequency of fused X-4 chromosomes would be expected in 2010 samples from southern populations.

Alternatively, studies of the inheritance of diapause in *D. americana* indicate that multiple alleles or loci contribute to diapause in southern populations with high frequencies of unfused X and 4th chromosomes (Chapter 2). Either heterozygosity for complementing low-diapause

alleles at the same locus or genotypes combining additive, diapause-promoting alleles at multiple loci could confer high diapause incidence without an increase in the frequency of fused X-4 chromosomes. Heterozygosity for complementing low-diapause alleles at the same locus is an unlikely explanation for the observed increase in diapause incidence. Homozygosity is expected to increase through inbreeding as a line is established in the laboratory, so high-diapause effects due to heterozygosity is unlikely to persist in isofemale lines (Kimura and Crow 1964).

Since diapause incidence did not change between samples among northern populations, this indicates that there is not a general tendency for isofemale lines to decrease diapause incidence under relaxed selection in laboratory conditions. It is also supportive of the notion that northern populations are already adapted to cold climate, so the 2009-2010 winter did not pose a novel selective pressure as it did for southern populations. In both pre-2010 and 2010 samples, a few northern lines exhibited low diapause incidence. This observation is consistent with low-diapause alleles segregating at low frequency in northern populations. Since rare alleles typically occur in heterozygotes, and low-diapause alleles appear to act in a recessive manner, these would be invisible to selection in natural populations, and produce low-diapause phenotypes in more homozygous laboratory lines.

It is notable that the IR population maintained an intermediate diapause incidence between samples. This observation is due to high variation in diapause incidence among lines, with a greater proportion of low-diapause lines than the other northern populations (Table 4.1). The IR site does not experience unusually warm winters relative to the other northern sites, and does not harbor a high proportion of the unfused X-4 chromosomes associated with low-diapause alleles (McAllister et al 2008, Chapter 2). Because mean diapause incidence did not change between samples allele frequency likely was unchanged by selection at this site. This study demonstrates rapid phenotypic change in response to a major selective event in natural populations of *D. americana*. That diapause incidence increased at southern sites in response to a particularly cold season is evidence that the capacity for diapause is an important component of fitness under cold conditions. The response to selection was likely facilitated by standing genetic variation within populations, and genetic variation at the selected locus/loci appears to remain following the selective event. Because high-diapause alleles have been associated with the X-4 fused chromosome, and these chromosomes harbor inversions that block recombination with unfused X and 4^{th} chromosomes, it is reasonable to hypothesize that the frequency of the X-4 fused chromosome would have increased along with diapause incidence in southern populations of *D. americana* following the 2009-2010 winter. Work to test this hypothesis is in progress in the McAllister laboratory.

Table 4.1 Sampling, diapause counts and diapause incidence of all isofemale lines.

Population (Latitude)	Line	Collected	# Sampled	# in Diapause	Diapause Incidence
RB (32.54)	RB05.40	2005	44	3	0.07
	RB08.04	2008	42	18	0.43
	RB10.04	2010	48	21	0.44
	RB10.06		46	27	0.59
	RB10.10		43	12	0.28
	RB10.12		36	15	0.42
	RB10.14		46	24	0.52
	RB10.16		32	30	0.94
	RB10.18		46	34	0.74
	RB10.20		43	30	0.70
	RB10.22		44	11	0.25
	RB10.26		45	25	0.56
	RB10.28		36	3	0.08
	RB10.54		47	36	0.77
DA (32.54)	DA06.02	2006	38	1	0.03
	DA06.08		43	1	0.02
	DA06.10		32	5	0.16
	DA06.12		46	19	0.41
	DA06.16		46	16	0.35
	DA06.18		30	4	0.13
	DA06.20		47	16	0.34
	DA06.24		17	11	0.65
	DA06.26		47	7	0.15
	DA06.34		42	4	0.10
	DA06.36		49	12	0.24
	DA06.40		38	2	0.05
	DA06.44		47	14	0.30
	DA10.02	2010	19	15	0.79
	DA10.04		44	33	0.75
	DA10.06		48	26	0.54
	DA10.08		47	40	0.85
	DA10.10		39	24	0.62
	DA10.14		19	13	0.68
	DA10.18		47	19	0.40
	DA10.20		45	23	0.51
	DA10.24		42	15	0.36
	DA10.26		34	31	0.91
	DA10.32		41	28	0.68
	DA10.34		44	33	0.75
	DA10.40		37	34	0.92

