THE CAUSES AND EFFECTS OF GENITAL HYPOALLOMETRY IN *DROSOPHILA*

By

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ABSTRACT

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The study of size covariation between traits has a long history of describing morphological variation. For over a century, scientists have recognized variation in the proportional size of traits, and have searched to explain the patterns from an evolutionary perspective. Research on the scaling relationships between traits, called allometries, has established the interaction of traits within an organism plays a crucial role in the adaptation of species to their environment. The evolutionary forces that give rise to changes in the proportional size of traits have been more difficult to elucidate.

Using the model organism, *Drosophila melanogaster*, I have focused my research on the scaling of male genitalia in relation to overall body size to explore proximate and ultimate causes of allometries in general. Most traits scale at or near a 1:1 ratio to overall body size, called isometry. In contrast, the male genitalia of many groups scale hypoallometrically to body size, remaining a constant size across a range of body sizes. Determining the factors that drive the atypical allometric relationship of the male genitalia promises to reveal principles of size control across all traits.

To investigate the developmental mechanisms underlying genital hypoallometry, I first compared the effects of genetic variation on genital traits (hypoallometric) to somatic traits (isometric). Previous research has shown that genital traits are less sensitive to environmental variation than somatic traits and here I demonstrate that genital traits are also less sensitive to variation in genetic factors that affect trait size. I

also showed that genitalia have low levels of developmental stability than somatic traits, measured as the response in trait size to stochastic developmental errors. Next, I used targeted gene expression of insulin-signaling genes in developing genital tissues of *Drosophila* to allometrically engineer male flies with extreme genital sizes. Females were exposed to males with different genital sizes, and demonstrated a preference for copulating, and fertilizing progeny, with males that had larger genitalia. To expand the scope of these results, a stochastic mathematical model of allometry evolution was designed that incorporated the developmental regulation of size. Results of simulated allometry evolution showed that the underlying factors controlling final trait size largely determine how scaling relationships respond to selection and evolve.

Collectively, my dissertation represents a significant step forward in our understanding of trait size regulation between covarying traits. Additionally, my research demonstrates the novel use of *Drosophila melanogaster* to modify existing levels of trait variation to test selection hypotheses. Scaling relationships between traits are an important component of morphological evolution that we can continue learning about only via multifaceted research as demonstrated here.

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After just over six years at Michigan State University working on my dissertation, I feel qualified to say several things about what it takes to complete a PhD in the life sciences. First, a good research program is essential. Second, perseverance in the face of constant failure, or at the very least, things constantly failing to go as planned. And third, surrounding yourself with good people. It is the third point that I wish to elaborate on in this section.

The journey to my PhD really started during my undergraduate career while doing research on subterranean insects at Hope College. For the first time in my life it was suggested to me by my research advisor, Thomas Bultman, that it might be possible for me to have a PhD someday. Ever since that little seedling of an idea was planted in my mind, I have worked toward making a PhD happen. Other professors at Hope College supported my aspirations and I thank K. Greg Murray and Kathy Winnet-Murray for reminding me that I am capable. Dr. Bultman provided me with so much support after introducing me to the concept of a PhD, including taking me to Switzerland in the summer of 2008 for some field research, and I will forever be in his debt for stoking the fire of research in me. Two other professors at Hope College had a very formative impact on my research, William Mungle and Wally Fu. Both forced me to stay the course over two semesters of challenging organic chemistry labs, and I have often measured my ability for hard work based on those two semesters.

Once I decided to attend Michigan State University, I was introduced to a new group of academic colleagues with whom I would develop as a scientist. To my wonderful committee members, thank you for your constant support during my time at MSU: Ian Dworkin, Jenny Boughman, Jim Miller and Mark Scriber. My first real contact with MSU was through my PhD mentor, Alex Shingleton, and I will always remember those initial meetings as I was introduced to the concept of proportional trait size and allometry. I will never meet a more intelligent, more dedicated, more unwilling to accept good-as-good-enough person, and I am so thankful for the tremendous impact he has had on me as a scientist and as a human being. Lastly, to Sue and Dick Hill, for their demonstration of how to be an educator in every sense of the word and how to balance work with life outside of the lab, thank you so much.

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TABLE OF CONTENTS

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER 1: General Introduction 1
Introduction 2
Background 2
Definitions of Allometry 3
Variation in Allometry 4
Summary of Dissertation 6
Summary of Chapter 2 7
Summary of Chapter 3 8
Summary of Chapter 4 8
General Methods 9
Study Subjects 9
Morphological Data Collection 11
Writing Style of This Dissertation 12

CHAPTER 2: The effect of genetic and environmental variation on genital size in male *Drosophila*: canalized but developmentally unstable 13
Introduction 14
Materials and Methods 17
Fly Stocks 17
Fly Rearing 18
Genetic Variation 18
Developmental Instability 18
Morphology 18
Analysis 19
Genetic Variation 19
Genetic Static Allometry 20
Developmental Instability 21
Results 23
Discussion 26

CHAPTER 3: Female preference and the hypoallometry of male genitalia in *Drosophila* 32
Introduction 33
Methods 40
Allometric Engineering 40
Fly Assays 43
Morphology 46
Analysis 47
Static Allometry 47

Mating Assay – Behavioral Traits 47
Mating Assay – Female Preference 48
Mating Assay – Reproductive Success 48
Developmental Stability 49
Results 50
Allometric Engineering 50
Static Allometry 52
Mating Assay – Behavioral Traits 52
Mating Assay – Female Preference 52
Developmental Stability 58
Discussion 62
APPENDIX 68

CHAPTER 4: Trait development and the evolution of allometries *in silico* 72
Introduction 73
Methods 79
Developmental Basis 79
Model 79
IndividualSA vs PopulationSA 82
Selection 82
Population Structure 86
Results 88
Discussion 96

CHAPTER 5: Conclusion and future directions 101
Summary of Chapters 102
Chapter 1 102
Chapter 2 102
Chapter 3 103
Chapter 4 104
Conclusion 105
Genital Hypoallometry in *Drosophila* 105
Future Directions 106

REFERENCES 108

LIST OF TABLES

Table A1. Pairwise comparisions by genotype of mating behaviors. No significant difference between genotypes for early stages of courtship. Significant differences in behaviors are indicated by (*). Direct competition regime was not analyzed because males could not be differentiated in video recording of mating trial. All *p*-values calculated by Tukey's HSD tests 71

Table A2. No relationship between the number of progeny sired and genital size. Linear regression of total number of offspring compared to genital size for each mating regime. No correlation or significant relationship between genital size and reproductive success across all males that sired offspring 71

LIST OF FIGURES

Figure 2.1. Genetic variation, allometric coefficient and fluctuating asymmetry of somatic and genital traits in male *Drosophila melanogaster*. (A) Genital traits had low levels of total genetic variation (light gray bars, CV_T) but not low levels of organ-autonomous genetic variation (dark gray bars, CV_I). The difference between total genetic variation and organ-autonomous variance is an estimate of genetic variation that is correlated with variation in other traits ('systemic' genetic variation). Columns with the same letter are not significantly different for total genetic variation (CV_T) using Tukey's HSD ($P > 0.05$). Traits do not differ for organ-autonomous genetic variation (CV_I) using Tukey's HSD ($P > 0.05$ for all) (B) The low systemic genetic variance of the genital traits reflected their low multivariate allometric coefficient compared to most somatic traits, although these differences are not significant for multiple comparisons (Tukey's HSD, $P>0.05$ for all). Grey horizontal line is isometry. (C) Genital traits did not show low levels of fluctuating asymmetry. Light grey bars, line 303, white bars, line 324, dark grey bars, line 335. Within a line, columns with the same letter are not significantly different for FA using Tukey's HSD (P 0.05). All error bars are 95% confidence intervals 24

