THE ROLE OF TEMPERATURE AND ADAPTIVE PHENOTYPIC PLASTICITY IN THE EVOLUTION OF *DROSOPHILA MELANOGASTER* MORPHOLOGICAL CLINES

A Dissertation Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment

of the Requirements of the Degree

Doctor of Philosophy

By

William Andrew Russey

August 2014

THE ROLE OF TEMPERATURE AND ADAPTIVE PHENOTYPIC PLASTICITY IN THE EVOLUTION OF *DROSOPHILA MELANOGASTER* MORPHOLOGICAL CLINES

William Andrew Russey

APPROVED:

Dr. W. Anthony Frankino, Chairman

Dr. Ricardo Azevedo

Dr. Blaine Cole

Dr. Gregg Roman

Dr. Stephen P. Roberts
University of Central Michigan

Dr. Dan E. Wells, Dean, College of Natural Sciences and Mathematics

ACKNOWLEDGEMENTS

First, I would like to thank my friends, professors, and committee whose guidance and support made this work possible. Dr. Tony Frankino, my principal investigator, who brought me into his lab and whose mentorship goes above and beyond what I ever thought I would receive from my advisor. His attention to detail has made me a better scientist, a better student, and a better teacher. Graduate school would have been a qualitatively different experience without you, and not for the better. Thank you for supporting me in the tough times and celebrating in the good.

Next, I would like to generally thank my committee for their guidance throughout my graduate career. I always walked away appreciative of my committee meetings and with an improved perspective on my project due to your feedback. Individually, I would like to thank Dr. Cole for forcing me into thought experiments and pushing methods and outcomes I had not considered. To Dr. Azevedo, thank you for encouraging me to learn the R programming language, helping me troubleshoot when I developed coding block, and for statistical guidance. To Dr. Roman, thank you for helping me appreciate *D. melanogaster* as an organism, and not simply an evolving object, as well as forcing me to think outside of my comfortable phenotype-centric box. Finally, I would like to thank Dr. Roberts for his extensive knowledge and input regarding insect flight dynamics and for being a great collaborator to work with.

A special thank you to the rest of the Ecology and Evolution department. There are so many who made this work and my sanity possible. I will do my best to thank you all here, but know there are many more. To Dr. Cole and Dr. Wiernasz, thank you so much for letting be an adjunct lab member and letting me attend your lab meetings. As the only graduate student in my own lab the last 3 years, having a small community to discuss science meant more to me than I can articulate here. Lab meetings gave me an

opportunity to explore and discuss literature a little, but not too far, out of my typical readings. Next, I want to thank my office-mates throughout the years. Dr. Lara Appleby(!), we started at U of H the same semester and from our first day through your graduation, your enthusiasm for science and life in general has never once ceased to amaze me. To Dr. Sujal Phadke, thanks for your professionalism in an office containing Eric Bakota and myself; that could not have been easy. As a senior graduate student you helped me immensely in dealing with graduate life. To Eric Bakota, currently you are the only other person on the planet who shares my experience of being a graduate student in the Frankino lab. Thanks for discussing everything in the lab except fruit flies, having a positive attitude, and willingness to chalk-talk through any problem. Lastly, I would like to thank Kristen Dimond, Jo Webb, Dane Jordan, and Hongan Long for your support, friendship, and general positivity toward science.

There were many, many undergraduates who made this work possible. Again, there are more than I can list here, but I will highlight those who went above and beyond what I ever expected. To Yasmeen Arastu, who worked with me for almost 4 years, I can not begin to express how important your contribution to this dissertation has been. You collected fire ants from their mounds, you caught jumping spiders, collected dung fly eggs off cow dung, imaged hundreds of fly wings, and most importantly, were always there when I needed help most. No other undergrad stayed as late or as often, and I cannot thank you enough. To Christine Sikes, simply stated, much of this work physically could not have happened without your hard work constructing the lab wind tunnel. You were a laboratory general in your years with the lab, and much of my work and Tony's would have suffered without you. To David Ha, you probably placed second in total laboratory hours. I will always be grateful for dedication and loyal volunteer service. To Larry Broome, while your work span was a bit shorter than some, you were extremely

dedicated and reliable in your time and efforts. While many others remain, in no particular order I would like to thank Naila Zafer, Jesus Vasquez, Kendall Mills, Stephanie Rice, Milhko Bravo, Emma Perez, and Yanni Liras.

Lastly I want to thank my family, many of whom may never understand exactly what it is I did for these 6 years, but never tried to dissuade me from pursuing what I was so eager to purse. To Zack, Ali, Cynthia, and Erik, you have all been as good of friends as family and provided so many great distractions from the grind of graduate life. To my parents Jim and Libby, who have provided nothing but love and support, thank you for encouraging me to pursue what I love. And finally, I want to thank my loving wife, Anne. In a season of late nights, worked weekends, missed vacations, and devastating experiments, you were my constant and unending source of love, friendship, support, and motivation. I truly could not have survived it all without you.

Thank you.

THE ROLE OF TEMPERATURE AND ADAPTIVE PHENOTYPIC PLASTICITY IN THE EVOLUTION OF *DROSOPHILA MELANOGASTER* MORPHOLOGICAL CLINES

An Abstract of a Dissertation

Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

William Andrew Russey

August 2014

ABSTRACT

Variation in morphology results in variation in ecologically relevant performances, which ultimately results in variation in fitness allowing for adaptive evolution. Task performances, such as flight ability, result from the proper scaling of and functional integration of numerous component traits. Morphological variation underlying ecologically relevant task performances can experience strong environmental effects in their expression, or phenotypic plasticity. Historically, the role phenotypic plasticity in adaptive evolution has been controversial, although it has garnered increased support in recent decades. *Drosophila* spp. are globally distributed and exhibit convergent morphological clines in flight morphology, and importantly, they also exhibit patterns of phenotypic plasticity consistent with these geographic patterns. In the work presented here, I examine if existing patterns of *D. melanogaster* flight morphology are adaptive regarding flight performance and fitness under the prediction of phenotype-environment matching, wherein the phenotype expressed in an environment enhances fitness in the predicted environment. In the work presented here, I demonstrate (i) phenotypic plasticity in *D. melanogaster* exhibits a pattern of adaptive phenotype-environment matching in which an induced phenotype is best-suited for flight at the temperature of development, (ii) the pattern of thermally-induced phenotypic plasticity facilitates the evolution of upwind flight performance at Cool and Warm flight temperatures, (iii) adaptive evolution by genetic accommodation is a dynamic process and the contribution of traits responding to selection vary and change over time, and finally, (iv) the adaptive pattern of phenotype-environment matching regarding flight performance is only partially realized as an increase to fitness, measured as survival in presence of predators. My dissertation work importantly demonstrates existing patterns of phenotype-environment matching in *D. melanogaster*, and demonstrates how this pattern facilitates adaptive

evolution by genetic accommodation in a complex phenotype that exhibits natural, continuous variation.

CONTENTS

CHAPTER 1. THE UTILITY AND EVOLUTION OF COMPLEX PHENOTYPES

1.1 The relationship among variance in morphology, performance, and fitness

The diversity of morphological form and its importance to our understanding of evolution are reflected in taxonomic nomenclature, where many taxonomic groups are named by Latin anatomical descriptions (e.g., Diptera, two-wing; Tetropod, four-foot; Actinopterygii, having rays-fins). However, it was not until a century after Darwin (1859) postulated the importance of heritable morphological variation in evolution that Arnold (1983) statistically formalized the relationship between morphology and fitness. In his seminal paper, Arnold expanded on Lande's (1979) multivariate selection theory by outlining statistical methods to describe how morphological variation results in performance variation, and performance variation results in fitness variation. His work was extended by Garland and Carter (1994) (see also, Garland and Losos 1994) to include direct effects of environmental variation on performance, and indirect effects exerted via environmentally induced variation in morphology. The relationships and variables connecting morphological variation to fitness variation continue to expand (Careau and Garland 2012). As this interconnected web of phenotypic variation grows, testing each relationship in a satisfactory way within the confines of a single experiment becomes exceedingly difficult. The central tenant remains the same: understanding how morphological variation produces variation in performance, and the variation in performance produces variation in fitness.

Complex phenotypes are those that are composed of many traits; these traits are arranged in hierarchies and suites that typically exhibit high degrees of internal covariation. Performance of ecological tasks, such as locomotor, courtship behaviors or prey handling and capture, are highly complex phenotypes and involve morphological,

physiological, and behavioral traits. The strong covariation within trait suites is presumably the product of selection for ecological function (Lande and Arnold 1983; Brodie III 1992; Bonine and Garland Jr. 1999; Klingenberg and Zaklan 2000; Baranzelli et al. 2014) and results in strong covariation among traits that comprise complex phenotypes within biological groups (e.g., species) relative to covariation between biological groups (Burkhardt and de la Motte 1985). Such covariation among functionally related morphological traits is reflected in the tight scaling relationships exhibited by most morphological traits. Although nearly a century of work documents these inter- and intra-group patterns in scaling relationship variation (e.g., Huxley 1932; Gould 1966; Emlen and Nijhout 2000; Pélabon et al. 2013), in most cases, little is known about how selection acts on relative trait size, or if the observed patterns of scaling relationship variation are truly adaptive.

Determining the adaptive nature of complex phenotypes can be difficult for three reasons. First, describing and comparing the patterns of covariation among elements of complex phenotypes can be challenging, because the scale at which component traits are described is partially subjective and methodological artifacts can profoundly influence experimental outcomes (Moczek 2006). For example, limb:body size scaling effects locomotor speed in lizards (Bonine and Garland Jr. 1999), but limb size is itself a complex function of the femur and tibia whose length and mass have important implications on locomotor kinematics (Blob and Biewener 1999). Describing the influence of hind limb length on locomotor performance thus may be affected by whether one studies the overall effect of length or the contribution of the individual component parts. Second, tight covariation among traits means there is an absence of phenotypic variants that can be used to determine how variation in each individual component trait,

or different patterns of covariance among component traits, might affect ecological performance, and ultimately, fitness. Third, the relationship between morphological variation, ecological performance, and fitness is typically unknown, obscure, or difficult to elucidate in ecologically relevant contexts (Wainwright and Reilly 1994; Frankino et al. 2009). Moreover, the relationships among morphology, performance and fitness variation are often environment-specific, further complicating experimental designs and interpretation of data.

There are two primary methods that can be used to overcome these challenges. First, experimental evolution can be used to test predictions about how selection acts on complex phenotypes in environments that differ in the phenotypes they favor (Garland Jr. and Rose 2009). If hypotheses regarding the adaptive nature of specific patterns of covariation are correct, then the experimental populations should evolve to express patterns of phenotypic (co)variation convergent with predictions derived from natural populations or calculated form functional principles. Predicted responses to selection provide strong evidence of adaptive value of the resultant phenotypes if the experimentally evolved lineages exhibit consistent patterns among component traits across replicates.

A second, complementary approach to overcome the challenge of elucidating effects of morphological variation on performance and fitness is that of allometric engineering, whereby individuals are manipulated experimentally to increase phenotypic variation in individual traits and to produce novel patterns of covariation among traits (Sinervo and Huey 1990; Sotherland et al. 1990; Sinervo 1992). These experimental populations can be used to measure how selection acts on traits individually and in combination. If novel

trait combinations fail to perform, or experience reduced relative fitness, then this provides evidence that natural patterns of covariation are adaptive (Andersson 1982; Greene et al. 1987; Sinervo and Huey 1990). In cases of presumptive adaptive phenotypic plasticity, the inducing-environment can be utilized to engineer individuals expressing a diversity of phenotypes that vary in patterns of trait covariation, and then these can be used to determine how these phenotypes perform across environments.

1.2 Phenotypic plasticity and adaptive evolution

Phenotypic plasticity is the ability of one genotype to express phenotypic variation in a manner that covaries with the environment. Adaptive phenotypic plasticity occurs when the covariance between phenotypic variance and environmental variance enhances performance, and thus fitness. The role of phenotypic plasticity in adaptive evolution has been of interest to evolutionary biologists for over a century (Baldwin 1896) and has become an expansive discipline in its own right (for reviews, Parsons et al. 1993; Pigliucci 2001; Dewitt and Scheiner 2004; West-Eberhard 2003; Whitman and Ananthakrishnan 2009). While not all phenotypic plasticity is necessarily adaptive (Smith-Gill 1983; Ghalambor et al. 2007; Morris and Rogers 2013), the simplest and most common prediction of adaptive phenotypic plasticity is a situation of phenotype-environment matching, wherein the developmental environment accurately signals the future selective environment and induces expression of the phenotype carrying the highest relative fitness in the anticipated selected environment (Moran 1992). Examples of phenotype-environment matching are common (e.g., Krueger and Dodson 1981; Lively 1986; Pfennig and Frankino 1997; DeWitt 1998; Rogers et al. 2002; Michel 2010), although plasticity can result in other complex patterns of fitness (Berven et al. 1979;

Conover and Schultz 1995; Marcil et al. 2006; Kingsolver and Huey 2008; Deere et al. 2012).

The predicted role of phenotypic plasticity on adaptive evolution has changed over time. Initially, phenotypic plasticity was viewed as impeding adaptive evolution because it reduces the phenotypic resemblance between parent and offspring, obscuring the relationship between genotypic and fitness variation (Wright 1931; Simpson 1953). More recently, phenotypic plasticity has been hypothesized to play a role in the adaptive evolution of populations by enabling a rapid response to selection and facilitating diversification among groups (Parsons et al. 1993; Pigliucci 2001; Dewitt & Scheiner, 2004; West-Eberhard, 2003; Whitman and Ananthakrishnan, 2009). Within this framework, the idea of genetic accommodation has come to enjoy a central position (e.g., Price et al. 1993; Pigliucci and Murren 2003; Braendle and Flatt 2006; Pigliucci et al. 2006; Moczek et al. 2011; Wund 2012; Schlichting and Wund 2014). Genetic accommodation describes the process through which adaptive genetic changes in the expression of a plastic phenotype occur. Such adaptive genetic changes result from the altered developmental regulation, either caused by a novel environment or mutations of large effect, that reveals cryptic genetic variation to selection (Gorur et al. 2005; Stasiuk et al. 2012). Genetic accommodation occurs when selection acts on this newly revealed variation to produce a novel plastic response, enhancing fitness. Most direct studies of genetic accommodation (e.g., Sollars et al. 2003; Suzuki and Nijhout, 2006; Waddington 1953, 1956) test for the evolved constitutive expression of a previously plastic phenotype, referred to as genetic assimilation.

To determine if genetic assimilation has occurred, and if the resulting pattern of plasticity is adaptive, fitness of environmentally-induced phenotypes should be quantified across the range of inducing environments in a full factorial design. Adaptation by genetic assimilation may result in either a generalist or specialist phenotype, depending on the relative fitness of the derived phenotype across selective environments (Table 1.1). In the context of these experiments, constitutive expression of traits that only increase fitness in a single environment constitute a specialist phenotype, whereas the constitutive expression of trait value that enhance fitness across all selective environments are interpreted as reflecting a generalist phenotype. Adaptation by genetic accommodation may not result in genetic assimilation, however, and may instead result in a pattern of phenotype-environment matching where fitness in an environment is dependent on the matching between the developmental and selective environments. It is essential to test phenotypes in environments other than the inducing environment to identify costs associated with phenotype-environment 'mismatching' (i.e., the fitness of individuals that develop in environments unassociated with the future or cued selective environment); otherwise, a phenotype with high fitness in multiple environments could be mistakenly viewed as a generalist. Although genetic accommodation and genetic assimilation are thought to be important evolutionarily, there have been no empirical tests of their importance using large populations and focal traits of known ecological importance that exhibit continuous phenotypic variation. Thus, general importance of genetic accommodation in natural populations remains an open question.

Table 1.1. Predicted outcomes of evolution by genetic accommodation to selection in environment A. Theoretical outcomes of a trait responding to selection in environment A when assayed in a full factorial manner post-selection. Organisms exhibit phenotypic plasticity such that trait values are determined by whether they develop in environment A or B. Fitness is determined in each environment for each phenotype in a full factorial design. The table assumes an increase in performance (fitness) in environment A resulting from selection in environment A (i.e., adaptive evolution follows development and selection in environment A). Fitness is described as "High" if the value in the focal cell is greater relative to that prior to evolution in environment A, whereas "Low" indicates a fitness equal to or lower than fitness prior to evolution. Genetic assimilation results from evolution by genetic accommodation and is thus implied, although genetic assimilation does not occur when only genetic accommodation is indicated.

Outcome	Development environment (A or B)	Selective environment (A or B)	Fitness (High or Low)	Mode of evolution
Specialist	A	A	High	Genetic Assimilation
	A	B	Low	
	B	B	Low	
Generalist	A	A	High	Genetic Assimilation
	A	B	High	
	B	B	High	
Phenotype-Environment Matching	A	A	High	Genetic Accommodation
	A	B	Low	
	B	B	High	

1.3 Temperature-induced plasticity as a tool to investigate morphological effects on performance and fitness

The effects of temperature on ectotherms are among the most extensively studied examples of phenotypic plasticity, affecting ecologically important traits such as longevity (Pearl 1928; Maynard-Smith 1963; Farmer and Sohal 1987), body size (Atkinson 1994; Atkinson and Sibly 1997), fecundity (Huey et al. 1995), lifetime progeny production (McCabe and Partridge 1997), and egg size (Azevedo et al. 1996). Additionally, temperature can greatly influence locomotor performance, and frequently exhibits genotype x environment interactions (e.g., Curtsinger and Laurie-ahlberg 1981; Bonine and Garland Jr. 1999; Gibert et al. 2001). These effects of temperature across diverse taxa indicate that temperature is perhaps the premier abiotic factor influencing adaptive evolution in ectotherms (Atkinson 1994; Atkinson and Sibly 1997; Gillooly et al. 2001). The diverse and numerous phenotypic effects of temperature, combined with the relative ease at which it can be manipulated in the laboratory, make temperature a powerful and practical experimental tool with which one can explore the roles of phenotypic plasticity and genetic accommodation on the adaptive evolution of complex phenotypes.

1.4 *Drosophila* as a model for adaptive evolution

For several reasons, *Drosophila* spp are well-suited as models for studying both the evolution of complex phenotypes and the role of phenotypic plasticity in adaptive evolution. First, they exhibit genetically-based clines in body and relative wing size. These morphological clines have been reported on each continent, but Antarctica (North America: Stalker and Carson 1947; Pegueroles et al. 1995; Huey et al. 2000; South America: Pegueroles et al. 1995; Gilchrist and Huey 2004; Europe: Pegueroles et al. 1995; Huey et al. 2000; Gilchrist and Huey 2004; Asia: Imasheva et al. 1994; Grotewiel

et al. 2005 and Africa: Pitchers et al. 2013) and across a number of *Drosophila* spp (e.g., *D. robusta*: Stalker and Carson 1947; *D. melanogaster*: Azevedo et al. 1998; *D. obscura*: Pegueroles et al. 1995; *D. subobscura*: Gilchrist and Huey 2004). Importantly, the geographic patterns are consistent with patterns of thermally-induced phenotypic plasticity in body size and relative wing size (e.g., James et al. 1997; French et al. 1998; Gilchrist and Huey 2004; Frazier et al. 2008). Both the clinal variation and plastic responses are consistent such that populations located in cool environments, or those that developed at cool temperature, exhibit large body size, and disproportionately large wings relative to flies from warm locales or that are developed at warm temperature. This pattern benefits at least some aspects of flight performance, at least in cool temperatures (Frazier et al. 2008), but far more data are needed to establish that these patterns are the result of adaptation for flight across environments as is often claimed (e.g., David and Capy 1988; Ayala et al. 1989; James et al. 1997; Azevedo et al. 1998; Gilchrist et al. 2001; Gilchrist and Huey 2004; Dillon and Frazier 2006; Frazier et al. 2008). The current support for adaptive phenotype-environment matching is consistent with predictions of flight dynamics; wing-beat frequency exhibits a positive linear relationship with temperature making flight at cool temperatures challenging (Curtsinger and Laurie-ahlberg 1981; Barnes and Laurie-Ahlberg 1986). The increase in wing size can move a greater volume of air per stroke and may compensate for low wing beat frequency at cool temperatures (Reed et al. 1942). Conversely, the increased power output requirements of flight at warm temperature needed to overcome lower air densities and increased air viscosity (Ellington 1984) appear to result from greatly increased wing beat frequencies (Barnes and Laurie-Ahlberg 1986).

Asserting these clines as adaptive seems intuitive, as morphology appears to compensate for flight challenges in a predictable manner, and flight performance as a mode of locomotion likely has many fitness effects (e.g., dispersal: Hoffmann et al. 2007). Much of the support for the adaptive nature of these clines comes from well-documented, rapidly evolving clines in *D. subobscura* recently introduced in North America (Ayala et al. 1989; Huey et al. 2000). My dissertation work importantly elucidates existing patterns of phenotype-environment matching in *D. melanogaster*, and demonstrating how this pattern facilitates adaptive evolution by genetic accommodation.

1.5 Overview

In this dissertation, I present a series of experiments testing the adaptive nature of phenotypic plasticity. I seek to determine the relationship between variation in morphology, performance and fitness, with the aim of understanding the possible adaptive nature of well-documented clines and patterns of phenotypic plasticity in *D. melanogaster* flight morphology and how genetic accommodation may facilitate their evolution. In the paragraphs that follow, I will briefly outline the goal, methods, and findings of four experiments designed to address the hypotheses unique to each chapter.

1.5.1 Chapter 2. Effects of environmental temperature on flight performance

In the first study, I examined the effects of temperature on development, flight performance, and senescence of flight performance in *D. melanogaster.* Flies were developed from egg to adult at either Cool (16^{o}C) or Warm (27^{o}C) and flown through a progressive velocity wind tunnel that quantifies the maximum headwind speed in which the average fliers (50th percentile) and top fliers (80th percentile) from a population can

achieve. Cool- and Warm-developed flies were assayed over the first 3 weeks post-eclosion at 16, 22, and 27^{o}C to determine thermal effects on senescence across flight environments. Cool-developed flies exhibited generally poor flight performance and no effect of age; thus, I could not identify an age of peak flight performance or the time frame of senescence in flight performance. Warm-developed flies, however, had maximal performance around 3 d post-eclosion. This declined sharply with age much younger than previously measured (Curtsinger and Laurie-ahlberg 1981; Lane et al. 2014). Both Cool- and Warm-developed flies exhibited superior flight at their temperature of development; i.e., exhibited phenotype-environment matching. These findings suggest the thermally-induced phenotypic plasticity during *D. melanogaster* development is adaptive when flight performance increases relative fitness. Additionally, my data demonstrate the importance of considering developmental temperature, assay temperature, and age when assaying ecological performance.

The format of this chapter differs from the others slightly as it is formatted for submission to the Journal of Experimental Biology. I am the primary author, and Dr. Stephen Roberts, Stephanie Rice (undergraduate), and Dr. W. Anthony Frankino are co-authors. A team of six undergraduates, led by Stephanie Rice, executed the experiment under my supervision. Dr. Frankino and Dr. Roberts provided feedback and edits to the manuscript, but I take full responsibility for any errors.

1.5.2 Chapter 3. The role of phenotypic plasticity in adaptive evolution

Here, I examined the role of thermally-induced phenotypic plasticity in adaptive evolution. Phenotypic plasticity allows for an organism to enhance its relative fitness by matching phenotype expression to environmental conditions. Historically, phenotypic

plasticity was believed to hinder a response to selection because it weakens the relationship between variation in genotype, phenotype and fitness. I tested whether phenotypic plasticity facilitated, or hindered, adaptive evolution by selecting for upwind flight performance at Cool (16^{o}C) and Warm (27^{o}C) flight temperatures in two separate experimental evolution designs. In Experiment I, all flies were developed at 21^{o}C. In Experiment II, flies were developed at either 16 or 27^{o}C and selected at their respective temperature of development. Selection was performed for 11 generations, at which time the degree to which flight performance, and flight-related traits, evolved between the two experimental designs was compared. The results show phenotypic plasticity in *D. melanogaster* is adaptive when development temperatures matched selective flight temperatures, although resultant morphological patterns did not evolve in the predicted manner. These findings are significant, as they indicate phenotypic plasticity and genetic accommodation facilitate adaptive evolution and likely encourage adaptive radiations and species diversification as observed in *Drosophila.*

I expect to submit this chapter as a manuscript to the journal Evolution this fall. Each experiment lasted roughly a calendar year and over 20 undergraduates contributed to this work dedicating an estimated 1300 man-hours in aggregate on selection days alone. I will be the primary author on the manuscript and Dr. W. Anthony Frankino the co-author. I have received feedback and edits from Dr. Frankino, but accept responsibility for any errors.

1.5.3 Chapter 4. Genetic accommodation promotes adaptive phenotype-environment matching in Drosophila melanogaster flight performance

Flight performance of laboratory stocks from differing source populations exhibit phenotype-environment matching in flight performance (Frazier et al. 2008, Chapter 2). I demonstrate in Chapter 3 that phenotypic plasticity clearly facilitates adaptive evolution, however, it was unclear if enhanced flight performance resulted from altered expression of plastic phenotypes. Evolved patterns of flight performance were compared following 7 and 11 generations of selection at Cool (16^{o}C) or Warm (27^{o}C) temperatures. I sought to determine if enhanced upwind flight performance resulted from specialization to the selected environment at the cost of performance in other environments, a generalist phenotype adept at flight in all environments, or an enhancement of phenotype-environment matching as exhibited by unselected populations. Cool- and Warm-selected flies were assayed for Cool and Warm flight performance in a modified 2 x 2 full factorial design. Evolved patterns of flight performance were dynamic, indicating the traits responding to selection changed over time. Flight performance patterns were qualitatively different between selected lineages following 7 generations of selection; Cool-selected flies exhibited a strong generalist phenotype, whereas Warm-selected flies exhibited a specialist phenotype. Following 11 generations of selection, however, enhanced phenotype-environment matching was observed in both Cool- and Warm-selected lineages with costs of development at temperature the alternate temperature. I conclude that genetic accommodation not only accelerated adaptation of flight performance, but also facilitated adaptive evolution of phenotype-environment matching.

I expect to submit this chapter as a manuscript to Proceedings of the Royal Society this fall. I will be primary author on the manuscript with Kendall Mills (undergraduate) and Dr.

W. Anthony Frankino as co-authors. Kendall Mills received undergraduate Thesis of the Year honors from the University of Houston for her contribution to the presented work. She led a team of 4 undergraduates under my supervision. Dr. Frankino provided feedback and edits on the presented work but I take responsibility for any errors.

1.5.4 Chapter 5. Does adaptive phenotype-environment matching in Drosophila flight performance enhance survival in presence of predators?

D. melanogaster body size, relative wing size, and wing shape are phenotypically plastic traits that exhibit adaptive phenotype-environment matching regarding flight performance. While variation in these morphological traits results in variation in performance, a direct relationship between flight performance and relative fitness has yet to be identified. In this study, I measured the strength and pattern of selection acting on flight performance using flies with experimentally enhanced variation in relative wing size. *D. melanogaster* were subject to predation by two predators with different hunting styles, twin-flagged jumping spiders (*Anasaitis canosa*) and yellow dung flies (*Scathophaga stercoraria*), across a range of temperatures (15, 18, 21 and 27^{o}C). Relative fitness of flies was predicted to be greatest when temperature of predation assay was most similar to the development temperature, i.e., phenotype-environment matching. My data only partially support this prediction; flies with relatively large wings exhibited lowest survival in presence of both predators even at Cool temperatures. Warm-developed flies, however, exhibited complex patterns of thermally-dependent survival. This finding lends partial support to claims of adaptive phenotypic plasticity. This chapter contains supplementary material separated to facilitate manuscript preparation.

I expect to submit this chapter as a manuscript to The Journal of Evolutionary Biology later this summer. I will be primary author on the manuscript, and Dr. Ian Dworkin, Naila Zafer (undergraduate), Yasmeen Arastu (undergraduate), and Dr. W. Anthony Frankino will be co-authors. Naila Zafer presented a portion of this work as a part of the STAR program. This project was executed with the help of four undergraduates. Dr. Dworkin provided significant statistical guidance and both he and Dr. Frankino have provided feedback and edits regarding the manuscript preparation. I take full responsibility for any errors.

1.6 Summary

The findings presented here provide mixed support for the hypothesis that morphological clines and thermally-induced phenotypic plasticity in *D. melanogaster* are adaptive. In support, temperature-induced phenotypic plasticity resulted in phenotype-environment matching for flight performance at Cool and Warm developmental temperatures, and importantly facilitated the adaptive evolution of flight performance. Genetic accommodation facilitated this adaptive evolution and enhanced the pattern of phenotype-environment matching. Exposure to predators, however, revealed limited support for adaptive phenotype-environment matching regarding relative fitness. I conclude that although the relationship between flight performance and fitness remains unclear, phenotypic plasticity facilitates adaptive evolution in *D. melanogaster*.

CHAPTER 2. EFFECTS OF ENVIRONMENTAL TEMPERATURE ON FLIGHT PERFORMANCE

2.1 Abstract

Temperature affects development, ecological performance, and senescence in ectotherms such as insects. In this study, *Drosophila melanogaster* were reared at Cool (16^{o}C) or Warm (27^{o}C) temperatures and flown through a custom wind tunnel during the first 3 weeks post-eclosion at Cool (16^{o}C), Moderate (22^{o}C), and Warm (27^{o}C) temperatures to determine effects of developmental and ambient temperature on upwind flight performance and its senescence. In general, Cool-developed flies were poor fliers and exhibited no detectable age-based variation or senescence in flight performance, whereas the flight performance in Warm-developed flies was highest in very young individuals and declined sharply with age. Both Cool- and Warm-developed flies exhibited superior flight at the temperature at which they developed, relative to flight at other temperatures and relative to flies that developed at other temperatures. This suggests that thermally-induced phenotypic plasticity during *D. melanogaster* development may be adaptive as it facilitates flight at the inducing temperature.

2.2 Introduction

In ectotherms, temperature can affect ecological performance in at least three ways. First, temperature variation during development can induce plastic responses in a variety of traits, e.g., development rate, adult size, physiology, behavior morphology, and life history (Whitman and Ananthakrishnan 2009). Such plasticity can result from inescapable effects of temperature on development or can reflect adaptive responses to the future selective environment as cued by developmental temperature; in this latter case of adaptive plasticity, individuals exhibit 'phenotype-environment matching' where

the phenotype expressed in an environment conveys higher fitness in that environment relative to other environments. Second, temperature affects the rate of senescence as reflected in a decline of physiological and/or ecological performance with age. Finally, temperature affects physiological performance via passive effects on enzyme-catalyzed reactions that in turn impacts ecological performance, e.g., wing beat frequency increases linearly with temperature, reducing flight ability at low temperatures (Barnes and Laurie-Ahlberg 1986). Although challenging, disentangling these various effects of temperature on ecological performance is critical to understanding the evolution of adaptive phenotypic plasticity, senescence and ecological performance in ectotherms.

Here, I test for independent affects of developmental temperature, performance temperature, and chronological age on *Drosophila melanogaster* flight performance. I use an enclosed progressive-velocity wind tunnel to discriminate populations of individuals based on their ability to overcome sustained headwinds over short flight distances. *D. melanogaster* is well suited to my study as it possesses presumably adaptive phenotypic plasticity in relative wing size and wing shape in response to rearing temperature (Debat et al. 2003; Frazier et al. 2008). My goals are to determine (i) the effects of temperature (rearing and acute) on patterns of senescence in flight performance, and (ii) if flight performance exhibits patterns consistent with adaptive phenotype-environment matching across developmental and flight temperatures. To achieve these goals, I measured and compared the wind speed that average (50^{th} percentile; WS_{50}) and strong (80^{th} percentile; WS_{80}) fliers were able to overcome from each fly population.

2.3 Results and discussion

Temperature during development had lasting effects on adult flight performance and interacted with flight temperature to affect flight ability and the pattern of flight senescence. Below, I first discuss performance of Cool- and Warm-developed flies before making comparisons between these two groups.

Neither age, flight temperature nor their interaction affected performance of average fliers (WS_{50}) and only flight temperature affected performance of the strongest fliers (WS_{80}) from the Cool (16^{o}C) development temperature (Table 1). A t-test on flight performance between development temperatures revealed Cool-developed flies produced relatively poor flight performance compared to Warm-developed flies (WS_{50} t=-3.37, d.f.=73.588, p=5.9x10^{-4}; WS_{80} t=-5.52, d.f.=73.957, p-value=2.3x10^{-7}) when considering all flight temperatures. Flies from Cool development temperature, however, differed in their performance across flight temperatures (Fig. 1), flying better at their Native temperature relative to the Alternate flight temperatures of 22^{o}C (WS_{50} p=7.1x10^{-4}; WS_{80} p=4.2x10^{-9}) and 27^{o}C (WS_{50} p=0.020; WS_{80} p=2.5x10^{-5}). Flight at these Alternate temperatures did not differ (WS_{50} p=0.90; WS_{80} p=0.16). Thus, despite generally poor flight performance across temperatures and no discernable age of peak flight performance (Fig. 1), Cool-developed flies exhibited adaptive phenotype-environment matching for flight temperature.

Flies from Warm (27^{o}C) development temperature exhibited different patterns of flight senescence across flight temperatures, experiencing a strong and rapid decline in flight performance shortly after eclosion for all but average fliers (WS_{50}) at the 16^{o}C flight temperature (Fig. 1; Table 1). Pair-wise comparisons of flight performance grouped by

Age confirm that flies 3 d post-eclosion had higher performance than 5 (WS_{50} $p=8.5x10^{-7}$; WS_{80} $p=2.2x10^{-5}$), 7 (WS_{50} $p=3.3x10^{-7}$; WS_{80} $p=9.0x10^{-8}$) and 10 d old flies (WS_{50} $p=1.7x10^{-7}$; WS_{80} $p=9.0x10^{-8}$), indicating that the strongest flight performance for Warm-developed flies occurs at 3 d post-eclosion. At 5 d post eclosion, flight performance of average fliers declines to *ca* 35% of peak performance at both Alternate 22°C and Native Warm flight temperatures. The strongest fliers (WS_{80}) show a similarly steep decline in performance, but at their Native flight temperature fell to 68% of peak performance at 5 d post eclosion and by day 7 had dropped to only 34% of peak performance (Fig. 1). The six-fold senescence of flight performance is more rapid and begins at an earlier age than has been found for other metrics of locomotor-related senescence in flies (e.g., wing beat frequency: Curtsinger and Laurie-ahlberg 1981; Lane et al. 2014; fall escape: Simon et al. 2006). Flight performance for the top flying (WS_{80}) Warm-developed flies was highest and exhibited the slowest senescence at their Native temperature (27°C), a pattern consistent with adaptive phenotype-environment matching.

Figure 2.1. Senescence of flight performance. Flight performance of average (A, B & C: WS_{50}) and strongest fliers (D, E & F: WS_{80}) into a progressive headwind is plotted against days since adult eclosion for flies from both development temperatures flown at three temperatures. Circles indicate average wind speed and error bars represent standard error. Males are represented as dashed lines with open circles; females are solid lines with filled circles. Line color indicates the development temperature, blue for Cool-developed and red for Warm-developed flies.