Table 4.1 Continued

DA (32.54)	DA10.42	2010	44	23	0.52
	DA10.44		38	11	0.29
	DA10.52		34	24	0.71
	DA10.58		45	41	0.91
	DA10.60		32	20	0.63
	DA10.62		33	25	0.76
	DA10.64		42	25	0.60
	DA10.66		42	27	0.64
	DA10.68		24	16	0.67
FP (34.19)	FP99.04	1999	49	0	0.00
	FP99.18		39	1	0.03
	FP99.28		47	5	0.11
	FP99.30		48	10	0.21
	FP99.32		36	13	0.36
	FP99.34		48	25	0.52
	FP99.36		15	0	0.00
	FP99.46		47	11	0.23
	FP99.50		47	13	0.28
	FP99.52		47	1	0.02
	FP99.56		47	39	0.83
	FP10.08	2010	45	41	0.91
	FP10.10		21	18	0.86
	FP10.14		36	20	0.56
	FP10.16		39	6	0.15
	FP10.18		66	42	0.64
	FP10.20		45	33	0.73
	FP10.24		55	40	0.73
	FP10.46		32	31	0.97
	FP10.54		39	30	0.77
	FP10.58		45	14	0.31
LR (34.71)	LR05.02	2005	30	14	0.47
	LR05.14		44	18	0.41
	LR05.18		47	5	0.22
	LR05.24		45	44	0.98
	LR05.26		47	36	0.77
	LR05.30		29	7	0.24
	LR05.32		47	8	0.17
	LR05.36		45	33	0.73
	LR05.38		47	28	0.60
	LR05.40		38	24	0.63
	LR10.04	2010	48	26	0.54
	LR10.08		43	30	0.70
	LR10.10		47	41	0.87
	LR10.14		37	32	0.86

Table 4.1 Continued

LR (34.71)	LR10.16	2010	40	22	0.55
	LR10.18		48	41	0.85
	LR10.20		47	34	0.72
	LR10.22		36	16	0.44
	LR10.24		47	27	0.57
	LR10.26		43	37	0.86
	LR10.28		47	45	0.96
	LR10.32		41	27	0.55
	LR10.34		45	39	0.87
	LR10.36		44	38	0.86
	LR10.38		14	8	0.57
	LR10.40		45	40	0.89
	LR10.42		46	46	1.00
	LR10.46		47	36	0.77
MK (38.93)	MK07.16	2007	45	45	1.00
	MK07.18		45	45	1.00
	MK07.22		45	36	0.80
	MK07.24		46	45	0.98
	MK07.36		46	44	0.96
	MK07.38		43	43	1.00
	MK07.40		42	31	0.74
	MK07.48		46	45	0.98
	MK07.60		47	38	0.81
	MK07.64		44	42	0.95
	MK10.04	2010	47	22	0.47
	MK10.06		47	43	0.91
	MK10.12		46	43	0.93
	MK10.16		46	46	1.00
	MK10.18		47	46	0.98
	MK10.22		43	43	1.00
	MK10.24		48	40	0.83
	MK10.34		43	39	0.91
	MK10.48		48	48	1.00
	MK10.56		49	44	0.90
DI (40.45)	DI05.02	2005	49	46	0.94
	DI05.06		39	31	0.79
	DI05.14		48	24	0.50
	DI05.54		48	35	0.73
	DI05.62		48	47	0.98
	DI05.82		13	11	0.85
	DI05.92		48	38	0.79
	DI10.04	2010	47	34	0.72
	DI10.08		37	13	0.35
	DI10.12		48	48	1.00