Figure 2.2 Model of a selection regime that alters the slope of the genital-body scaling relationship while maintaining genital-autonomous genetic variation. Selection is for proportionally smaller genitalia in large males and proportionally larger genitalia in small males (black arrows). Implicit to such a regime is that there is selection or constraint maintaining variation in body size (gray arrows) 25

Figure 3.1. *Elav-GAL80* expressed with *Poxn* rescues wild-type mating behavior. Courtship latency of IIS manipulated flies (*Poxn > dFOXO.3x)* had significantly longer courtship latency than control male flies (*Poxn > UAS-GFP*). Coexpression of an *Elav-GAL80* that prevented expression of GAL4 in neuronal tissues recovered wild-type courtship latency (*Elav-GAL80:Poxn > FOXO.3x*). The symbol (a) denotes significance (Tukey's HSD $p < 0.0001$) 42

Figure 3.2. IIS perturbation using *Poxn* changes size of the genitalia only. Size of the posterior lobe of the genital arch was significantly smaller than the large or wild-type genital size classes (Tukey HSD < 0.001, represented by symbol 'a'). There were no significant differences in size of the wings or puparial between the genital size classes (Tukey HSD > 0.05). Data for genital size classes were pooled by the IIS transgene present in the fly (large: *InR.CA*, wild-type: *UAS-GFP*, small: *dFOXO.3x*) because there was no difference in size between flies with or without the *Ubi-GFP* (Tukey HSD > 0.05). Error bars represent SEM 51

Figure 3.3. Males with larger genitalia are more likely to mate in single male regime. Logistic regression of male mating success (probability = 1.0) or failure (probability = 0) by log-transformed genital size of males. Females were more likely to mate with males that had larger genitalia ($p = 0.009$). Males with small genitalia were able to successfully

mate with females, but did so at a lower frequency than males with larger genital sizes. Histogram bars in grey (top) show frequency of successfully copulation and black bars (bottom) show frequency of failure to copulate by genital size. Line represents logistic regression probability curve 53

Figure 3.4. Males with larger genitalia are more likely to sire offspring in single mating regime. Logistic regression of a female fertilizing (probability = 1.0) or not fertilizing (probability = 0) eggs by log-transformed genital size. Females were more likely to fertilize eggs using sperm from males with larger genitalia (p = 0.001). Females fertilized eggs using sperm form males with small genitalia, but at a lower frequency than sperm from males with larger genital sizes. Histogram bars in grey (top) show frequency of successful fertilization and black bars (bottom) show frequency of failure to fertilize by genital size. Line represents logistic regression probability curve 54

Figure 3.5. Males with larger genitalia were more likely to mate in the direct competition regime. Logistic regression of a male mating success (probability = 1.0) or failure (probability = 0) by log-transformed genital siz. Females were more likely to mate with males that had larger genitalia (p = 0.002). Males with small genitalia were able to successfully mate with females, but did so at a much lower frequency than males with larger genital sizes. Histogram bars in grey (top) show frequency of successful copulation and black bars (bottom) show frequency of failure to copulate by genital size. Line represents logistic regression probability curve 56

Figure 3.6. Males with larger genitalia were more likely to sire progeny in the indirect competition regime. Logistic regression of female fertilizing (probability = 1.0) or not fertilizing (probability = 0) eggs by log-transformed absolute genital size. There was a significant positive relationship between genital size and fertilization probability (p = 0.031). Sperm from males with small genitalia was used to fertilize eggs, but at a much lower frequency than sperm from males with larger genital sizes. Histogram bars in gray (top) show frequency of fertilization and black bars (bottom) show frequency of failure to fertilize by genital size. Line represents logistic regression probability curve 57

Figure 3.7. Fluctuating asymmetry (FA) is higher in genital traits than the wings in the single male regime. There was no difference in FA values by trait across the genital size classes, but there was a difference between the traits. FA was calculated using the FA10b value which accounts for measurement error in log-transformed traits. Error bars are 95% confidence intervals 59

Figure 3.8. No difference in FA between flies that mated and did not mate in the direct competition regime. Error bars are 95% confidence intervals 60

Figure 3.9. No difference in FA between flies that sired progeny and those that did not in the indirect competition regime. Error bars are 95% confidence intervals 61

Figure 3.10. Response in genitalia and wings to IIS manipulation to make large and small traits. Wing significantly increased and decreased in size in response to up-regulating (large: *NP6333>InR.CA*) and down-regulating (small: *NP6333>InR.DN*^{25}) IIS in those tissues (Tukey's HSD < 0.05). Posterior lobe of the genital arch only decreased in size in response to down-regulating IIS (small: *Poxn>dFOXO.3x*) but did not increase in size when IIS was up-regulated (large: *Poxn>InR.CA*) suggesting the genitalia are insensitive to positive nutritional growth regulators or are at a maximum size in response to nutrition. Error bars are 95% confidence intervals. Control flies were > *UAS-GFP* for both traits 65

Figure A1. Complete mating assay consisting of 18 – 35mm x 10mm mating chambers with mating pairs visible in each chamber 69

Figure A2. There was a difference in allometric coefficient between, but not within, traits. The allometric coefficient was lower in the genitalia thatn the wing, as expected. he allometric coefficients do not vary across size class, but are different between traits. Error bars are 95% bootstrap confidence intervals from 1000 replicates 70

Figure 4.1. Population static allometry and the individual static allometry. (A) A hypothetical populationSA demonstrating the relationship between covarying traits T1 and T2 comprised of 25 individuals, represented by unique colors. (B) Individuals express only one phenotype (colored circle) of the possible individualSA possible for their genotype (colored lines) based on the environmental conditions they are exposed to during development 78

Figure 4.2. Patterns of individualSA. (A, A') When $\mu_G = 0$, the populationSA appears normally distributed (A) and the individualSA have equal levels of variation as the size of T1 increases or decreases (A') creating a bowtie shaped pattern. (B, B') When $\mu_G > 0$, the populationSA appears similar in distribution to populations with $\mu_G = 0$, including similar slope and covariation between traits T1 and T2 (B), but the underlying individual-level static allometries exhibit more variation as the size of T1 increases (B') producing a speedometer shaped pattern. (C, C') When $\mu_G < 0$, the population-level static allometry has the same appearance and statistical properties as populations with $\mu_G > 0$ or $\mu_G = 0$, but the individual-level static allometries exhibit more variation as the size of T1 decreases (C') creating a broomstick shaped pattern 84

Figure 4.3. Four selection regimes: Stabilizing selection (A), Directional selection (B), Proportional selection (C) and Correlational selection (D). Grey points indicate selected individuals red points indicate individuals removed from the population prior to reproduction. The positive form of selection is shown in B-D with negative selection for those types removing individuals from the opposite orientation in the population 87