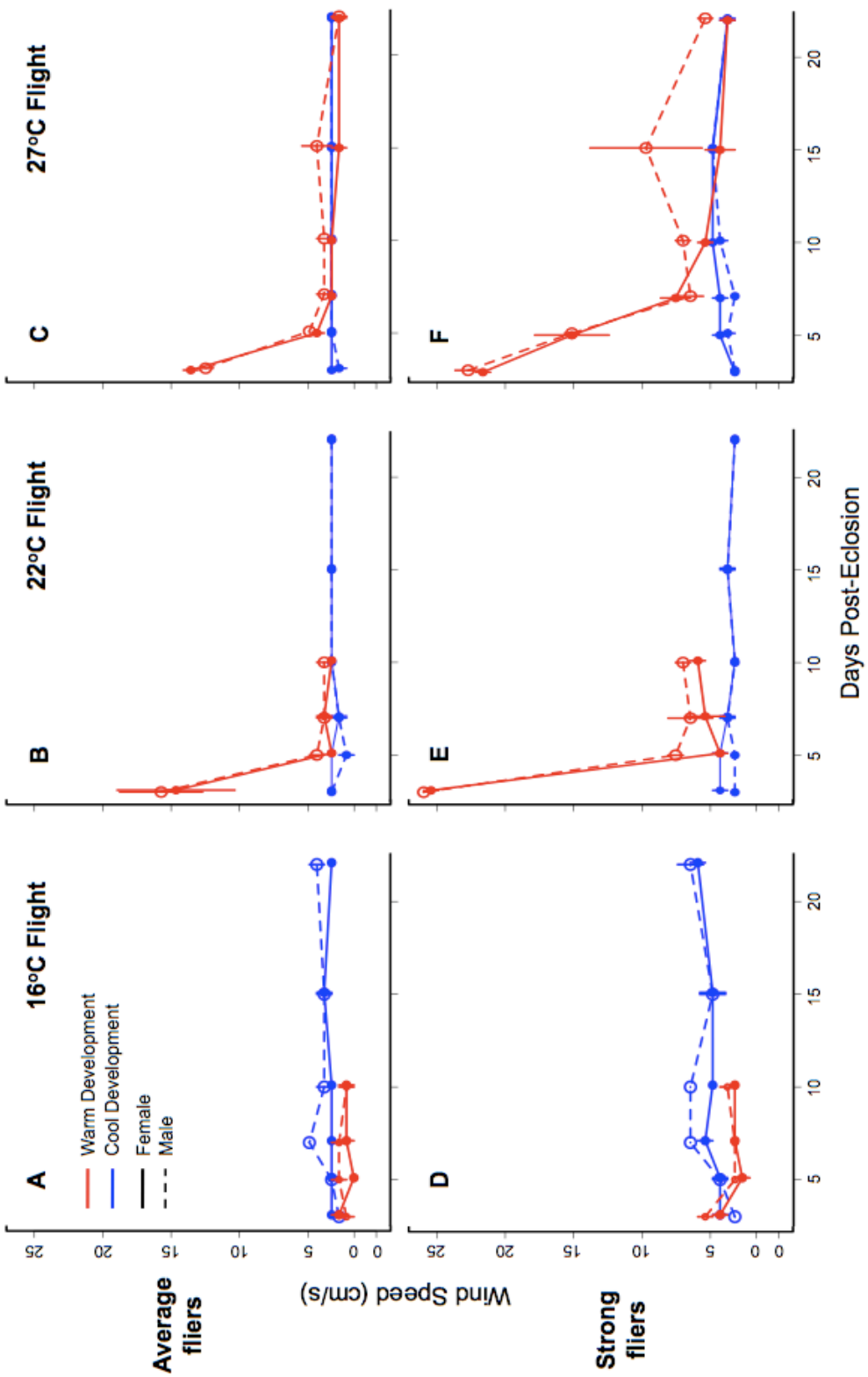

A
16°C Flight
B
22°C Flight
C
27°C Flight
D
E
F
Warm Development
Cool Development
Female
Male
Average fliers
Strong fliers
Wind Speed (cm/s)
Days Post-Eclosion

Table 2.1: Analysis of deviance on age by flight performance. I tested for an effect of age, flight temperature, and their interaction on flight performance of average (WS_{50}) and strong (WS_{80}) fliers under the model. Analysis of deviance was performed separately for each development temperature. Significant ($\alpha<0.05$) effects are indicated by asterisk (*).

		d.f.	Deviance	*P*-value
Cold-developed flies				
WS_{50}	Null			
	Age	5	0.481	0.993
	Flight Temp (FT)	2	0.881	0.644
	Age : FT	10	0.905	0.999
WS_{80}	Null			
	Age	5	1.817	0.874
	Flight Temp (FT)	2	6.338	0.042
	Age : FT	10	3.636	0.962
Warm-developed flies				
WS_{50}	Null			
	Age	3	68.678	<0.001
	Flight Temp (FT)	2	37.257	<0.001
	Age : FT	6	10.872	0.092
WS_{80}	Null			
	Age	3	109.07	<0.001
	Flight Temp (FT)	2	86.342	<0.001
	Age : FT	6	23.369	<0.001

Adaptive phenotype-environment matching requires that the phenotypes expressed convey superior performance in the inducing environments relative to other environments. My data indicate that the flies from both Cool and Warm development temperatures fit this pattern, exhibiting superior flight at their Native flight temperatures relative to the Alternate flight temperatures. Moreover, flies from each development temperature fly better at their Native flight temperature than flies that develop at other development temperatures; Cool-developed flies exhibit significantly higher flight performance than Warm-developed flies at Cool flight temperature (WS_{50} $p=5.4x10^{-6}$; WS_{80} $p=2.2x10^{-4}$) whereas Warm-developed flies are superior to Cool-developed flies at Warm flight temperatures (WS_{50} $p=9.6x10^{-4}$; WS_{80} $p=1.7x10^{-7}$). Thus, I have strong evidence that development temperature induces a phenotype that facilitates flight at the Native temperature, supporting hypotheses suggesting thermally-induced phenotypic variation in *Drosophila* flight morphology is adaptive.

Temperature exerts strong effects on development, aging, and ecological performance in ectotherms. In this study, I investigated the relationship between temperature of development and senescence in flight performance using a newly designed, progressive velocity wind tunnel. I found that that development temperature has strong effects on flight performance, and for Warm-developed flies, interacts with flight temperature to affect performance such that rapid senescence is revealed within the first 5-7 days of adult life. While previous work demonstrates adaptive phenotype-environment matching at Cold flight temperatures (Frazier et al. 2008), the study was criticized for a lack of evidence supporting adaptive phenotype-environment matching at warmer temperatures (Kingsolver and Hedrick 2008). These data address such criticisms by comparing Cool- and Warm-developed flies across different air temperatures in a full factorial design. I

document two lines of evidence for adaptive phenotype-environment matching (A) flight performance was best at air temperatures that matched developmental temperatures, and (B) for a given air temperature (Warm or Cool), flies at their Native developmental temperature achieve greater flight performance and outperform flies of another development temperature. My findings are consistent with those of Frazier (2008) at Cool temperatures and extend support of adaptive plasticity to Warm temperatures. I encourage investigators to consider explicitly the thermal conditions experienced during development and experimental assays when exploring adaptive phenotypic plasticity, ecological performance and senescence in ectotherms. Failure to do so may significantly impact findings and interpretations dependent on chosen age and temperatures.

2.4 Materials and methods

2.4.1 Study system

D. melanogaster used in this experiment were from a laboratory stock population founded 25 generations prior from several hundred females collected from Fenn Valley Vineyards (Fennville, MI, USA; N 42^{o} 34', W 86^{o} 14') and maintained as a large (N>2000 flies) free-mating colony on a 12:12 L:D cycle. To determine the pattern of senescence across flight temperatures, I assessed flight performance at six chronological ages (3, 5, 7, 10, 15, and 22 d post-adult eclosion). Flies were reared from egg to adult and held post-eclosion at one of two development temperatures [Cool (16^{o}C) or Warm (27^{o}C)] and flown at each of three flight temperatures (16, 22, and 27^{o}C) in a 2x3 full factorial design (development temperature x flight temperature). Flight trials at each development-temperature/flight-temperature/adult-age combination were replicated three times (1,000 eggs/replicate; ~700 flies), and trials began at 0900 in randomized order. Trials where flies were flown at their development temperature (e.g., flies reared

and flown at 16^{o}C) are referred to as having been flown at their “Native” temperature, whereas flies flown at temperatures other than their development temperature are referred to as having been flown at an “Alternate” temperature.

2.4.2 Wind tunnel

A contained, progressive velocity wind tunnel of new custom design was used to assay *D. melanogaster* flight performance over short distances. Detailed description of the design and function will be provided elsewhere. In brief, the tunnel consists of a series of 15 small (~63 sq cm) collection chambers attached by 14 short 4 cm dia, 8 cm long passageways coated with Fluon™ so that insects must fly through the passageways from chamber to chamber to advance up the tunnel. *Drosophila* are motivated to fly via a combination of positive phototaxis, positive chemotaxis, and negative geotaxis. Between collection chambers, air speed steps up linearly at each passageway such that the headwind is weakest in the starting gate (0 cm/s) and strongest in the final passageway (26cm/s). The tunnel is capable of phenotyping the flight ability of hundreds of flies in a single trial, during which the flies self-sort based on flight ability for 15 min. At the conclusion of a trial, the tunnel is flooded with CO_2 and anesthetized flies are removed from the collection chambers and counted by sex. The resulting distribution of flies across collection chambers reflects the upwind flight ability of the population. I use the wind speed that average (50th percentile; WS_{50}) and strong (80th percentile; WS_{80}) fliers were able to overcome to quantify and compare flight ability of each development temperature/flight temperature/age combination.

2.4.3 Flight trials at Native temperature

To identify the pattern of senescence in flight performance and determine if the pattern differs in sidereal time across thermal environments, I assayed upwind flight performance of flies at their Native temperature. Within a development temperature, all eggs were collected on the same date and flight performance assays were conducted as each age cohort reached the appropriate target chronological age for testing (3, 5, 7, 10, 15, and 22 d). At 16^{o}C, flies from the stock population eclose over a period of four days beginning on the 21st day after oviposition whereas at 27^{o}C flies eclose within a 24 h window on the 9th day after oviposition. Thus, although flies developed at 16^{o}C eclose over 4 d, they originate from eggs laid within a 24 h window. Therefore, 'age' refers to the oldest adults eclosing from a given lot over 4 d.

2.4.4 Flight trials at Alternate temperature

To determine the effect of development temperature on flight performance at other flight temperatures and to test for adaptive phenotype-environment matching, I tested flies from both development temperatures at two Alternate flight temperatures (22^{o}C and the temperature at which they did not develop). I staggered egg collection from the stock population over several days to yield cohorts of flies differing in post-metamorphic age on the same date. Logistical constraints meant that I had to conduct assays for different development/flight temperature combinations on separate days. However, each target age (3, 5, 7, 10, 15, and 22 d post-eclosion) for a particular development/flight temperature combination was assayed on the same date. Logistic constraints prevented me from collecting all Cool-developed eggs in a single cohort. Thus, to decrease variation in post-eclosion ages around each target age for the Cool-developed flies, I collected eggs in two cohorts (n=21,000 eggs/cohort/flight temperature) on two

consecutive days and sampled adults eclosing from these such that half were at the target age and the remaining half were ±1 d off the target age. Based on results from Native temperature flight trials, I did not test ages 15 or 22 d Warm-developed flies because senescence had occurred by 10 d post-eclosion (see Results and Discussion).

2.4.5 Analyses

To test for effects of sex on flight performance, I performed an analysis of deviance within development temperature with flight performance (WS_{50}, WS_{80}) as my response variables as indicated by the collecting chamber representing the 50^{th} or 80^{th} percentile of upwind flight ability. Sex, flight temperature and age were main effects, and all interactions were tested.

WS ~ sex * flight temperature * age

Using segment number rather than wind speed as the response variable was necessary because variation in chamber progression at Cool temperature was not reflected in wind speed variation in lowest performance; many flies were not successful flying upwind and thus have an upwind flight performance of 0 cm/sec, however, this wind speed corresponds to both the release gate and collection chamber number one. Thus, using collection chamber number rather than wind speed enables me to distinguish between flies that did not leave the release gate versus those that were unable to overcome the weakest headwind. Both segment and wind speed increase linearly with distance and results are thus interchangeable. I found no effect of sex for Cool- (WS_{50}: $F_{1,107}$=0.018, p=0.89; WS_{80}: $F_{1,107}$=0.056, p=0.81) or Warm-developed flies (WS_{50}: $F_{1,71}$=0.288, p=0.59; WS_{80}: $F_{1,71}$=0.802, p=0.37), nor any significant interactions with age or flight temperature (p's>0.82). Thus, sexes were pooled in all analyses, but are plotted separately in Figure 2.1.

I tested for differences in flight performance by comparing WS_{50} and WS_{80} among groups. Performance of flies from each development temperature was analyzed separately since the Warm and Cool Alternative flight temperature trials differed in the number of age cohorts tested. To test for effects of age (senescence) and flight temperature on flight performance, I performed an analysis of deviance on the collection chamber corresponding to the WS_{50} and WS_{80} as the response variables with flight temperature and age as main effects and an interaction term using *anova* in R (R Core Team 2013) on a generalized linear model fit with a negative binomial distribution using *glm.nb* (MASS package, Venables and Ripley 2002). Post-hoc t-tests were performed on WS_{50} and WS_{80} using *t.test* in R (R Core Team 2013). I adjusted for multiple comparisons using a Bonferroni correction, significance level was adjusted to $\alpha=0.0167$ for flight temperature comparisons and $\alpha=0.0083$ for age comparisons. To determine the effect of development temperature on flight performance across all flight temperatures, a one-sided t-test was performed on the performance of Cool and Warm-developed flies using only age cohorts represented in all trials (3-10 d post-eclosion). To compare the rate of senescence between Warm-developed flies at the 22^{o}C Alternate and 27^{o}C Native flight temperatures, one-sided t-tests were performed on the WS_{50} and WS_{80} of 3 d versus 5 and 7 d old flies within treatments.

CHAPTER 3. THE ROLE OF PHENOTYPIC PLASTICITY IN ADAPTIVE EVOLUTION

3.1 Abstract

Historically, the role phenotypic plasticity in adaptive evolution has been controversial. Plasticity reduces the covariation among genotypic, phenotypic, and fitness variation, and may thus hinder the response to selection. Conversely, phenotypic plasticity can expose new phenotypic variation to selection and may thereby promote adaptive evolution. *Drosophila* spp. exhibit thermally-induced phenotypic plasticity in morphological traits associated with flight; these responses are widely believed to promote flight at the inducing temperature, although few data address this hypothesis directly. To test empirically if phenotypic plasticity inhibits or facilitates adaptive evolution, I subjected experimental populations of *D. melanogaster* to selection on flight performance in the presence and absence of thermally-induced phenotypic plasticity and compared the rate of adaptation between groups. When flies developed at an intermediate temperature that did not match flight temperature, populations did not respond to selection during 11 generations of selection. However, lineages experiencing a developmental temperature matching their flight temperature responded rapidly to selection, exhibiting dramatic increases in flight performance. My data demonstrate a strong, facilitatory role of phenotypic plasticity in adaptive evolution, suggesting that phenotypic plasticity promotes diversification.

3.2 Introduction

The role of phenotypic plasticity in adaptive evolution has been of interest to evolutionary biologists for over a century (Baldwin 1896). Phenotypic plasticity, the ability of one genotype to express multiple phenotypes across environments, was historically thought to hinder adaptive evolution by reducing the phenotypic resemblance between

parent and offspring, thereby obscuring the relationship between genetic variation and fitness (Wright 1931; Simpson 1953). Over time however, the adaptive potential of phenotypic plasticity has received much theoretical and empirical support, and the study of adaptive phenotypic plasticity has become an expansive discipline in its own right (for reviews, Parsons et al. 1993; Pigliucci 2001; Dewitt and Scheiner 2004; West-Eberhard 2003; Whitman and Ananthakrishnan 2009).

The debate over the adaptive nature of plasticity centers on the idea that a genetically diverse population with corresponding high phenotypic variation will exhibit high variation in fitness when there is no plasticity. When a population exhibits adaptive phenotypic plasticity, however, a genetically diverse population will produce low phenotypic variation relative to the genetic variation (Moran 1992); increasing mean fitness in the induced environment while reducing phenotypic variation. Thus, in a population exhibiting adaptive phenotypic plasticity for fitness-related traits, selection can be weaker and adaptive evolution may proceed more slowly due to reduced heritability, low phenotypic variation, and low variation in fitness (Scheiner and Lyman 1989). More recently, the idea that phenotypic plasticity facilitates adaptive evolution by genetic accommodation has received much theoretical support (e.g., Moran 1992; West-Eberhard 2003, 2005; Pfennig et al. 2010; Moczek et al. 2011).

Genetic accommodation is the adaptive genetic change of the regulation and form of a plastic trait that follows the exposure of cryptic genetic variation (Gibson and Dworkin 2004) resulting from a novel developmental environment or a mutation of large effect (Pigliucci and Murren 2003; West-Eberhard 2003, 2005; Braendle and Flatt 2006; Moczek et al. 2011; Schlichting and Wund 2014). Acting on this newly exposed variation,

selection can favor the evolution of new phenotypes; eventually the population is expected to constitutively express the derived, newly favored phenotype in the absence of the developmental perturbation that initially revealed the variant. Using relatively small laboratory populations, genetic accommodation has been shown to be a potent process underlying evolution of morphological traits of unknown ecological importance (Waddington 1953, 1956; Bateman 1957) or modifying the expression of mutations of major effect (Sollars et al. 2003; Suzuki and Nijhout 2006). However, the ability of genetic accommodation to work on continuously varying, ecologically relevant traits in large populations remains an open question and its general evolutionary importance in quantitative traits in natural populations is unknown.

Ecological performance traits, such as locomotion, prey capture, etc., are composed of multiple component parts that function as a single unit in an ecological context. Such trait suites typically exhibit high internal covariation, the disruption of which may have important costs to performance, and thus fitness (Brodie III 1992). Performance and behavior often respond to selection more readily than individual morphological traits (Blomberg et al. 2003; Rhodes and Kawecki 2009), as these mediate morphological effects on fitness (Arnold 1983) and may evolve through “multiple adaptive solutions” including, but not limited to, the individual morphological traits themselves (Garland et al. 2011; Careau and Garland 2012). For example, lizard locomotor performance can be enhanced by changing limb length (Bonine and Garland Jr. 1999), muscle fiber type (Bonine et al. 2005), thermal physiology (Bauwens et al. 1995), or combinations thereof.

Performance of ecologically relevant tasks relies on the functional coupling of diverse suites of morphological, physiological, or behavioral traits (Brodie III 1992; Svensson

and Friberg 2007). Tight covariation among functionally related morphological, physiological, and behavioral traits can impair the independent response of traits to selection (Cheverud 1984; Arnold 1992; Schluter 1996; Arnold et al. 2008). However, novel environments may disrupt typical development, releasing cryptic variation to produce new patterns of trait (co)variation and thus produce adaptation via genetic accommodation (Hayden et al. 2011; Iwasaki et al. 2013). Although thought to be an important process, little direct empirical evidence for such a scenario exists (but see Adams and Huntingford 2004; Suzuki and Nijhout 2006; Ledon-Rettig et al. 2008).

Among other reasons, experimental evolution studies provide a powerful tool to examine evolutionary histories and trajectories of ecologically relevant traits because of their power to isolate and control the developmental and selective environments separately. In the case of genetic accommodation, this means investigators can study populations where phenotypic plasticity is expressed or absent. Thus, experimental evolution can be used to isolate the effects of phenotypic plasticity on adaptive evolution and diversification via genetic accommodation in ecologically relevant traits. Moreover, replication of experimental populations founded from a single stock of known evolutionary history removes the confounding effects and low statistical power that can plague studies of natural populations.

Drosophila spp. have long been considered an exemplar of adaptive evolution and adaptive phenotypic plasticity for three reasons: (i) they exhibit convergent geographic clines in flight morphology (Imasheva et al. 1994; James et al. 1997; Azevedo et al. 1998; Gilchrist and Huey 2004; Liefting et al. 2009; Pitchers et al. 2013; Bhan et al. 2014), (ii) they exhibit patterns of thermally-induced phenotypic plasticity consistent with

this geographic pattern (Debat et al. 2003; Gilchrist and Huey 2004; Pitchers et al. 2013), and (iii) these morphological patterns are purportedly adaptive for increasing flight ability at local temperatures (Ayala et al. 1989; Starmer and Wolf 1989; Azevedo et al. 1998; Huey et al. 2000; Gilchrist and Huey 2004; Dillon and Frazier 2006; Frazier et al. 2008). Both the latitudinal clines and developmental plasticity exhibit similar morphological patterns; low developmental temperatures result in large bodies and disproportionately large wings, as well as a narrowing of the intervein region between the 2nd and 3rd longitudinal veins, a broadening of the posterior lobe and distal margin of the wing as well as a shortened cross veins (Debat et al. 2003).

In particular, the replicated, and independently evolved convergent clines in body size and relative wing size have received much attention (e.g., Imasheva et al. 1994; Azevedo et al. 1998; Gilchrist and Huey 2004; Bhan et al. 2014), as they are thought to offer strong evidence of selection along a thermal gradient favoring the same phenotypic outcome through selection on flight performance (David and Capy 1988; Ayala et al. 1989; Huey et al. 2000). However, empirical evidence supporting the adaptive nature of these clines and patterns of plasticity are scant. Cool-temperature-induced flight morphology compensates for physiological challenges of flight at cool temperatures under laboratory conditions (Frazier et al. 2008; Chapter 2). Although Cool-developed flies exhibit superior flight performance compared to Warm-developed flies at low temperatures (Frazier et al. 2008; Chapter 2), no other empirical evidence exists that connects the geographic or plastic patterns in flight morphology to flight performance across temperatures (but see Chapter 2). Moreover and critically, the relationships among phenotypic, performance and fitness variation have not been established in this system.

In this study, I conducted two experiments to determine the role of phenotypic plasticity in the adaptive evolution of *Drosophila melanogaster* flight performance at Cool (16^{o}C) and Warm (27^{o}C) flight environments. In Experiment I ("No Plasticity"), I reared all flies at an intermediate temperature (22^{o}C). This treatment produced flies with a range of phenotypic variance intermediate to the purportedly adaptive phenotypic range for these flight temperatures. In Experiment II ("Plasticity"), flies were developed *and* flown at Cool or Warm temperatures, allowing the expression of the presumably adaptive range of flight morphology for these flight temperatures. This "Plasticity" treatment may allow for evolution of extreme flight phenotypes by genetic accommodation; exposure of variation appropriate to each temperature may facilitate adaptive evolution at that temperature. I compared the evolutionary rates and outcomes between the two designs. If Experiment I exhibits a greater response to selection than Experiment II, then phenotypic plasticity has an inhibitory effect on adaptive evolution. Conversely, if the response to selection were greater in Experiment II, then this would provide evidence that plasticity has a positive role in adaptive evolution. If populations in both experiments evolve at similar rates and to the same degree, then I would assert plasticity has little, or no effect on adaptive evolution.

The morphologies of flies in Experiment I are predicted to evolve towards patterns exhibited in natural populations, e.g., relatively large wings in Cool-selected flies. Morphological predictions for Experiment II are less clear, although morphology will be quantified under Cool and Warm developmental conditions for both selective-temperature treatments to assess evolved patterns in plasticity. Investigations into the morphology are underway, and mechanisms underlying adaptation of flight performance

are assessed in Chapter 5. To my knowledge, this is the first study in which a complex trait such as flight performance is allowed to evolve both with and without induced phenotypic plasticity.

3.3 Materials and Methods

3.3.1 D. melanogaster stock population

The population used in this study was founded in 2010 from several hundred females collected in East Lansing, MI (for full details Parigi et al*., in press*). The colony has been maintained in the Frankino lab as two large intercrossed free-mating 0.3 m^3 cages of several thousand flies, developed each generation under a range of densities, food availabilities, nutrition, egg collection ages and temperatures to discourage adaptation to a single laboratory condition. Flies were reared in this manner for 25 generations prior to Experiment I, and 40 generations prior to Experiment II. There was no significant difference between male and female flight performance in the parental (F0) or evolved (F11) generations (see Results), thus analyses are restricted to females as they were the only sex subject to selection in the experimental design. To determine if the slopes describing the pattern of thermally-induced plasticity in morphology differed between the starting populations for Experiments I & II, I reared flies as described below and performed an ANCOVA with log wing area as the response variable; log pupae area as the covariate and Experiment as a main effect as is common in studies of scaling relationships (e.g., Egset et al. 2011; Gotoh et al. 2014).

3.3.2 Progressive velocity wind tunnel

The wind tunnel used is a custom-design that is used to phenotype the maximum upwind flight ability of *Drosophila.* A full description of which is provided in Sikes et al*.,*

(*in prep*). The tunnel consists of a series of 4 cm dia, 8 cm long passageways coated with Fluon™ to prevent terrestrial locomotion, that connect 15 collecting chambers into which the flies self-sort based on their upwind flight ability. Air is drawn through the tunnel by vacuum that creates a negative pressure, which is stepped down at each collection chamber. This results in a headwind that is weak at the start of the tunnel but increases linearly over the 2 m length of the unit. The shape of the tunnel maintains laminar airflow and wind speed can be adjusted precisely in real time via a potentiometer that controls the vacuum. Flies are released at the low-airspeed end of the tunnel and are motivated to fly into the headwind through a combination of negative geotaxis (the unit is placed at a 35° angle), positive chemotaxis, and positive phototaxis. After 15 min of flight time, the tunnel is flooded with CO_2 and the flies retrieved from the collection chambers. Weak fliers remain in the chambers close to starting point whereas stronger fliers overcome the increasing headwind and make it to the more distant collection chambers. Because total air intake is read in real time by an anemometer and the decline in headwind speed is linear between each collection chamber, the maximum upwind flight ability can be calculated for individual flies based on their resting location in the tunnel and the air intake volume. Air speed within the wind tunnel was increased at generations 3 and 7 by 2.5 cm/s (Final max wind speed 31.8 cm/s) to enhance the strength of selection. The distribution of these flies across collection chambers describes the flight performance of a population and is easily quantified and visualized as a histogram (e.g., Fig. 3.1).

3.3.3 Experiment I: "No Plasticity"

To test the degree and rate of adaptation when phenotypic plasticity does not produce presumably adaptive phenotypes, populations of flies were selected for upwind flight

performance at 16 or 27°C but reared at the intermediate temperature of (22°C). Thus, flies only experienced their selective temperature during performance assays. The parental generation (F0) consisted of 24,000 eggs at a density of 250 eggs per 25ml of media, equally divided between the two Flight Temperature Treatments. Within a Flight Temperature Treatment, eggs were assigned pseudo-randomly among four replicate artificial selection lineages and two Motivation Control lineages (described below) for a total of 2,000 eggs per lineage (*ca* 1,500 flies). Adults were held in free-mating cages by replicate until the day of a flight trial, 7-10 d post-eclosion. On the day of an assay, flies were subjected to chill coma (4°C) for 5 min so they could be collected from population cages. They were then allowed to recover for one hour in dry 250 ml bottles before a flight trial, to enhance performance in the wind tunnel (Weber 1996). At the conclusion of a trial, flies were anesthetized (CO_2), recovered from the collecting chambers, and counted. Only the best performing 20% of females were retained (average=137 females). Eggs were collected from these each generation. At generation 9, selection pressure was increased by 5% (Avg=103 females).

For each Flight Temperature Treatment, I had two Motivation Control lines that were treated identically to Flight Temperature Treatment lines except the vacuum on the wind tunnel was not engaged and thus they experienced no headwind during a flight trial. These Motivation Control lineages thus experienced the same stimuli to motivate them to proceed through the tunnel, although flight was not required. This allowed me to determine if the degree to which any response to selection was due to changes in response to stimuli versus flight ability. At generation 13, a total of 2,000 eggs (*ca* 1400 flies) per replicate were collected for each Motivation Control lineage, halved, and then used to assay these controls independently with and without a headwind. A t-test was

used to compare the median flight performance between Motivation Controls flown with and without wind to assess the degree to which enhanced performance resulted from increased motivation versus improved flight ability (see Analyses).

Each generation, additional sets of 250 eggs were collected from a replicate from both Flight Temperature Treatments and used to quantify any indirect morphological response to selection on flight performance. Focal lines were chosen in a repeating sequence such that the pupae size, wing size and wing shape of paired lines from both Flight Temperature Treatments were quantified every 4th generation, providing a sliding window of morphological measurements across the experimental timeline (details provided below). At generations 3, 7 and 11, the morphology of all lines from both Flight Temperature Treatments were quantified, however, these data have not been fully analyzed at this time due to the weak response to selection on flight (see Results).

3.3.4 Experiment II: Thermally-induced developmental plasticity

Methods regarding egg collection, flight trials, and selection are the same as in Experiment I. The protocols in Experiment II, however differed from Experiment I in two important ways. First, fly development temperature matched Flight Temperature Treatment, allowing for selection to act on a range of phenotypes expressed via phenotypic plasticity presumed to be adaptive for flight at each temperature. Second, two Developmental Control lineages per Flight Temperature Treatment were added to control for changes specific to development at 16 or 27^{o}C (Partridge et al 1994; James and Partridge 1995). Twice the typical number of eggs (4,000 per gen/Flight Temperature Treatment combination) was collected for these Developmental Controls, with half assayed for upwind flight performance. I then haphazardly selected 20% of

females from the remaining half that had not experienced the wind tunnel. Temperature dramatically effects development time in *Drosophila* (James and Partridge 1995; James et al. 1997), thus the Warm-selected lines underwent selection at roughly twice the rate as did the Cool-selected lines. Because of this, generation 11 was chosen as an endpoint for selection *a priori*. For logistical reasons, half of Developmental and Motivation Controls replicates were dropped at generation 8. To determine the extent to which motivation affected flight performance adaptation, flight performance of Motivational Controls was assayed at generation 12 (Cool) and 18 (Warm) in the presence and absence of a headwind. Selection, however, was ended following 11 generations of selection.

3.3.5 Quantifying and comparing flight performance

I calculated the maximum wind speed overcome by the 50^{th} percentile (WS_{50}) of each replicate population and used this to compare the flight performance among groups. Because the distribution of flight performance in my assays tends to be right skewed, this metric better describes the flight performance of a lineage than the average, which is more sensitive to extreme values. To determine the degree to which flight performance evolved within each Experiment, I compared the WS_{50} at generation 11 to that of the parental (F0) population with a one-sided t-test within a Flight Temperature. To test for differences in flight performance between sexes, an ANOVA on WS_{50} with Generation, Flight Temperature Treatment, and Sex as main effects and all interactions revealing no significant sex effects (Experiment I, $p \geq 0.224$; Experiment II, $p \geq 0.394$; Table 3.1) regarding flight performance; thus, I restricted analyses to females only as they were subject to direct selection.

3.3.6 Testing for effects of phenotypic plasticity on the evolution of flight performance

Realized heritability of flight performance was estimated as twice the slope of the regression of WS_{50} over the cumulative selection differential (Falconer and Mackay, 1996). Slopes that deviate from zero indicate a response to selection. Realized heritabilities were compared between Experiment I and II using one-sided t.test in R (R core development team) within Flight Temperature Treatments to illustrate evolutionary impact of developmental plasticity in flight performance adaptation to Cool and Warm environments.

3.3.7 Quantifying and comparing morphology

I tested for a correlated response to selection in flight morphology by comparing wing size, body size, and wing shape between Flight Temperature Treatments. Wing to body size scaling can be estimated for a population easily from live, intact flies in three steps (Stillwell et al. 2011). First, pupae are imaged using a microscope attached to a digital camera and computer. Second, the wing of the eclosed adult is gently pressed between two pieces of glass and imaged similarly (Weber 1988; Houle et al. 2003; Stillwell et al. 2011). Third, 14 hard landmarks are placed manually on the fly wing, and custom software automatically places 24 sliding landmarks along the wing margin and calculates the areas of the pupa and the wing as the pixel count of their silhouettes. Major axis regression (MAR) is used to describe the relationship between the log-log transformed size data (Frankino et al. 2009). Wing shape is summarized using geometric morphometrics and compared using standard statistical methods. Comparing body size, wing size, and wing shape of flies from the selected lineages to the control lineage will identify morphological evolution in response to selection for upwind flight ability.

To determine if allometric relationships in the flight selected lineages differed from Developmental Controls, slopes and the intercepts of the wing:body size scaling relationship were compared by ANCOVA on log wing size as the response variable, with log body size as the covariate and Generation as the main effect (Egset et al. 2012; Gotoh et al. 2014). If slopes do not differ significantly between the flight selected lineages and Developmental Controls, then the morphological response to selection is due largely to selection in the developmental environment and not selection imposed by flight performance. The morphological data set for Experiment I consists of a sliding window of four replicate lineages measured at generations 8, 9, 10, and 11. Data for Experiment II consists of Cool Flight Temperature Treatment generation 7 and Warm Flight Temperature Treatment generation 12, both of which were reared at their selected temperature and will provide preliminary glimpse at evolution of morphology with developmental plasticity in response to selection on upwind flight performance. Data collection is currently underway which will assess patterns of morphology in all evolved lineages.

To analyze shape differences between evolved lines and parental generations as described above, Principle Components Analysis (PCA) and Canonical Variate Analysis (CVA) were conducted to describe absolute and relative changes in shape, respectively. This combination of analyses allows the comparison of absolute shape variation with PCA among groups (Zelditch et al. 2012), and quantify the shape variation which best discriminates among groups using CVA (Albrecht 1980) with cross-validation to reduce bias of the discriminate function (Lachenbruch 1967). Together, these analyses facilitate comparison of absolute and relative shape among known groups (Klingenberg and Monteiro 2005; Zelditch et al. 2012). Wings were Procrustes transformed (Rohlf and

Slice 1990) to remove effects of rotation, alignment, and size on shape analyses. All shape analyses used a common consensus image for Procrustes transformations and a common PC space was used within PCA and CVA comparisons. Common consensus image was a randomly chosen 22^{o}C developed wing from Experiment I. As with body and wing size measures, the wing shape of the starting populations of Experiment I and II were compared to detect if they differed significantly in their initial wing shape. Flies developed at 22^{o}C were predicted to evolve morphologies more similar to flies developed 16 or 27^{o}C in response to selection for flight performance in Experiment I. This prediction was tested using a t-test on the four Mahalanobis distances between the selected lineages of each Flight Temperature Treatment and the F0 population wing shape of both 21^{o}C developed flies and flies developed at their respective selected temperature. The Mahalanobis distance describes the difference among group means accounting for within group variance, (Klingenberg and Monteiro 2005), akin to number of standard deviations of an observation from mean. Thus, a larger Mahalanobis distance indicates a greater distinction between shape among lineages. Changes in wing shape between evolved and F0 populations (differences along CV1 and CV2) are described using the nomenclature of Birdsall (et al. 2000; see also, Debat et al. 2003). Finally, the contribution of each trait to evolution of the scaling relationship intercept and slopes was made by comparing the evolved univariate distributions of traits to the F0 population distribution with t-tests.

3.4 Results

3.4.1 No effect of sex

Flight performance did not differ significantly between males and females. ANOVA's with WS_{50} as the response variable, and Sex, Flight Temperature Treatment and generation

as main effects and all interactions revealed no significant effect of Sex or any Sex interactions for both Experiment I & II (Table. 3.1). Sexes were thus pooled for all analyses that follow.