Table 4.1 Continued

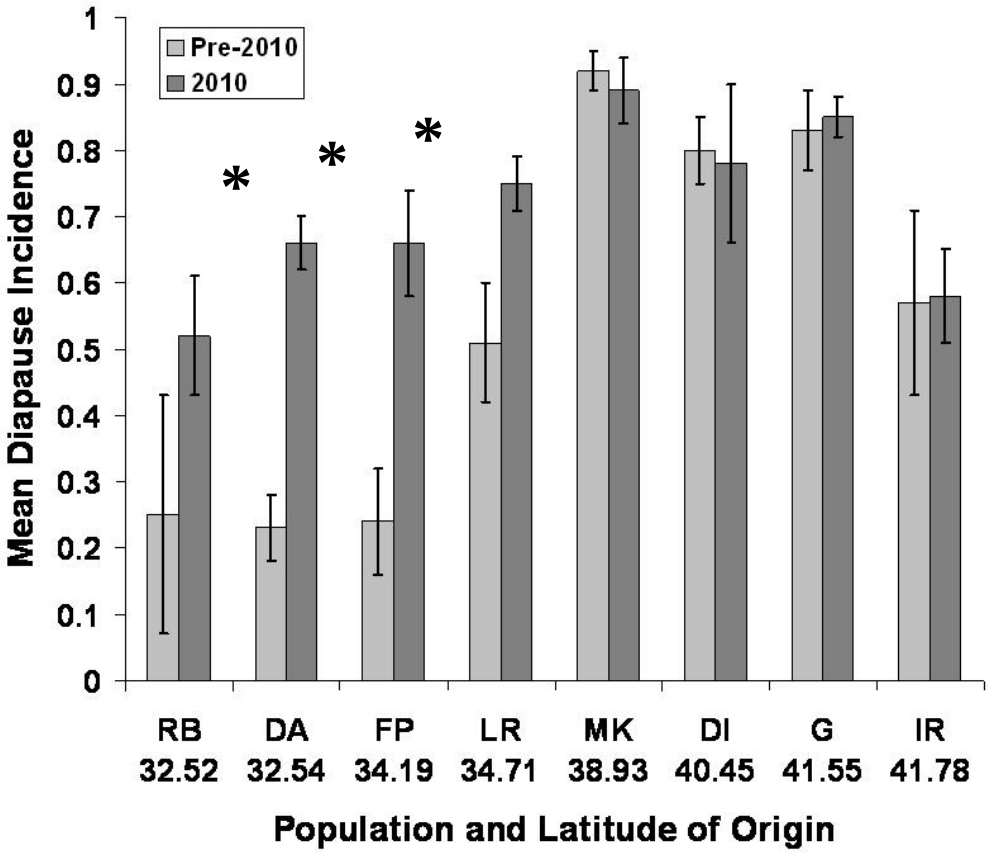
DI (40.45)	DI10.14	2010	47	39	0.83
	DI10.16		47	46	0.98
G (41.55)	G96.04	1996	28	15	0.54
	G96.11		17	17	1.00
	G96.12		44	30	0.68
	G96.13		48	45	0.94
	G96.40		48	34	0.71
	G96.45		47	46	0.98
	G96.47		24	22	0.92
	G96.48		14	12	0.86
	GK10.08	2010	31	24	0.77
	GK10.10		47	35	0.74
	GK10.12		43	40	0.93
	GK10.14		47	42	0.89
	GK10.18		42	41	0.98
	GK10.20		46	37	0.80
	GK10.22		48	40	0.83
	GK10.24		47	43	0.91
	GK10.26		48	44	0.92
	GK10.28		47	36	0.77
IR (41.78)	IR04.32	2004	24	6	0.25
	IR04.34		47	34	0.72
	IR04.96		38	35	0.92
	IR04.118		47	24	0.51
	IR04.126		13	1	0.08
	IR04.134		17	16	0.94
	IR10.02	2010	48	40	0.83
	IR10.08		48	42	0.88
	IR10.12		19	3	0.16
	IR10.22		48	29	0.60
	IR10.30		46	34	0.74
	IR10.32		28	13	0.46
	IR10.48		41	31	0.76
	IR10.50		47	44	0.94
	IR10.56		15	11	0.73
	IR10.58		47	35	0.74
	IR10.60		33	22	0.67