Figure 4.4. Evolution of populationSA for ten generations of selection on bowtie populations ($\mu_G = 0$). Inset plots display the change in the allometric coefficient (α) through time (generation). (A) The individualSA pattern when $\mu_G = 0$. Generations 0 –

10 represented by colored lines blue – red. The effect on the populationSA from stabilizing selection (B), positive and negative directional selection (C,C'), positive and negative proportional selection (D,D') and positive and negative correlational selection (E,E') 90

Figure 4.5. Evolution of populationSA for ten generations of selection on speedometer populations ($\mu_G > 0$). Inset plots display the change in the allometric coefficient (α) through time (generation). (A) The individualSA pattern when $\mu_G > 0$. Generations 0 – 10 represented by colored lines blue – red. The effect on the populationSA from stabilizing selection (B), positive and negative directional selection (C,C'), positive and negative proportional selection (D,D') and positive and negative correlational selection (E,E') 91

Figure 4.6. Evolution of populationSA for ten generations of selection on broomstick populations ($\mu_G < 0$). Inset plots display the change in the allometric coefficient (α) through time (generation). (A) The individualSA pattern when $\mu_G < 0$. Generations 0 – 10 represented by colored lines blue – red. The effect on the populationSA from stabilizing selection (B), positive and negative directional selection (C,C'), positive and negative proportional selection (D,D') and positive and negative correlational selection (E,E') 92

Figure 4.7. Initial (blue) and final (red) bowtie populations ($\mu_G = 0$) after 10 generations of selection. All lines are of the corresponding colored populationSA calculated using major axis regression. Represented are stabilizing selection (B) positive and negative directional selection (C, C') positive and negative proportional selection (D, D'), positive and negative correlational selection (E, E') 93

Figure 4.8. Initial (blue) and final (red) speedometer populations ($\mu_G > 0$) after 10 generations of selection. All lines are of the corresponding colored populationSA calculated using major axis regression. Represented are stabilizing selection (B) positive and negative directional selection (C, C') positive and negative proportional selection (D, D'), positive and negative correlational selection (E, E') 94

Figure 4.9. Initial (blue) and final (red) broomstick populations ($\mu_G < 0$) after 10 generations of selection. All lines are of the corresponding colored populationSA calculated using major axis regression. Represented are stabilizing selection (B) positive and negative directional selection (C, C') positive and negative proportional selection (D, D'), positive and negative correlational selection (E, E') 95

CHAPTER 1:

General Introduction

Introduction

Background

Understanding why organisms are the size and shape that they are has been a focus of research for well over a century. How populations adapt to their environment through morphological diversification is of great interest to evolutionary biologists, driven by the visually apparent variation in body plan across taxonomic groups (Peters 1983, Wagner and Lynch 2010). Some of the most common classes of adaptations by organisms to their environment are phenotypic modification, and modifications to the degree of integration between traits within an organism (Schmidt-Nielsen 1984, Brown and West 2000, Klingenberg and Marugan-Lobon 2013). Much remains to be discovered about how integrated traits in a population respond to selection. Specifically, how is selection affecting change in the phenotypes of covarying traits in a population? To what degree does the development of tissues constrain or facilitate response to selection? How can we elucidate the selection pressures acting on populations and generate predictions for the evolutionary outcomes from different forms of selection? Research on the evolution of morphology spans many disciplines and includes behavioral, developmental, genetic, and physiological research. The goal of this dissertation is to use a multifaceted approach to test questions of morphological evolution, first through empirical research on a specific trait size/body size relationship and more broadly through theoretical work that uses a mathematical model to explore how selection can change the relationships between traits within an organism.

The evolution of size and shape springs from how traits are related to one another developmentally and functionally (Calder 1984). As organisms adapt to their

environments, the maintenance of trait functionality is a potentially powerful mechanism for their survival. The balance between maintaining utility and adapting to new conditions often hinges on the relative size of traits to one another (Pelabon et al. 2014). For example, the size of a flying insect's wing needs to be large enough to provide sufficient lift for a given body size to fly, but not so large as to be energetically costly to generate and maintain. An observable pattern throughout natural systems is that much of the morphological diversity across taxa can be distilled to variation in the proportional size of the same or similar traits (Thompson 1942, Gould 1966, Emlen and Allen 2003).

Definitions of Allometry

How traits are developmentally connected to one another can be described in several contexts. Julian Huxley (1932) first used the term allometry to refer to any trait that deviated from maintaining a 1:1 consistent size ratio with body size. Presently, allometry is often used to refer to the study of any scaling relationships between two traits (Gould 1966, Pelabon et al.). Implicit to the concept of allometry is that there is covariation between the two traits being studied, indicative of a biologically meaningful link such as shared developmental control mechanisms or a behavioral integration of traits that must work in conjuction with one another for survival and reproduction.

There are three categories of allometries that describe different aspects of trait covariation: evolutionary, ontogentic and static allometries (Cock 1966, Gould 1966, Cheverud 1982). An evolutionary allometry compares the scaling relationships between traits across related taxanomic groups at the same developmental stage (typically

species or populations). An ontogenetic, or growth, allometry compares individuals of the same species across developmental stages. The third type of allometry is a static allometry and compares the size of traits in individuals of the same species all at the same developmental stage. Static allometries most often compare a trait to overall body size of an organism but can be applied to any co-varying traits within an organism. The source of size variation further distinguishes the classification (Shingleton et al. 2007). For traits that vary due to environmental conditions, such as nutrition levels or temperature, the comparison is referred to as an environmental static allometry. Phenotypic variation that is due to genetic variation in organisms that have all been exposed to the same environmental conditions are called genetic static allometries.

Variation in Allometry

Allometric relationships are often described mathematically using the allometric equation $y = bx^{\alpha}$ which relates two traits, x and y (Huxley 1932). The allometric equation is often log-transformed, making the relationship a linear comparison between traits and scale-independent for comparison across trait scales (Kerkhoff and Enquist 2009). As a result of the log-transformation, the allometric equation becomes linear as $\log(y) = \alpha * \log(x) + \log(b)$, where the slope is defined by α, the allometric coefficient, and the allometric intercept is log(b). Much of the morphological variation between pairs or sets of traits can be encapsulated by changes in these two parameters (Gould 1966). For instance, traits with an allometric coefficient (α) of 1 are classified as isometric. Any increase in the size of one trait results in a proportional change in the associated trait. Traits with $\alpha > 1$ are classified as hyperallometric and increase disproportionately in size

with their associated trait. Traits with $\alpha < 1$ are classified as hypoallometric and remain more or less the same size across a range of their associated trait sizes. The intercept term describes the relative size of a trait that is independent of the associated trait. An increase or decrease in only the intercept of the allometric equation results in relatively larger or smaller traits across a range of the covarying trait, respectively (Shingleton 2012).