Table 3.1. ANOVA shows no effect of Sex as main effect or interactions. Using the *step* function in R, models were simplified using Akaike's Information Criterion (AIC) backward form the full model. The cutoff for Experiment I was AIC=155.0, and for Experiment II AIC=201.7.Sex was removed and ANOVA was performed with female performance as response variable and Generation and Flight Temperature Treatment as main effects and an interaction term. Experiment I model reduced to Generation as an explanatory main effect of performance ($F_{3,28}$=2.52, p=0.078). Experiment II model showed significant effect of generation ($F_{3,24}$=11.9, p=5.5x10^{-5}), but not temperature ($F_{1,24}$=1.07, p=0.311) or the interaction ($F_{3,24}$=2.47, p=0.086).

		d.f.	Sum sq.	Mean sq.	F-value	*P-value*
Experiment I	Gen	3	128.0	42.67	4.071	0.0116
	Flight temp (FT)	1	7.000	6.960	0.664	0.4193
	Sex	1	19.00	19.03	1.816	0.1841
	Gen:FT	3	17.20	5.740	0.546	0.6519
	Gen:Sex	3	39.70	13.24	1.263	0.2976
	FT:Sex	1	4.400	4.360	0.416	0.5221
	Gen:FT:Sex	3	45.50	15.19	1.450	0.2401
	Residuals	48	503.1	10.48		
Experiment II	Gen	3	1547	515.6	23.47	<0.0001
	Flight temp (FT)	1	24.80	24.80	1.127	0.2938
	Sex	1	11.70	11.70	0.534	0.4685
	Gen:FT	3	368.0	122.7	5.585	0.0023
	Gen:Sex	3	28.30	9.40	0.430	0.7324
	FT:Sex	1	3.400	3.400	0.156	0.6948
	Gen:FT:Sex	3	66.90	22.30	1.015	0.3942
	Residuals	48	1054	22.0		

3.4.2 Experiment I: "No Plasticity"

No significant increase in flight performance was found after 11 generations of selection for increased flight performance in either the Cool (t=-1.82, d.f.=3, p=0.917) or Warm (t=-0.713, d.f.=3, p=0.736) flight-selected lineages (Fig 1A-H). Realized heritabilities for each replicate ranged between 0.006-0.068 for Cool and 0.009-0.070 for Warm Flight Temperature Treatments. No individual replicate had a slope significantly different from 0 (p's ≥ 0.062), nor did the average realized heritability differ significantly from 0 (Fig. 2C) for Cool (h^2=0.047, SD=0.034, t=1.258, p=0.237) and Warm (h^2=0.032, SD=0.020, t=1.63, p=0.134) Flight Temperature Treatments. Such consistency among replicates indicates the lack of response to selection is real, and did not likely occur by chance.

Similar to the flight selected lineages, the motivation control lines similarly showed no significant increase in progression through the wind tunnel (Cool: t=-6.6, d.f.=1, p=0.952; Warm: t=-1.22, d.f.=1, p=0.781). Motivation control lines flown at wind speeds matching generation 11 selected lineages did not differ significantly in their performance relative to when they were tested without a headwind (Cool Selected: d.f.=1, t=-1.00, p=0.5; Warm Selected, d.f.=2, t=0, p=1.0), although this may be attributed to high variance between 2 replicates for both Temperature Treatments. Realized heritabilities for Cool Motivation Controls were 0.065 and 0.031, and neither differed significantly from 0 (p=0.234 and 0.528). Realized heritabilities for Warm Motivation Controls were 0.058 and 0.083, although only the latter was significant (p=0.315, 0.043). The average realized heritabilities of Cool (h^2=0.010, SD=0.021, t=-0.238, p=0.817) or Warm (h^2=0.072, SD=0.019, t=-1.94, p=0.081) Motivation Controls did not differ from 0 (data not shown). In sum, the similarity of flight performance between F0 and F11 generations and the

realized heritabilities indistinguishable from zero indicate no response to selection in Experiment I.

3.4.3 Experiment II: Thermally-induced developmental plasticity

Flight performance responded significantly to selection when flies were reared at their flight temperature (Fig 3.1I-L & Q-T). When comparing the WS_{50} of the F0 and F11 populations, I found significant flight performance adaptation for both Cool (t=4.10, d.f.=3, p=0.013) and Warm Temperature Treatments (t=2.91, d.f.=3, p=0.031). I was unable to test for significantly different flight performances between generation 11 and F0 Motivation and Development Controls within Flight Temperature Treatment due presence of only one replicate. However, tests with Flight Development Temperatures pooled show that selected lines had evolved significantly greater flight performance than Developmental Controls (t=4.9274, d.f.=8.938, $p=4.1x10^{-4}$), but Motivation Controls exhibited a marginally significant trend (t=2.448, d.f.=1.864, p=0.072) that is likely of biologically significance. Mean realized heritabilities for the selected lines were greater than those found in Experiment I (Fig. 2F) for both Cool (h^2=0.162, SD=0.024, t=7.70, $p=1.6x10^{-5}$) and Warm (h^2=0.139, SD=0.040, t =3.48, p=0.006) Flight Temperature Treatments. Realized heritabilities ranged from 0.102-0.291 for Cool and 0.089-0.176 for Warm Flight Temperature Treatments, and each replicate differed significantly from zero (Cool: p's ≤ 0.003; Warm: p's ≤ 0.049).

Fig 3.1. Frequency distribution by wind tunnel segment of *D. melanogaster.* Plots of the average frequency distributions for Flight Temperature Treatments ("selected"; n=4) and Developmental Controls ("Control"; n=2) of female *Drosophila melanogaster* by Generation and Flight Temperature Treatment for both Experiment I (A-H) and Experiment II (I-X). Average WS_{50} is reported underneath panel letter with arrow indicating where that value falls on the distribution. Colored bars indicate the distribution of top 20% performers that are selected. Error bars represent standard error. (X) The 11^{th} generation Warm Control for Experiment II consists of a single replicate and thus no error bars are present. In Experiment I, Cool- and Warm-selected flies were assayed on the same day for a given generation. In Experiment II, Cool-selected and Cool Controls were assayed on the same day, and Warm-selected and Warm Controls were assayed on the same day, but Cool and Warm lineages were assayed as separately as generations progressed at different rates.

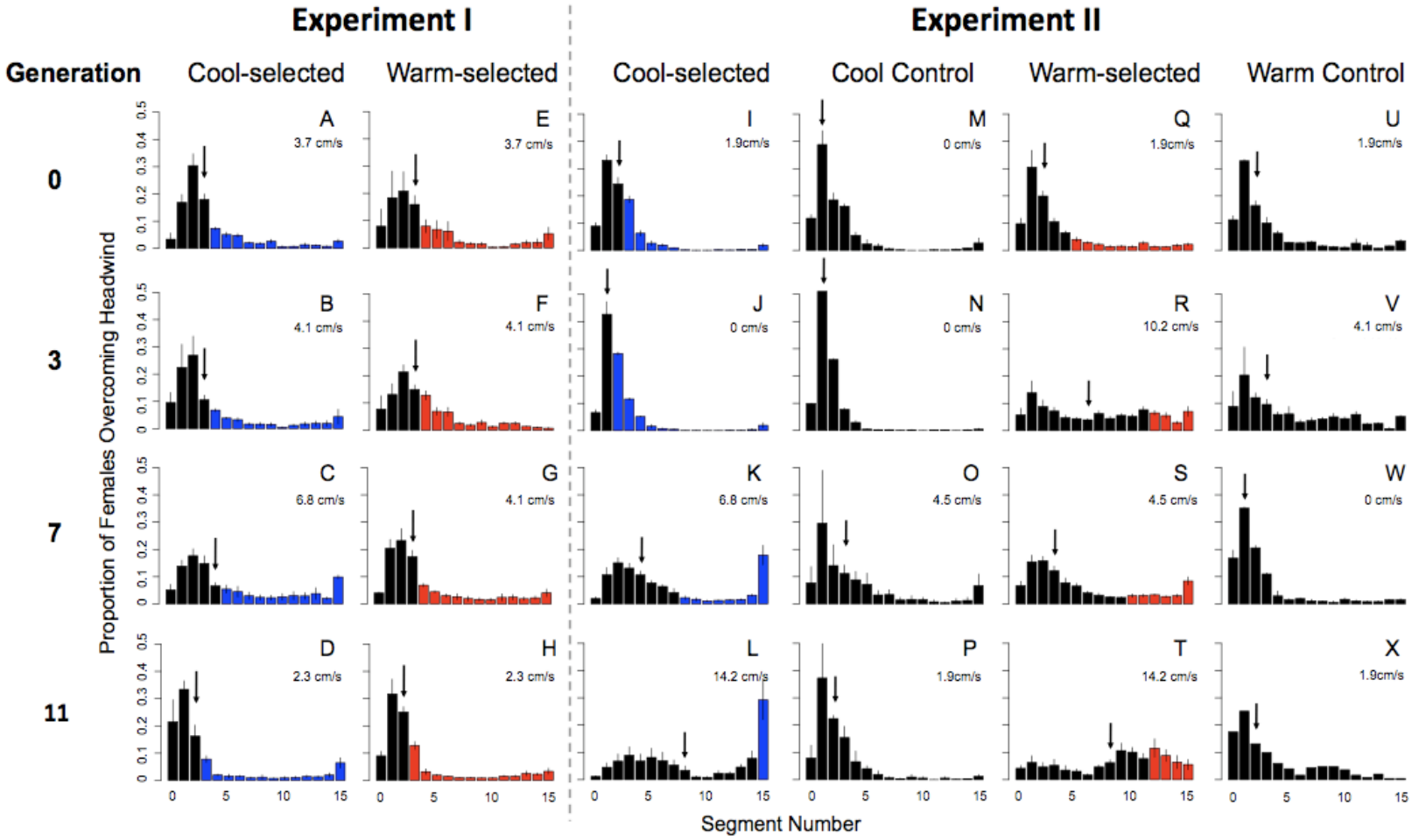
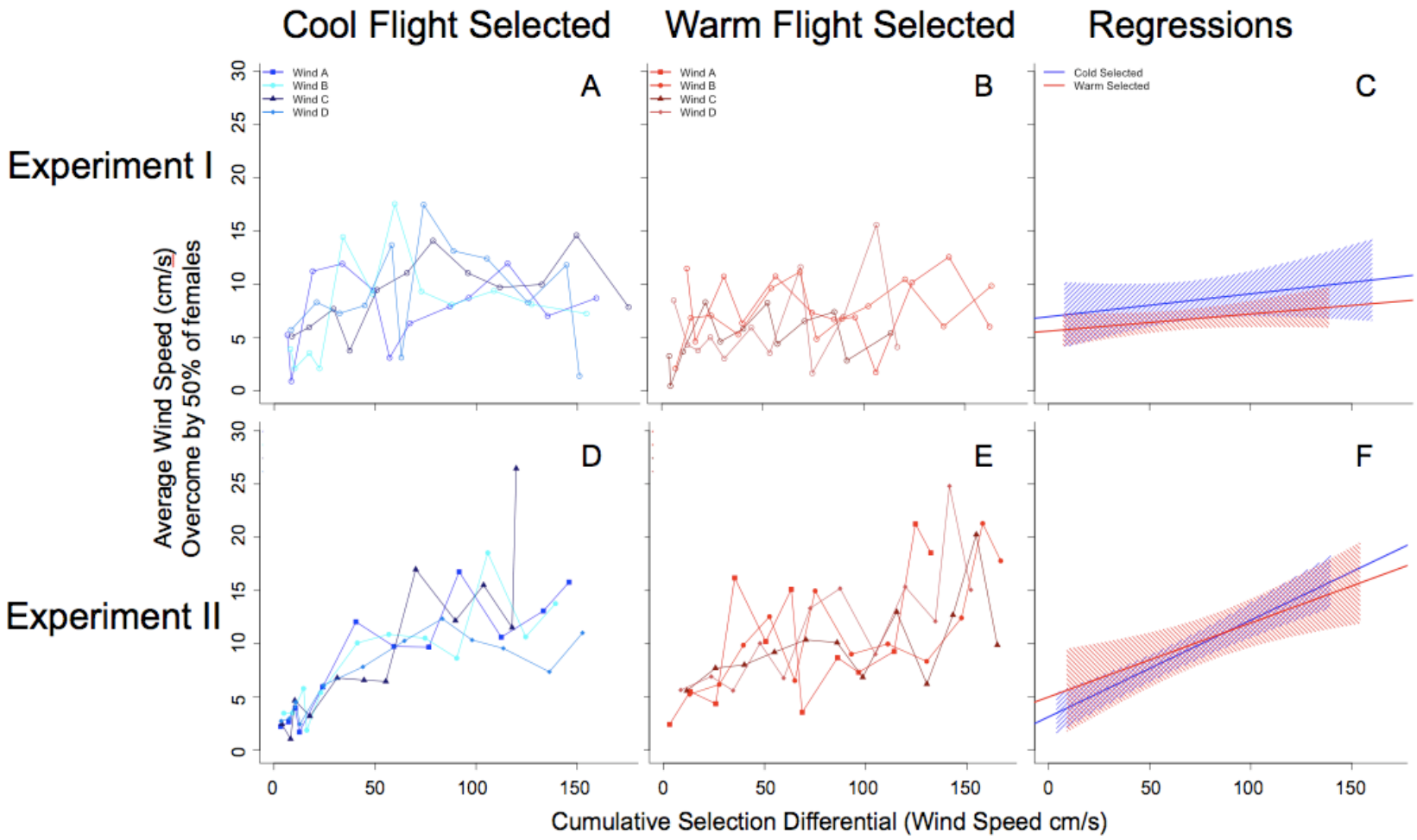

Experiment I
Experiment II
Generation
Cool-selected
Warm-selected
Cool-selected
Cool Control
Warm-selected
Warm Control
0
3
7
11
A 3.7 cm/s
E 3.7 cm/s
I 1.9cm/s
M 0 cm/s
Q 1.9cm/s
U 1.9cm/s
B 4.1 cm/s
F 4.1 cm/s
J 0 cm/s
N 0 cm/s
R 10.2 cm/s
V 4.1 cm/s
C 6.8 cm/s
G 4.1 cm/s
K 6.8 cm/s
O 4.5 cm/s
S 4.5 cm/s
W 0 cm/s
D 2.3 cm/s
H 2.3 cm/s
L 14.2 cm/s
P 1.9cm/s
T 14.2 cm/s
X 1.9cm/s
Proportion of Females Overcoming Headwind
0 0.1 0.2 0.3 0.4 0.5
0 5 10 15
Segment Number

Figure 3.2. Realized Heritability of Flight Performance (WS_{50}). Realized heritabilities are plotted for Experiment I (A, B, C) and Experiment II (D, E, F). Individual replicates are denoted by a unique hue of blue (Cool Flight Selected, panels A & D) or red (Warm Flight Selected, panels B & E). The average regression of response and cumulative selection differential are plotted for Experiment I (C) and Experiment II (F) with hatched areas indicating 95% confidence area of slope.


Cool Flight Selected
Warm Flight Selected
Regressions
Experiment I
Experiment II
Average Wind Speed (cm/s)
Overcome by 50% of females
Cumulative Selection Differential (Wind Speed cm/s)
Wind A
Wind B
Wind C
Wind D
Cold Selected
Warm Selected
A
B
C
D
E
F
0
5
10
15
20
25
30
0
50
100
150

Assays on Motivation and Developmental Controls reveal the response of flight performance to selection resulted in part from increased motivation, but could not be attributed to adaptations to developmental environment. Following 6 generations of relaxed selection I tested the 18th generation Warm Flight Temperature Treatment in which the presence of headwind had no significant effect on flight performance (t=0.781, d.f.=1.91, p=0.52). The Cool temperature Motivation Controls tested the subsequent generation (12) show no significant difference in performance in presence or absence of wind (t=-0.6, d.f.=1.85, p-value=0.614). Together, these findings indicate that the response to selection is due to changes in both flight ability and response to the stimuli. Realized heritabilities for the Motivation Controls were lower than the flight selected lines. Cool Motivation Control realized heritabilities were 0.142 and 0.100; both were significant (p=0.018, 0.039). Warm Motivation Control realized heritabilities were 0.028 and 0.032, neither of which were significant (p=0.597, 0.712). Average realized heritability for Cool Motivational Controls was significant (h^2=0.148, SD=0.020, t=3.69, p=0.004), although Warm Motivational Control was not (h^2=0.008, SD=0.021, t=0.178, p=0.862). Realized heritabilities of Cool flight-selected flies were not significantly greater than those of the Motivational Controls (t=1.43, d.f.=3.97, p=0.114), although Warm flight-selected flies did exhibit greater realized heritabilities than Warm Motivational Controls (t=5.43, d.f.=3.06, p-value=0.006).

3.4.4 Quantifying and comparing evolution of flight ability

Realized heritabilities were roughly four-fold greater when Development Temperature matched Flight Temperature (Experiment II, Fig. 2F) than when they did not (Experiment I, Fig. 2C). Both Flight Temperature Treatments in Experiment II had significantly greater realized heritabilities than those from Experiment I (t-test: Cool, t=3.403, d.f.=3.71

p=0.015; Warm, t=3.88, d.f.=5.80, p=0.004). Additionally, the flight performance of flies in Experiment II was greater than the flight performance of flies in Experiment I for Cool (t=3.12, d.f.=3, p=0.026) and Warm (t=5.17, d.f.=3, p=0.007) Flight Temperature Treatments.

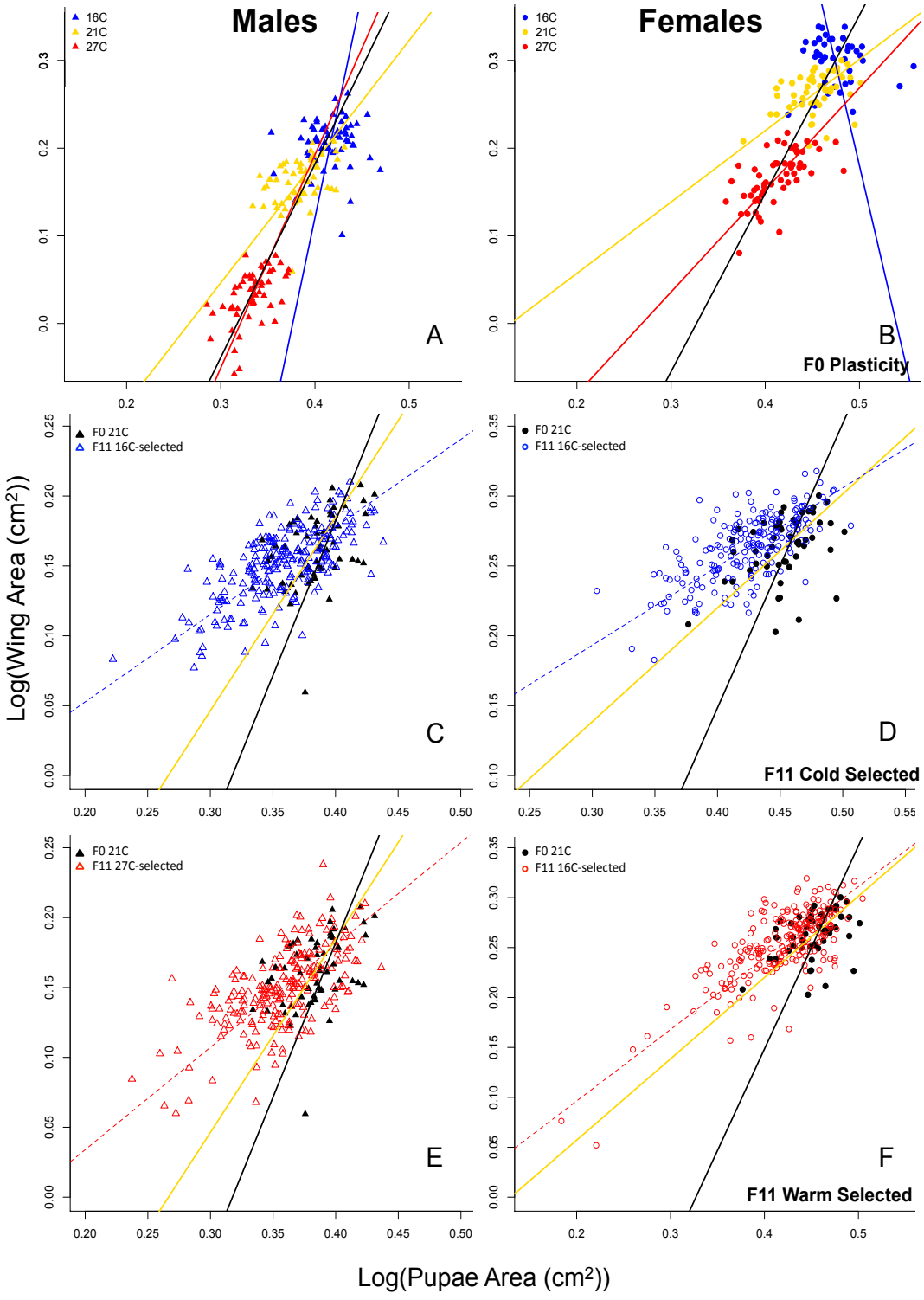
3.4.5 Quantifying and comparing morphology

I conducted a series of tests to determine if the differing responses to selection could have resulted from differences in variation among the parental (F0) populations. ANCOVA revealed no effect of starting population regarding the thermally-induced plasticity in morphology among the F0 populations of Experiments I and II (d.f.=1, F=0.002, p=0.968). PCA on the parental generation reveals substantial absolute shape variation in the thermally-induced plasticity in morphology (Procrustes distance=0.372). CVA revealed the two parental generations could be effectively discriminated ($p<1.0x10^{-4}$) although the Procrustes distance explaining the relative shape change was minimal (Procrustes distance=$6.6x10^{-4}$, ~1.7% of absolute shape variation) and well within a typical range of intraspecific variation in *D. melanogaster* (Klingenberg and Zaklan 2000). Thus, I do not attribute differences in response to selection to differences between the F0 populations. Correlations within each development temperature were lowest for Cool developed flies and increased with temperature for both Experiment I [r^2=(Cool: $1.7x10^{-4}$; Intermediate: 0.153; Warm: 0.399), p=(Cool: 0.860; Intermediate: $4.5x10^{-3}$; Warm: $1.1x10^{-7}$)] and Experiment II [r^2=(Cool: 0.208; Intermediate: 0.587; Warm: 0.549), p=(Cool: $1.2x10^{-3}$; Intermediate: $3.3x10^{-8}$; Warm: $5.8x10^{-5}$)]. Thus all correlations were significant except the Cool developed flies in Experiment I.

3.4.6 Experiment I: correlated evolution of morphology to selection on flight performance

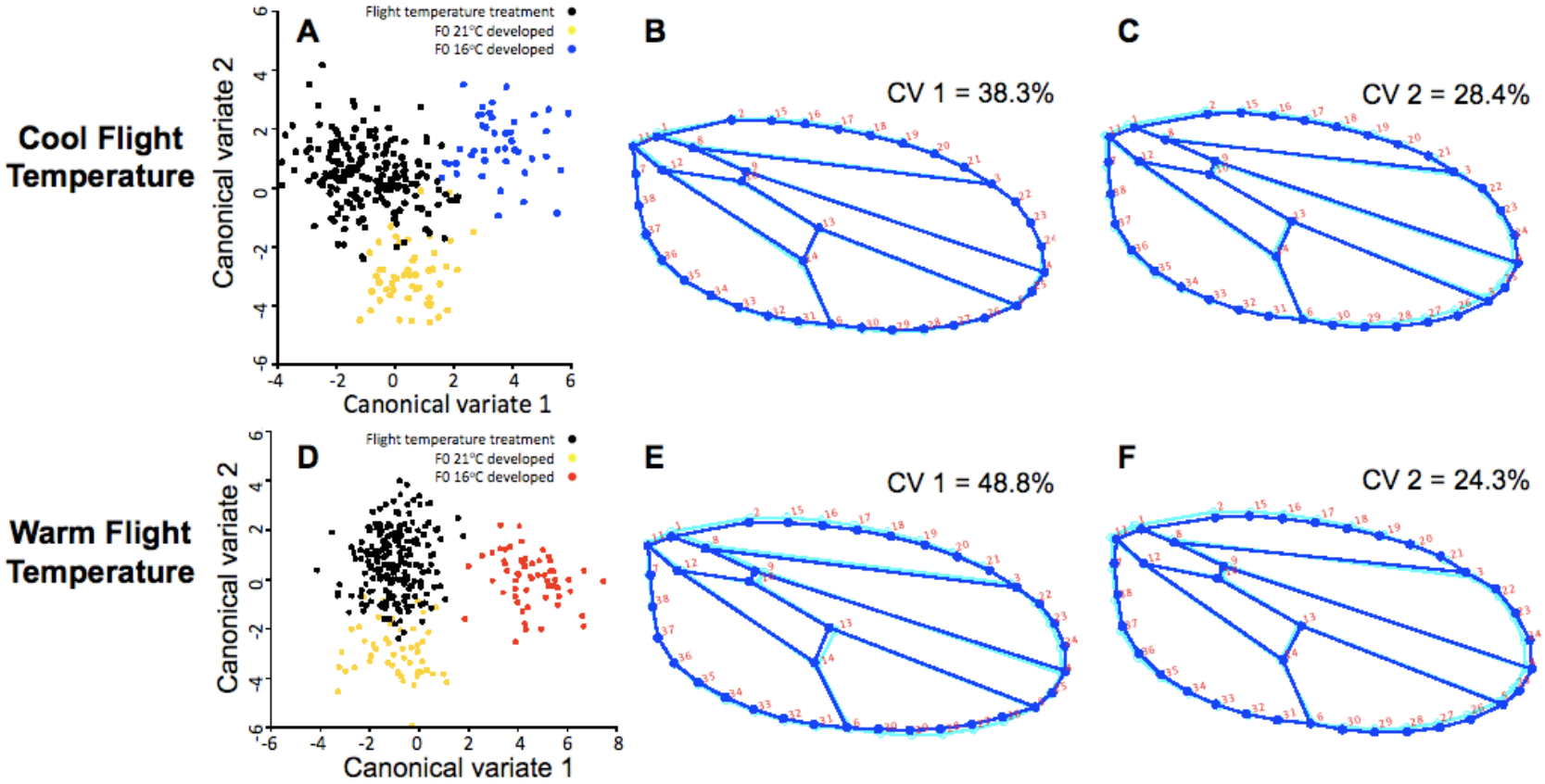
Following 11 generations of selection, I found no significant change in slope for either Cool ($F_{1,243}$=0.694, p=0.406; r^2=0.456, p=< $1.0x10^{-16}$) or Warm ($F_{1,262}$=2.68, p=0.103; r^2=0.571, p<$1.0x10^{-16}$) Flight Temperature Treatments although both had significant increases in intercept (Cool p=$4.23x10^{-5}$; Warm p=0.015; Fig. 3.3) and trended toward hypoallometry. A conservative Bonferroni-corrected α of 0.0125 was used for the univariate t-tests of morphology. Mean body size decreased significantly from the F0 population ($\bar{x}_0$=0.453, SD_0=0.025) significantly in both Cool ($\bar{x}_{8:11=}$0.424, $SD_{8:11=}$0.035, t=6.74, d.f.=108, p=$7.9x10^{-10}$; Fig. 3.3D) and Warm ($\bar{x}_{8:11=}$0.419, $SD_{8:11=}$0.047, t=7.22, d.f.=146, p=$2.6x10^{-11}$; Fig. 3.3F) Flight Temperature Treatments after 11 generations. This likely explains the increased intercept of the scaling relationship. Wing size did not differ significantly for Cool Flight Temperature Treatment ($\bar{x}_{8:11=}$0.263, $SD_{8:11=}$0.024, t=0.024, d.f.=79.9, p=0.981; Fig. 3.3D) evolved flies relative to F0 flies ($\bar{x}_0$=0.263, SD_0=0.23), but were nearly significantly smaller under the Warm Temperature Treatment ($\bar{x}_{8:11=}$0.253, $SD_{8:11=}$0.036, t=2.49, d.f.=118, p=0.014; Fig. 3.3F). Motivational Controls were collected for morphological analysis, but have not yet been quantified.

Figure 3.3. Experiment I Morphological response to selection for increased flight performance. (A & B) Allometric relationships are plotted for the initial parental population. Colors points represent individuals developed throughout ontogeny at 16 (Blue), 21 (gold) or 27^{o}C (red). Solid colored lines represent the slope of MAR and describe the allometric relationship for each rearing treatment. The solid black lines indicate the aggregate allometric relationship across development treatments. Solid lines are consistent across all panels for the given sex. (C & D) The F_{11} Cool Flight Temperature Treatment (Blue) plotted against the parental (F_0) population (Black). Dashed colored lines represent the allometric relationship of the evolved lineages. (E & F) The F_{11} Warm Flight Temperature Treatment (Red) plotted the parental (F_0) population.


Males
Females
16C
21C
27C
A
B
F0 Plasticity
F0 21C
F11 16C-selected
C
D
F11 Cold Selected
F11 27C-selected
E
F
F11 Warm Selected
Log(Wing Area (cm²))
Log(Pupae Area (cm²))

Selection for flight performance at Cool flight temperatures was predicted to produce wing shapes similar to flies that develop at Cool temperature and selection at Warm flight temperatures to produce wing shapes similar to flies that develop at Warm temperature. CVA on the four late generation Cool Flight Temperature Treatment lineages and F0 population developed at either 16 or 21^{o}C revealed that the Mahalanobis distance was smaller between selected lines and the F0 population developed at 21^{o}C, than the presumptive target 16^{o}C morphology (t=2.41, d.f.=5.98, p=0.026), although the distances were large enough to classify distinct groups (mean distance from 16^{o}C=5.11, from 21^{o}C=4.31) the average Procrustes distance between derived and parental shape was low (Procrustes distance=0.012; Fig. 3.4B & C). For the Warm Flight Temperature Treatment, the Mahalanobis distances were significantly smaller in relation to 21^{o}C flies of the F_0 population (t=-5.98, d.f.=3.52, p=0.003) than the presumptive target 27^{o}C morphology. As with the Cool Temperature Treatment, the Mahalanobis distances were large enough to distinguish groups (mean distance from 21^{o}C=4.39, from 27^{o}C=5.89) although the absolute shape change was low (Procrustes distance=0.011; Fig. 3.4E & F). In both cases, this constitutes a failure of my prediction. The shape variation was much higher within groups relative to between groups for both Flight Temperature Treatments, indicating a large environmental effect and offering little support for adaptive or correlated evolution of morphology. Shape change was best discriminated from F_0 population developed at 21^{o}C by CV2 for both Cool- and Warm-selected treatments (Fig. 3.4A & D). In both cases this resulted in lengthening of the distal tip of the wing, broadening of the wing margin proximally, a slight proximal movement of the Anterior Cross Vein and a slight distal movement of the Posterior Cross Vein (Fig. 3.4C & F).

Figure 3.4. Experiment I shape change between F0 and evolved lineages. For orientation, proximal is to the left, anterior is at the top. (A,D) Plots of CVA discriminating Flight Temperature Treatments from F_0 populations developed at 21^{o}C and the selected environment. (B, C, E & F) Wireframes illustrate shape differences between the consensus (cyan) and flight-selected lines (dark blue). Shape differences are shown with a scale factor of 10 to aid visualization of subtle shape differences.

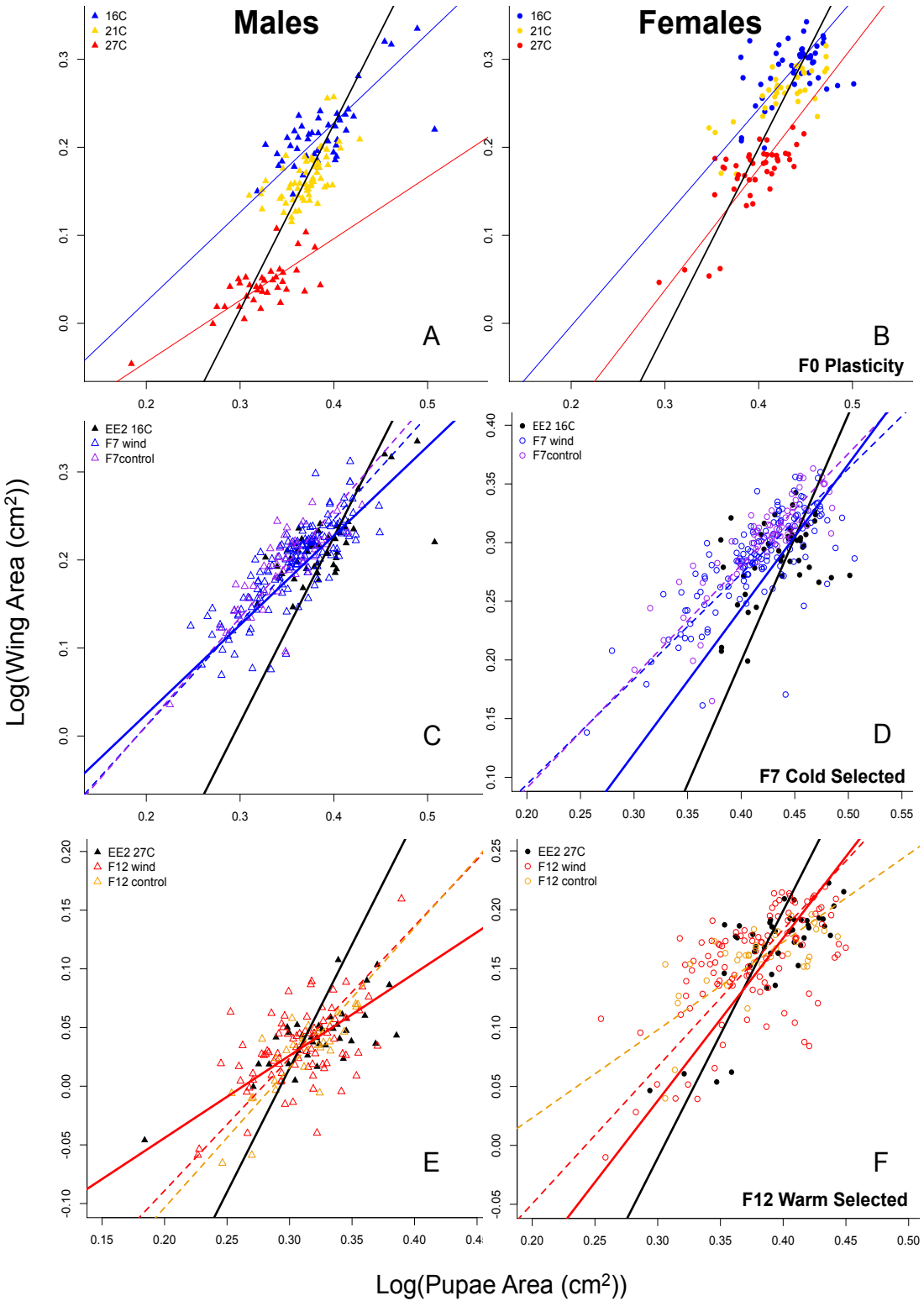

Cool Flight Temperature
Warm Flight Temperature
A
B
C
D
E
F
Flight temperature treatment
F0 21°C developed
F0 16°C developed
Canonical variate 1
Canonical variate 2
CV 1 = 38.3%
CV 2 = 28.4%
CV 1 = 48.8%
CV 2 = 24.3%

3.4.7 Experiment II: correlated evolution of morphology to selection on flight performance

While data have been collected for both generation 7 and 12 for both Flight Temperature Treatments in Experiment II, only generation 7 of Cool and generation 12 of Warm Flight Temperature Treatments are currently quantified (Fig. 3.5). Following 7 generations of selection, the slope of Cool Flight Temperature ($F_{1,181}$=1.81, p=0.179; r^2=0.577, p< $1.0x10^{-16}$) and Cool Motivation Control ($F_{1,99}$=2.71, p=0.103; r^2=0.726, p< $1.0x10^{-16}$) flies did not differ significantly from parental F_0 population (Fig. 3.5D), although the intercepts had increased significantly (Cool Flight Temperature: $F_{1,182}$=7.06, p=0.009; Cool Motivation Control: $F_{1,100}$=10.62, p=0.001). The Developmental Control lines, however, did differ significantly in slope from the parental generation ($F_{1,112}$=4.88, p=0.029; r^2=0.728, p=< $1.0x10^{-16}$) becoming hypoallometric relative to F_0 population (Fig. 3.5D). I found no significant difference in the evolved Warm Flight Temperature Treatment slope ($F_{1,147}$=2.65, p=0.106; r^2=0.351, p=< $2.3x10^{-11}$) or intercept ($F_{1,148}$=0.006, 0.940; Fig. 5F). However, the slope of the scaling relationship of both Motivation ($F_{1,82}$=5.31, p=0.024; r^2=0.222, p=< $1.8x10^{-3}$) and Development Control ($F_{1,79}$=5.69, p=0.020; r^2=0.447, p=< $4.4x10^{-6}$) groups had become hypoallometric relative to F_0 population (Fig. 4F). Interestingly, the slopes describing the scaling relationships among the evolved Flight Temperature Treatments, Motivational Controls and Development Controls did not differ significantly for either Cool ($F_{2,257}$=1.02, p=0.364) or Warm ($F_{2,179}$=0.463, p=0.630) flight-selected lines, although intercepts for Cool-selected lineages did differ significantly ($F_{2,179}$=0.46, p=0.019). Such consistency among the evolved morphological scaling relationships indicates that Development Temperature strongly affects the observed morphological changes.