Table 4.2 Comparison of mean diapause incidence between samples of each population.

Population	Latitude	Pre-2010			2010			Sum of Squares	F	p-value
		N	Mean	SE	N	Mean	SE			
RB	32.54	2	0.25	0.18	12	0.52	0.07	0.129	2.11	0.172
DA	32.54	13	0.23	0.05	22	0.66	0.04	1.534	49.27	<0.0001*
FP	34.19	11	0.24	0.08	10	0.66	0.08	0.957	14.2	0.0001*
LR	34.71	10	0.51	0.09	18	0.75	0.04	0.374	8.26	0.008*
MK	38.93	10	0.92	0.03	10	0.89	0.05	0.004	0.24	0.630
DI	40.45	7	0.80	0.05	5	0.78	0.12	0.001	0.03	0.866
G	41.55	8	0.83	0.06	10	0.85	0.03	0.003	0.18	0.677
IR	41.78	6	0.57	0.14	11	0.58	0.07	0.049	0.67	0.426

Number of lines sampled, mean diapause incidence and standard error of the mean is reported for pre-2010 and 2010 samples of each population. * denotes significant difference between samples.

Figure 4.1 Mean diapause incidence of pre-2010 and 2010 samples of each population. Error bars represent standard errors of means. * denotes p<0.05



CHAPTER 5

CONCLUSIONS

Darwin's (1859) original description of evolution by natural selection is based upon four postulates, all of which must hold for adaptation to occur. There must be phenotypic variation among individuals, phenotypic variation must be at least partially heritable, there must be variation in reproductive success among individuals, and the probability of reproductive success must be affected by phenotype. Gould and Lewontin's (1979) criticism of "the adaptationist programme" highlighted the problem of researchers asserting that traits were adaptive in the absence of evidence of fitness effects or selection. The development of methods to both directly measure fitness and indirectly identify past selective events addressed this problem (e.g. Kingsolver et al 2001, McDonald and Kreitman 1991, Otto 2000). However, techniques solely identifying the fitness effects of phenotypes leave out the heritability component of evolution by natural selection. Further, those identifying genetic targets of selection omit the phenotype component, and cannot directly show fitness effects. Therefore, Barrett and Hoekstra (2011) argue for the necessity of demonstrating the three-way interaction between fitness, genotype, and phenotype to declare a trait adaptive.

Although there are ample available methods to study genotype-phenotype interactions in a wide variety of species, connecting these to fitness in the natural environment is technically difficult and/or resource

intensive in most species. An additional challenge is to predict which traits have heritable variation and important fitness effects. One approach to select putatively adaptive traits for further study is to identify convergent traits among unrelated lineages living in similar environments (Endler, 1986). This study employed this approach in cold climate *Drosophila* to address the general hypothesis that shared traits among species sharing similar climate and habitat distributions are candidates for adaptations to shared selective pressures.

Multiple *Drosophila* species have independently colonized cold climates and are exposed to similar selective pressures in these environments (Gilbert *et al* 2001, Kellermann *et al* 2012, Danks 2002). Under similar environments, populations of cold climate *Drosophila* commonly exhibit parallel phenotypic clines. Traits associated with cold climate in multiple *Drosophila* species were examined for evidence that they are adaptive in the North American endemic species *D. americana*. Traits investigated were reproductive diapause, wing length, starvation resistance, body mass, and lipid content.