The causes of deviation from the norm of isometry are intimately tied to the general principles of growth control. Proximate causes for different allometric relationships involve a modification of the mechanisms that link environmental and genetic size regulators with individual trait growth (Shingleton et al. 2005, 2007). For example, hyperallometric traits could be the result of hyper-sensitivity to growth cues in response to environmental inputs (such as nutrition levels) resulting in disproportionate growth as compared to traits that have similar sensitivities to growth cues as the body in general (Emlen et al. 2012). Hypoallometric traits, on the other hand, are less sensitive to growth factors resulting in a more constant size in the face of environmental variation (Tang et al. 2011). The ultimate causes for different scaling relationships lie in the types of selection that can give rise to allometries, although the effects of specific types of selection continue to be a question with no clear answer (Bonduriansky and Day 2003, Kodric-Brown et al. 2006, Eberhard et al. 2009, Cayetano et al. 2011, Arendt and Fairbairn 2012, Berger et al. 2012, Fromhage and Kokko 2014, Pelabon et al. 2014). Theoretical predictions suggest that the same scaling relationships can be the possible outcomes of different forms of selection, and that different forms of selection can result

in the same scaling relationships. While this very well may be the case, the field of allometric research is in need of an updated framework that synthesizes our understanding of trait development with the evolution of proportional trait size to continue moving forward.

Summary of Dissertation

My doctoral dissertation research focuses on how scaling relationships evolve and the integral role they play in developing morphological diversity. My dissertation consists of four chapters following this introductory chapter. The first research chapter, Chapter 2, describes the phenomenon of genital hypoallometry within male *Drososphila melanogaster* and suggests possible causes for the atypical scaling relationship observed in male genitalia across arthropods. Chapter 3 is a test for the type of selection acting on male genitalia in *D. melanogaster* using the genetic tools available to manipulate genital size beyond what would be found in natural populations. Chapter 4 outlines a mathematical model developed to test the effects of different forms of selection on populations and generate predictions of how allometries can evolve. Chapter 5 is a conclusion chapter that comments on the causes of genital hypoallometry in *Drosophila*.

Collectively, my dissertation presents a novel approach to the ongoing question of how allometries evolve and attempts to test theories using a known biological example of hypoallometry. My dissertation is among the first to use the GAL4-UAS system in *Drosophila* to test questions about selection on trait size by artificially modifying phenotypic variation without physical manipulation. In total, my dissertation represents a

significant step forward in understanding how phenotypic traits have evolved into the sizes observed today, and how they will continue to evolve in the future.

Summary of Chapter 2

The genitalia of most male arthropods scale hypoallometrically with body size, that is, they are typically the same size among large and small individuals in a population. Such scaling is expected to arise when genital traits show less variation than somatic traits in response to factors that generate size variation among individuals in a population. Nevertheless, there have been few studies directly examining the relative sensitivity of genital and somatic traits to factors that affect their size. Such studies are key to understanding genital evolution, and more generally, the evolution of morphological scaling relationships. Previous studies indicate that the size of genital traits in male *D. melanogaster* show a relatively low response to variation in environmental factors that affect trait size. In this chapter the size of genital traits in male fruit flies is shown to also exhibit a relatively low response to variation in genetic factors that affect trait size. Importantly, however, this low response is only to genetic factors that affect body and organ size systemically, not those that affect organ size autonomously. Furthermore, it is shown that the genital traits do not exhibit low levels of developmental instability, i.e. the response to stochastic developmental errors that also influence organ size autonomously. These results are discussed in the context of current hypotheses on the proximate and ultimate mechanisms that generate genital hypoallometry.

Summary of Chapter 3

Across a large portion of the animal kingdom, male genital size remains near constant among individuals in a population, despite considerable phenotypic variation in body size and the size of other organs. Several competing hypotheses have been proposed to explain the selective pressures underlying this commonly observed phenomenon. However, a lack of experimental data makes it difficult to distinguish between the different hypotheses. For any test of how selection is acting using a natural population, morphological variation that exceeds the natural range of size is required. To satisfy this requirement, “allometric engineering” in *D. melanogaster* was used to modify a nutrient-sensing pathway in specific tissues and produce males with extreme genital morphologies. Males of different genital sizes were then used to assay the effects of genital size on various measures of reproductive success. The results of these experiments indicate females prefer males with larger genital sizes as compared to smaller genitalia for copulation and fertilization but are able to copulate with males of any genital size. Females choose to fertilize eggs at the same frequency regardless of the genital size of the male from which the sperm came. The ability to engineer male flies with smaller but not larger genitalia, despite a demonstration of increase and decrease in wing size due to perturbation of the same nutrient-sensing signals, suggests male genitalia may be less sensitive to signals of increasing size or at a maximum size in response to nutrition-based growth regulators.

Summary of Chapter 4

How scaling relationships evolve is an ongoing question in the field of morphological evolution. Theory predicts that the effects of selection on scaling

relationships is difficult to anticipate, and empirical work has done little to resolve the question while focusing on exaggerated traits typical of secondary sexual characteristics. In chapter 4 the understanding of development in holometabolous insects is used to construct a stochastic mathematical model that simulates populations of individuals with a set of hypothetical covarying traits that are free to evolve in response to different types of selection. The developmental parameters included in the model represent general classes of growth factors that are known to control trait development. The results of simulations show that the underlying growth factors have a profound effect on the predicted response to selection. Based on which mechanisms are controlling size, populations can have different possible responses to selection on trait size. It is possible for the same scaling relationships to be the result of multiple selection regimes. Conversely, it is also possible for the same type of selection to result in multiple scaling relationships based entirely on how the individuals in a population are able to respond to selection. Additionally, the results suggest scaling relationships that are ostensibly identical can respond to the same selection pressure in different ways. The underlying developmental control mechanisms that are responsible for regulating trait size, and the covariation between integrated traits, is a primary factor in the determination of how scaling relationships evolve.

General Methods

Study Subjects

Drosophila melanogaster are a well-recognized model system for a variety of biological applications. They are holometabolous insects, defined as progressing through three distinct life stages: larva, pupa and adult (Klowden 2007). *D.*

melanogaster are often reared under standard laboratory conditions, accepted to be 25°C and either 24 hr constant light exposure or 12 hr : 12 hr light : dark cycles (Stocker and Gallant 2008). At 25°C, the *D. melanogaster* life cycle takes nine days from egg to sexually mature adult making them highly amenable to research that requires large numbers of individuals (Stocker and Gallant 2008). A standard cornmeal/molasses fly food was made for all flies at Michigan State University as needed.

One of the most powerful genetic tools available to *D. melanogaster* is the GAL4/UAS system, which allows controlled expression of transgenes in flies (Duffy 2002). GAL4/UAS is a transcriptional regulation system isolated from the yeast *Saccharomyces cerevisiae*. GAL4 encodes a protein that binds to the Upstream Activating Sequence (UAS) and initiates transcription of a gene associated with the UAS. GAL4 is expressed in a tissue or temporal specific manner, and when combined with a UAS transgene construct in a single fly, allows for targeted expression of a gene that is localized spatially and/or temporally. There are a number of GAL4 and UAS transgenes that are publicly available, having been created and screened for expression through the use of a P-transposase promoter. An additional level of expression control is possible through the use of the protein GAL80 that is also expressed in a tissue specific manner and competitively binds to GAL4 to prevent any transcriptional activity (Elliot and Brand 2008). While many more genetic tools are possible using *D. melanogaster*, the GAL4/UAS system and the GAL80 protein were the primary techniques used in this dissertation.