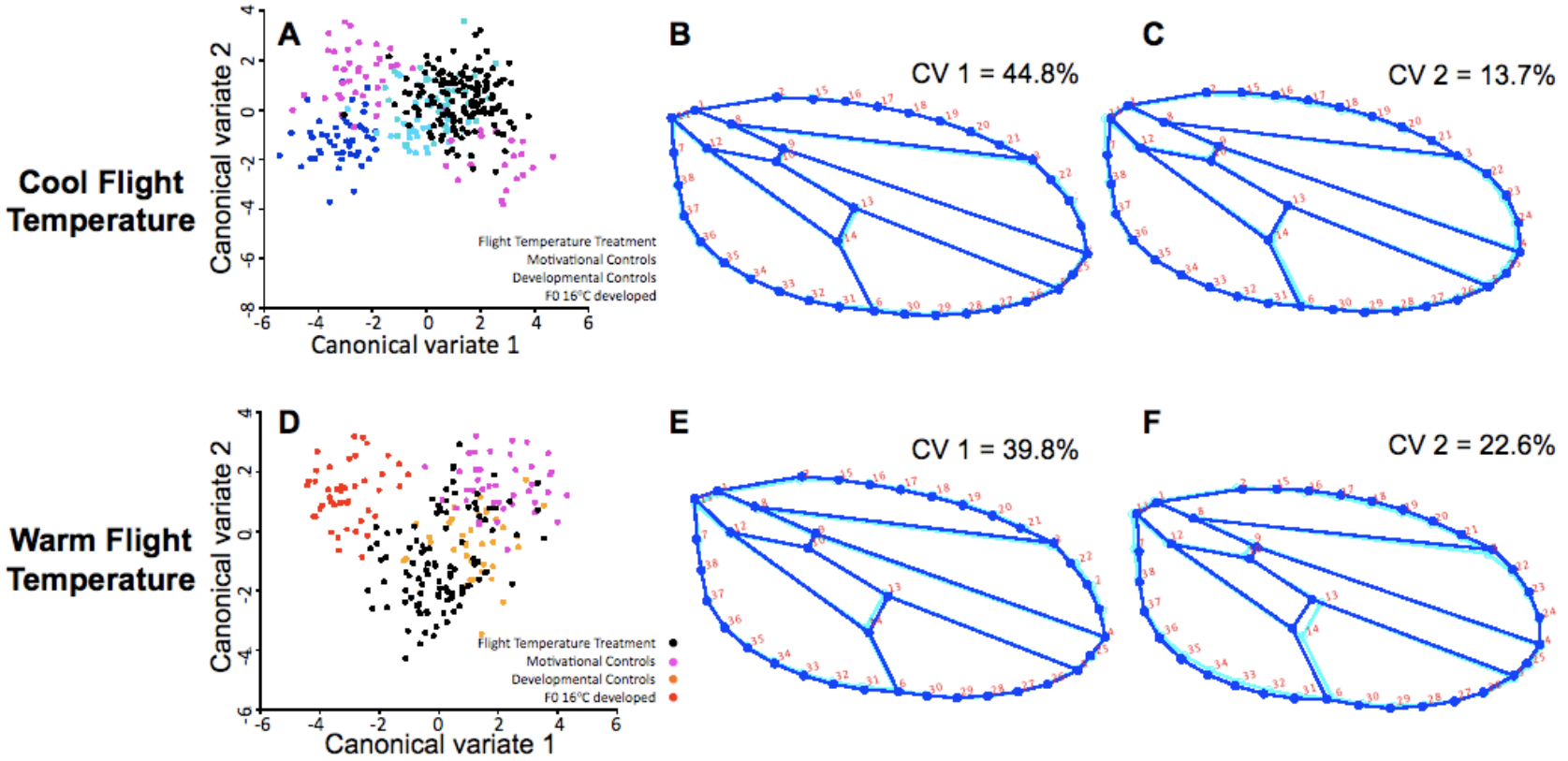
Univariate tests on morphology reveal morphology did not evolve in the predicted manner. Mean body size decreased significantly (Bonferroni α=0.004) for both Cool ($\bar{x}_7$=0.414, SD_7=0.044, t=4.06, d.f.=120.8, p=4.3x10^{-5}) and Warm ($\bar{x}_{12}$=0.375, SD_{12}=0.41, t=3.15, d.f.=106.9, p=0.001) Flight Temperature Treatments from the body size of the F_0 population (Cool: $\bar{x}_0$=0.437, SD_0=0.029; Warm: $\bar{x}_0$=0.394, SD_0=0.032). Both Warm Motivation Controls (Cool: $\bar{x}_7$=0.422, SD_7=0.039, t=2.25, d.f.=99.3, p=0.013; Warm: $\bar{x}_{12}$=0.375, SD_{12}=0.031, t=2.81, d.f.=83.6, p=0.003) also decreased in body size, but body size in Development Controls did not differ significantly (Cool: $\bar{x}_7$=0.421, SD_7=0.041, t=2.42, d.f.=113.8, p=0.008; Warm: $\bar{x}_{12}$=0.379, SD_{12}=0.038, t=2.00, d.f.=72.3, p=0.024). Mean wing size did not differ significantly between the F_0 population (Cool: $\bar{x}_0$=0.289, SD_0=0.032; Warm: x_0=0.168, SD_0=0.040) or generation 7 Cool Flight Temperature Treatment ($\bar{x}_7$=0.286, SD_7=0.040, t=0.512, d.f.=99.7, p=0.610) or the generation 12 Warm Flight Temperature Treatment ($\bar{x}_{12}$=0.154, SD_{12}=0.045, t=1.91, d.f.=92.2, p=0.059). Neither Motivation Controls (Cool: $\bar{x}_7$=0.294, SD_7=0.034, t=-0.846, d.f.=99.9, p=0.400; Warm: $\bar{x}_{12}$=0.160, SD_{12}=0.032, t=1.07, d.f.=82.54, p=0.287) nor Development Controls (Cool: $\bar{x}_7$=0.301, SD_7=0.039, t=-1.81, d.f.=110.7, p=0.073; Warm: $\bar{x}_{12}$=0.157, SD_{12}=0.031, t=1.44, d.f.=80.4, p=0.153) differed significantly in wing size from F_0 population.

Figure 3.5. Morphological response to selection for increased flight performance with plasticity. (A & B) Allometric relationships are plotted for the initial parental population. Colors points represent individuals developed throughout ontogeny at 16 (Blue), 21 (gold) or 27^{o}C (red). Solid colored lines represent the slope of MAR and describe the allometric relationship for each rearing treatment. The solid black lines indicate the aggregate allometric relationship across development treatments. Solid lines are consistent across all panels for the given sex. (C & D) The F_7 Cool Temperature Treatment (Blue) plotted against Development Controls (Purple) and the parental (F_0) Cool-Developed population (Black). Dashed colored lines represent the allometric relationship of the derived lineages. (E & F) The F_{12} Warm Flight Temperature Treatment (Red) plotted against Development Controls (Orange) and the parental Warm-Developed population (Black). Dashed colored lines represent the allometric relationship of the evolved lineages.


Males
Females
16C
21C
27C
A
B
F0 Plasticity
EE2 16C
F7 wind
F7control
C
D
F7 Cold Selected
EE2 27C
F12 wind
F12 control
E
F
F12 Warm Selected
Log(Wing Area (cm²))
Log(Pupae Area (cm²))

CVA between the Cool-developed F_0 population and the generation 7 Cool flight-selected lineages revealed significant Mahalanobis distances ($p<1.0x10^{-4}$) large enough to distinguish between groups (Average Mahalanobis distance: Flight Temperature Treatment=5.06; Motivation Control=4.84; Development Control=4.22). The absolute shape difference between F_0 and generation 7 Cool Flight Temperature Treatment flies was very small and within the range of intraspecific variation (Procrustes distance: Flight Temperature Treatment=0.009; Motivation Control=0.008; Development Control=0.006; Fig. 3.6). The subtle differences in shape were significant for Flight Temperature Treatment ($p<0.004$) and Motivation Control lines ($p<0.029$), but the Development Controls did not differ ($p>0.071$). Shape change, while subtle, was thus statistically significant although perhaps not biologically relevant. Evolution of Cool Flight Temperature Treatment flies was best discriminated by CV1 (Fig. 3.6A), a slight proximal movement of the anterior cross vein, distal movement of the posterior crossvein, and anterior movement of longitudinal vein 2 (Fig 3.6B).

Figure 3.6. Experiment II shape change among F0 and evolved treatment and control lines. For orientation, proximal is to the left, anterior is at the top. (A,D) Plots of CVA discriminating Flight Temperature Treatments from F_0 populations developed at the selected environment. (B, C, E & F) Wireframes illustrate shape differences between the consensus (cyan) and flight-selected lines (dark blue). Shape differences are shown with a scale factor of 10 to aid visualization of subtle shape differences.


Cool Flight Temperature
A
Canonical variate 2
Canonical variate 1
Flight Temperature Treatment
Motivational Controls
Developmental Controls
F0 16°C developed
B
CV 1 = 44.8%
C
CV 2 = 13.7%
Warm Flight Temperature
D
Canonical variate 2
Canonical variate 1
Flight Temperature Treatment
Motivational Controls
Developmental Controls
F0 16°C developed
E
CV 1 = 39.8%
F
CV 2 = 22.6%

For the Warm-evolved lineages, Mahalanobis distances were significant from F_0 populations for each lineage (Flight Temperature Treatment Average=4.62, $p<0.020$; Motivation Control=5.27, $p<1.0x10^{-4}$; Development Control=4.78, $p=0.001$) and sufficient to discriminate groups (Fig. 3.6). The consistent separation among the various evolved lineages again indicates that the developmental environment affects morphological evolution, although absolute changes to wing shape were minimal and may lack biological significance. Procrustes distances among Warm-selected lineages exhibit similarly small differences in shape change (Procrustes distance: Flit Temperature Treatment=0.010; Motivational Control=0.010; Developmental Control=0.0086). Evolution of Warm Flight Temperature Treatment flies was similarly best discriminated by CV1 (Fig. 3.6D). Changes in shape resulted mostly from distal movement of the posterior crossvein, and minor anterior movement of longitudinal veins 1, 2, and 3 (Fig 3.6E).

3.5 Discussion

In this study, I examined the role of phenotypic plasticity in facilitating adaptive evolution of flight performance. Flight performance is a composite phenotype affected by variation in numerous morphological, physiological, and behavioral component traits, each of which potentially could contribute to the response to selection. Two artificial selection experiments were conducted for 11 generations, distinguished by whether populations developed at an intermediate 21^{o}C or the temperature at which flight performance was measured (16 or 27^{o}C). Conditions where development and flight temperatures were matched exhibited a strong response to selection for increased flight performance in both Cool and Warm Flight Temperature Treatments, whereas populations that developed at temperatures that did not match flight temperatures exhibited no response

to selection (Figs. 3.1, 3.2). The nearly 4-fold increase in the realized heritability of flight performance in Experiment II suggests a strong, positive effect of phenotypic plasticity in adaptive evolution (Fig. 3.2).

Comparison of flight performance among the selected lineages and the various controls reveals the degree to which the response to selection in Experiment II was due to thermally-induced changes in flight ability, responses to the motivating stimuli, and responses to developmental conditions. For example, the lack of a statistically significant increase in flight performance in Developmental Controls in Experiment II indicates the increased flight performance did not result from thermally-induced phenotypes alone, but by selection acting on the phenotypes induced by such development. However, evolved patterns of locomotor performance in the Motivational Controls were complex. Realized heritabilities were significant only for Cool Motivation Control lines in Experiment II, where the development temperature matched the selective temperature. Although these controls did not experience a headwind, they did experience selection for a response to all three motivating stimuli. Motivational Controls evolved increased flight performance, but exhibited flight distributions that did not differ in the presence or absence of a headwind. This likely indicates that the increased flight performance in Cool-selected lines is due, in part, to increased motivation or response to the stimuli. This “motivation” could result from increased activity levels or increased sensitivity to motivating stimuli, or both. Studies aimed at disentangling these potential responses are planned. In addition to the well-documented morphological plasticity described above, *D. melanogaster* exhibit plasticity in many physiological and life-history traits (e.g., development time: James and Partridge 1995; wing beat frequency: Barnes and Laurie-Ahlberg 1986; Frazier et al. 2008; fecundity: (Cooper et al. 2010); protein expression: Robinson et al.

2010). It is interesting that the Motivational Controls failed to evolve in Experiment I, where developmental conditions did not match flight conditions; this suggests that developmental plasticity played an important role in all aspects of the response to selection. Many other traits in *Drosophila* may be similarly dependent on phenotypic plasticity to adapt rapidly to new environments.

The striking lack of response in Experiment I is exacerbated by the potential for performance to evolve via “multiple adaptive solutions” (Garland et al. 2011; Careau and Garland 2012). Selection on performance allows adaptation to occur through combinations of change in morphological, physiological, or behavioral traits, and yet *D. melanogaster* exhibited no response to selection when development temperatures did not match flight temperatures. Interestingly, the responses to selection among replicates within each Flight Temperature Treatment in Experiments I & II appear consistent despite this flexibility. This similarity in the response to selection is reflected in the realized heritabilities for each replicate in Experiment II (which all differed statistically from zero), and those in Experiment I (which were not distinguishable from zero). Such a consistent response to selection among replicates within experiments may reflect biases imposed on the response to selection that result from patterns of genetic variation in and covariation among traits (Schluter 1996; Arnold et al. 2008), or similar effects of phenotypic variation among traits. Alternatively, this pattern could be the product of the existence of a single 'best' evolutionary solution to the selection imposed by my experiment; this may be particularly true as selection on performance was strong whereas it was likely relatively reduced for other traits (e.g., development time, but see below), reducing the impact of fitness trade-offs that might be important in nature. Unfortunately, the specific suites of traits facilitating flight performance adaptation have

not yet been identified. My data indicate the responses involve behavioral and perhaps physiological and morphological traits as well. Once the phenotypic basis of the response to selection has been elucidated, it may prove fruitful to use the tools available for *Drosophila* to dissect the genetic basis of adaptation in this experiment.

Temperature fluctuates seasonally in many biomes where *D. melanogaster* occurs. Thus, natural populations are unlikely to receive such consistent selection for enhanced flight performance as that applied here, as different temperatures presumably favor different flight phenotypes. Within each geographic population in nature, seasonal fluctuations may push the favored phenotype about some multivariate mean value in a predictable manner; this would explain the maintenance of phenotypic plasticity overlaid onto the geographic clines. Additionally, fluctuating temperatures during development may enhance phenotypic variation on which selection can act. *D. melanogaster* are highly sensitive to temperature during ontogeny, and temperature shifts during larval and pupal development can affect trait expression, including adult flight morphology, and thereby alter typical patterns of covariation among traits relative to that observed when they developed in constant conditions (French et al. 1998; Chapter 5). Phenotypic plasticity appears to keep phenotypic variation within a range suitable for flight performance adaptation. As *D. melanogaster* expanded its range to new environments with novel seasonal and thermal patterns, plasticity would facilitate the production of genetically differentiated clines by genetic accommodation over time. I was unable to test specifically for genetic differentiation resulting from genetic accommodation, however, samples from generation 12 are frozen and I expect will be the subject of future investigations. This method of adaptation would explain rapid range expansion in *Drosophila* spp. and may explain the repeated evolution of morphological clines (David

and Capy 1988; Huey et al. 2000). Such clinal morphology is not exclusive to *Drosophila,* however, and may be of particular importance to other insects in which the generation:season ratio is high and Bergmann clines are typically observed (Blanckenhorn and Demont 2004; Chown and Gaston 2014). Bergmann clines, in which body size increases at higher latitudes, are common in ectotherms (Atkinson 1994; Atkinson and Sibly 1997).

Flight performance adaptation did not produce the predicted morphological patterns in flight-selected treatments or controls (Fig. 3.3). Slopes of the wing:body size scaling relationship in Flight Temperature Treatments did not differ significantly from the F_0 population under similar developmental conditions, although both Developmental Controls and Warm Motivation Controls did differ significantly in their slopes. Importantly, the evolved treatment and control slopes did not differ significantly from each other, indicating that the morphological response of all lineages favored hypoallometry, a pattern much different from this near-isometry exhibited in the F_0 population and those found in natural populations. All evolved slopes were significant ($p<0.005$), indicating that the trend toward hypoallometry is real. Biologically, this means that wing size does not necessarily change with body size (i.e., the wings became less plastic relative to the body). The reduced response in flight-selected lineages relative to Developmental Controls may indicate the patterns of wing:body size scaling in F_0 population were beneficial to flight, and selection for flight morphology resisted response of selection to development temperature.

Mean body size decreased in both Experiments among all treatment and control lines (Figs. 3.3 & 3.4). This finding is in accord with predicted response to selection for Warm-

flight in Experiment I since body size and temperature are negatively correlated (e.g., James et al. 1997), but contrary to Cool-flight predictions; Cool development increases body size in multi-year selection studies (Partridge et al. 1994; Bochdanovits and de Jong 2003). Cool development temperatures slow development of flies (James and Partridge 1995) and result in a population eclosing mostly over a four-day window (see Chapter 2). Cool-developed flies were assayed 7 days post-eclosion, timing that may have favored faster development and thus decreased body size. In Experiment II, the magnitude of decreased body size was greatest in Flight Temperature Treatments, indicating that flight performance into a headwind may also favor small body size. This comparison can be made in Experiment I once morphology of Motivation Controls is quantified, although I do not expect a morphological response given that flight performance did not evolve.

The response of wing shape to selection in Experiment I was predicted to produce shapes more similar to Cool and Warm temperature-induced morphologies relative to 21^{o}C induced morphology (Fig. 3.5). This prediction was not met, as both Cool and Warm Flight Temperature Treatments remained closer in shape to F_0 population developed at 21^{o}C; this is perhaps not surprising given that flight performance did not evolve in this experiment. Despite the striking difference in evolved flight performance between Experiments, the degree of absolute change in wing shape were similar and minimal, ~2-3% of total shape variation, in both Experiment I and II, indicating that wing shape likely did not respond to selection for flight performance into a headwind. The prediction for wing shape change in Experiment II was less clear. Cool Flight Temperature Treatment flies had wing shapes similar to Developmental Controls (Fig. 3.6A), thus evolved wing shape changes were most likely due to temperature of

development. Warm Flight Temperature Treatments, however, did separate partially from Developmental Controls (Fig. 3.6D), although shape change is again minimal and likely not relevant biologically (Fig. 3.6E, F). The difference in shape between generation 0 and 11 is<25% of overall shape variation and thus exhibits substantial environmental variation (Breuker et al. 2006). In all cases, the difference in shape change is well below the typical range of intraspecific variation (Klingenberg and Zaklan 2000) and could thus be explained by bottleneck effects of selection.

My data show unambiguously that phenotypic plasticity facilitates adaptive evolution by enhancing the response to selection. The inability of *Drosophila* to evolve enhanced flight performance via predicted morphological adaptation at Cool and Warm flight temperatures was surprising given the evolvability of body size (Bochdanovits and de Jong 2003), wing size (Vijendravarma et al. 2011), and the slope (Stillwell et al. 2014) and intercept (Frankino et al. 2005; Frankino et al. 2007) of wing:body size scaling relationships. Thermally-induced plasticity in traits other than the morphological ones measured here appear to play an important role in facilitating adaptive evolution of flight performance. For example, the changes in the ultrastructure of indirect flight muscle can have significant impact on *Drosophila* flight performance (Miller et al. 2008). Phenotypic plasticity resulting from developmental temperatures is widespread in ectotherms, and such plasticity appears to facilitate adaptive evolution to novel environments and may have explanatory power regarding diversification across the broad range of thermal environments inhabited by ectotherms.

CHAPTER 4. GENETIC ACCOMMODATION PROMOTES ADAPTIVE PHENOTYPE-ENVIRONMENT MATCHING IN *DROSOPHILA MELANOGASTER* FLIGHT PERFORMANCE

4.1 Abstract

Genetic accommodation is the process by which adaptive genetic changes in expression of a plastic phenotype occur. These adaptive genetic changes result from altered developmental regulation that reveals cryptic genetic variation, either caused by a novel environment or mutation of large effect, to selection. This process results in a new range of phenotypic variation better adapted to the novel environment. Genetic accommodation has been shown to occur for novel phenotypes resulting from unnatural developmental environments or those involving mutations of large effect in small, experimental laboratory populations. Evolution through genetic accommodation has not yet been examined empirically for an ecologically relevant trait that shows variation in natural populations. In this study, I investigate the role of genetic accommodation in facilitating the adaptation of *Drosophila melanogaster* flight performance following 7 and 11 generations of selection on upwind flight ability at Cool and Warm temperatures. I found the patterns and costs of thermal specialization for flight to change over time. Evolved patterns were consistent with predictions of adaptive phenotypic plasticity; flies exhibited phenotype-environment matching, where populations have greatest relative flight performance at their developmental temperature. Both Cool- and Warm-selected lineages exhibited increased flight performance in thermal environments when matched to their development temperature. Significant costs of phenotype-environment mismatching were detected, indicating that enhanced flight performance did not result simply from selection for a strong generalist flight phenotype. My data provide empirical support for the hypothesis that genetic accommodation facilitates the evolution of

adaptive patterns of phenotype-environment matching in an ecologically relevant trait such as flight performance, and suggest it is important for adaptation and diversification in general.

4.2 Introduction

When heritable variation is present in the expression of a plastic trait, and patterns of selection vary across environments, adaptive phenotypic plasticity can evolve in regulation or form. The process by which phenotypic plasticity evolves adaptive genetic change in phenotypic expression in this manner is called genetic accommodation, and it had received relatively sparse attention until recently (West-Eberhard 2003, 2005; Braendle and Flatt 2006; Pigliucci et al. 2006; Crispo 2007; Moczek et al. 2011; Schlichting and Wund 2014). While the hypothesis that genetic accommodation facilitates adaptive evolution has received challenges (e.g., Orr 1999; De Jong 2005), empirical evidence is amassing in its support. Phylogenic analyses on traits with clear ecological relevance such as skull (Adams and Huntingford 2004) and gut morphology (Ledon-Rettig et al. 2008), support this hypothesis by demonstrating that populations with a genetically differentiated, derived phenotype resulted from ancestral populations with plastic trait expression. Unfortunately, most direct experimental support for genetic accommodation comes from experiments using novel phenotypes with unclear ecological function (e.g., cross vein presence: Waddington 1953; Bateman 1957; bithorax phenotype: Waddington 1956) or from studies of laboratory populations with single mutations of large effect (e.g., Sollars et al. 2003; Suzuki and Nijhout 2006). It is unclear how findings of these studies translate into the general importance of genetic accommodation in natural populations. Direct experimental tests of the importance of

genetic accommodation in adaptation of polygenic plastic traits of ecological importance are lacking.

Most, if not all, traits are phenotypically plastic to some degree, altering expression in response to environmental variation. Not all cases of phenotypic plasticity are adaptive; some may simply be passive physiological or developmental responses to the environment (Smith-Gill 1983). Plasticity is adaptive, however, when the developmental environment accurately predicts, or covaries with, the future selective environment and induces a phenotype that exhibits greater fitness in the selective environment relative to alternative inducible phenotypes. Such enhanced fitness may manifest within phenotype across selective environments, as well as within an environment across phenotypes. The pattern of increased relative fitness resulting from covariation between phenotype expression and the selective environment is called adaptive phenotype-environment matching (Moran 1992). Determining the adaptive nature of plasticity is an expansive field in and of itself (for reviews Pigliucci 2001; Dewitt and Scheiner 2004; Whitman and Ananthakrishnan 2009), but relatively few studies investigate directly the role of genetic accommodation in facilitating the evolution of adaptive phenotypic plasticity.

Adaptive genetic change of a plastic phenotype by genetic accommodation can be simplified to three predicted outcomes; a decrease, increase, or no resultant change to expression of plasticity. Genetic assimilation, which has received the most empirical attention, is when the expression of a novel, induced phenotype becomes canalized through selection such that it becomes insensitive to environmental variation and expressed constitutively (reviewed in Braendle and Flatt 2006). Thus, genetic assimilation favors a dramatic reduction in phenotypic plasticity. In this way, genetic

assimilation may result in a generalist or specialist phenotype, depending on benefits and costs associated with constitutive phenotypic expression across environments. Alternatively, genetic accommodation can favor increased phenotypic plasticity that enhances precision of phenotype-environment matching. Such a response is predicted in variable environments that continue to expose genetic variation for phenotypic variation across environments that favor different combinations or values. The evolution of phenotype-environment matching is typically attributed to the benefits of proper matching (Schlichting 1986; West-Eberhard 1989; Boratyński et al. 2014), although the costs of mismatching may be just as important in producing this pattern (Lively 1986; DeWitt 1998). To appropriately distinguish among the various outcomes of adaptive evolution by genetic accommodation, a population must be developed, and fitness tested, across a range of phenotype-inducing environments.

Drosophila are established model organisms for the study of genetic accommodation (Waddington 1953, 1956; Bateman 1957; Sollars et al. 2003), due mostly to practicality and precedence. In addition, natural patterns of phenotypic variation in ecologically relevant morphologies of *Drosophila* make them well-suited to studies of genetic accommodation. *D. melanogaster* exhibit genetically-based clines (e.g., Imasheva et al. 1994; James et al. 1997; Azevedo et al. 1998; Gilchrist and Huey 2004; Liefting et al. 2009; Bhan et al. 2014) and thermally-induced phenotypic plasticity (e.g., James et al. 1997; French et al. 1998; Gilchrist and Huey 2004; Frazier et al. 2008) in body size and relative wing size. The clinal and plastic patterns are consistent such that populations located in cool environments, or flies developed under cool conditions, exhibit large body size and disproportionately large wings. The existence of this phenotypic plasticity and these geographic patterns are typically explained by adaptationist arguments regarding

how these phenotypes are believed to be matched to flight performance in the thermal environment in which they occur. There exists some empirical evidence, however, that supports the argument for adaptive phenotypic plasticity for flight at Cool (Frazier et al. 2008) and Warm (Chapter 2) temperatures. In my previous work, I demonstrate that thermally-induced plasticity facilitates the adaptive evolution of flight performance (Chapter 3), which did not occur absent development temperatures matched to flight temperatures. Here, I explore the dynamics of flight performance evolution resulting from genetic accommodation.

In this study, I test upwind flight performance of populations that were developed and assayed at matched and unmatched temperatures after 7 and 11 generations of selection for increased flight performance at Cool (16^{o}C) and Warm (27^{o}C) temperatures. Both Cool- and Warm-selected flies rapidly evolved superior flight performance at their respective selected temperature (Chapter 3), however, it is unclear if their responses to selection resulted from change in traits that enhance flight performance in general (i.e., that produce a generalist flyer that performs well in all thermal environments), or adaptations specific to the selected flight temperature. Such specialization may occur either through enhanced phenotype-environment matching (i.e., enhanced phenotypic plasticity), or by genetic assimilation, where traits that benefit flight at the selected temperature become constitutively expressed and reduce flight performance at alternative temperatures.

To investigate these issues, flies of both Cool- and Warm-flight selected lines were developed and assayed for flight performance at both temperatures in a modified 2 x 2 full factorial design. Using this design, I address the following questions: (i) Did evolved

increases in flight performance result from the production of a strong generalist or specialist phenotype as predicted by genetic assimilation? (ii) Does a strong generalist phenotype result form genetic assimilation or from enhanced phenotype-environment matching? This question is most readily apparent if a cost is present when development temperature does not match assay temperature. Finally, (iii) did selection at the flight-challenging Cool temperature produce stronger fliers than selection at the more flight-permissive Warm temperatures? I found that flies selected at Cool and Warm temperatures each exhibited patterns of flight performance predicted by genetic assimilation at generation 7, but genetic accommodation resulted in enhanced phenotype-environment matching by generation 11.

4.3 Materials and Methods

4.3.1 Overview

The *D. melanogaster* populations used here are the product of artificial selection for development and upwind flight performance at either Cool (16^{o}C) or Warm (27^{o}C) temperatures (Chapter 3). The temperature at which a fly lineage was artificially selected is referred to as its Native temperature, and that at which it was not selected referred to as its Alternate temperature. Assays of upwind flight performance were performed following 7 and 11 generations of artificial selection under 3 development/flight temperature combinations. Native Treatment refers to tests of flies that were developed and assayed at their Native temperature; these serve as a reference for the performance of flies in other treatments. Alternate Treatment refers to flies reared and assayed at their Alternate temperature; these were used to determine the degree to which increased flight performance at the Native temperature resulted from genetic assimilation. Finally, Mismatched Treatment refer to flies that were reared at their Native temperature, but

assayed at their Alternate temperature; flight performance of these flies distinguishes between a strong generalist flight phenotype and a phenotype-environment matching pattern of trait expression.

4.3.2 Study populations

To examine patterns in the adaptive evolution of *D. melanogaster* flight performance, I reared and selected flies for headwind flight performance for 11 generations using a custom built, progressive-velocity wind tunnel (Chapter 3). Experimental lineages were developed and selected at either 16 or 27^{o}C. The strongest flying (top 20%) females were selected to produce each subsequent generation. For the control lines that experienced similar development and handling conditions, 20% of females were selected haphazardly. Their were four replicate treatment lineages and two control lineages for both selective temperatures, each line consisting of 2,000 flies/generation. By generation 7, flight performance diverged 8.51 (Cool) and 3.25 (Warm) SD from starting population and 1.17 (Cool) and 2.34 (Warm) SD from controls. By generation 11, flight performance had diverged further to 17.2 (Cool) and 11.8 (Warm) SD from the starting populations and diverged *ca* 5 SD from thermal-development controls (see Chapter 3 for details). Below, flies selected for upwind flight performance at cool temperature are called Cool-selected flies whereas those selected for improved flight performance at warm temperature are referred to as Warm-selected flies.

4.3.3 Wind tunnel

The wind tunnel is 2m in length and is composed of 15 uniform (~63 sq cm) collection chambers, a start gate and end-piece which contains a strong LED light source and yeast/beer mixture to provide positive phototactic and chemotactic stimuli to motivate

flies to progress through the wind tunnel. Additionally, inclining the tunnel 35^{o} stimulates negative geotactic behavior. The headwind is weakest at the start gate (0 cm/s), and increases linearly up to the final segment (31.8 cm/s). This device can phenotype the flight performance of hundreds of flies in a single trial. Each collection chamber is connected to the next by a short 4 cm dia, 8 cm long passageway coated with Fluon™ so that insects must fly through the passageway to advance up the tunnel. Flight assays lasted 15 minutes, after which time each chamber was flooded with CO_2 to anesthetized flies and prevent further movement. Flies were then removed and counted by chamber to produce a distribution of flight ability for each population.

4.3.4 Flight Performance Trials

For each Native, Alternate, and Mismatched Treatment, 1,000 eggs were collected from each of four replicate selected treatment lineages and two control lines, except for the generation 11 Warm-selected flies, in which only one control was available. Flies were reared at a density of 25 eggs/ml of standard fly media. Flies were reared through ontogeny and held post-adult eclosion at their Native (Native and Mismatched Treatments) or Alternate (Alternate Treatment) temperatures until flight was assayed. Flight performance was assayed at the peak flight performance age for a given development temperature; 3 d post-eclosion following Warm development 27^{o}C and 7 d post-eclosion following Cool development 16^{o}C (Chapter 2). Flies were collected into empty 100 ml bottle following brief <10 min chill coma at 5^{o}C. To induce mild desiccation to enhance response to motivating stimuli and allow for recovery from chill coma (Weber 1996), flies were held without food for 1 (Warm) or 2 (Cool) h before being released into the wind tunnel *en masse* at the start of a trial.

4.3.5 Analyses

Flight at cool temperatures is challenging physiologically (Frazier et al. 2008; Chapter 2) and therefore produces a right-skewed distribution of flight performance. Thus, I analyzed the wind speed that the 50th percentile (WS_{50}) of flies that were able to overcome for each replicate, as this metric accurately describes the performance of a skewed distribution. The mean WS_{50} of the four replicates was used to compare performance of Native, Alternate and Mismatched Treatments groups within and between Cool- and Warm-selected lines.

(i) *Distinguishing between generalist and specialist*. To determine if flight-selected lineages evolved a specialist or generalist phenotype as predicted by genetic assimilation, I compared the WS_{50} of the Native and Alternate Treatments within the Cool- or Warm-selected flies. A generalist phenotype exhibits strong flight performance in both Cool and Warm environments when development and assay temperatures match, whereas a specialist performs well only at the selected temperature.

(ii) *Distinguishing between genetic assimilation and phenotype-environment matching*. To differentiate between a strong generalist phenotype and performance increases resulting from enhanced phenotype-environment matching, the WS_{50} of the Mismatched Treatment was compared to WS_{50} of Native and Alternate Treatments within Cool or Warm-selected flies. A strong generalist did not suffer a cost of development temperatures that did not match assay temperatures, thus traits beneficial to flight were constitutively expressed.

(iii*) Comparing performance of Cool and Warm selected flies*. Multiple comparisons were made across selection regimes to determine if selection in the relatively challenging Cool temperature produced stronger fliers than selection at relatively permissive. Cool-selected lines exhibited a stronger flight phenotype if their flight performance exceeds Warm-selected lines across flight temperatures.

All analyses are conducted via one-sided *t.test* (R Core Team 2013) with a Bonferroni correction for multiple comparisons within each hypothesis test. Analyses were conducted separately for generations 7 and 11 and results were compared to see how the basis of the response to selection may have changed as evolution proceeded. . Control lineage flies were assayed, however, as they are not included formally in analyses as they are not critical to my tests, but they are included in Figure 4.1 as a visual of baseline flight performance.