I primarily focused on investigating the genotype-phenotype-fitness interaction for the reproductive diapause trait in *D. americana*. Reproductive diapause is broadly shared among cold climate *Drosophila* species (Chapter 2). It is typically triggered by the seasonal environmental cues of cold temperature and shortening daylength (Danks 2002). The diapause state in *Drosophila* arrests ovarian development in

sexually immature females, which delays reproduction, slows the metabolism, reducing energy and oxygen usage, and also confers increased resistance to cold, desiccation, and starvation (Danks 2002).

In Chapter 1, I investigated the association between clinal genetic variation and reproductive diapause in *D. americana*. My results were consistent with a dominant allele linked to the fused X-4 chromosome conferring high diapause incidence. These results link genetic variation known to be under selection with variation in a putatively adaptive trait. X-linkage of the diapause trait is shared between *D. americana* and its closest Old-World relative, *D. lummei* (Lumme and Keranen 1978). This is significant, because the presumed ancestral allele conferring high diapause incidence has become associated with the derived X-4 fusion, supporting the hypothesis that derived chromosomal rearrangements maintain variation adaptive to cold-climate in *D. americana* (Evans et al 2007, Mena 2009).

Additional loci also appear to contribute to control of diapause in *D. americana*, especially in lines derived from southern populations, where unfused X and 4^{th} chromosomes are common. Crosses between two low diapausing lines can produce offspring with high diapause incidence. This observation is consistent with relaxed selection on the diapause trait in warmer environments. Of further interest is that there is no evidence of clinal variation of either *timeless* or *couch potato* in *D. americana* (McAllister and Evans 2006, Mena 2009). Sequence variation in *timeless*

is associated with variation in diapause incidence in *D. melanogaster* and *D. triauraria*, while variation in *couch potato* is associated with variation in diapause incidence in *D. melanogaster* and *D. montana* (Tauber *et al* 2007, Sandrelli *et al* 2007, Schmidt *et al* 2008, Kankare *et al* 2010, Yamada and Yamamoto 2011). This is further evidence that the genetics underlying variation in diapause differ among different *Drosophila* species.

In Chapter 2, I observed that latitude of origin is significantly associated with diapause incidence among isofemale lines of *D. americana* derived from populations spanning a broad geographical range. Further, my observations revealed that diapause incidence is quite variable among isofemale lines derived from southern populations, and almost universally high among isofemale lines derived from northern populations. These observations are consistent with selection favoring high diapause incidence in cold environments, and relaxed selection on the trait in warmer environments.

In Chapter 3, I investigated the response of diapause to temporal changes in environment. I observed that following the record cold winter of 2009-2010, diapause incidence significantly increased in three of four southern populations of *D. americana*, and did not increase in any of four northern populations. These observations are consistent with a selective advantage of diapause under cold climate conditions. Presence of genetic and phenotypic variation for diapause incidence in southern populations of *D. americana* allowed a rapid and strong phenotypic response to a

changing environment (Lande and Shannon 1994, Barrett and Schluter 2008).

D. americana has a large effective population size and substantial gene flow among populations, allowing for genetic variation in the species (McAllister 2002, Caletka & McAllister 2004, McAllister et al 2008). Both the presence of fused X-4 chromosomes in Southern populations and known gene flow between populations promote the hypothesis that the frequency of the X-4 fused chromosome would have increased along with diapause incidence in southern populations of *D. americana* following the 2009-2010 winter. This hypothesis is being independently tested in the McAllister laboratory.