Fly strains used in laboratory experiments were based on the standard isogenic lab strain *Samarkand* (*Sam*) or on wild caught lines from Raleigh, NC (*Ral*). Flies were obtained from the Bloomington Drosophila Stock Center (BDSC) at Indiana University unless otherwise noted. Controlling for the possible confounding effects of different genetic backgrounds on the expression of a gene is a crucial step in any study manipulating gene expression (Chari and Dworkin 2013). To minimize the effect of genetic background, all transgenes were introgressed into the *Sam* line for a minimum of five generations. The introgression of transgenes into a common genetic background is designed such that a gene of interest is repeatedly inserted into a genetically homogenous line. After a sufficient number of generations, only the transgene that is traceable through genetic markers should be present in the otherwise homogenous background.

Morphological Data Collection

All morphological trait measurements were dissected using micro forceps in 75% ethanol. Flies used in any behavioral assays were reared socially isolated as individuals in 2 mL microfuge tubes. Because all growth is fixed during the pre-adult stages in holometabolous inects, puparial case size is tightly correlated to adult body size (Chiang and Hodson 1950). Therefore, puparial case area was used as an estimate of body size whenever possible. To trace puparial cases to their respective adult fly, flies were allowed to eclose in marked 2 mL microfuge tubes and the puparial cases were saved for measurement.

Writing Style of This Dissertation

My dissertation is the direct result of collaboration with others who have contributed critical pieces to the work. I could not have completed this body of work without their direct input and guidance on my research. In recognition of their efforts, I will use the first person plural and not the first person singular for the remainder of the dissertation.

CHAPTER 2:

Dreyer, AD and Shingleton, AW. 2011. The effect of genetic and environmental variation on genital size in male *Drosophila*: canalized but developmentally unstable. *PloS One* Vol. 6(12): pp.e28278

Introduction

Within a population or species, variation in body size is expected to be accompanied by approximately equivalent variation in the size of individual morphological traits. Such covariation is necessary to maintain correct body proportion across the range of body sizes observed in animal populations. A notable exception to this pattern, however, is the relationship between genital size and body size in arthropods. The genitalia of most arthropods are more-or-less the same size in both large and small individuals (Eberhard et al. 1998, Bernstein and Bernstein 2002, Funke and Huber 2005, Hosken et al. 2005, Kawano 2006). Consequently, smaller males have proportionally larger genitalia than larger males. While the phenomenon is most obvious in males, it has also been observed in female arthropods (Eberhard et al. 1998, Palestrini et al. 2000, Uhl and Vollrath 2000, Tatsuta et al. 2007), as well as some mammals (Patterson 1982, Oosthuizen and Miller 2000, Eberhard 2009).

The scaling relationship between two traits among individuals of the same developmental stage in a population is called a static allometry, and is typically described using the allometric equation, $y = bx^{\alpha}$, where *x* and *y* are the size of two traits (Huxley and Teissier 1936). Log transformation of this equation produces the simple linear equation $\log(y) = \alpha \log(x) + \log(b)$, and log-log plots of the size of different traits among individuals in a population typically reveal linear scaling with a slope of α, called the allometric coefficient (Huxley and Teissier 1936). When $\alpha = 1$, the relationship between *x* and *y* is called isometry, with the ratio of *y* to *x* remaining constant across a range of *x*. When $\alpha < 1$ or > 1 the relationship is hypo- or hyperallometric, respectively,

with relative size of *y* decreasing (hypoallometry) or increasing (hyperallometry) with an increase in *x*. Fundamental to the concept of allometry is that x and y covary; that is the factors that generate variation in *x* also generate variation in *y.* The allometric coefficient therefore captures the extent to which these factors affect *y* relative to *x*. If a factor that generates size variation affects both traits equally, then *y* will scale isometrically to *x* (assuming that all size variation is due to the factor). If the factor has a lesser or greater effect on *y* than *x*, *y* will scale with *x* hypo- or hyperallometrically respectively (Shingleton et al. 2007).

The observed hypoallometry of the male genitalia in arthropods suggests that genital traits are relatively insensitive to the factors that generate size variation among individuals in a population. Size variation may be generated by environmental variation (plasticity), genetic variation and developmental instability (variation due to stochastic developmental perturbations within an individual, (Van Valen 1962, Palmer and Strobeck 1986, Nijhout 2003). Consequently, we might expect the genitalia to be environmentally canalized, genetically canalized and/or developmentally stable. Here, we define canalization as the property of a trait to resist genetic or environmental variation (Waddington 1961, Stearns et al. 1995, Debat 2001), and developmental stability as the property of a trait to resist stochastic developmental perturbations that generate fluctuating asymmetry (FA) in a bilaterally symmetrical organism (Parsons 1990, Debat 2001). Previous studies have demonstrated that the male genitalia of *Drosophila* are environmentally canalized, at least with respect to developmental

nutrition, temperature and larval crowding (Shingleton et al. 2009). It is unclear, however, whether they are also genetically canalized and developmentally stable.

In contrast to our lack of understanding of the developmental mechanisms that underlie genital hypoallometry, there are a number of hypotheses as to its adaptive significance (Arnqvist 1997, Bonduriansky and Day 2003, Hosken and Stockley 2004). A general theme of many of these hypotheses is that there is stabilizing selection on male genital size, either because females are physically unable to mate with males bearing inappropriately-sized genitalia (Dufour 1936) or because females prefer males with genitalia of a specific size (Eberhard et al. 1998). Alternatively, hypoallometry may arise because there is directional selection on increased genital size that is strong in small males but weak or absent in large males (Eberhard et al. 2009). These different hypotheses, while not mutually exclusive, serve to emphasize the observation that the form of selection on genitalia can be difficult to infer from patterns of allometry (Bertin and Fairbairn 2007).

Elucidating the proximate mechanisms that generate genital hypoallometry may help clarify the ultimate evolutionary processes that cause it. This is because different evolutionary hypotheses suggest different patterns of genetic and environmental variation in genital size. For example, if genital hypoallometry were a consequence of elevated levels of stabilizing selection on genital size, we would expect to see a reduction in the level of genetic variation in genital size relative to other traits: that is they should be genetically canalized (Falconer and Mackay 1996, Eberhard 2009). We

might also expect the genitalia to be environmentally canalized and developmentally stable (Scharloo et al. 1967, Kaufman et al. 1977, Gavrilets and Hastings 1994, Falconer and Mackay 1996, Eshel and Matessi 1998, Gibson and Wagner 2000), but see (Siegal and Bergman 2002).

Here we measure the level of genetic variation and developmental stability in genital and somatic traits in *D*. *melanogaster*. Consistent with our understanding of the mechanisms that generate morphological scaling relationships, we find that genital traits are genetically canalized. However, the genitalia are only canalized with respect to genetic factors that affect the size of all organs in the body systemically. Genital traits are not canalized with respect to genetic factors that affect the size of individual organs autonomously. Further, we find that genital traits are not developmentally stable as indicated by elevated levels of fluctuating asymmetry relative to some somatic traits. We discuss these findings in light of current theories of genital evolution and argue that stabilizing selection on genital size alone is insufficient to explain their hypoallometric relationship with body size.

Materials and Methods

Fly Stocks

Male flies were from 38 of the Core40 isogenic wild-type lines from the *Drosophila* Genetic Reference Panel (DGRP).