4.4 Results

4.4.1 Sex effect

I conducted a series of t-tests to determine if there were any differences in WS_{50} between sexes in our assays. Tests on each selection regime separately reveal Cool-selected (generation 7: t=-0.209, d.f.=22.0, p=0.836; generation 11: t=1.42, d.f.=28.7, p=0.168) and Warm-selected (generation 7: t=0.498, d.f.=18.2, p=0.625; generation 11: t=-0.379, d.f.=9.73, p=0.713) sexes did not differ significantly. Thus, sexes were pooled in all subsequent analyses.

4.4.2 Distinguishing between generalist and specialist

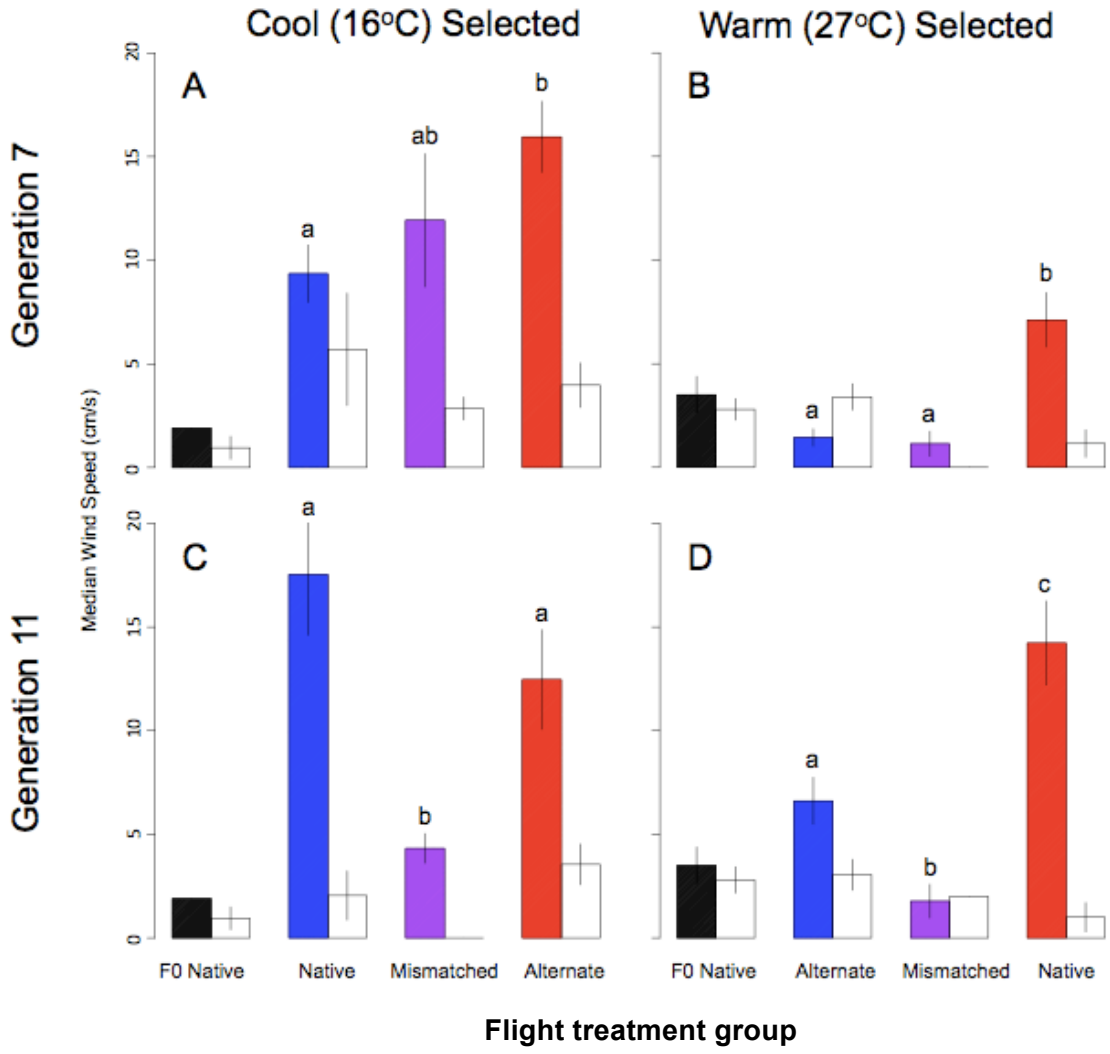
To determine if the evolution of increased flight performance to Cool or Warm environments resulted in a generalist or specialist phenotype as would result from genetic assimilation, I compared the flight performance of Native and Alternate Treatments within Cool- and Warm-selected lineages (Fig. 4.1; α=0.0125). Specialization of flight performance would be indicated by a strong performance at Native temperature, but not at Alternate temperature, whereas a generalist phenotype was flight-proficient at both temperatures. Flight performance of Cool-selected Native Treatment flies was significantly less than performance of Alternate Treatment flies at generation 7 (t=-2.99, d.f.=13.4, p=0.0051; Fig. 4.1A), indicating Cool-selected flew significantly better at Warm temperature. Although flight performance at Native temperature continued to increase, flight performance of Native Treatment flies was not significantly greater than Alternate Treatment flies at generation 11 (t=1.34, d.f.=13.5, p=0.102; Fig. 4.1C) indicating Cool-selected flies exhibited a pattern typical of a generalist phenotype. The flight performance of Warm-selected Native Treatment flies was significantly higher than the flight performance of the Alternate Treatment at generation 7 (t=-4.09, d.f.=8.40, p=0.0016; Fig. 4.1B) and generation 11 (t=-3.26, d.f.=11.0, p=0.0038; Fig. 4.1D). Thus, only Warm-selected flies show evidence favoring specialization of flight performance at generation 7 and 11, but the difference between performance at Native and Alternate Treatments declined by generation 11 (Fig. 4.1D).

4.4.3 Distinguishing between genetic assimilation and phenotype-environment matching

To differentiate generalist phenotypes and those that exhibited enhanced phenotype-environment matching, I compared the flight performance of Mismatched Treatments to both Native and Alternate Treatments within Cool- and Warm-selected lineages (Fig.

4.1; α =0.0062). Adaptive phenotype matching is evident when performance of Mismatched Treatment flies is lower than the performance of Native and Alternate Treatment flies, i.e., there exists a cost of mismatching development and selective temperatures. A strong generalist phenotype is indicated when flight performance is strong, and does not differ significantly across treatments. Flight performance of Cool-selected Mismatched Treatment flies did not differ significantly from performance of Native (t=-0.736, d.f.=9.52, p=0.760) or Alternate Treatments (t=-1.10, d.f.=10.7, p=0.149) at generation 7 (Fig. 4.1A). By generation 11 (Fig. 4.1C), flight performance of Cool-selected Mismatched Treatment flies became significantly lower than flight performance of Native (t=4.38, d.f.=7.82, p=0.0012) and Alternate (t=-3.23, d.f.=8.21, p=0.0058) Treatment flies. At generation 7 (Fig. 4.1B), flight performance of Warm-selected Mismatched Treatment flies did not differ significantly from Alternate Treatment flies (t=0.407, d.f.=12.5, p=0.345), but was significantly lower than Native Treatment flies (t=-4.11, d.f.=9.81, p=0.0011). By generation 11 (Fig. 4.1D), flight performance of Warm-selected Alternate Treatment flies became significantly greater than performance of Mismatched Treatment flies (t=3.46, d.f.=12.6, p=0.0022), although remained significantly less than Native Treatment performance (t=-5.68, d.f.=9.17, $p=1.4x10^{-4}$). Together, these results show at generation 7, Cool-selected lines exhibited a generalist pattern of adaptation, and Warm-selected lines exhibited a pattern typical of specialists. After 11 generations of selection for increased flight performance, both Cool- and Warm-selected lineages exhibited a pattern of trait expression resulting in phenotype-environment matching.

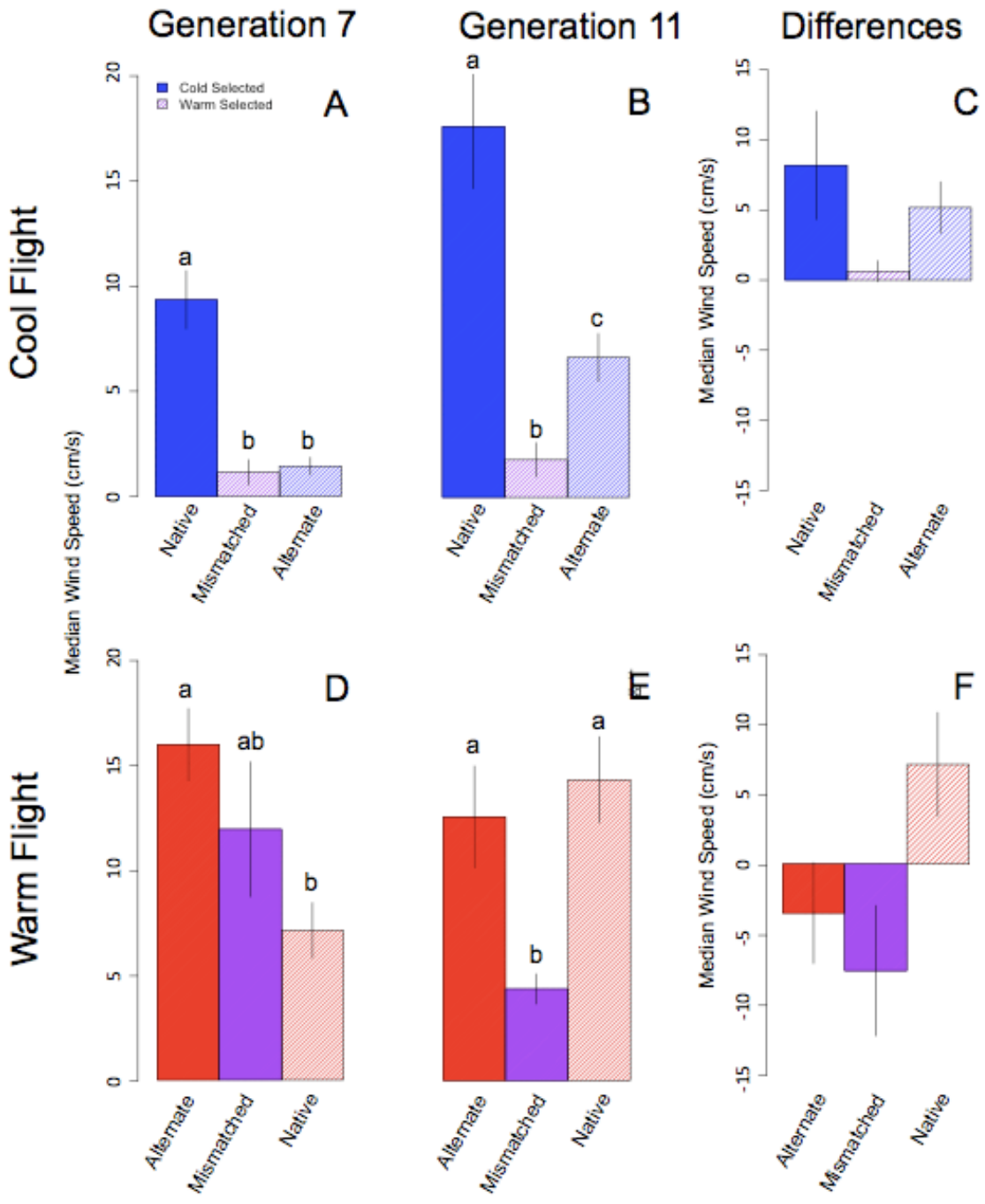
Figure 4.1. Evolution of Plasticity and Cost of Phenotype-Environment Mismatch: Flight performance within lineages selected for Cool and Warm flight performance at generations 7 and 11 with sexes pooled. Each colored bar is the mean headwind speed that 50% of the tested population achieved (WS_{50}) for four replicate selected lineages. Open bars are the mean of two replicate control lineages except for Warm-selected Generation 11 in which only one control was used. Lines indicate standard error and lower case letters indicate significant differences within panel. The flight performance of the founding population is displayed in black for reference. In instances where no bar is visible, the mean WS_{50} was measured as 0 cm/s. Cool- and Warm-selected lineages were assayed separately as generation times differ significantly between the treatments. F0 Native treatments were assayed on the same day, prior to a response to selection.


Cool (16°C) Selected
Warm (27°C) Selected
Generation 7
Generation 11
Median Wind Speed (cm/s)
A
B
C
D
a
ab
b
a
a
b
a
b
a
b
c
F0 Native
Native
Mismatched
Alternate
F0 Native
Alternate
Mismatched
Native
Flight treatment group

4.4.4 Comparing evolved performance of Cool- and Warm-selected flies

To determine if selection at a relatively challenging Cool temperature resulted in stronger fliers than selection at a relatively permissive Warm temperature, I compared flight performance of Native Treatment flies to Alternate and Mismatched Treatments across selective temperatures (Fig. 2; α=0.0062). Such a determination was made based on the performance of Cool-selected flies relative to Warm-selected flies across treatments. I found the Cool-selected Native Treatment flies outperformed the Warm-selected Alternate (t=5.46, d.f.=8.28, $p=2.7x10^{-4}$) and Mismatched (t=5.42, d.f.=9.57, $p=1.7x10^{-4}$) Treatment flies at generation 7 (Fig. 4.2A) and generation 11 (Alternate: t=3.47, d.f.=9.07, p=0.0035; Mismatch: t=5.18, d.f.=8.07, $p=4.1x10^{-4}$; Fig. 4.2B). The flight performance of Warm-selected Native Treatment flies was lower than Cool-selected Alternate Treatment flies (t=4.07, d.f.=13.1, $p=6.4x10^{-4}$), but did not differ significantly from performance of Cool-selected Mismatched Treatment (t=1.38, d.f.=9.30, p=0.09921) (Fig. 4.2D). By generation 11 (Fig. 4.2E), flight performance of Warm-selected Native Treatment flies increased such that it no longer differed significantly from Cool-selected Alternate Treatment flies (t=-0.558, d.f.=13.6, p-value=0.293), and was then significantly greater than that of the Cool-selected Mismatched Treatment flies (t=-4.59, d.f.=8.69, p-value=0.0014). Ultimately, these results demonstrate that while both Cool- and Warm-selected lineages exhibit phenotype-environment matching at generation 11, Cool-selected flies express greater flight performance and thus a stronger degree of adaptation.

Figure 4.2. Comparison of flight performance of Cool and Warm-flight selected lineages. Comparisons of flight performance between Cool- and Warm-selected lines based on flight temperature by generation with sexes pooled. Each colored bar is the mean headwind speed that 50% of the tested population achieved (WS_{50}) among four replicate selected lineages. Lines indicate standard error and lower case letters indicate significant differences. Panels (C & F) illustrate the evolved difference in flight performance between generations 7 and 11.


Generation 7
Generation 11
Differences
Cool Flight
Warm Flight
Cold Selected
Warm Selected
A
B
C
D
E
F
Median Wind Speed (cm/s)
Native
Mismatched
Alternate
Flight Treatment Group

4.5 Discussion

4.5.1 Overview

Rearing flies at their flight temperature facilitated evolution of upwind flight performance (Chapter 3). Here, I sought to determine how this adaptation occurred. I tested flight performance of flies following 7 and 11 generations of selection for upwind flight performance at Cool or Warm temperatures. I reared the evolved flies at their Native and Alternate temperatures, and compared their flight performances to determine if flies evolved a specialist or a strong generalist phenotype. To differentiate between the evolution of constitutively expressed flight-facilitating traits and phenotype expression dependent on development temperature, I compared performance of Mismatched Treatments to the Native and Alternate Treatments within each selected temperature. Finally, to determine if selection at a relatively challenging Cool-temperature resulted in stronger fliers than selection at a relatively permissive Warm temperature, I compared Native Treatments to Mismatched and Alternate Treatments across selected lineages. Patterns of performance at generation 7 were consistent with predictions of genetic assimilation, as flight phenotypes appeared to be constitutively expressed. As selection continued, however, both Cool- and Warm-selected flies exhibited a pattern of phenotype-environment matching by generation 11, indicating that the traits responding to selection changed over time. Flight performance of Cool-selected flies was generally greater than Warm-selected flies, indicating a stronger response to selection in the challenging Cool-temperature. Together, these data show genetic accommodation can maintain and improve phenotype-environment matching in a polygenic, ecologically relevant trait.

4.5.2 Shifting targets of genetic accommodation

Flight at Cool temperatures is challenging, and relatively few individuals can generate and sustain flight at or below 16^{o}C prior to selection (Frazier et al. 2008; Chapter 2; Fig. 2.1A,C). Traits that enhanced upwind flight performance in the flight-challenging Cool temperature may be more likely to improve flight performance across environments than those evolved at the flight-permissive Warm environment. My data support this hypothesis as the Cool-selected lines exhibited nearly twice the flight performance when developed and flown at Warm temperature than the Warm-selected lines at generation 7, and did not differ significantly from Warm-selected lines at generation 11. Warm-selected lineages, however, remained poor fliers at Cool temperature at generation 7, and although performance increased by generation 11 at Cool temperature, it remained significantly less than that of Cool-selected flies.

The differing responses of Cool- and Warm-selected flies might be due to differences in flight-related physiology. Wing beat frequency exhibits a negative association with temperature (Curtsinger and Laurie-ahlberg 1981; Barnes and Laurie-Ahlberg 1986), but the square of wing beat frequency is directly proportional to lift production, the force perpendicular to the direction traveled that is typically associated with countering weight (Ellington 1984). Therefore, increasing wing beat frequency to improve Cool flight could also improve Warm flight by generally increasing lift. Additionally, flies selected in the absence of a headwind for 11 generations to select for increased motivation toward stimuli exhibited no difference in performance when tested with and without wind (Chapter 3). This finding indicates the increased performance across temperatures, particularly at generation 7, may result from increased attraction to stimuli, or perhaps a greater propensity for ambient movement.

The Warm-selected lines exhibited enhanced flight performance by generation 7 without significant increases in flight performance at Cool temperature, regardless of their development temperature. This pattern would be expected if traits which facilitate flight in a permissive Warm temperature become genetically assimilated and their expression was detrimental or neutral to performance in Cool temperatures, or alternatively, if adaptations for Warm-flight are only expressed by development in Warm temperature. Regardless, selection for Warm flight performance resulted in a specialist phenotype for Warm temperature at generation 7. The differing response in Cool- and Warm-selected flies indicates the initial response to selection for flight performance in Cool and Warm temperatures likely involved different traits or different patterns of contribution by the same traits. By generation 11, Warm-selected flies increased flight performance at Cool temperature when development temperature matched assay temperature, a pattern consistent with phenotype-environment matching. Morphology of Warm-selected lines did not evolve in a manner predicted to enhance Cool-flight based on natural geographic patterns (Chapter 3). Flies selected for progression through the wind tunnel without a headwind at Warm temperature did not exhibit the same increases in motivation as those selected at Cool temperature (Chapter 3), thus the increased performance at Cool temperature is unlikely the result of increased motivation. Unfortunately, I was unable to identify the specific traits targeted by selection, although the traits are likely to be physiological.

4.5.3 Addressing criticisms of genetic accommodation

Two of the primary criticisms of adaptive evolution by genetic accommodation is that most examples of genetic accommodation involve “phenotypic deviants” with unknown

ecological relevance, and that a “lack of convincing examples” exist, more specifically examples of traits that naturally exhibit continuous phenotypic variation (De Jong 2005). *Drosophila* spp are globally distributed and exhibits convergent clines in flight morphology (Imasheva et al. 1994; James et al. 1997; Azevedo et al. 1998; Gilchrist and Huey 2004; Liefting et al. 2009; Bhan et al. 2014) and consistent patterns of phenotypic plasticity in flight morphology (e.g., James et al. 1997; French et al. 1998; Gilchrist and Huey 2004; Frazier et al. 2008). *Drosophila* are highly plastic organisms, exhibiting phenotypic plasticity in not just morphological measures described above, but also many physiological traits (e.g., development time: James and Partridge 1995; wing beat frequency: Barnes and Laurie-Ahlberg 1986; Frazier et al. 2008; and protein expression: Robinson et al. 2010). Thus, the phenotypically plastic traits that responded to selection, and evolved by genetic accommodation, are likely numerous and may underlie the generation of diversity in this globally distributed taxa. Thermally-induced phenotypic plasticity in flight morphology is purported to be adaptive and enhance flight performance at local temperatures (Ayala et al. 1989; Azevedo et al. 1998; Huey et al. 2000; Frazier et al. 2008) by increasing wing area at low temperatures to compensate for low wing beat frequencies at low temperatures (Reed et al. 1942; Ellington 1984; Frazier et al. 2008). Locomotor performance is of ecological relevance for mobile organisms, and the adaptive increase in upwind flight performance was facilitated by genetic accommodation. Work remains, however, to identify the specific morphological, physiological, or behavioral traits that underlie this adaptation.

4.5.4 Perspective and future directions

This study is the first to demonstrate genetic accommodation in a complex phenotype with clear ecological relevance. Most direct investigations genetic accommodation focus

on a particular stress-induced morphological trait of unclear ecological importance (e.g., Waddington 1953, 1956; Bateman 1957; Sollars et al. 2003), or mutations of large effect (Suzuki and Nijhout 2006). While this approach provides powerful tests demonstrating proof of concept, such narrow focus may be biased toward finding genetic assimilation, as the traits selected for increased expression are initially rare by design and are exclusively morphological phenotypes. Here, I selected on flight performance, a complex phenotype which results from the interactions of multiple morphological, physiological, and behavioral traits, permitting "multiple adaptable solutions" (Garland et al. 2011; Careau and Garland 2012). Investigators of genetic accommodation, particularly in regard to origination of novelty, may benefit from focusing on such traits that allow for more evolutionary outcomes (West-Eberhard 2005). While my work captured adaptive evolution and associated costs in real time, further empirical work is necessary to determine the extent to which adaptation occurs by genetic accommodation in the wild. Adaptive evolution by genetic accommodation may well be responsible for the convergent morphological clines and documented adaptive radiations of *Drosophila.*

4.5.5 Summary

In this study, I demonstrated the role of genetic accommodation in facilitating the adaptive evolution of flight performance across temperatures. The patterns in flight performance were dynamic, exhibiting dramatic and temperature-dependent differences between generation 7 and 11 in the response to selection. Cool-selected flies initially developed into a strong generalist flight phenotype, but as Cool-flight performance continued to respond to selection, the pattern evolved a pattern of phenotype-environment matching. Conversely, Warm-selected flies were specialized for Warm-flight by generation 7, before exhibiting adaptive phenotype-environment matching by

generation 11. Efforts to identify specific traits responding to selection are underway and will likely further our understanding of the mechanisms underlying genetic accommodation. A focus on polygenic, ecologically relevant complex traits such as flight performance, rather than uncommon stress-induced phenotypes, will aid future investigations of adaptation by genetic accommodation by allowing for more dynamic responses to selection. Such studies are needed, as increased empirical evidence of ecologically important traits would address some of the major criticisms of a likely widespread evolutionary process.

CHAPTER 5. DOES ADAPTIVE PHENOTYPE-ENVIRONMENT MATCHING IN *DROSOPHILA* FLIGHT PERFORMANCE ENHANCE SURVIVAL IN PRESENCE OF PREDATORS?

5.1 Abstract

Adaptive phenotypic plasticity results when individuals express one of many alternative phenotypes that convey the highest fitness in the local environment. The resultant pattern of covariation between phenotype expression and environment is called adaptive phenotype-environment matching. Deviations from this pattern, or phenotype-environment mismatching, are maladaptive and reduce fitness. *Drosophila melanogaster* exhibit convergent, genetically-based clines and thermally-induced phenotypic plasticity in relative wing size. These patterns are widely believed to be adaptive, although few data explicitly test their effects on fitness. Here, I test for phenotype-environment matching in wing phenotype expression in *D. melanogaster* across thermal environments in the presence of two different predators with differing hunting styles. I found little support for adaptive phenotype-environment matching; across all temperatures, flies developed at cool temperatures exhibit greater mortality in the presence of predators than flies developed at warm temperatures. Mortality patterns exhibited by flies developed at warm temperatures are more complex, and depend on both predator and thermal environment. I found little support for the idea that the geographic patterns and phenotypic plasticity in *D. melanogaster* flight morphology enhance survival in the presence of predators. My data thus suggest these patterns are best explained by the action of other selective agents on performance.

5.2 Introduction

Phenotype-environment matching is the special case of adaptive phenotypic plasticity in which phenotypes induced by developmental environment confer greater relative fitness than alternative inducible phenotypes (see for reviews: Moran 1992; Dewitt et al. 1998; West-Eberhard 2005; Moczek et al. 2011). While conceptually similar to the Beneficial Acclimation Hypothesis which predominately describes facultative physiological responses, particularly those within a given life stage (Wilson and Franklin 2002; Deere and Chown 2006), phenotype-environment matching typically refers to non-reversible morphological plasticity. While there are many examples of phenotype-environment matching (e.g., Krueger and Dodson 1981; Lively 1986; Pfennig 1990; Brakefield et al. 1996), a number of examples exist that contradict this conceptually intuitive pattern (e.g., Berven et al. 1979; Leroi et al. 1994; Conover and Schultz 1995; Sibly et al. 1997; Gibert et al. 2001). It is therefore imperative that the relationships among morphological and fitness variation be determined across environments explicitly in cases of putative adaptive plastic responses (Huey et al. 1999).

Morphological variation generates variation in ecological performance, which in turn produces variation in fitness (Arnold 1983). Thus, selection acts on morphological variation, at least in part, based on how morphological traits affect the execution of ecologically relevant tasks (Losos 1990; Brodie 1992; Garland and Losos 1994; Adams and Rohlf 2000). Ecological function favors proper scaling of traits across the range of body size (Emlen and Nijhout 2000; Kawano 2000), presumably as a result of correlational selection on joint trait values for proper function (Brodie 1992; Frankino et al. 2009; Kawano et al. 2012). The resulting patterns of strong covariation make assessing the fitness contribution of variation in individual traits difficult. However,

temporal variation in the environment (Moran 1992) coupled with temporal variation in sensitivity among traits to environmental cues (Shingleton et al. 2007; Baranzelli et al. 2014), can be used as an experimental tool to enhance the range of phenotypic variance in plastic traits. This experimentally enhanced phenotypic plasticity can be used to tease apart the contribution of individual traits to performance, and ultimately fitness.

Drosophila body size is determined late in larval ontogeny, following the gut purge before pupation (Shingleton et al. 2007). Wing size, however is determined during wing imaginal disk growth, which begins in earnest just before and continues through the pupal stage (Madhavan and Schneiderman 1977; Shingleton et al. 2008). Thus, thermal variation during this period affects wing size independently of body size. Therefore, manipulating temperature during the pupal stage affects wing size after body size has been determined, providing a way to experimentally alter wing size independently from body size. *Drosophila* spp. are thus well suited as a model for studying adaptive phenotype-environment matching because their body size, relative wing size, and wing shape (i) are easily quantified (e.g., Robertson 1959; Weber 1990; Houle et al. 2003; Mezey and Houle 2005; Stillwell et al. 2011) and manipulated experimentally (e.g., James et al. 1997; Frazier et al. 2008), (ii) likely have many fitness effects (e.g., Ewing 1964; Hoffmann et al. 2007; Menezes et al. 2013), (iii) exhibit genetically-based clines (e.g., Imasheva et al. 1994; James et al. 1997; Azevedo et al. 1998; Gilchrist and Huey 2004; Liefting et al. 2009; Pitchers et al. 2013; Bhan et al. 2014), (iv) exhibit thermally-induced phenotypic plasticity within populations (e.g., James et al. 1997; French et al. 1998; Debat et al. 2003; Gilchrist and Huey 2004; Frazier et al. 2008), and finally, (v) these geographic and plastic patterns are similar to those found in many holometabolous

insects (e.g., antlion, Scharf et al. 2009; speckled wood butterfly, Vandewoestijne and Van Dyck 2011).

Whether the result of genetic differentiation among populations or thermally-induced plastic responses within populations, *D. melanogaster* that develop at Cool temperatures have large bodies and wings that are larger relative to the body (i.e., have lower wing loading) as compared to flies developed in warm temperatures (e.g., James et al. 1997; French et al. 1998; Gilchrist and Huey 2004; Frazier et al. 2008). This ubiquitous pattern of phenotype-environment matching of flight related morphology enhances local flight performance (Frazier et al 2008; Chapter 2), and is thus predicted to enhance fitness. In fact, the convergent geographic clines and thermally-induced phenotypic plasticity in flight-related morphology of *Drosophila* is widely cited as evidence for adaptation to flight in different thermal environments (e.g., David and Capy 1988; Ayala et al. 1989; James et al. 1997; Azevedo et al. 1998; Gilchrist et al. 2001; Gilchrist and Huey 2004; Dillon and Frazier 2006; Frazier et al. 2008). Unfortunately, data addressing this hypothesis are scant; tests explicitly determining the fitness effects of variation in relative wing size in *Drosophila* spp. are few and deal mainly with phenotypic effects on dispersal (e.g., Hoffmann et al. 2007; Bhan et al. 2014), take off performance (e.g., Dillon and Frazier 2006; Frazier et al. 2008), or sexual selection (e.g., Ewing 1964; Sisodia and Singh 2004; Menezes et al. 2013).

The effect of variation in *Drosophila* relative wing size on survival in the presence of predators remains to be determined (but see DeNieu et al. 2014, *in press*). Numerous studies show adaptive plasticity resulting directly from predator-based cues in developmental environment (Krueger and Dodson 1981; Lively 1986; DeWitt 1998; Auld

and Relyea 2010). Here I am testing for a more indirect relationship. The environmental cue of interest is temperature during development, which is not in itself a direct indicator of predator type or quantity. The prediction is straightforward. Phenotype-environment matched morphology enhances local flight performance, and generally increased flight performance should improve ability to escape or avoid predators.

Here, I conduct a series of experiments to determine if thermally-induced phenotypic plasticity in relative wing size and wing shape affects survival of *D. melanogaster* in the presence of predators in a thermally dependent manner. I assayed survival in presence of the terrestrial hunting jumping spider, *Anasaitis canosa*, and the aerially hunting *Scathophaga stercoraria*. Phenotype-environment matching was predicted, where fly survival will be greatest at their flight morphology-inducing temperature. The timed development manipulation of wing phenotype expression described here, allowed experimental decoupling of body and wing morphology phenotypes. The increased variation in relative wing size enables the testing for independent contributions of body size, wing size and wing shape to survival across temperatures.

5.3 Materials and methods

5.3.1 Overview

In this study, I manipulated the temperatures of larval and pupal development to produce three qualitatively distinct flight-morphology groups that vary in their wing loading (WL), referred to as WL Phenotype Class throughout. Each WL Phenotype Class was experimentally produced by developing larvae at a moderate (21^{o}C) temperature, and then transferring pupae to either Cold (15^{o}C), Moderate (21^{o}C), or Warm (27^{o}C) temperatures. The resulting WL Phenotype Classes are referred to as Low, Mid, and

High WL Phenotype Classes, respectively. Mixed populations of males from these WL Phenotype Classes were subjected to predation imposed by either *A. canosa* (twin-flagged jumping spider) or *S. stercoraria* (yellow dung fly). Predation assays were conducted at four temperatures encompassing developmental temperatures used to produce each of three WL Phenotype Classes in a 4x3 factorial design. The temperature at which predation occurred is referred to as Predation Temperature throughout. A Cool (18^{o}C) Predation Temperature was added as 15^{o}C approaches the lower bound of flight ability in *D. melanogaster* (Lehmann 1999), whereas 18^{o}C is flight-permissive, albeit challenging (Frazier *et al*; 2008). The relative fitness of each WL Phenotype Class was estimated from their mortality in presence of each predator type. I predicted phenotypic plasticity to be adaptive and exhibit phenotype-environment matching such that fly mortality would be lowest for the WL Phenotype Class developed under conditions most similar to the Predation Temperature.

5.3.2 Experimental D. melanogaster prey populations

Flies used were derived from a natural population of *D. melanogaster*, originally collected at Fenn Valley Vineyards (Fennville, Michigan GPS coordinates: 42.578919, -86.144936) in 2010 (for full details, Parigi et al*., in prep*). My culture of this stock population is maintained at more than 2,000 individuals per generation reared at a variety of temperatures and food densities to resist adaptation to a single lab environment (Chapter 1). For each experiment, eggs were collected off 25% grape juice-agar plates, and placed in cohorts of 50 into vials containing 7ml of fly food until pupal transfer, described below. This density minimizes effects of larval competition (Shingleton et al. 2007; Myers and Frankino 2012). The duration of the pupal stage is inversely related to temperature. To decrease variation in post-eclosion age, egg

collection was staggered such that eggs of the Low and Mid WL Phenotype Classes were collected 10 and 4 d prior to eggs of the High WL Phenotype Class flies. This produced WL Phenotype Classes of the same age on a given trial day. Prey populations consisted of only 3-7 day old *D. melanogaster* males to guard against effects of female mass changes resulting from egg development or oviposition, which would alter wing loading during the Predation Trials.

5.3.3 Predators

Two predators with different hunting styles were used to test the generality of the effect of flight performance on fitness. *A. canosa*, is a terrestrial, stalk and ambush predator that specializes in hunting terrestrial invertebrates such as ants (Jackson and Olphen 1991). These spiders exhibit the typical hunting behavior of saltacids that hunt flies in the wild, e.g. *Phidipus sp.* (Jackson and Pollard 1996); They approach and leap on a fly from any direction and typically puncture the fly on the dorsal thorax near wing base (laboratory observations). My second predator used was the yellow dung fly, *S. stercoraria*, which hunts on the wing. These predatory flies feed preferentially on Diptera roughly half their size (Failes et al. 1992). Both predators were maintained on diets consisting of mostly of flightless (*Curly*), white-eye (*W1118/Fm7*) *D. melanogaster* mutants and established successful breeding laboratory colonies, indicating *D. melanogaster* were sufficient in nutrition and a viable target for predation. Access to flight capable *D. melanogaster* was limited prior to trials to avoid predator training.

A. canosa were collected from within the city of Houston, TX USA (N 29° 43' 17.3964", W 95° 20' 35.4978") and maintained individually in the laboratory on a diet of ants (*Solenopsis invicta*) and the mutant *D. melanogaster* described above. I constructed

size-matched male-female spider dyads for use in each Predation Trial to reduce the probability of cannibalism (laboratory observation). To motivate spiders to hunt, they were not fed for two weeks prior to a predation trial.

S. stercoraria were collected near Syracuse NY, USA and used to establish a lab colony, maintained in 120 ml vials at 22^{o}C on a diet of mutant *D. melanogaster* (described above) and occasionally supplemented with *D. pseudoobscura and D. persimilis*. In these small rearing vials, flight is minimized for both predator and prey. Thus, my rearing regime likely did not provide training for predators in how to effectively hunt *D. melanogaster* during flight in an open arena. Pairs of sexually immature male *S. stercoraria* were used in each Predation Trial as they have higher prey consumption rates than females or sexually mature males (Failes et al. 1992). Additionally, males can relentlessly attempt mating and often mate-guard upon success, reducing predator-prey encounter rates in mixed-sex predator dyads. Due to lack of availability of males, however, pairs of female *S. stercoraria* were used in the 18^{o}C predation treatment as described below. I were unable to conduct trials with this predator at 27^{o}C due to its high mortality above 25^{o}C (Blanckenhorn 1998).