Taken together, these results demonstrate a genotype-phenotype-fitness interaction for reproductive diapause in *D. americana*, supporting the prediction that the capacity for reproductive diapause is adaptive to cold climate in this species (Figure 5.1). A genotype-phenotype interaction is shown by the association between high diapause incidence and a selectively maintained clinal distribution of the X-4 chromosomal fusion. Diapause incidence is significantly associated with latitude among *D. americana* populations. This indirectly supports a genotype-phenotype interaction, given the latitudinal distribution of the X-4 chromosomal fusion. It also supports a phenotype-fitness interaction, since variation in the trait is associated with variation in climate over the species range. A phenotype-fitness interaction is more directly shown by the increase in

diapause incidence in southern populations following a record cold winter, consistent with response of the trait to changing selective pressure.

In Chapter 2, I also investigated whether there was evidence that additional traits shared by cold climate *Drosophila* species are adaptive to cold climate in *D. americana*. My observations revealed that, among isofemale lines representing a broad geographical range, latitude was significantly associated with wing length, and starvation resistance, but not with body mass and lipid content. Covariance between traits and environment is supportive of, but is not conclusive evidence for the prediction that wing length and starvation resistance are adaptive to cold climate.

Based on traits covarying with environment among multiple cold climate *Drosophila* species, I predicted that there would be evidence that diapause, wing length, starvation resistance, body mass, and lipid content are adaptive to cold climate in *D. americana*. I could demonstrate a genotype-phenotype-fitness interaction for diapause, and could show covariance with latitude for wing length and starvation resistance. However, there was not evidence that body mass and lipid content are adaptive to cold climate in *D. americana*. These results lead me to conclude first, that by considering which traits are putatively adaptive in species subject to similar selective pressures, candidate traits to investigate as potential adaptations can be effectively chosen.

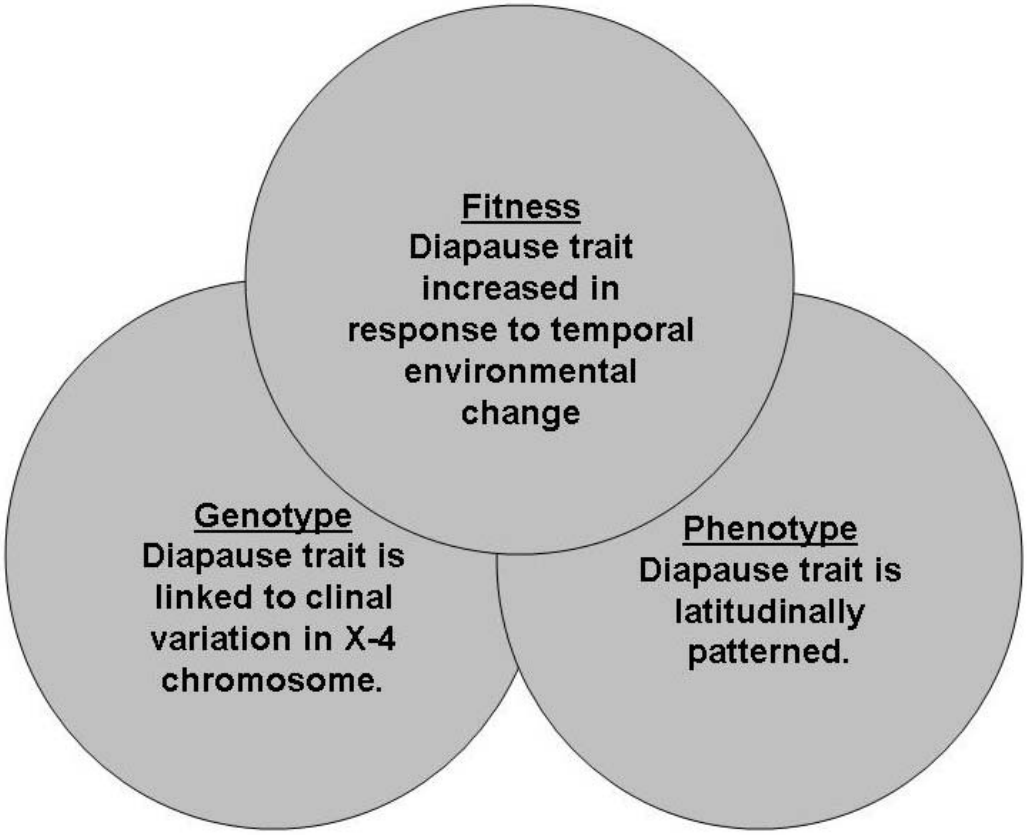
The results of this study also show that *D. americana* can be an effective model in studying adaptation. That the species is broadly distributed, harbors substantial genetic variation, and has defined regions of geographically structured variation are all factors which aid in demonstrating relationships between genotype, phenotype, and fitness. *D. americana* does offer a unique opportunity to investigate the potential action of sex-specific selection in combination with adaptation to climate. Because known clinally distributed sequence variation is X-linked in *D. americana*, it can respond to selection favoring female fitness over male fitness (Rice 1984, Gibson et al 2002, Evans et al 2007). The impact of sexual antagonism on these traits can be investigated by measuring response to sex-specific selection on these traits in the laboratory (Morrow et al 2008).