Fly Rearing

Genetic Variation

Genetic variation was assayed among the Core40 isogenic DGRP lines. Larvae from each line were reared in vials at low density (≤ 50 larvae) on standard cornmeal:molasses medium at 25˚C in constant light. We collected, dissected and measured males from at least three vials per line, totaling ten males per line.

Developmental Instability

We used three of the Core40 DGRP lines to assay developmental instability (lines 303, 324, 335). Larvae were reared at low density (≤ 50 larvae) in ten vials per line, as described above. We selected, dissected and measured five males from each vial, totaling 50 males per line.

Morphology

Five organs were dissected from each male fly: three somatic traits (the wing, the femur of the first leg, and the maxillary palp) and two genital traits (the posterior lobe of the genital arch and the anal plate). Organs were mounted in dimethyl hydantoin formaldehyde for imaging. Organ measurements were taken as area for the wing, the maxillary palp, the posterior lobe of the genital arch, and the anal plate and as the length of the femur, using a Leica DM6000B compound microscope and Retiga 200R digital camera. We also measured a fourth somatic trait, thorax length, as the distance between the attachment of the neck to the posterior tip of the scutellum using a Leica MZ16FA dissecting microscope and a Leica DFC250 digital camera. Images were

analyzed using ImagePro. All linear measurements were squared prior to analysis to convert them to the same dimension as area measurements. All data were then natural log transformed to allow the fitting of the linear allometric equation. For the measurement of FA, we measured the wing, femur, maxillary palp and posterior lobe of the genital arch from both sides of the fly three times, and calculated measurement error using the methods of Palmer and Strobeck (Palmer and Strobeck 2003).

Analysis

Genetic Variation

We fit the data to the following linear model:

$$Y = u + G + e$$

where *Y* is the morphological measurement, *u* is the intercept term, *G* is the effect of line (random factor) and *e* is remaining non-genetic variation. We used the *lmer* function in the *lme4* package in R (R-Development-Core-Team 2009) to extract the variance components using REML for *G*, which is a measure of the total genetic variation of *Y,* here referred to as V_T. Each V_T was then converted into a coefficient of variation (CV_T) using the formula $CV_T = \sqrt{e^{V_T} - 1}$ (Wright 1952). CV_T was used as a measure of a trait's total genetic variation.

We reanalyzed the data but statistically controlled for variation in other traits by including them as covariates in our model. This allowed us to estimate the amount of

genetic variation in a trait that was orthogonal to and independent of variation in all other traits, that is a trait's organ-autonomous genetic variation. The final model was:

$$Y = u + A + B + ... + G + e$$

where *A, B etc.* are the size of all other traits. We then extracted the variance components for *G,* (V_I) which is a measure of the organ-autonomous genetic variation of *Y*, here referred to as V_I, from the analysis. We used V_I to calculate the organ-autonomous coefficient of variation (CV_I) using the formula $CV_I = \sqrt{e^{V_I} - 1}$.
Each dataset was sampled with replacement to generate 1000 bootstrap datasets, which were analyzed and used to construct a 95 percent confidence interval of each trait's total (CV_T) and organ-autonomous (CV_I) genetic variation.

Genetic Static Allometry

The allometric coefficient of the genetic static allometry (where size variation is solely a consequence of genetic variation) was calculated from the mean log-transformed trait measurements for each line. We used these data to calculate the variance-covariance matrix for traits among lines, and extracted the first eigenvector from this matrix using the *svd* function in the base package of R (R-Development-Core-Team 2009). The allometric coefficient is reflected by the loadings of the first eigenvector. Isometry occurs when all loadings of the vector equal $1/\sqrt{n}$, where *n* is the number of traits measured. Multiplying the loadings by $\sqrt{n}$ gives the bivariate allometric coefficient for each trait against a measure of overall body size (Klingenberg 1996). We

used a random-variable bootstrap method to generate 95 percent confidence intervals for the allometric coefficients for each trait (Shingleton et al. 2009).

Developmental Instability

Here we define developmental instability as the imprecision that results from developmental noise, the random developmental processes that cause a trait to deviate from its expected growth trajectory given its genotype and environment. Conversely, the capacity of the growing organ to counteract developmental noise is defined as developmental stability. Fluctuating asymmetry is therefore a measure of developmental instability, and is reduced in organs that are developmentally stable. We used the FA10b index, which corrects for measurement error, to quantify fluctuating asymmetry for the wing, maxillary palp, femur and posterior lobe of the genital arch (Palmer and Strobeck 2003). To calculate FA10b we fit the repeated measurement of each trait to the following model:

$$Y = u + S + I + SI + e$$

where *u* is the intercept term, *S* is the effect of body side, left or right (fixed factor), *I* is the effect of the individual (random factor), *SI* is the interaction between individual and side and *e* is measurement error. We used the *lmer* function in the *lme4* package in R (R-Development-Core-Team 2009) to estimate the variance components for *SI* (σ^2_{SI}), which is used to calculate FA10b:

$$FA10b = 0.798\sqrt{2\sigma^2_{SI}}$$

We used the *MCMCglmm* function in the *MCMCglmm* package in R (R-Development-Core-Team 2009) to generate values of 95 percent support for each trait's level of fluctuating asymmetry. We used a prior equal to the variation in wing size measurements to generate parameter estimates and compared the results to those using a non-informative prior and found no difference in parameter estimates.

All traits were tested for antisymmetry and directional asymmetry by assaying the distribution of trait size on the right (R) and left (L) side of an individual. For antisymmetry we tested the (R – L) distribution for normality and for directional asymmetry we compared the mean of the signed (R – L) to zero (Palmer and Strobeck 2003). (R – L) for almost all traits was normally distributed (Shapiro-Wilk test, $P > 0.004$ with Bonferonni correction). The only exceptions were the maxillary palps of line 303 and wings of line 335 ($P<0.004$ for both). Plotting R versus L for the size of both these traits suggested three maxillary palp measurements from line 303 and seven wing measurements from 335 were outliers. Removal of these data normalized the distribution of (R – L) for both these traits, although their inclusion had no effect on the analysis (not shown). The maxillary palp of line 324 also showed evidence of slight directional asymmetry with mean (R – L) deviating significantly from zero (t-test, $p<0.004$ with Bonferoni correction). However, the mean (R – L) was less than *FA*4*a* (where $FA4a = 0.798\sqrt{\sigma^2_{(R-L)}}$,), and so any directional asymmetry was considered to be a consequence of developmental instability (Palmer and Strobeck 2003).

Results

The total amount of genetic variation (CV_T) was lower for the genital traits (genital arch and anal plate) than for the somatic traits (wing, maxillary palps and thorax) (Figure 2.1A), although when correcting for multiple comparisons this reduction in genetic variation was significant only for the genital arch (Tukey's HSD, $p < 0.05$). In contrast, none of the traits differed in their level of organ-autonomous genetic variation (CV_I) – that is the amount of genetic variation in trait size that is not correlated with variation in the size of other traits – when correcting for multiple comparisons (Tukey's HSD, $p > 0.05$) (Figure 2.1B). These results suggest that very little of the variation in genital trait size is a response to variation in genetic factors that affect all traits systemically. It is the response to these systemic genetic factors that controls the slope of an organ's scaling relationship with body size on a genetic static allometry: traits with low response should scale hypoallometrically with body size. Correspondingly, we found that the genital traits were significantly more hypoallometric to overall body size than most somatic traits (Figure 2.1B). Interestingly, the femur of the first leg, like the genital traits, displayed both low levels of genetic variation and scaled more hypoallometrically to overall body size than other somatic traits (Figures 2.1A & 2.1B).