5.3.4 Effects of developmental temperature on morphology

To determine the morphological effects of development temperature and my developmentally timed thermal manipulation of phenotype expression, 600 eggs (200/WL Phenotype Class) were collected from the colony and divided them into two groups: Constant or Shifted Temperature of development. Constant Temperature eggs were reared to adulthood at one of three temperatures (15, 21, or 27^{o}C), producing the typical pattern of covariation in wing to body size. Shifted Temperature flies were

subjected to a developmentally-timed thermal treatment at the pupal stage (Shingleton et al. 2007). Late in the 3rd larval instar, larvae will cease feeding and exit food substrate for pupation. Vials of developing larvae were checked twice daily for these crawling larvae. Fly larvae were gently transferred using a damp paintbrush from larval-food vials held at 21°C to fresh food vials held at 15, 21 or 27°C. To estimate the wing:body scaling relationships, larvae were instead housed individually with unique numerical identifiers until eclosion at which time wings were imaged. Larvae that had not left food, or had already formed pupal casing were not transferred. This manipulation allows for temperature to effect wing size after flies achieve final body size.

Wing:body size scaling was estimated from live, intact flies (Stillwell et al. 2011). Pupae were imaged using a Leica MZ125 microscope attached to a Leica DFC290 digital camera and computer. Once eclosed, the adult wings were pressed between two pieces of glass using a vacuum powered 'wing grabber' and imaged similarly (Weber 1988; Houle et al. 2003; Stillwell et al. 2011). Custom software that works within ImagePro (6.2) automatically calculates the areas of the pupa and the wing as the pixel count of their silhouettes and is available upon request. Major axis regression (MAR) is used to describe the relationship between the log transformed size data for each treatment/sex combination (Frankino et al. 2009). Univariate means of body size and wing size were compared using t.test in R (R Core Team 2013).

5.3.5 Predation trials

Five replicate predation cages were set up for each Predation Trial. Prey populations for each replicate Predation Trial consisted of 20 flies from each WL Phenotype Class (60 flies/replicate) produced via the Shifted Temperature manipulation described above.

Each WL Phenotype Class was marked with minute amounts of a WL Phenotype Class-specific fluorescent powder on the ventral thorax and the articulating coxa. These marks enabled identification of WL Phenotype Class during each census. Colors (green, orange, or pink) were unique to a WL Phenotype Class within a replicate, but distributed across replicates such that WL Phenotype Class/color combinations were equally represented. *D. melanogaster* were marked at least 24 h prior to start of each Predation Trial; during the intervening 24 h, the flies remove powder from all but the deepest crevices of their joints and the articulation between coxa and thorax; the remaining trace powder is visible only under a microscope for ~7 days (*personal observation*).

Due to the visual acuity of jumping spiders, a preliminary test was conducted in which 6 replicates of 60 flies developed at 22^{o}C were divided into 3 groups of 20 and marked with fluorescent powder as described above. One-way ANOVA on mortality with powder color as a main effect revealed no significant effect of color after 48 h of exposure to *A. canosa* ($F_{1,16}$=0.591, p=0.453). Subsequently, to test if flourescent marking had an effect on mortality in presence of predator relative to unmarked flies. I tested 3 replicates of 25 orange-marked and 25 unmarked *D. melanogaster* in presence of *A. canosa* and identified no significant difference in mortality (t-test: d.f.=3.67, t=2.01, p=0.121). Hence I am confident that neither marking in general nor specific colors affected the results.

Mixed prey populations composed of 20 marked flies of each WL Phenotype Class were released into white nylon 0.3m^3 Bugdorm^{TM} cages containing three juice plates with yeast paste 1 h before the start of a Predation Trial. For *S. stercoraria* Predation Trials, small cotton balls soaked in 10% sucrose was also added to cages to prevent desiccation of the predators. Predators were placed under an inverted 50 mm diameter x

15 mm petri dish and allowed to acclimate to test environments for 1 h. Following this acclimation period the petri dish was removed and trials began. All treatments began at 13:00, 4 h after the start of the light cycle, and lasted 48 h at 12:12 L:D.

5.3.6 Experiment I: mortality by WL Phenotype Class

The goal of this Experiment was to determine if phenotype-environment matching of morphology to temperature confers enhanced relative fitness measured as survival in presence of predators. Prey populations were censused three times during each 24 h cycle: at dawn (0800), mid-day (1300), and dusk (1900). At each census, predators were removed briefly from cages. Dead flies were aspirated from the cage and viewed under a microscope to identify the WL Phenotype Class via the florescent powder traces and to verify that the fly had died as a result of predation. To guard against frequency-dependent effects on WL Phenotype Class-specific mortality, each dead fly was replaced with a live, marked male from the same WL Phenotype Class. Once the dead flies had been replaced, predators were released back into the cages as before, only without the 1 h acclimation period. Census of each replicate took *ca* 30 min. Mortality curves did not diverge or cross (see Supplemental Materials). Thus, only mortality at the end of the experiment was analyzed, as mortality rates appear to be constant through the experiment.

5.3.7 Experiment II: multivariate selection on morphology

An additional Experiment (II) was conducted to quantify pattern and intensity of selection on wing size, body size and wing shape. The experimental design was very similar to that in Experiment I, however, the right wing from each fly was imaged and wing shape quantified prior to each Predation Trial and dead flies were not replaced at each census.

Surviving flies were collected following 48 h of selection and quantified their wing shapes a second time to identify individuals by “wingerprinting”. Subtle variation in wing shape among individuals in these defined populations allowed the identification of flies that survived, and by subtraction those that did not from the pool of the starting population of flies. From these data, estimates of the selection imposed on these morphological traits were made independently. Detailed methods are provided in the Supplemental Materials.

5.3.8 Analyses

To test for adaptive phenotype-environment matching, I fit a generalized linear model of the form (Mortality ~ WL Phenotype Class*Predation Temperature) to test for effects of Predation Temperature, WL Phenotype Class and their interaction on the total fly mortality within each predator type. There was no significant effect of the body:wing size interaction or quadratic selection effects and thus they were dropped from the model. Mortality counts were distributed normally (Shapiro-Wilks: W=0.975, p=0.258). To identify differences in mortality among WL Phenotype Classes within Predation Temperature, I used Tukey HSD test for post-hoc multiple comparisons. Tests were followed with one-way ANOVA on mortality within each Predation Temperature with WL Phenotype Class as the main effect. When significant effects were detected, ANOVA was followed with Tukey HSD to determine differences among WL Phenotype Classes. All tests were performed in R statistical software (R Core Team 2013).

5.4 Results

5.4.1 Effects of developmental temperature on morphology

Manipulations of developmental temperature affected mean body size, wing size, and pattern of covariation between these traits (Fig. 5.1). Body size and wing size of male flies reared under my Constant Development Treatment exhibit a negative relationship with temperature, a pattern typical of natural populations. Body size differed between Experiments in the expected direction; my Shifted Temperature treatment decreased body size of Low WL Phenotype Class (Constant: x=0.421, SD=0.022; Shifted: x=0.400, SD=0.023; t=-5.90, d.f.=47.6, $p=3.67x10^{-7}$), and increased body size of High WL Phenotype Class (Constant: x=0.312, SD=0.024; Shifted: x=0.372, SD=0.027; t=5.00, d.f.=47.7, $p=8.18x10^{-6}$) after controlling for changes in body size between Mid WL Phenotype Classes in Shifted and Constant Development Treatments. Wing size did not differ between Low WL Phenotype Classes (Constant: x=0.236, SD=0.025; Shifted: x=0.238, SD=0.021; t=-1.70, d.f.=47.6, p=0.096), although did significantly increase in the High WL Phenotype Class (Constant: x=0.051, SD=0.025; Shifted: x=0.132, SD=0.024; t=6.46, d.f.=54.5, $p=2.98x10^{-8}$) after controlling for changes between Mid WL Phenotype Classes. Despite this increase in wing size, it remained significantly smaller than the Mid WL Phenotype Class (Mid WL Phenotype Class: x=0.217, SD=0.022; t=-3.97, d.f.=45.8, $p=2.5x10^{-4}$). Although the slope describing the scaling relationship fit to the bivariate means did not quite differ significantly (ANCOVA: $F_{1,5=}13.462$, p=0.067), the manipulation did significantly increase relative wing size in Low WL Phenotype Class (Constant: x=0.619, SD=0.032; Shifted: x=0.688, SD=0.032: d.f.=54.4, t=8.41, $p=1.0x10^{-11}$) and relative wing size in High WL Phenotype Class (Constant: x=0.514, SD=0.021; Shifted: x=0.576, SD=0.021: d.f.=75.9, t=13.0, $p=2.2x10^{-16}$). Despite this increase in relative wing size in both WL Phenotype Classes, variance in relative wing size

increased by 20.7% (Constant: $\sigma^2=2.55x10^{-3}$; Shifted: $\sigma^2=3.09x10^{-3}$), my analyses mostly failed to detect significant selection gradients on morphological traits, methods and results for Experiment 2 are provided in detail in the Supplemental Materials.

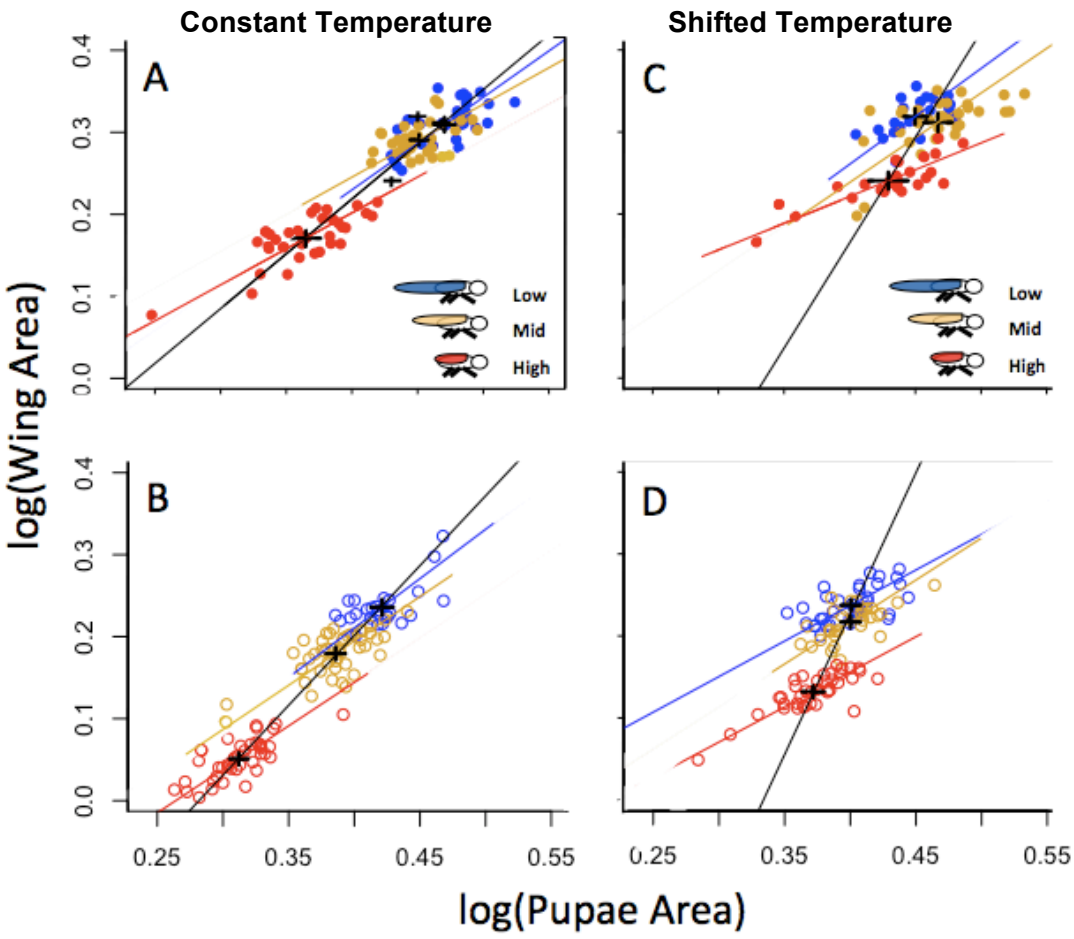


Fig. 5.1: Thermal plasticity of relative wing size. Manipulating temperature at pupal stage affects wing size far more than body size, producing three distinct WL Phenotype Classes. (A & B) Constant Temperature flies were reared from pupae to adult under constant thermal environment. (C & D) Shifted Temperature flies were developed from egg up to pupal stage at 21^{o}C before being moved to 15, 21 or 27^{o}C to increase variation in relative wing size. Circles are colored according to temperature of pupation; blue (15^{o}C), yellow (21^{o}C) and red (27^{o}C). Lines indicate wing-body size scaling (MAR) for each sex at each temperature. Black crosses indicate bivariate mean within development-temperature groups with 95% confidence intervals. Black lines indicate the MAR for all three rearing classes pooled, For Constant development temperature, female (A) slope=1.33 and male (B) slope= 1.70. For Shifted development temperature, female (C) slope=2.57 and male (D) slope=1.14.

5.4.2 Mortality by WL Phenotype Class: predation by A. canosa

ANOVA revealed significant effects of WL Phenotype Class and Predation Temperature, but not their interaction, on mortality in the presence of the terrestrially hunting spider (Fig. 5.2A; Table 1), thus failing to support adaptive phenotype-environment matching. Post-hoc Tukey HSD test on mortality indicated that flies of Low WL Phenotype Class (i.e., low wing loading) had significantly higher mortality rates than those from the Mid (diff=-4.55, CI[-7.52, -1.58], p=0.0016) and High (diff=3.65, CI[0.676, 6.62], p=0.013) WL Phenotype Classes (Fig 5.2B). There was no significant difference in mortality between flies between Mid and High WL Phenotype Class (diff=-0.90, CI[-3.87, 2.07], p=0.746), indicating a significant fitness cost of low wing loading in the presence of *A. canosa*. For Predation Temperatures, Tukey HSD test revealed Cold Predation Temperatures were significantly lower than Moderate (Diff=5.20, CI[1.42, 8.98], p=0.0033) and nearly significant from Cool (Diff=3.47, CI[-0.313, 7.25], p=0.083) Predation Temperatures. No other Predation Temperatures differed significantly in their mortality (p's ≥ 0.149).

Table 5.1. ANOVA of morphological and environmental effects on *D. melanogaster* survival in presence of *A. canosa.*

	d.f.	Sum Sq.	Mean Sq.	F-value	p-value
Phenotype Class	2	232.2	116.1	7.677	0.0013
Predation Temperature	3	210.7	70.22	4.643	0.0063
Phen Class:Pred Temp	6	66.80	11.14	0.736	0.6228
Residuals	48	726.0	15.13		

Table 5.2. ANOVA of morphological and environmental effects on *D. melanogaster* survival in presence of *S. stercoraria.*

	d.f.	Sum Sq.	Mean Sq.	F-value	p-value
Phenotype Class	2	218.0	109.0	5.129	0.0115
Predation Temperature	2	145.5	72.77	3.423	0.0446
Phen Class:Pred Temp	4	161.4	40.35	1.898	0.1341
Residuals	33	701.5	21.26		

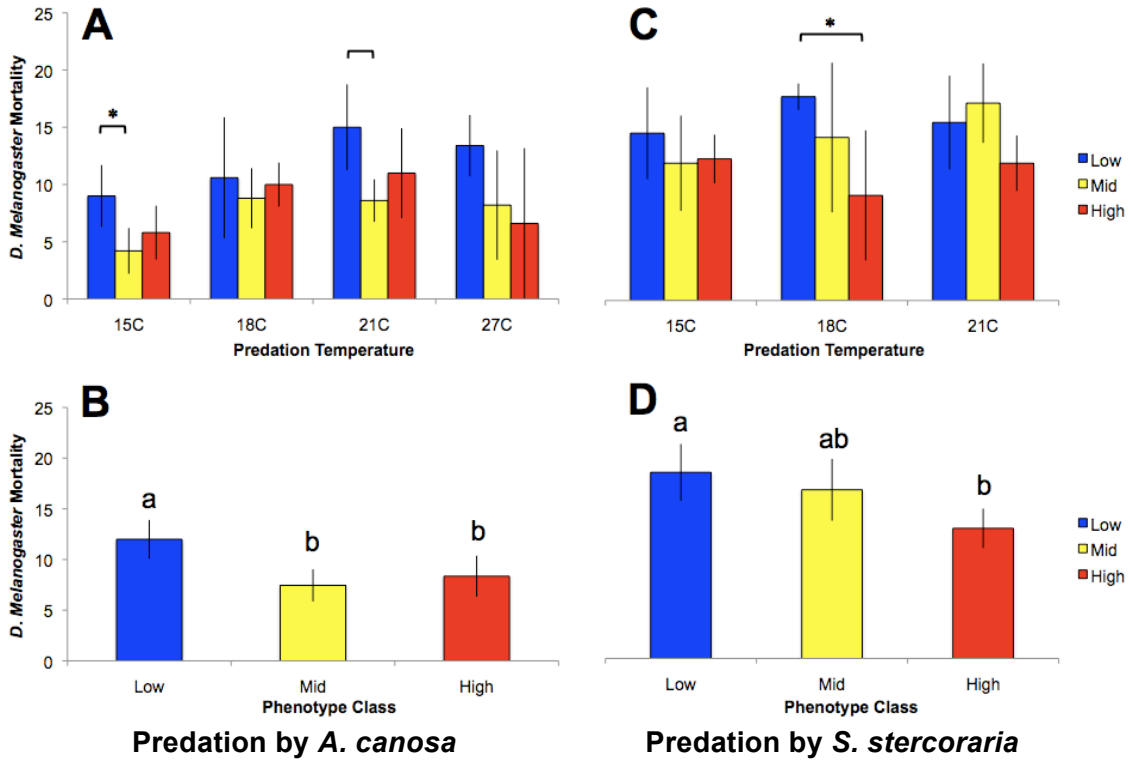


Fig. 5.2. Mortality of flies from three WL Phenotype Classes subject to predation by *A. canosa* and *S. stercoraria* at various temperatures. (A & C) Columns indicate mean mortality for each relative wing size class within a Predation Temperature; error bars represent 95% confidence intervals. Brackets with asterisks indicate statistically significant differences ($p<0.05$). Brackets without asterisk indicate differences near statistical significance ($p<0.06$). (B & D) Columns indicate average mortality across all flight environments, lowercase letters denote significant differences among groups ($p<1x10^{-5}$)

To examine patterns of phenotype-environment matching in mortality within Predation Temperature, one-way ANOVAs were performed on mortality with WL Phenotype Class as the main effect. There was a significant effect of WL Phenotype Class on mortality at the Cold temperature ($F_{2,12}$=4.09, p=0.044), and post-hoc tests revealed that Low WL Phenotype Class exhibit significantly greater mortality than Mid WL Phenotype Class (Diff=-4.8, CI[-9.36, -0.241], p=0.039), but not High WL Phenotype Class (Diff=3.2, CI[-1.36, 7.76], p=0.189). Mortality did not differ between Mid and High WL Phenotype Classes (Diff=-1.6, CI[-6.16, 2.96], p=0.629). There was no significant effect of WL Phenotype Class on mortality within Cool ($F_{2,12}$=0.586, p=0.572), Moderate ($F_{2,12}$=3.66, p=0.057), or Warm ($F_{2,12}$=1.99, p=0.179) Predation Temperatures.

5.4.3 Mortality by WL Phenotype Class: Predation by S. stercoraria:

In the presence of yellow dung flies, Predation Temperature and WL Phenotype Class significantly affected *D. melanogaster* mortality*,* although their interaction did not (Fig. 5.2B; Table 3). Within Predation Temperature, High WL Phenotype Class flies had significantly lower mortality than Low (Diff=5.50, CI[1.22, 9.78], p=0.0093) WL Phenotype Class, although the difference between Mid and Low flies (Diff=-1.93, CI[-6.20, 2.35], p=0.517) and Mid and High (Diff=3.57, CI[-0.704, 7.85], p=0.116) was not significant. Within Trial Temperatures, fly mortality was significantly lower at Cold than Cool Predation Temperature (Diff=4.67, CI[0.285, 9.05], p=0.035). The difference between Cold and Moderate (Diff=2.27, CI[-1.86, 6.40], p=0.380) or Cool and Moderate (Diff=-2.40, CI[-6.78, 1.98], p=0.382) Predation Temperatures was not significant.

One-way ANOVAs on mortality with WL Phenotype Class as main effect were performed to examine patterns of phenotype-environment matching within Predation Temperatures.

WL Phenotype Class did not significantly effect mortality at Cold ($F_{2,12}$=0.815, p=0.466) or Moderate ($F_{2,12}$=2.39, p=0.134) Predation Temperatures, but did have a significant effect at the Cool ($F_{2,9}$=4.428, p=0.046) Predation Temperature. Within Cool Predation Temperature, mortality of the Low WL Phenotype Class was significantly higher than the High WL Phenotype Class (Diff=11.5, CI[0.657, 22.3], p=0.038). Mortality did not differ between Low and Mid (Diff=-4.75, CI[-15.6, 6.09], p=0.470) or Mid and High (Diff=6.75, CI[-4.09, 17.6], p=0.244) WL Phenotype Classes.

5.5 Discussion

The putative adaptive nature of thermally-induced phenotypic plasticity of body size, wing size, and wing shape in *D. melanogaster* was assessed by determining if temperature-specific trait expression affected survival in the presence of terrestrially and aerially hunting predators across thermal environments. If the patterns of phenotype expression *Drosophila* exhibit across thermal gradients are adaptive for flight performance, then flies should exhibit a pattern of phenotype-environment matching in fitness. Thus, flies should have the highest survival, and lowest mortality, at Predation Temperatures closest to their phenotype-inducing Developmental Temperature. In addition to providing an empirical example of adaptive plasticity, such a finding would be consistent with the hypothesis that patterns of genetic differentiation among geographic populations of flies are adaptations for flight performance in different thermal environments (e.g., Ayala et al. 1989; Azevedo et al. 1998; Gilchrist and Huey 2004).

Contrary to predictions, relatively large-winged flies, i.e., those possessing morphology typical of populations throughout cool developmental temperatures or high latitudes, had the lowest survival at nearly all temperatures tested. This is particularly surprising at

lower temperatures where the Low WL Phenotype Class is more capable at generating flight than the other WL Phenotype Classes (Frazier et al. 2008; Chapter 2). Individuals with relatively small wings, i.e., those possessing morphology typical of populations throughout low latitudes and warm developmental temperatures, exhibit a more complex pattern of survival. Mortality of relatively small winged flies was generally lowest at warm temperatures, but the difference between Mid and High WL Phenotype Classes was not statistically significant (Fig. 5.2, Table 5.2). The inability to detect a significant difference is due in part to high variance in predation levels among predator pairs. Patterns of *D. melanogaster* mortality were similar in presence of both the aerially hunting *S. stercoraria* and terrestrially hunting *A. canosa*. I attribute the unexpected finding of poor survival in the Low WL Phenotype Class flies to (i) interactions between development and performance temperatures on activity, or (ii) interactions between performance temperature and flight kinematics. Below, each explanation is addressed in turn.

5.5.1 Interactions between development and performance temperatures on activity

The effects of thermally-induced developmental plasticity on other aspects of *Drosophila* locomotor performance are complicated. *Drosophila* walking speeds generally decline with ambient temperature (Dillon and Frazier 2006), however, flies from different source populations demonstrate peak walking performance at performance temperatures consistent with phenotype-environment matching (Gibert et al. 2001). Walking speed of male *D. melanogaster* is associated positively with some aspects of fitness (e.g., mating success: Partridge *et al,* 1987; Gilchrist *et al,* 1997), but has not been directly tested with regard to predator escape or avoidance. Fast walking speeds and burst locomotion may attract more attention from visually triggered predators and decrease fitness if locomotor speeds are insufficient for predator escape.

5.5.2 Interactions between performance temperature and flight kinematics

D. melanogaster are sufficiently small that metabolic heat produced from flight muscles is dissipated immediately, and the internal temperature of the fly is that of the ambient environment (Harrison and Roberts 2000). This impacts flight performance as wing beat frequency increases with ambient temperatures (Reed et al. 1942; Curtsinger and Laurie-ahlberg 1981; Barnes and Laurie-Ahlberg 1986) and mean lift generated by wings is directly proportional to the square of wing-beat frequency (Ellington 1984). Thus, low temperatures reduce the ability of flies to generate lift. The thermally-induced increase in wing size at cold development temperatures is cited as an adaptation to overcome low wing-beat frequencies at cold temperatures by increasing power output (e.g., Barnes and Laurie-Ahlberg 1986; Starmer and Wolf 1989; David et al. 1994; Azevedo et al. 1998; Frazier et al. 2008). The volume of air moved per wing stroke is proportional the product of wing length and wing area (Reed et al. 1942), the increased volume of air moved per stroke may compensate for reduced wing beat frequencies. However, the lower wing-beat frequency of large wings (Frazier et al. 2008) may be costly at warm temperatures during predator escape if insufficient lift is produced, resulting in reduced take-off performance.

Another potential interaction of temperature on flight could result from induced asymmetries in morphology during development. Asymmetries in forelimb length, although not wing veins, affected both survival of *Musca domestica* in presence of *S. stercoraria* as well as the prey-capture success of *S. stercoraria* (Swaddle 1997). Fluctuating asymmetry resulting from environmental stress is common in insects (for review: Beasley et al. 2013) and fluctuating asymmetry in *D. melanogaster* wing length

increases at stressful (e.g., ≤ 13^{o}C and>27^{o}C) developmental temperatures (Imasheva et al. 1997). Although these temperatures are outside of the range used in the current study, my developmental manipulations may have produced asymmetries in the wing or other traits that may have affected my results. If the switch to cold temperature (16^{o}C) during pupal stage is more stressful than a switch to warm pupation temperature (27^{o}C), then the Low WL Phenotype Class flies may exhibit greater asymmetries in traits that develop during the pupal stage such as limbs and wings (Shingleton et al. 2007). Such asymmetric morphologies would be predicted to be ill-suited for predator escape.

5.5.3 Multivariate selection on morphology

In Experiment II, selection gradients were calculated for body size, wing size, and wing shape and found little evidence of selection. The only morphological trait that associated with survival was wing shape (PC1; Fig. 5.S5), and then only for flies in presence of *A. canosa* at Warm Predation Temperatures (Fig. 5.S2 and S3). The Shifted Temperature developmental manipulation produced an expanded range of phenotypic variance (Fig. 5.1) with which selection can be detected, thus I am confident that morphology had limited effects on survival. The patterns of mortality were remarkably similar across Predation Temperatures and between predator types, however, the underlying cause of mortality may differ in each case. For example, enhanced locomotor ability of cold-developed flies at cold temperatures may attract more attention form predators, whereas surface area of large cold-developed wings may reduce wing beat frequency and may be ill-suited for predator escape at warmer performance temperatures. In addition, traits not considered here (e.g., limb length, thoracic mass) may have responded to my manipulation of development temperature and affected performance and survival across performance temperatures.

There are many competing hypotheses that pertain to the geographic and plastic patterns of variation in flight morphology and physiology exhibited by *Drosophila* spp. and other dipterans (e.g. hotter is better: Gilchrist and Huey 2001; optimal development temperature: Barnes and Laurie-Ahlberg 1986). Tests of these hypotheses often use different performance metrics, even if the morphological traits under study are the same, producing mixed results and perhaps placing support for the hypotheses at odds across studies. Determining the fitness effects of morphology and physiology are complicated; any measured effect is dependent, in part, on development environment, the assay environment and the trait assayed. Depending on environmental conditions, some traits may show positive, negative, or no relationship between phenotype and fitness variation. It is therefore vital that studies investigating the fitness effects of trait variation test across a breadth of developmental and assay environments in a full factorial experimental design (Huey et al. 1999). Through such experiments, investigators can tease apart the independent contributions of induced phenotypes in an ecologically relevant spectrum of environments. Using this approach, my data demonstrate that such studies can reveal surprising relationships among morphological and fitness variation, or lack thereof.

This study addressed a long-held hypothesis that thermally-induced phenotypic plasticity in *Drosophila* wing loading is adaptive, improving flight performance across thermal environments. The data provide little support for this pattern of adaptive phenotype-environment matching in the context of predator avoidance: fly morphology is correctly matched at warmer performance temperatures (i.e., confers enhanced survival) but not at colder temperatures. Thermal conditions affect the development of adult morphology,

but the precise traits that promote survival of *D. melanogaster* in presence of terrestrially and aerially hunting predators remain unclear (but see DeNieu et al. 2014). Other aspects of flight performance (e.g., flight endurance) or flight-related morphology (e.g., thoracic muscle mass) not measured here might be important targets of selection. However, censuses revealed mortality patterns to be consistent through time and frequency-independent (Fig. 5.S6, 5.S7). Thus, while I was unable to identify the precise targets of selection, I believe the robust pattern of mortality observed is a real effect of developmental temperature on adult performance. These findings suggest further testing is needed before thermally-induced morphology in *Drosophila* can be described as adaptive phenotypic plasticity. Changes in performance, even if perceived to be ecologically relevant, may not necessarily translate into realized fitness benefits and should not be assumed.

5.6 Supplemental materials

As described briefly in the Methods, an additional experiment was conducted to allow estimation of the pattern and strength of selection on body size, wing size, and wing shape. Trials were conducted as in Experiment I, except that fly wing shapes were quantified before and at the end of a Predation Trial. Dead flies were not replaced, and only censused at 48hr. Using unique wing shape variation to identify individuals that survived exposure to the predators (and, those that did not by subtraction from the starting population) allows estimation of the selection gradient experienced by each trait in each treatment environment.

5.6.1 Materials and methods

Rearing of WL Phenotype Classes was identical to that described in Experiment I with the following exceptions. As larvae began to pupate, they were removed from food vials and imaged using a Leica DFC290 digital camera on Leica MZ125 microscope set to 16x and placed into a uniquely numbered 1.5 ml plastic epitube with 0.5 ml of standard fly food media. Epitubes were then punctured with a 23-gauge needle to allow gas exchange (Stillwell et al. 2011) and incubated at the assigned temperature. Pupae area was calculated from the pixel count of the pupal silhouette. Within 48 h of eclosion, the right wing of each fly was imaged using the same Leica equipment set to 32x. Wing images were acquired without damaging the wing using a vacuum powered 'wing grabber' (Weber 1988; Houle et al. 2003; Stillwell et al. 2011). After wing imaging, flies were marked with fluorescent powder as in Experiment I and placed into vials in groups of 20 flies of the same WL Phenotype Class 24 h before trials began. Flies were released into 0.3 m^3 BugDorms™ 1 h prior to the release of predators to allow for dispersal and acclimation to the environment. Dead flies were aspirated out of cages at

24 h and 48 h. After 48 h in presence of predators, the surviving *D. melanogaster* were removed from by cooling cages to 4^{o}C to induce chill comas. WL Phenotype Class of flies was identified by their fluorescent color and identified to individual by reimaging and re-quantifying shape of the wing.

5.6.1.1 Image capture

Wing images were landmarked using custom software in which 14 type I landmarks are placed manually, and 24 sliding semi-landmarks were automatically placed evenly along the wing margin between hard landmarks (Fig. S5.1), allowing for quantification of wing curvature. TpsRelw (Rohlf 2013) was used to conduct Procrustes superimposition to simplify the description of shape within each Predation Trial by aligning wing images by rotation, translation and scale to remove effects of size (Rohlf and Slice 1990). All Procrustes transformations were performed using the same consensus wing image. The transformed landmark coordinates were output as a weight matrix to provide a unique shape ID for each wing. This technique provides a unique identifier for each fly similar conceptually to a human fingerprint. PC space was defined using all starting flies across trials. To make shapes and PC scores comparable across trials, weight matrices of surviving flies were multiplied by the eigenvectors of the pooled starting populations for each predator/temperature combination.

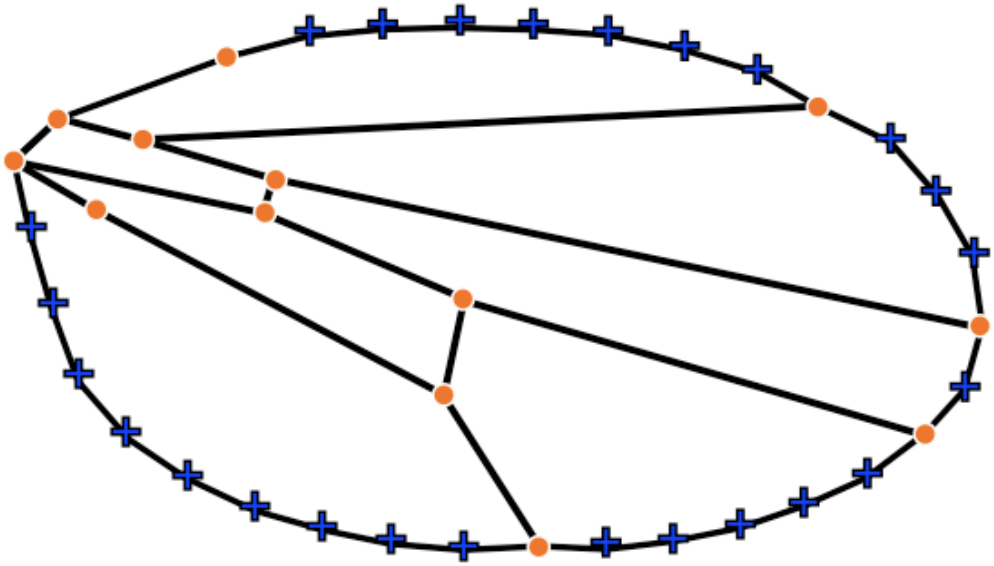


Figure S5.1: Landmarks used for Quantifying Wing Shape: Typical wing after analysis by custom software. A total of 14 type I landmarks (orange circles) are placed manually and 24 semi-landmarks (blue crosses) are placed automatically around wing margin between hard landmarks.

5.6.1.2 Wingerprinting

I had two goals for the morphometric work described below; (i) to develop a method of wingerprinting to identify live flies with intact wings and (ii) to quantify multivariate selection by predation. To match flies that survived Predation Trials to the starting population, I calculated the Procrustes distance, or the square root of sum of squares differences of all Procrustes transformed landmarks, between each surviving wing and each starting fly of the same WL Phenotype Class within that Predation Trial. WL Phenotypes Class was not marked in the 27^{o}C *A. canosa* Predation Trial so surviving wing shape was compared among all 60 starting individuals. Distance between each pair is then ranked from smallest to largest and a vector containing the nearest match (smallest difference) for each survivor is created. My custom 'wingerprinting' method was written in R (available in Dryad), and correctly matched 89.89% flies in a cohort of 212 known individuals. Matching failures are largely due to occasional damage to the wing during the experiment and usually can be resolved by manual inspection of the respective wings.

I used the first two principle components (PC1 and PC2) that account for 40.6% of total shape variation as shape variables in multivariate analysis. PC1 accounts for 27.19% of shape variation and is best described as a broadening of the proximal wing margins anteriorly and posteriorly, distal movement of both anterior and posterior crossveins, and a shortening of the distal wing margin. PC2 accounts for 18.67% of shape variation and is best described by a broadening of the proximal wing margin on the posterior side, broadening of intervein region D, proximal movement of both anterior and posterior crossveins, and shortening of the distal wing margin. Shape variables were generated as partial warp scores from the thin-plate spline (Bookstein 1991) and two standard uniform

components (Rohlf and Bookstein 2003) to capture both the uniform and non-uniform aspects of shape variation in a weight matrix of Procrustes-transformed landmark coordinates. Principle Component Analysis (PCA) of the Shifted Development Treatment (see Materials and Methods of main article) was used to generate a PC space, in which each Predation Trial was oriented to facilitate comparisons. This was accomplished by multiplying the eigenvector matrix by the weight matrix of each treatment.