D. americana will be most generally useful as a model for the study of adaptive evolution in the context of comparative studies with other cold climate *Drosophila* species. Investigating genotype, phenotype, and fitness interactions in the same traits among diverse species will allow identification of patterns of similarities and differences in adaptive traits. Sufficiently broad sampling among these species will provide power to detect the influence of various factors in shaping suites of traits adaptive to environment. Recent results from laboratory evolution of *E. coli* indicate that adaptive evolution continues to gradually refine fitness over many generations following the initial response to a novel environment (Barrick

et al 2009). Comparison between short-term adaptation of species such as *D. melanogaster* and long-term adaptation of species such as *D. americana* to cold-climates allows investigation of this process in nature.

Whole-genome information is available for representatives of all major *Drosophila* lineages, and tools for investigating genotype-phenotype interactions have been developed for *D. melanogaster* and other species. *Drosophila* are therefore perhaps uniquely well-suited to investigating the role of physical and functional organization of the genome in constraining evolution. Linkage relationships among genes are known to constrain adaptation (Betancourt and Presgraves 2002, Crombach and Hogeweg 2007, Springman et al 2005) and adaptive associations among loci have been of sustained interest for decades (Dobzhansky 1970; Kirkpatrick and Barton 2006). There is substantial variation in linkage relationships among genes among the major *Drosophila* lineages (Bhutkar *et al* 2008). Additionally, the relative importance of *cis* or *trans* mutations' contributions to phenotypic evolution is actively discussed and investigated in a variety of species including *Drosophila* (*e.g.* Arnold 1992, Hansen 2006, Hoekstra and Coyne 2007, Carroll 2008). Comparisons among *Drosophila* lineages could be of unique value in studying the less well-known hypothesis that karyotypic evolution contributes to phenotypic evolution by changing the physical arrangement of genes relative to one another (*e.g.* Wilson *et al* 1974, Ranz *et al* 2007).

Figure 5.1 Summary of evidence supporting the genotype-phenotype-fitness interaction of reproductive diapause in *D. americana*.



APPENDIX

Table A.1 Number of females dissected, found to be in diapause, and the measured diapause incidence of each line of *D. virilis* group species.

Species	*Line*	*# Dissected*	*# In Diapause*	*Diapause Incidence*
D. lummei	1011.01	28	28	1.00
D. lummei	1011.07	38	37	0.97
D. lummei	1011.08	29	28	0.97
D. lummei	207	76	76	1.00
D. virilis	V46	32	0	0.00
D. virilis	308151	80	1	0.01
D. virilis	iHWE	29	1	0.03
D. virilis	Y5615	40	28	0.70
D. borealis	PG05.02	73	71	0.97

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