Although the genital traits were genetically canalized with respect to factors that affect organ size systemically, they did not show low levels of developmental instability. In contrast, within each of the three lines examined, the maxillary palp and the genital arch had significantly higher levels of fluctuating asymmetry than either the wing or the femur (Tukey's HSD $p > 0.05$) (Figure 2.1C).

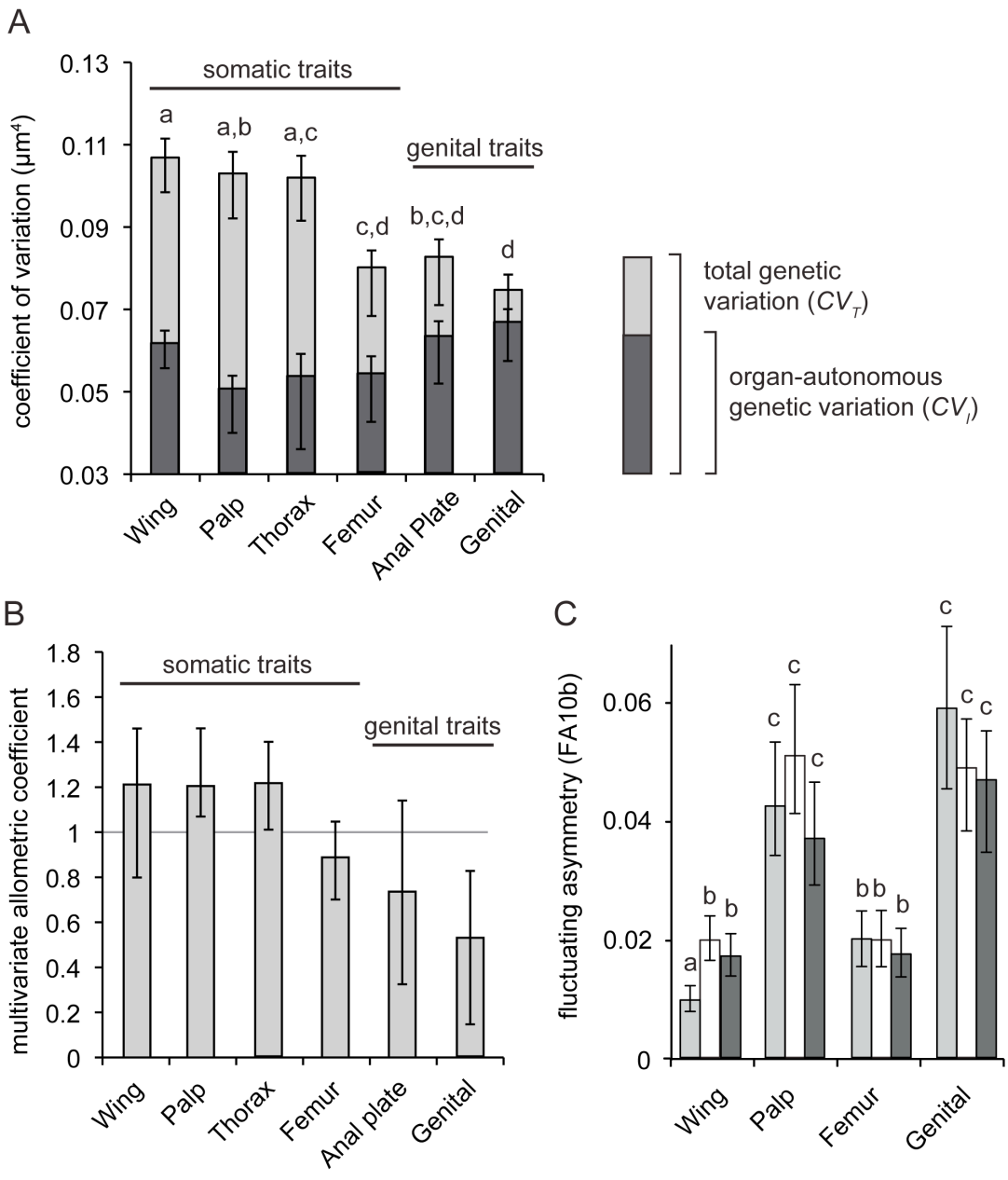

Figure 2.1. Genetic variation, allometric coefficient and fluctuating asymmetry of somatic and genital traits in male *Drosophila melanogaster*. (A) Genital traits had low levels of total genetic variation (light gray bars, CV_T) but not low levels of organ-autonomous genetic variation (dark gray bars, CV_I). The difference between total genetic variation and organ-autonomous variance is an estimate of genetic variation that is correlated with variation in other traits ('systemic' genetic variation). Columns with the same letter are not significantly different for total genetic variation (CV_T) using Tukey's HSD ($P > 0.05$). Traits do not differ for organ-autonomous genetic variation (CV_I) using Tukey's HSD ($P > 0.05$ for all) (B) The low systemic genetic variance of the genital traits reflected their low multivariate allometric coefficient compared to most

Figure 2.1 (con't). somatic traits, although these differences are not significant for multiple comparisons (Tukey's HSD, $P>0.05$ for all). Grey horizontal line is isometry. (C) Genital traits did not show low levels of fluctuating asymmetry. Light grey bars, line 303, white bars, line 324, dark grey bars, line 335. Within a line, columns with the same letter are not significantly different for FA using Tukey's HSD (P 0.05). All error bars are 95% confidence intervals.

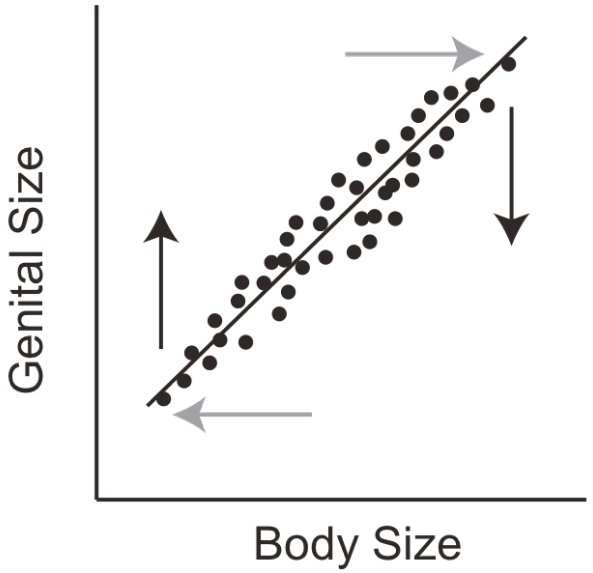

Figure 2.2 Model of a selection regime that alters the slope of the genital-body scaling relationship while maintaining genital-autonomous genetic variation. Selection is for proportionally smaller genitalia in large males and proportionally larger genitalia in small males (black arrows). Implicit to such a regime is that there is selection or constraint maintaining variation in body size (gray arrows).