5.6.2 Analyses

5.6.2.1 Linear regressions

The independent contribution of body size, wing size, PC1 and PC2 of wing shape on relative fitness was assessed by multivariate regression in R (R Core Team 2013). Interactions and non-linear terms had no statistically significant effects, so they were dropped from the model.

$$\text{relative fitness} \sim \text{body size} + \text{wing size} + PC1 + PC2$$

This analysis estimates selection gradients within each Predation Temperature for each predator (Fig. S5.2). Body size and wing size were standardized to a x=0 and SD=1 using the *scale* function in R (R Core Team 2013). PC's were centered to x=0.

5.6.2.2 Shape analyses

To determine if wing shape (PC1, PC2) effected survival, I used two-block Partial Least Squares (PLS) regression (Klingenberg and Zaklan 2000; Dworkin et al. 2011) to perform a singular value decomposition on the matrix of covariance of shape and

relative fitness. The method is similar to PCA, but where PCA extracts shape variables (PC's) that are uncorrelated with each other within a matrix (e.g., matrix of landmarks), PLS performs a singular value decomposition to identify axes of maximal covariation between matrices, where the first axis has the highest covariation and each subsequent axis has less in turn. In this study, PLS was used to identify the combination that maximally covaried between a matrix of principle components, summarizing shape, and a matrix of relative fitness. Results were best summarized as the RV coefficient as it is analogous to the correlation coefficient (Robert and Escoufier 1976) when comparing variation and covariation matrices (Dworkin et al. 2011). The RV coefficient is calculated by dividing the total amount of covariation between matrices by the total variation contained within the two matrices. Ultimately, this analysis provides a correlation statistic between wing shape and relative fitness (Fig. S5.3 & S5.4). Code was written using R 2.15.0 (R Core Team 2013), modified from Dworkin (et al. 2011) and is available on Dryad.

5.6.3 Results (Details in figure captions)

The typical prediction of adaptive phenotypic plasticity is phenotype-environment matching where induced phenotypes will exhibit enhanced fitness relative to alternative phenotypes in that same environment. For example, Cold-developed flies are predicted to have superior survival (fitness) at Cold temperatures relative to Warm temperatures, and should have superior survival at Cold temperatures relative to flies developed at other temperatures. The data lend little support to this hypothesis. *D. melanogaster* with an experimentally enhanced range of relative wing size were exposed to terrestrial- and aerially-hunting predators at several temperatures. However, linear regressions of body size, wing size, and wing shape (PC1 and PC2) on survival indicate only one statistically

significant effect of morphology on survival; PC1 of wing shape in the presence of the *A. canosa* at the 27^{o}C Predation Temperature (Fig. S5.2, S5.3 & S5.5). This finding was verified using PLS analysis, which revealed a significant correlation between wing shape and survival. The lack of significant morphological effects is surprising, given the clear differences in survival among my WL Phenotype Classes (see Fig. 2). Thus, I attribute high mortality of Low WL Phenotype Class flies to thermally-induced changes in physiology, behavior, or perhaps yet unmeasured aspects of morphology such as foreleg size (DeNieu et al. 2014).

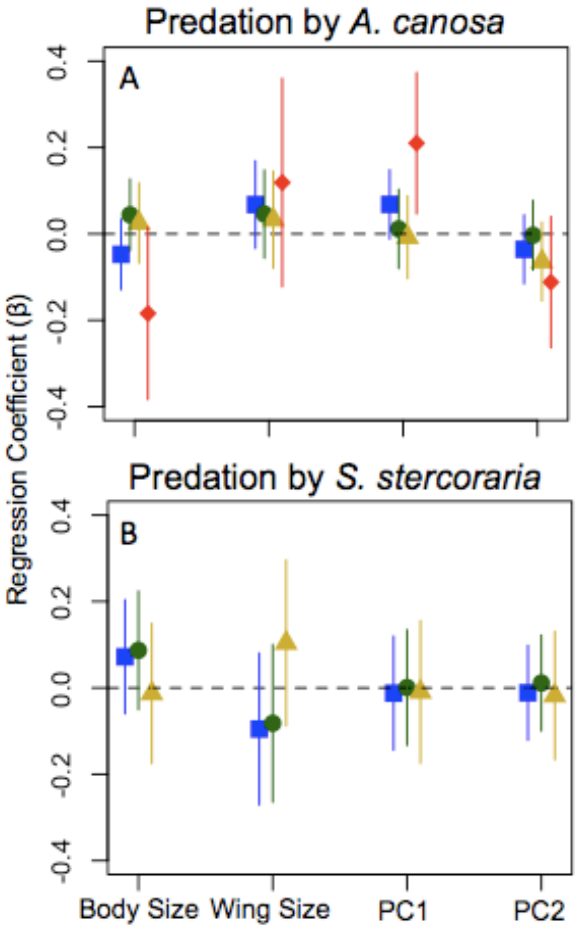


Figure S5.2: Regression coefficients of linear regression: relative fitness (*w*) ~ body size + wing size + PC1 + PC2. Body size and wing size were standardized to a mean=0 and std=1. Diamonds indicate regression coefficient (β) for body size, wing size, PC1 and PC2 of flies preyed upon by either *A. canosa* or *S. stercoraria* at a range of temperatures. Colors indicate Predation Temperature: blue squares (15^{o}C), green circles (18^{o}C), gold triangles (21^{o}C) and red diamonds (27^{o}C). Lines indicate 95% confidence intervals. The only significant coefficient is PC1 of wing shape under predation by *A. canosa* at 27C (p=0.012). No other coefficients are significantly different than 0. Thus, I have limited evidence of wing shape affecting survival in presence of a predator.

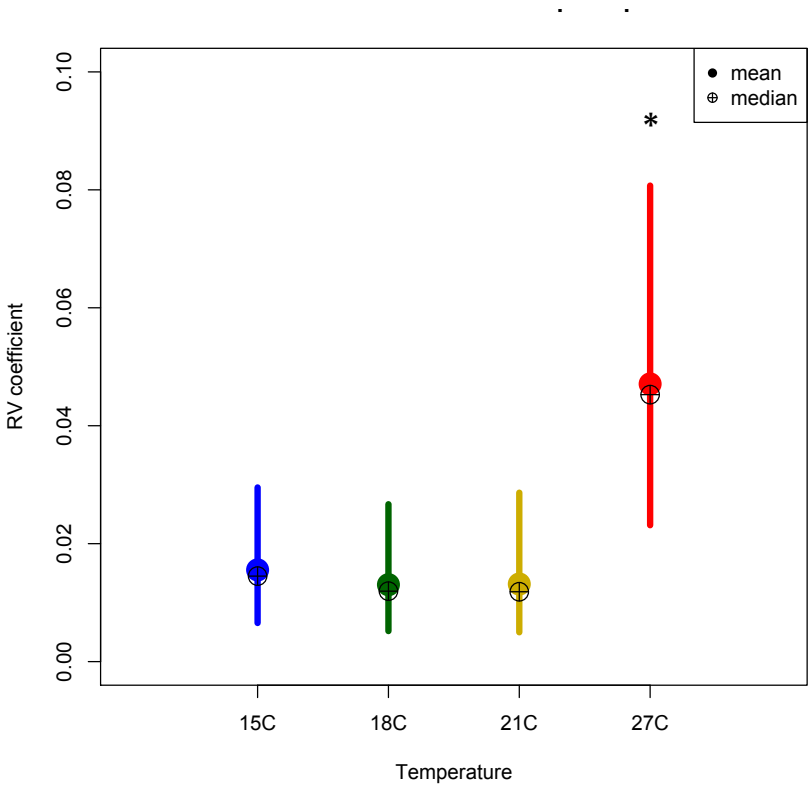


Figure S5.3: Correlation (RV coefficient) between wing shape and relative fitness of survivors in presence of spiders. Circles indicate the mean RV coefficient of 1,000 bootstrapped values; the lines indicate the 95% confidence interval. Color of circle and lines indicate the Predation Temperature. Black crossed-circles indicate the median. Asterisk (*) indicates the RV coefficient for Warm treatment is significantly different that 0 ($p=2.0x10^{-4}$), evidence of a significant relationship between wing shape and survival.

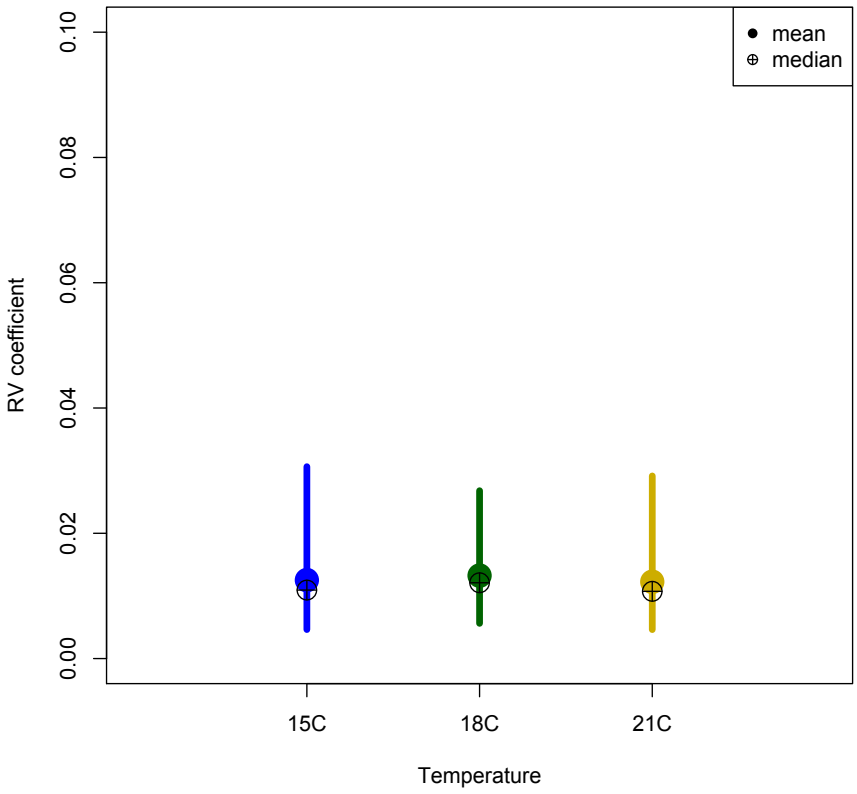


Figure S5.4: Correlation (RV coefficient) between wing shape and relative fitness of survivors in presence of dung flies. Circles indicate the average RV coefficient of 1,000 bootstrapped values; the lines indicate the 95% confidence interval. Color of circle and lines indicate the Trial Temperature. Black crossed-circles indicate the median. None of the RV coefficients are significantly different than 0, thus I did not find any significant relationships between wing shape and survival.

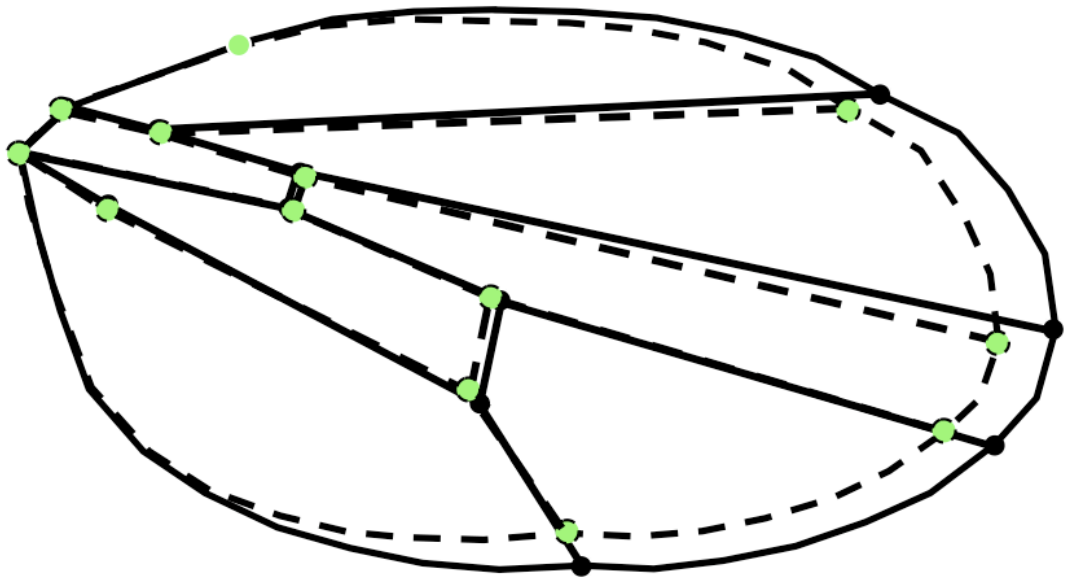


Figure S5.5: Wireframe of *D. melanogaster* shape differences between predation survivors and mean shape of starting population. Survivors in presence of *A. canosa* at 27^{o}C are indicated by green dots and black dashed lines where starting population is represented by black dots and solid black lines. Landmarks on the proximal edge of wing align wing shapes. A general shortening of the distal wing margin and narrowing of anterior and posterior edges best describes shape of survivors. Additionally, there is a slight distal movement of proximal cross vein and proximal movement of the distal cross vein.

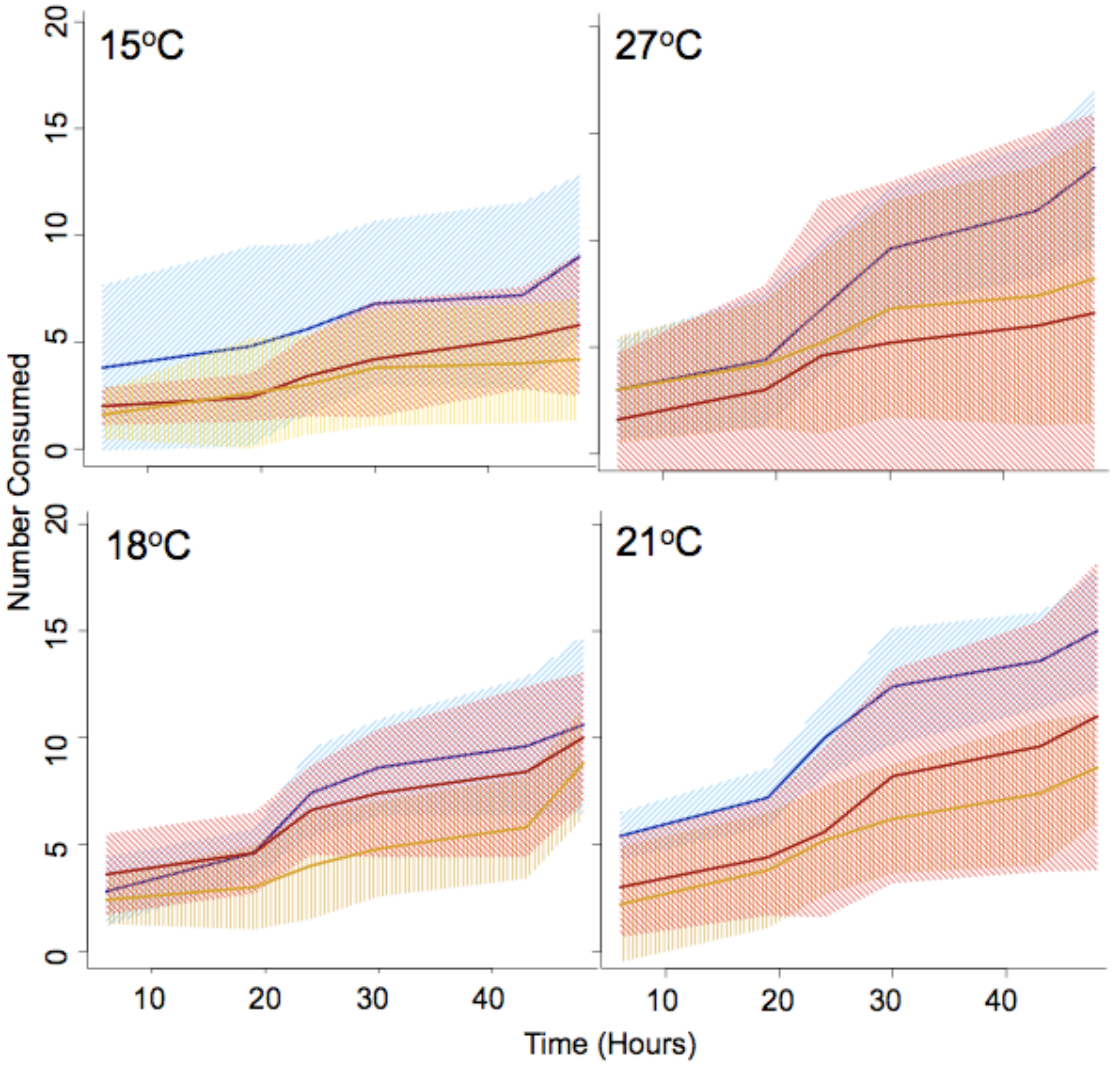


Figure S5.6: Time course of *Drosophila* mortality in presence of *A. canosa* over 48 h in Experiment I. Solid lines indicate average mortality (n=5), and shaded regions indicate 95% confidence intervals at each census (0800, 1300 and 1900). Colors indicate WL Phenotype Class with blue, yellow and red indicating Low, Mid and High WL Phenotype Class respectively. The rank order of mortality appears relatively constant over the duration of the trials, but was not formally tested.

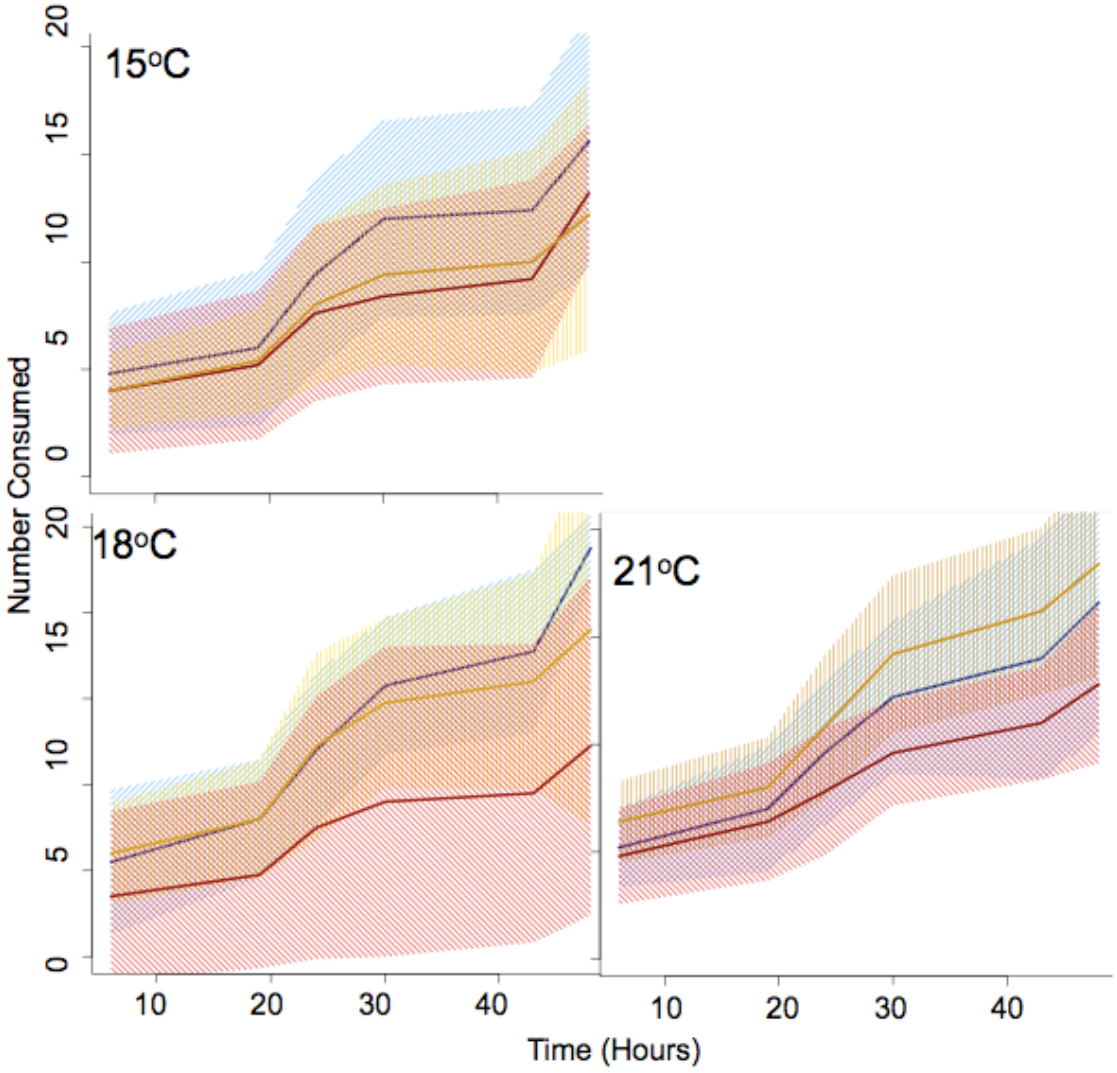


Figure S5.7: Time course of *Drosophila* mortality in presence of *S. stercoraria* over 48 h in Experiment I. Solid lines indicate average mortality (n=5), and shaded regions indicate 95% confidence intervals at each census (0800, 1300 and 1900). Colors indicate WL Phenotype Class with blue, yellow and red indicating Low, Mid and High WL Phenotype Class respectively. The rank order of mortality appears relatively constant over the duration of the trials, but was not formally tested.

CHAPTER 6.CONCLUSIONS: PHENOTYPIC PLASTICITY IN *DROSOPHILA MELANOGASTER* FACILITATES ADAPTIVE EVOLUTION

6.1 Overview

Drosophila spp. exhibit geographic variation and thermally-induced phenotypic plasticity in traits related to flight performance. Despite a paucity of data directly addressing the consequences of this variation, these patterns are viewed as being an exemplar of adaptive evolution. Motivated by these biological patterns in need of explanation, I sought to determine if the clinal variation and phenotypic plasticity exhibited by these flies was adaptive. I also sought to elucidate the more general role of phenotypic plasticity as a facilitator or inhibitor of adaptive evolution. In a series of tests, I demonstrate the adaptive nature of phenotypic plasticity in a variety of ways. Together, my experiments lead to the following conclusions: (i) thermally-induced phenotypic plasticity in *D. melanogaster* exhibits a pattern of adaptive phenotype-environment matching wherein the expressed phenotype is best-suited for flight at the temperature of development, (ii) this phenotypic plasticity facilitates the evolution of upwind flight performance at Cool and Warm flight temperatures, (iii) adaptive evolution by genetic accommodation is a dynamic process and the contribution of traits responding to selection vary and change over time, and finally, (iv) the adaptive pattern of phenotype-environment matching in flight performance is only partially realized as an increase in fitness, measured as survival in presence of predators. The results supporting these findings are provided below in brief and followed by general conclusions; see referenced chapters for full details.

6.2 Chapter 2. Effects of environmental temperature on flight performance

Temperature exerts myriad effects on the expression and ecological performance of phenotypes in ectotherms. Here, I sought to determine how developmental temperature affects phenotype expression, ecological performance and senescence of performance across temperatures. In particular, I tested for adaptive phenotype-environment matching, where the phenotype expressed in a particular environment conveys higher fitness in that environment relative to other phenotypes. In two ways, I found that *D. melanogaster* exhibit adaptive phenotype-environment matching in how flight performance is affected by developmental and ambient flight temperatures. First, within a phenotype induced at a developmental temperature, flight performance was best at that same temperature relative to others. Second, within a given flight temperature, flies which developed at that same temperature exhibited superior flight performance relative to flies developing at other temperatures. These complementary patterns of phenotype-environment matching in flight performance may translate into increased reproductive success in natural populations, as developmental and flight temperatures are likely often similar. A direct relationship between flight performance and mating success has yet to be examined, however, another measure of locomotor performance, walking speed, is correlated with increased mating success in *Drosophila* (Partridge et al. 1987; Sisodia and Singh 2004). The link between walking speed and mating success is partially attributed to an ability to pursue females. Under this assumption, males with enhanced flight performance should benefit similarly. Moreover, upwind or prolonged flight performance ability may affect other fitness components such as dispersal (Hoffmann et al. 2007), foraging ability, etc., and strong performance may be reflective of a generally physiologically robust phenotype. Thus, flight performance likely has multifaceted fitness effects.

Cool development temperatures induced a generally poor-flight phenotype, and I was thus unable to identify a peak age of flight performance for flies that developed at low temperature. Cold development affected flight performance across temperature appeared in a manner that appeared pathological, however, Cool-developed flies still outperformed the Warm developed flies at Cool flight temperature and responded strongly to selection on flight performance (see Chapter 3 below), suggesting this is not the case. Warm-developed flies, conversely, showed increased flight performance and a reduced rate of senescence when assayed at their development temperature. Ultimately, my data illustrate the important effects of temperature on development, ecological performance, and senescence in ectotherms and illustrate the evolutionary and practical importance of considering each when assaying ecological performance.

6.3 Chapter 3. The role of phenotypic plasticity in adaptive evolution

Phenotypic plasticity can obscure the relationship between genetic and phenotypic variation across environments, impeding the response to selection - or it can produce variants on which selection can act to facilitate adaptive evolution and diversification. Where along the continuum most instances of plasticity fall remains an open question. I conducted two complimentary artificial selection experiments to distinguish between these alternative roles of plasticity in the adaptive evolution of upwind flight performance at two temperatures. When they developed at a moderate temperature (i.e., in the *absence* of flight-environment specific thermal plasticity), *D. melanogaster* selected for upwind flight performance at Cool and Warm temperatures exhibited no response to selection. However, when development temperature matched flight temperature (i.e., in the *presence* of flight-environment specific thermal plasticity), the response to selection

was rapid. Realized heritabilities of flight performance were nearly four times greater when development temperature matched the flight temperature at which selection occurred than when development and flight temperature did not match. Unfortunately, the basis of this response to selection is unclear. Only body size, wing size, and wing shape were explicitly considered here, but a more thorough analysis of morphological, physiological and behavioral traits across a range of temperatures (Huey et al. 1999) may elucidate how my experimental populations evolved increased flight ability. If the traits that respond to selection can be identified, the levels of phenotypic plasticity in these traits could be examined in natural populations to improve our understanding of how natural patterns of phenotypic plasticity enhance fitness and promote diversification.

Body size, wing size and relative wing size respond rapidly to artificial selection and thus exhibit heritable variation (e.g. Weber 1990; Santos et al. 1997; Partridge et al. 1999; Frankino et al. 2007; Teuschl et al. 2007), and all have clear effects on *Drosophila* flight kinematics (Reed et al. 1942; Ellington 1984; Barnes and Laurie-Ahlberg 1986). Based on the geographic patterns of morphological variation observed in nature and working from first aerodynamic principles, I predicted that morphology would respond indirectly to selection on flight performance, producing phenotypes well suited to flight at Cool or Warm temperature when flies developed at moderate temperatures (Experiment I). Predictions of the indirect response of body size, relative wing size and wing shape to selection on flight performance were less clear for the experiment in which development temperatures matched selective temperatures (Experiment II). Predictions for wing shape were especially troublesome given that shape variation among clines is better explained by environmental variation rather than genetic differences among populations (Pitchers et al. 2013). In both artificial selection experiments, however, morphologies

consistent with those observed in nature typical of 16 and 27^{o}C did not evolve. Surprisingly, body size decreased significantly across treatments and controls in both experiments, indicating that our rearing protocol likely favored small body size independent of development temperature. This may result from my collection of eggs from mass laying events, which may have favored females that produced smaller, more numerous eggs would have enjoyed a disproportionate representation in the next generation (but see Schwarzkopf et al. 1999).

The fitness consequences of body size are complex, and likely differ across environments. For example, *D. melanogaster* artificially selected for large body size exhibited increased lifetime reproductive success, survival and daily progeny production (McCabe and Partridge 1997). Large body size also increases mating success in *Drosophila* (Partridge et al. 1987; Sisodia and Singh, 2004). However, high temperatures increase development rate and reduce time to maturation, and such rapid development ultimately results small adult body size while reducing generation times and increasing fitness by increasing population growth (for review Kingsolver & Huey, 2008). It is possible that any number of these fitness effects may initially experience stronger selection in natural populations than selection on flight performance. Thus, body size may respond more rapidly to selection than flight-specific traits such as (relative) wing size. Both the Cool- and Warm-selected lines that responded to selection trended toward hypoallometry of wing-body size scaling, and experienced a significant decrease in body size. These findings are significant as they indicate that body size and wing size are not necessarily constrained to evolve in tandem. In nature, selection may thus first focus primarily on body size, with wing phenotypes adapting later, improving aspects of flight

performance - including those not selected for here (e.g., hovering, maneuverability, take-off, or endurance).

In sum, my data from this chapter fail to support the hypothesis that morphological clines are adaptive, but neither do they reject the hypothesis. While my morphological data fails to support the hypothesis, the qualitatively different responses to identical selective agents when morphology was presumably matched (via development temperature) to flight temperatures, versus when it was not, strongly supports the hypothesis that natural patterns of thermally-induced plasticity are adaptive and facilitate adaptive evolution.

6.4 Chapter 4. Genetic accommodation promotes adaptive phenotype-environment matching in *Drosophila melanogaster* flight performance

Genetic accommodation is the process by which development in a novel environment releases cryptic variants that are then subject to selection. Over time, selection shapes these new variants, refining their expression. At first, expression of the novel phenotype is plastic such that a return to the original developmental environment will produce an atavistic phenotype, however, over time expression of the derived phenotype will become 'genetically assimilated', i.e., constitutively expressed across all environments. Although the idea of evolution by genetic assimilation/accommodation enjoys great appeal, few if any data address this in a natural system. Here I examined the experimental lineages from Chapter 3 at different time points to determine if the populations were evolving via genetic assimilation and accommodation.

I found that the traits responded to selection by genetic accommodation, and that they varied in their contributions over time and enhanced patterns of phenotype-environment

matching. After 7 generations of selection, Cool- and Warm-flight selected lineages exhibited qualitatively different patterns of performance across flight temperatures. Cool-selected lineages exhibited a strong generalist flight phenotype; as flight performance increased at Cool temperatures over the generations, they also became more flight-proficient at the Warm temperature regardless of temperature of development. In contrast, Warm-selected flies exhibited a specialist phenotype, as flight performance increased only when Warm-developed and flown at Warm temperatures as they had been selected. By generation 11, however, both Cool- and Warm-selected lines exhibited phenotype-environment matching with a cost to flight performance when development temperature did not match flight temperature.

Investigations of the morphological basis of these effects are in progress and thus the results presented here are preliminary; the thesis contains morphological results for generation 7 Cool-selected flies and generation 11 Warm selected flies only. Once all morphological data are analyzed, a change in morphological response may be evident. However, preliminary investigations into which traits may have responded to selection reveal that the targets were not the predicted morphological characters typically associated with enhanced flight performance at thermal extremes. Complex phenotypes, such as flight performance, are likely to first evolve by behavioral modification and physiological changes prior to morphological responses (Garland and Losos 1994; Garland et al., 2011). Physiological traits which increase flight performance and may have responded to selection include wing beat frequency (Barnes and Laurie-Ahlberg, 1986) or enhanced ultrastructure of indirect flight muscle (Miller et al. 2008), Studies are currently underway to investigate these physiological changes.

6.5 Chapter 5. Does adaptive phenotype-environment matching in *Drosophila* flight performance enhance survival in presence of predators?

Morphological variation produces variation in ecological performance, and this performance variation results in variation in relative fitness (Arnold 1983; Garland and Losos 1994; Careau and Garland 2012). Despite strong evidence of phenotype-environment matching which resulted in enhanced flight performance in *D. melanogaster* (Chapters 2 & 4), there was only partial support for phenotype-environment matching regarding relative fitness. Survival of *D. melanogaster* with artificially enhanced variation in relative wing size was examined in presence of terrestrial (*Anasaitis canosa*) and aerial (*Scathophaga stercoraria*) hunting predators at Cold, Cool, Moderate, and Warm temperatures (15, 18, 21, and 27^{o}C respectively). Flies with relatively large wings, morphology typical of Cool populations and development temperatures, exhibited the highest mortality across nearly all predator/temperature combinations. However, relatively small winged flies exhibited the lowest mortality at warmer temperatures consistent with phenotype-environment matching. These results contrast with results of Chapter 2 where *D. melanogaster* exhibited phenotype-environment matching even at Cool flight temperatures. Apparently, the subtle but significant increase in performance I observed was not great enough to promote survival. This contradiction may be explained as an artifact of the developmental manipulation. Flies used in the predation assays were not developed entirely at Cool temperatures, but only during the pupal stage. Increased flight performance of Cool-developed flies at Cool temperatures may result from effects of temperature on larval development when adult body size is determined. The artificially increased relative wing size may have resulted in wings too large for efficient take-off or flight performance. It is currently unclear which specific traits resulting from Cool-development enhance Cool-temperature flight performance. Alternatively, the

targets, pattern and intensity of selection may differ across temperatures. For example, the superior performance of Warm-developed flies at Warm-flight temperatures (Chapter 2) may facilitate predator escape whereas Cool-developed flies are incapable of such escape. Conversely, at Cool flight temperatures, where activity levels are low for flies from all development treatments, increased activity of Cool-developed flies may attract more attention from visually stimulated predators in a generally static population. Thus, across flight temperatures, the pattern of survival between thermally-induced phenotypes may be similar, but the causes of mortality could differ dramatically.

Predation may be a relatively weak selective force in natural populations compared to other fitness measures affected by wing phenotypes such as dispersal (Hoffmann et al. 2007) or mate acquisition (Ewing 1964; Menezes et al. 2013). If predation were responsible for creating or reinforcing natural patterns of geographic variation or thermally-induced plasticity in flight morphology, I would expect stronger patterns of phenotype-environment matching for fitness. Determining what aspects of fitness most directly benefit from flight performance in natural populations will deepen our understanding of fruit fly ecology and help determine what shapes the morphological clines in *Drosophila*.

6.6 Summary

The role of phenotypic plasticity in facilitating adaptive evolution has recently received much attention (Pigliucci 2001; West-Eberhard 2003; Dewitt and Scheiner 2004; Whitman and Ananthakrishnan 2009). My dissertation work contributes significantly to this field by elucidating existing patterns of phenotype-environment matching in *D. melanogaster* and demonstrating how this pattern facilitates adaptive evolution by

genetic accommodation. The lack of a morphological evolution in response to selection on flight performance was surprising given the independently derived, convergent morphological patterns in flight morphology across *Drosophila* (e.g., Imasheva et al. 1994; James et al. 1997; Azevedo et al. 1998; Bhan et al. 2014). The hypothesis that these natural morphological patterns are adaptive and result from selection on flight performance remains to be directly demonstrated. While my data fail to support the hypothesis that geographic clines in flight-morphology result from selection for flight performance in different thermal environments, they demonstrate clearly that phenotypic plasticity can facilitate the evolution of the clinal patterns. The identification of the traits that responded to artificial selection for increased flight performance, coupled with investigations of these traits in natural populations, might provide the evidence necessary to determine if evolution of the genetically based morphological clines in *D. melanogaster* result from genetic accommodation of phenotypically plastic traits. Thermally-induced plasticity is widespread in ectotherms, and insects in particular. Thus, it is conceivable that thermally-induced phenotypic plasticity is of primary importance in facilitating adaptive evolution in a changing environment on a broad phylogenetic scale. If the fitness effects of flight performance can be elucidated, then the repeated evolution of independently derived morphological clines in *Drosophila,* which are likely facilitated by adaptive phenotypic plasticity, will truly be “a grand experiment in evolution” (Ayala et al. 1989).

REFERENCES

Adams, C. E., and F. A. Huntingford. 2004. Incipient speciation driven by phenotypic plasticity? evidence from sympatric populations of arctic charr. Biol. J. Linn. Soc. 81:611–618.

Adams, D. C., and F. J. Rohlf. 2000. Ecological character displacement in Plethodon : Biomechanical differences found from a geometric morphometric study. Proc. Natl. Acad. Sci. 97:4106–4111.

Albrecht, H. 1980. Multivariate analysis and the study of form, with special reference to canonical variate analysis. Am. Zool. 20:679–693.

Andersson, M. 1982. Female choice selects for extreme tail length in a widowbird. Nature 299:818–820.

Arnold, S. J. 1992. Constraints on phenotypic evolution. Am. Nat. 140:S85–S107.

Arnold, S. J. 1983. Morphology, performance and fitness. Am. Zool. 23:347–361.

Arnold, S. J., B. Reinhard, P. A. Hohenlohe, B. C. Ajie, and A. G. Jones. 2008. Understanding the evolution and stability of the G-matrix. Evolution. 62:2451–2461.

Atkinson, D. 1994. Temperature and organism size - A biological law for ectotherms? Adv. Ecol. Res. 25:1–58.

Atkinson, D., and R. M. Sibly. 1997. Why are organisms usually bigger in colder environments? Making sense of a life history puzzle. Trends Ecol. Evol. 12:235–239.

Auld, J. R., and R. a. Relyea. 2010. Adaptive plasticity in predator-induced defenses in a common freshwater snail: altered selection and mode of predation due to prey phenotype. Evol. Ecol. 25:189–202.

Ayala, F. J., L. Serra, and A. Prevosti. 1989. A grand experiment in evolution: the *Drosophila subobscura* colonization of the Americas. Genome 31:246–255.

Azevedo, R. B. R., V. French, and L. Partridge. 1996. Thermal evolution of egg size in *Drosophila melanogaster*. Evolution. 50:2338–2345.

Azevedo, R. B. R., A. C. James, J. McCabe, and L. Partridge. 1998. Latitudinal variation of wing: Thorax size ratio and wing-aspect ratio in *Drosophila melanogaster*. Evolution (N. Y). 52:1353–1362.

Baldwin, J. M. 1896. A new factor in evolution. Am. Nat. 30:441–451.

Baranzelli, M. C., A. N. Sersic, and A. A. Cocucci. 2014. The search for Pleiades in trait constellations : functional integration and phenotypic selection in the complex flowers of *Morrenia brachystephana* (Apocynaceae). J. Evol. Biol. 27:724–736.

Barnes, P. T., and C. C. Laurie-Ahlberg. 1986. Genetic variability of flight metabolism in *Drosophila melanogaster*. III. Effects of GPDH allozymes and environmental temperature on power output. Genetics 267–294.

Bateman, K. G. 1957. The genetic assimilation of four venation phenocopies. Genetics 56:443–474.

Bauwens, D., T. Garland Jr., A. M. Castilla, and R. Van Damme. 1995. Evolution of sprint speed in Lacertid lizards: morphological, physiological and behavioral covariation. Evolution (N. Y). 49:848–863.

Beasley, D. A. E., A. Bonisoli-Alquati, and T. A. Mousseau. 2013. The use of fluctuating asymmetry as a measure of environmentally induced developmental instability: A meta-analysis. Ecol. Indic. 30:218–226.

Berven, K. A., D. E. Gill, and S. J. Smith-Gill. 1979. Countergradient selection in the green frog, *Rana clamitans*. Evolution. 33:609–623.

Bhan, V., R. Parkash, and D. D. Aggarwal. 2014. Effects of body-size variation on flight-related traits in latitudinal populations of *Drosophila melanogaster*. J. Genet. 93:103–12.
Birdsall, K., E. Zimmerman, K. Teeter, and G. Gibson. 2000. Genetic variation for positioning of wing veins in *Drosophila melanogaster*. Evol. Dev. 2:16–24.
Blanckenhorn, W. U. 1998. Adaptive phenotypic plasticity in growth, development and body size in the yellow dung fly. Evolution. 52:1394–1407.
Blanckenhorn, W. U., and M. Demont. 2004. Bergmann and converse bergmann latitudinal clines in arthropods: two ends of a continuum? Integr. Comp. Biol. 44:413–24.
Blob, R., and A. Biewener. 1999. In vivo locomotor strain in the hindlimb bones of *Alligator mississippiensis* and *Iguana iguana*: implications for the evolution of limb bone safety factor and non-sprawling limb posture. J. Exp. Biol. 202:1023–1046.
Blomberg, S. P., T. Garland Jr., and A. R. Ives. 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. Evolution. 57:717–745.
Bochdanovits, Z., and G. de Jong. 2003. Experimental evolution in *Drosophila melanogaster*: interaction of temperature and food quality selection regimes. Evolution. 57:1829–1836.
Bonine, K. E., and T. Garland Jr. 1999. Sprint performance of phrynosomatid lizards, measured on high-speed treadmill, correlates with hindlimb length. J. Zool. 248:255–265.
Bonine, K. E., T. T. Gleeson, and T. Garland. 2005. Muscle fiber-type variation in lizards (Squamata) and phylogenetic reconstruction of hypothesized ancestral states. J. Exp. Biol. 208:4529–4547.
Bookstein, F. L. 1991. Morphometric Tools for Landmark Data: Geometry and Biology. Cambridge University Press.
Boratyński, Z., J. C. Brito, J. C. Campos, M. Karala, and T. Mappes. 2014. Large spatial scale of the phenotype-environment color matching in two cryptic species of african desert jerboas (*dipodidae*: *jaculus*). PLoS One 9:e94342.
Braendle, C., and T. Flatt. 2006. A role for genetic accommodation in evolution? BioEssays 28:868–873.
Brakefield, P. M., J. Gates, D. Keys, F. Kesbeke, P. J. Wijngaarden, A. Monteiro, V. French, and S. B. Carroll. 1996. Development, plasticity and evolution of butterfly eyespot patterns. Nature 384:236–242.
Breuker, C. J., J. S. Patterson, and C. P. Klingenberg. 2006. A single basis for developmental buffering of *Drosophila* wing shape. PLoS One 1:e7. Public Library of Science.
Brodie III, E. D. 1992. Correlational selection for color pattern and antipredator behavior in the garter snake. Evolution. 46:1284–1298.
Burkhardt, D., and I. de la Motte. 1985. Selective pressures, variability, and sexual dimorphism in stalk-eyed flies (*Diopsidae*). Naturwissenschaften 72:204–206.
Careau, V., and T. Garland. 2012. Performance, personality, and energetics: correlation, causation, and mechanism. Physiol. Biochem. Zool. 85:543–571.
Cheverud, J. M. 1984. Quantitative genetics and developmental constraints on evolution by selection. J. Theor. Biol. 110:155–171.
Chown, S. L., and K. J. Gaston. 2014. Exploring links between physiology and ecology at macro-scales: the role of respiratory metabolism in insects. Biol. Rev. Camb. Philos. Soc. 74:87–120.

Conover, D., and E. T. Schultz. 1995. Phenotypic similarity and the evolutionary significance of countergradient variation. Trends Ecol. Evol. 10:248–252.
Cooper, B. S., M. Czarnoleski, and M. Angilletta Jr. 2010. Acclimation of thermal physiology in natural populations of *Drosophila melanogaster*: a test of an optimality model. J. Evol. Biol. 23:2346–2355.
Crispo, E. 2007. The Baldwin Effect and genetic assimilation: Revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. Evolution. 61:2469–2479.
Curtsinger, J. W., and C. C. Laurie-ahlberg. 1981. Genetic variability of flight metabolism in *Drosophila melanogaster*. I. Characterization of power output during tethered flight. Genetics 98:549–564.
Darwin, C. 1859. On the Origin of Species. John Murray, London.
David, J., and P. Capy. 1988. Genetic variation of *Drosophila melanogaster* natural populations. Trends Genet. 4:106–111.
David, J. R., B. Moreteau, J. P. Gauthier, G. Petavy, A. Stockel, and A. G. Imasheva. 1994. Reaction norms of size characters in relation to growth temperature in *Drosophila melanogaster*: an isofemale lines analysis. Genet. Sel. Evol. 26:229–251.
De Jong, G. 2005. Evolution of phenotypic plasticity: patterns of plasticity and the emergence of ecotypes. New Phytol. 166:101–118.
Debat, V., M. Begin, H. Legout, and J. R. David. 2003. Allometric and nonallometric components of *Drosophila* wing shape respond differently to developmental temperature. Evolution. 57:2773–2784.
Deere, J. a, and S. L. Chown. 2006. Testing the beneficial acclimation hypothesis and its alternatives for locomotor performance. Am. Nat. 168:630–644.
Deere, K. a, G. F. Grether, A. Sun, and J. S. Sinsheimer. 2012. Female mate preference explains countergradient variation in the sexual coloration of guppies (*Poecilia reticulata*). Proc. Biol. Sci. 279:1684–90.
DeNieu, M., W. Pitchers, and I. Dworkin. 2014. Adaptation to a novel predator in *Drosophila melanogaster*: How well are we able to predict evolutionary responses? Cold Spring Harbor Labs Journals. http://dx.doi.org/10.1101/005322
DeWitt, T. J. 1998. Costs and limits of phenotypic plasticity: Tests with predator-induced morphology and life history in a freshwater snail. J. Evol. Biol. 11:465–480.
Dewitt, T. J., and S. M. Scheiner (eds). 2004. Phenotypic Plasticity: Functional and Conceptual Approaches. Oxford University Press, Inc.
Dewitt, T. J., A. Sih, and D. S. Wilson. 1998. Costs and limits of phenotypic plasticity. TRENDS Ecol. Evol. 13:77–81.
Dillon, M. E., and M. R. Frazier. 2006. *Drosophila melanogaster* locomotion in cold thin air. J. Exp. Biol. 209:364–371.
Dworkin, I., J. a Anderson, Y. Idaghdour, E. K. Parker, E. a Stone, and G. Gibson. 2011. The effects of weak genetic perturbations on the transcriptome of the wing imaginal disc and its association with wing shape in *Drosophila melanogaster*. Genetics 187:1171–1184.
Egset, C. K., G. H. Bolstad, G. Rosenqvist, J. A. Endler, and C. Pe. 2011. Geographical variation in allometry in the guppy (*Poecilia reticulata*). J. Evol. Biol. 24:2631–2638.
Egset, C. K., T. F. Hansen, A. LE Rouzic, G. H. Bolstad, G. Rosenqvist, and C. Pélabon. 2012. Artificial selection on allometry: change in elevation but not slope. J. Evol. Biol. 25:938–948.

Ellington, C. P. 1984. The aerodynamics of hovering insect flight. VI. Lift and power requirements. Philos. Trans. R. Soc. B Biol. Sci. 305:145–181.
Emlen, D. J., and H. F. Nijhout. 2000. The development and evolution of exaggerated morphologies in insects. Annu. Rev. Entomol. 45:661–708.
Ewing, A. W. 1964. The influence of wing area on the courtship behaviour of *Drosophila melanogaster.* Anim. Behav. 12:316–320.
Failes, E. S., A. Canada, and O. Canada. 1992. Predatory behaviour of *Scatophaga stercoraria* under laboratory conditions. Entomophaga 37:205–213.
Farmer, K. J., and R. S. Sohal. 1987. Effects of ambient temperature on free radical generation, antioxidant defenses and life span in the adult housefly, *Musca domestica*. Exp. Gerontol. 22:59–65.
Frankino, W. A., A. W. Shingleton, and D. J. Emlen. 2009. Experimental approaches to studying the evolution of morphological allometries. Pp. 419–478 *in* T. Garland and M. R. Rose, eds. Experimental Evolution: Concepts, Methods, and Applications. University of California Press.
Frankino, W. A., B. J. Zwaan, D. L. Stern, and P. M. Brakefield. 2007. Internal and external constraints in the evolution of morphological allometries in a butterfly. Evolution 61:2958–70.
Frankino, W. A., B. J. Zwaan, D. L. Stern, and P. M. Brakefield. 2005. Natural selection and developmental constraints in the evolution of allometries. Science. 307:718–720.
Frazier, M., J. Harrison, S. Kirkton, and S. Roberts. 2008. Cold rearing improves cold-flight performance in Drosophila via changes in wing morphology. J. Exp. Biol. 211:2116–2122.
French, V., M. Feast, and L. Partridge. 1998. Body size and cell size in Drosophila: the developmental response to temperature. J. Insect Physiol. 44:1081–1089.
Garland Jr., T., and M. R. Rose (eds). 2009. Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments. University of California Press, Berkeley, California.
Garland, T., S. a Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proc. Biol. Sci. 278:574–81.
Garland, T., and J. B. Losos. 1994. Ecological Morphology of Locomotor Performance in Squamate Reptiles. Pp. 240–302 *in* P. C. Wainwright and S. M. Reilly, eds. Ecological Morphology: Integrative Organismal Biology. University of Chicago Press.
Ghalambor, C. K., J. K. McKay, S. P. Carroll, and D. N. Reznick. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Funct. Ecol. 21:394–407.
Gibert, P., R. B. Huey, and G. W. Gilchrist. 2001. Locomotor performance of Drosophila melanogaster: interactions among developmental and adult temperatures, age, and geography. Evolution 55:205–209.
Gibson, G., and I. Dworkin. 2004. Uncovering cryptic genetic variation. Nat. Rev. Genet. 5:681–690.
Gilchrist, G. W., and R. B. Huey. 2001. Parental and developmental temperature effects on the thermal dependence of fitness in *Drosophila melanogaster*. Evolution. 55:209–214.

Gilchrist, G. W., and R. B. Huey. 2004. Plastic and genetic variance in wing loading as a function of temperature within and among parallel clines in *Drosophila subobscura*. Integr. Comp. Biol. 44:461–470.
Gilchrist, G. W., R. B. Huey, and L. Serra. 2001. Rapid evolution of wing size clines in *Drosophila subobscura*. Genetica 112-113:273–286.
Gillooly, J. F., J. H. Brown, G. B. West, V. M. Savage, and E. L. Charnov. 2001. Effects of size and temperature on metabolic rate. Science. 293:2248–2251.
Gorur, G., C. Lomonaco, and A. Mackenzie. 2005. Phenotypic plasticity in host-plant specialisation in *Aphis fabae*. Ecol. Entomol. 30:657–664.
Gotoh, H., H. Miyakawa, A. Ishikawa, Y. Ishikawa, Y. Sugime, D. J. Emlen, L. C. Lavine, and T. Miura. 2014. Developmental link between sex and nutrition; doublesex regulates sex-specific mandible growth via juvenile hormone signaling in stag beetles. PLoS Genet. 10:e1004098.
Gould, S. J. 1966. Allometry and size in ontogeny and phylogeny. Biol. Rev. 41:587–638.
Greene, E., L. J. Orsak, and D. W. Whitman. 1987. A tephritid fly mimics the territorial displays of its jumping spider predators. Science 236:310–2.
Grotewiel, M. S., I. Martin, P. Bhandari, and E. Cook-Wiens. 2005. Functional senescence in *Drosophila melanogaster*. Ageing Res. Rev. 4:372–397.
Harrison, J. F., and S. P. Roberts. 2000. Flight respiration and energetics. Annu. Rev. Physiol. 62:179–205.
Hayden, E. J., E. Ferrada, and A. Wagner. 2011. Cryptic genetic variation promotes rapid evolutionary adaptation in an RNA enzyme. Nature 474:92–5.
Hoffmann, A. A., E. Ratna, C. Sgrò, M. Barton, M. Blacket, R. Hallas, S. De Garis, and A. Weeks. 2007. Antagonistic selection between adult thorax and wing size in field released *Drosophila melanogaster* independent of thermal conditions. J. Evol. Biol. 20:2219–2227.
Houle, D., J. Mezey, P. Galpern, and A. Carter. 2003. Automated measurement of *Drosophila* wings. BMC Evol. Biol. 3:25.
Huey, R. B., D. Berrigan, G. W. Gilchrist, and J. C. Herron. 1999. Testing the adaptive significance of acclimation: a strong inference approach. Am. Zool. 39:323–336.
Huey, R. B., G. W. Gilchrist, M. L. Carlson, D. Berrigan, and L. Serra. 2000. Rapid evolution of a geographic cline in size in an introduced fly. Science. 287:308–309.
Huey, R. B., T. Wakefield, W. D. Crill, and G. W. Gilchrist. 1995. Within- and between-generation effects of temperature on early fecundity of *Drosophila melanogaster*. Heredity. 74 (Pt 2):216–23.
Huxley, J. S. 1932. The problems of relative growth. Methuen & Co., London.
Imasheva, a G., O. a Bubli, and O. E. Lazebny. 1994. Variation in wing length in Eurasian natural populations of *Drosophila melanogaster*. Heredity. 72:508–514.
Imasheva, A. G., V. Loeschcke, L. A. Zhivotovsky, and O. E. Lazebny. 1997. Effects of extreme temperature on phenotypic variation and developmental stability in *Drosophila melanogaster* and *Drosophila buzzatii*. Biol. J. Linn. Soc. 61:117–126.
Iwasaki, W. M., M. E. Tsuda, and M. Kawata. 2013. Genetic and environmental factors affecting cryptic variations in gene regulatory networks. BMC Evol. Biol. 13:1–14.
Jackson, R. R., and A. Van Olphen. 1991. Prey-capture techniques and prey preferences of *Corythalia canosa* and *Pystira orbiculata*, ant-eating jumping spiders (Araneae, Salticidae). J. Zool. 223:577–591.

Jackson, R. R., and S. D. Pollard. 1996. Predatory behavior of jumping spiders. Annu. Rev. Entomol. 41:287–308.
James, A. C., R. B. R. Azevedo, and L. Partridge. 1997. Genetic and environmental responses to temperature of *Drosophila melanogaster* from a latitudinal cline. Genetics 146:881–890.
James, A. C., and L. Partridge. 1995. Thermal evolution of rate of larval development *Drosophila melanogaster* in laboratory and field populations. J. Evol. Biol. 8:315–330.
Kawano, K. 2000. Genera and allometry in the stag beetle family Lucanidae, Coleoptera. Ann. Entomol. Soc. Am. 93:198–207.
Kawano, S. M., W. C. Bridges, H. L. Schoenfuss, T. Maie, and R. W. Blob. 2012. Differences in locomotor behavior correspond to different patterns of morphological selection in two species of waterfall-climbing gobiid fishes. Evol. Ecol. 27:949–969.
Kingsolver, J. G., and T. L. Hedrick. 2008. Biomechanical acclimation: flying cold. Curr. Biol. 18:R876.
Kingsolver, J. G., and R. B. Huey. 2008. Size, temperature, and fitness: three rules. Evol. Ecol. Res. 10:251–268.
Klingenberg, C. P., and L. R. Monteiro. 2005. Distances and directions in multidimensional shape spaces: implications for morphometric applications. Syst. Biol. 54:678–688.
Klingenberg, C. P., and S. D. Zaklan. 2000. Morphological integration between developmental compartments in the *Drosophila* wing. Evolution. 54:1273–1285.
Krueger, D. a., and S. I. Dodson. 1981. Embryological induction and predation ecology in *Daphnia pulex*. Limnol. Oceanogr. 26:219–223.
Lachenbruch, P. A. 1967. An almost unbiased method of obtaining confidence intervals for the probability of misclassification in discriminant analysis. Biometrics 23:639–645.
Lande, R. 1979. Quantitative genetic analysis of multivariate evolution, applied to brain: body size allometry. Evolution. 33:402–416.
Lande, R., and S. J. Arnold. 1983. The measurement of selection on correlated characters. Evolution. 37:1210–1226.
Lane, S. J., W. A. Frankino, M. M. Elekonich, and S. P. Roberts. 2014. The effects of age and lifetime flight behavior on flight capacity in *Drosophila melanogaster*. J. Exp. Biol. 217:1437–43.
Ledon-Rettig, C. C., D. W. Pfennig, and N. Nascone-Yoder. 2008. Ancestral variation and the potential for genetic accommodation in larval amphibians: Implications for the evolution of novel feeding strategies. Evol. Dev. 10:316–325.
Leroi, A. M., A. F. Bennett, and R. E. Lenski. 1994. Temperature acclimation and competitive fitness: An experimental test of the beneficial acclimation assumption. Proc. Natl. Acad. Sci. 91:1917–1921.
Liefting, M., A. A. Hoffmann, and J. Ellers. 2009. Plasticity versus environmental canalization: Population differences in thermal responses along a latitudinal gradient in *Drosophila serrata*. Evolution. 63:1954–1963.
Lively, C. M. 1986. Predator-induced shell dimorphism in the acorn barnacle *Chthamalus anisopoma*. Evolution. 40:232–242.
Losos, J. B. 1990. The evolution of form and function: Morphology and locomotor performance in West Indian Anolis lizards. Evolution. 44:1189–1203.

Madhavan, M. M., and H. A. Schneiderman. 1977. Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila melanogaster*. Roux's Arch. Dev. Biol. 183:269–305.
Marcil, J., D. P. Swain, and J. a Hutchings. 2006. Countergradient variation in body shape between two populations of Atlantic cod (*Gadus morhua*). Proc. Biol. Sci. 273:217–23.
Maynard-Smith, J. 1963. Temperature and the rate of ageing in poikilotherms. Nature 199:400–402.
McCabe, J., and L. Partridge. 1997. An interaction between environmental temperature and genetic variation for body size for the fitness of adult female *Drosophila melanogaster*. Evolution. 51:1164–1174.
Menezes, B. F., F. M. Vigoder, A. A. Peixoto, J. Varaldi, and B. C. Bitner-mathé. 2013. The influence of male wing shape on mating success in *Drosophila melanogaster*. Anim. Behav. 85:1217–1223.
Mezey, J. G., and D. Houle. 2005. The dimensionality of genetic variation for wing shape in *Drosophila melanogaster*. Evolution. 59:1027–1038.
Michel, M. J. 2010. Spatial dependence of phenotype-environment associations for tadpoles in natural ponds. Evol. Ecol. 25:915–932.
Miller, M. S., P. Lekkas, J. M. Braddock, G. P. Farman, B. A. Ballif, T. C. Irving, D. W. Maughan, and J. O. Vigoreaux. 2008. Aging enhances indirect flight muscle fiber performance yet decreases flight ability in *Drosophila*. Biophys. J. 95:2391–2401.
Mirth, C. K., and A. W. Shingleton. 2012. Integrating body and organ size in *Drosophila*: recent advances and outstanding problems. Front. Endocrinol. 3:1–13.
Moczek, A. P. 2006. A matter of measurements: challenges and approaches in the comparative analysis of static allometries. Am. Nat. 167:606–11; discussion 612–8.
Moczek, A. P., S. Sultan, S. Foster, C. Ledón-Rettig, I. Dworkin, H. F. Nijhout, E. Abouheif, and D. W. Pfennig. 2011. The role of developmental plasticity in evolutionary innovation. Proc. Biol. Sci. 278:2705–13.
Moran, N. A. 1992. The evolutionary maintenance of alterantive phenotypes. Am. Nat. 139:971–989.
Morris, M. R. J., and S. M. Rogers. 2013. Overcoming maladaptive plasticity through plastic compensation. Curr. Zool. 59:526–536.
Myers, E. M., and W. A. Frankino. 2012. Time in a bottle: The evolutionary fate of species discrimination in sibling *Drosophila* species. PLoS One 7:e31759.
Orr, H. A. 1999. An evolutionary dead end? Science. 285:343–344.
Parsons, P. A., R. B. Huey, J. G. Kingsolver, R. E. Lenski, A. F. Bennett, F. G. Howarth, F. S. Chapin III, K. Autumn, F. Pugnaire, A. A. Hoffmann, and M. Watson. 1993. Supplement: evolutionary resposnes to environmental stress. Am. Nat. 142:S1–S113.
Partridge, L., B. Barrie, K. Fowler, and V. French. 1994. Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. Evolution. 48:1269–1276.
Partridge, L., A. Ewing, and A. Chandler. 1987. Male size and mating success in *Drosophila melanogaster*: the roles of male and female behaviour. Anim. Behav. 35:555–562.
Partridge, L., R. Langelan, K. Fowler, B. J. Zwaan, and V. French. 1999. Correlated responses to selection on body size in *Drosophila melanogaster*. Genet. Res. 74:43–54.

Pearl, R. 1928. Rate of Living. Alfred A. Knopf, Inc, New York.
Pegueroles, G., M. Papaceit, A. Quintana, A. P. Guillén, and L. Serra. 1995. An experimental study of evolution in progress: clines for quantitative traits in colonizing and Palearctic populations of *Drosophila*. Evol. Ecol. 9:453–465.
Pélabon, C., G. H. Bolstad, C. K. Egset, J. M. Cheverud, M. Pavlicev, and G. Rosenqvist. 2013. On the relationship between ontogenetic and static allometry. Am. Nat. 181:195–212.
Pfennig, D. W. 1990. The adaptive significance of an environmentally-cued developmental switch in an Anuran tadpole. Oecologia 85:101–107.
Pfennig, D. W., and W. A. Frankino. 1997. Kin-mediated morphogenesis in falcultatively cannibalistic tadpoles. Evolution. 51:1993–1999.
Pfennig, D. W., M. A. Wund, E. C. Snell-Rood, T. Cruickshank, C. D. Schlichting, and A. P. Moczek. 2010. Phenotypic plasticity's impacts on diversification and speciation. Trends Ecol. Evol. 25:459–67.
Pigliucci, M. 2001. Phenotypic Plasticity: Beyond Nature and Nurture. Johns Hopkins University Press, Baltimore, MD.
Pigliucci, M., and C. J. Murren. 2003. Perspective: Genetic assimilation and a possible evolutionary paradox: can macroevolution sometimes be so fast as to pass us by? Evolution 57:1455–64.
Pigliucci, M., C. J. Murren, and C. D. Schlichting. 2006. Phenotypic plasticity and evolution by genetic assimilation. J. Exp. Biol. 209:2362–7.
Pitchers, W., J. E. Pool, and I. Dworkin. 2013. Altitudinal clinal variation in wing size & shape in African *Drosophila melanogaster*: One cline or many? Evolution. 67:438–452.
Price, T., M. Turelli, and M. Slatkin. 1993. Peak shifts produced by correlated response to selection. Evolution. 47:280–290.
R Core Team. 2013. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria.
Reed, S. C., C. M. Williams, and L. E. Chadwick. 1942. Frequency of wing-beat as a character for separating species races and geographic varieties of *Drosophila*. Genetics 27:349–361.
Rhodes, J. S., and T. J. Kawecki. 2009. Behavior and Neurobiology. Pp. 263–300 *in* T. Garland Jr. and M. R. Rose, eds. Experimental Evolution: Concepts, Methods, and Applications. University of California Press, Berkeley, California.
Robert, P., and Y. Escoufier. 1976. A unifying tool for linear multivariate statistical methods: The RV-Coefficient. J. R. Stat. Soc. Ser. C 25:257–265.
Robertson, F. W. 1959. Studies in quantitative inheritance. Xiii. Interrelations between genetic behavior and development in the cellular constitution of the *Drosophila* wing. Genetics 44:1113–1130.
Robinson, R. a S., J. F. Kellie, T. C. Kaufman, and D. E. Clemmer. 2010. Insights into aging through measurements of the *Drosophila* proteome as a function of temperature. Mech. Ageing Dev. 131:584–90.
Rogers, S. M., V. Gagnon, and L. Bernatchez. 2002. Genetically based phenotype-environment association for swimming behavior in lake whitefish ecotypes (*Coregonus clupeaformis Mitchill*). Evolution 56:2322–9.
Rohlf, F. J. 2013. tpsRelw - Thin-plate splines relative warp analysis. http://life.bio.sunysb.edu/morph/soft-tps.html
Rohlf, F. J., and F. L. Bookstein. 2003. Computing the uniform component of shape variation. Syst. Biol. 52:66–69.

Rohlf, F. J., and D. Slice. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. Syst. Zool. 39:40–59.
Santos, M., D. J. Borash, A. Joshi, N. Bounlutay, and L. D. Mueller. 1997. Density-dependent natural selection in *Drosophila*: evolution of growth rate and body size. Evolution. 51:420–432.
Scharf, I., I. Filin, D. Ben-Yehoshua, and O. Ovadia. 2009. Phenotypic plasticity and variation in morphological and life-history traits of antlion adults across a climatic gradient. Zoology 112:139–150.
Scheiner, S. M., and R. F. Lyman. 1989. The genetics of phenotypic plasticity I. Heritability. J. Evol. Biol. 2:95–107.
Schlichting, C. D. 1986. The evolution of phenotypic plasticity in plants. Annu. Rev. Ecol. Evol. Syst. 17:667–93.
Schlichting, C. D., and M. a Wund. 2014. Phenotypic plasticity and epigenetic marking: an assessment of evidence for genetic accommodation. Evolution 68:656–72.
Schluter, D. 1996. Adaptive Radiation Along Genetic Lines of Least Resistance. Evolution. 50:1766–1774.
Schwarzkopf, L., M. W. Blows, and M. J. Caley. 1999. Life-history consequences of divergent selection on egg size in *Drosophila melanogaster*. Am. Nat. 154:333–340.
Shingleton, A. W., W. A. Frankino, T. Flatt, H. F. Nijhout, and D. J. Emlen. 2007. Size and shape: the developmental regulation of static allometry in insects. BioEssays 29:536–548.
Shingleton, A. W., C. K. Mirth, and P. W. Bates. 2008. Developmental model of static allometry in holometabolous insects. Proc. R. Soc. London. Biol. Sci. 275:1875–1885.
Sibly, R. M., L. Winokur, and R. H. Smith. 1997. Interpopulation variation in phenotypic plasticity in the speckled wood butterfly, *Pararge aegeria*. Oikos 78:323–330.
Simon, A. F., D. T. Liang, and D. E. Krantz. 2006. Differential decline in behavioral performance of *Drosophila melanogaster* with age. Mech. Ageing Dev. 127:647–51.
Simpson, G. G. 1953. The Baldwin Effect. Evolution. 7:110–117.
Sinervo, B. 1992. Allometric engineering: A causal analysis of natural selection on offspring size. Science. 258:1927–1930.
Sinervo, B., and R. B. Huey. 1990. Allometric engineering: An experimental test of the causes of interpopulational differences in performance. Science. 248:1106–1109.
Sisodia, S., and B. N. Singh. 2004. Size dependent sexual selection in *Drosophila ananassae*. Genetica 121:207–17.
Smith-Gill, S. J. 1983. Developmental plasticity: developmental conversion versus phenotypic modulation. Am. Zool. 23:47–55.
Sollars, V., X. Lu, L. Xiao, X. Wang, M. D. Garfinkel, and D. M. Ruden. 2003. Evidence for an epigenetic mechanism by which Hsp90 acts as a capacitor for morphological evolution. Nat. Genet. 33:70–4.
Sotherland, P. R., J. A. Wilson, and K. M. Carney. 1990. Naturally-occuring allometric engineering experiments in avian eggs. Am. Zool. 30:A86.
Stalker, H. D., and H. L. Carson. 1947. Morphological variation in natural populations of *Drosophila robusta* sturtavant. Evolution. 1:237–248.
Starmer, W. T., and L. L. Wolf. 1989. Causes of variation in wing loading among *Drosophila* species. Biol. J. Linn. Soc. 37:247–261.

Stasiuk, S. J., M. J. Scott, and W. N. Grant. 2012. Developmental plasticity and the evolution of parasitism in an unusual nematode, *Parastrongyloides trichosuri*. Evodevo 3:1–14.
Stillwell, R. C., I. Dworkin, A. W. Shingleton, and W. A. Frankino. 2011. Experimental manipulation of body size to estimate morphological scaling relationships in *Drosophila*. J. Vis. Exp. e3162.
Stillwell, R. C., W. A. Frankino, I. Dworkin, and A. W. Shingleton. 2014. Tipping the scales: evolution of allometric slope independent of average trait size. *in review*
Suzuki, Y., and H. F. Nijhout. 2006. Evolution of a polyphenism by genetic accommodation. Science. 311:650–652.
Svensson, E. I., and M. Friberg. 2007. Selective predation on wing morphology in sympatric damselflies. Am. Nat. 170:101–12.
Swaddle, J. P. 1997. Developmental stability and predation success in an insect predator-prey system. Behav. Ecol. 8:433–436.
Teuschl, Y., C. Reim, and W. U. Blanckenhorn. 2007. Correlated responses to artificial body size selection in growth, development, phenotypic plasticity and juvenile viability in yellow dung flies. J. Evol. Biol. 20:87–103.
Vandewoestijne, S., and H. Van Dyck. 2011. Flight morphology along a latitudinal gradient in a butterfly: do geographic clines differ between agricultural and woodland landscapes? Ecography. 34:876–886.
Venables, W. N., and B. D. Ripley. 2002. Modern applied statistics with S. Fourth Edition. Springer, New York.
Vijendravarma, R. K., S. Narasimha, and T. J. Kawecki. 2011. Plastic and evolutionary responses of cell size and number to larval malnutrition in *Drosophila melanogaster*. J. Evol. Biol. 24:897–903.
Waddington, C. H. 1953. Genetic assimilation of an acquired character. Evolution. 7:118–126.
Waddington, C. H. 1956. Genetic assimilation of the bithorax phenotype. Evolution. 10:1–13.
Wainwright, P. C., and S. M. Reilly (eds). 1994. Ecological Morphology: Integrative Organismal Biology. University of Chicago Press, Chicago.
Weber, K. E. 1988. A system for rapid morphometry of whole, life flies. *Drosoph*. Inf. Serv. 67:96–101.
Weber, K. E. 1996. Large genetic change at small fitness cost in large populations of *Drosophila melanogaster* selected for wind tunnel flight: Rethinking fitness surfaces. Genetics 144:205–213.
Weber, K. E. 1990. Selection on wing allometry in *Drosophila melanogaster*. Genetics 126:975–989.
West-Eberhard, M. J. 2003. Developmental Plasticity and Evolution. Oxford University Press, Inc, New York.
West-Eberhard, M. J. 2005. Phenotypic accommodation: Adaptive innovation due to developmental plasticity. J. Exp. Zool. 304:610–618.
West-Eberhard, M. J. 1989. Phenotypic plasticity and the origins of diversity. Annu. Rev. Ecol. Evol. Syst. 20:249–278.
Whitman, D. W., and T. N. Ananthakrishnan (eds). 2009. Phenotypic Plasticity of Insects: Mechanisms and Consequences. Science Publishers, Enfield.
Wilson, R. S., and C. E. Franklin. 2002. Testing the beneficial acclimation hypothesis. Trends Ecol. Evol. 17:66–70.
Wright, S. 1931. Evolution in Mendelian populations. Genetics 16:97–159.

Wund, M. a. 2012. Assessing the impacts of phenotypic plasticity on evolution. Integr. Comp. Biol. 52:5–15.
Zelditch, M. L., D. L. Swiderski, and H. D. Sheets. 2012. Geometric Morphometrics for Biologists: A Primer. Academic Press, London.