Discussion

Elucidating the causes of the unusual scaling relationship between genital size and body size in arthropods is an active but unresolved area of research (Eberhard et al. 1999, Green 1999, Huber 2003, Bertin and Fairbairn 2007, Eberhard 2009). The goal of our study was to begin to explore the proximate mechanisms that underlie genital hypoallometry, specifically the response of male genital size to genetic variation and to stochastic developmental errors.

The slope of a scaling relationship between body and organ size captures the extent to which factors that generate variation in body size also generate variation in organ size (and vice versa). Consequently, traits that scale hypoallometrically to body size, such as the genitalia, are expected to show low levels of variation in response to genetic and environmental factors that affect both body and organ size. Previous studies have shown that, as expected, genital traits show low levels of variation in response to environmental factors that affect body and organ size; the genitalia are thus environmentally canalized (Shingleton et al. 2009). Our data show that genital traits also show low levels of variation in response to genetic factors that affect body and organ size, that is the genitalia are genetically canalized (Figure 2.1A). Importantly, however, genital traits do not show low levels of variation in response to genetic factors that autonomously affect their size (Figure 2.1A). These genetic factors presumably affect organ size at the level of individual organs and not through systemic mechanisms. The genitalia also do not appear to show low levels of variation in response to environmental factors that affect organ size autonomously. Fluctuating asymmetry (FA)

arises through stochastic perturbations in the developmental process at the molecular, chromosomal and epigenetic level (Parsons 1990) and is, by definition, not coordinated across the body. Implicit to the concept of FA is that, since both sides of a bilateral organism are influenced by identical genes, non-directional differences between the two sides must be environmental in origin (Waddington 1942). FA can therefore be considered a reflection of environmental variation that acts at the level of individual organs (and tissues within those organs) rather than through systemic mechanisms. Our finding that FA for genital traits is the same or higher than for somatic traits suggests that genital traits do not have reduced sensitivity to environmental factors that act autonomously on organs or tissues.

Our results suggest that there are two broad classes of developmental mechanisms that regulate organ size in *Drosophila*: (1) systemic mechanisms that regulate organ and body size as a whole, for example the level of circulating growth hormone; and (2) organ autonomous mechanisms that affect the size of organs individually, for example the expression of genes that pattern individual organs. The genitalia appear to have reduced their response to the former but not the latter affectors of size (Figure 1A). This is to be expected. The slope of an organ-body size scaling relationship captures the extent to which factors that generate variation in body size also generate variation in organ size; the evolution of hypoallometry (or hyperallometry) should therefore involve changes in the response of an organ to these factors.

How the genitalia reduce their response to systemic regulators of size is unclear but is an area of active research. For example, the developing genitalia are insensitive to

changes in insulin signaling, the primary developmental mechanism through which nutrition regulates growth in all animals (Shingleton et al. 2005). Changes in nutrition during development affect the level of circulating insulin-like peptides that in turn affects the rate of cell proliferation in growing tissues. Because the growth rate of the genitalia is relatively insensitive to changes in insulin-signaling, their final size is less sensitive to changes in nutrition and the genitalia are nutritionally canalized (Shingleton et al. 2009). Importantly, insulin-insensitivity could also account for the genetic canalization of the genitalia and the low slope of their genetic static allometry. Genetic variation in body size has been linked to allelic variation within the insulin-signaling pathway (Sutter et al. 2007). Organs that are insensitive to changes in insulin-signaling caused by nutritional variation should also be insensitive to changes in insulin-signaling caused by genetic variation. More generally, if genetic variation in body and organ size is primarily mediated by genes involved in the environmental regulation of size, then environmental and genetic canalization may reflect the same developmental processes.

A deeper understanding of the developmental mechanisms that underlie the genetic and environmental canalization of *Drosophila* genitalia will help clarify the adaptive significance of their low allometric slope. There are a number of alternative hypotheses to account for genital hypoallometry (Arnqvist 1997). The ‘lock-and-key hypothesis’ argues that male genitalia need to be of a particular size in order to physically fit with the female genitalia, with strong stabilizing selection for genitalia of an intermediate size (Eberhard 1985). The ‘one-size-fits-all’ hypothesis is similar but proposes that there is stabilizing sexual selection rather than natural selection for genitalia of an intermediate

size, with females favoring males with such genitalia (Eberhard 1985). These models have been criticized more recently, in part because empirical studies have revealed directional selection on genital size in male water striders (*Aquarius remigis*) despite being hypoallometric to body size (Bertin and Fairbairn 2007). In response to this criticism the models have been extended from their original implications of stabilizing selection to include directional selection. Specifically, hypoallometry may result if positive directional selection on genital size is more intense for small males than large males (Eberhard et al. 2009).

Our data suggest that genital hypoallometry is not a consequence of stabilizing selection on genital size alone. Stabilizing selection should not only reduce the genetic variation in genital size that is correlated with variation in the size of other traits but also the genetic variation in genital size that is organ autonomous (Falconer and Mackay 1996, Eberhard et al. 2009), which we did not find. Further, stabilizing selection might also be expected to reduce the developmental instability of the genital traits (Gavrilets and Hastings 1994) but see (Siegal and Bergman 2002), also not supported by our data. Rather, the finding that the genitalia are only canalized with respect to genetic and environmental factors that generate systemic variation in body and organ size suggests that selection for hypoallometry has targeted the mechanisms that regulate the response of the genitalia to these factors. These mechanisms ultimately regulate the relationship between genitalia and body size (Dreyer and Shingleton 2011).

What form of selection would target these mechanisms preferentially? One hypothetical selection regime favors large genitalia in small males and small genitalia in large males (Figure 2.2). Such selection is not expected to reduce genital-autonomous genetic variation. This is because alleles that make the genitalia autonomously large will be selected against in large males but selected for in small males, maintaining overall genetic variation. The inverse is true for alleles that make the genitalia autonomously small. In contrast, alleles that reduce the relative sensitivity of the genitalia to systemic genetic and environmental regulators of organ size will be favored in both large and small males. Implicit to this selection regime is the assumption that variation in body and somatic trait size is maintained, either through selection or constraint (Figure 2.2). Directional selection on genital size that is more negative in large males than small males, or more positive in small males than large males, should similarly target genes that influence the relationship between genitalia and body size and change the slope of their scaling relationship (Eberhard et al. 2009). However, like stabilizing selection, such directional selection might be expected to also reduce organ-autonomous genetic variation in the genitalia (Bulmer 1976, Crnokrak and Roff 1995). On the other hand, of all morphological traits genital traits may be most closely related to fitness. Fitness traits seem to have elevated levels of variance (Merila and Sheldon 1999) and this may counter the effects of directional selection on organ-autonomous genetic variation in genital size.

Interestingly, the femur of the first leg of male *Drosophila,* like the genital traits, also showed low levels of total genetic variation and scaled hypoallometrically to body size.

The first legs of male *Drosophila* carry the sex-combs, thought to be used for grasping the female genitalia prior to intromission (Cook 1977, Ng and Kopp 2008). One hypothesis for the reduced total genetic variation of the femur therefore is that similar selective pressures are acting on the first leg and genitalia in male *Drosophila*.

In general, the slope of allometric scaling relationships is a multivariate trait that reflects variation in organ size, body size and the relationship between the two. Specifically, it describes the extent to which environmental or genetic factors influence trait size relative to body size. Implicit to the concept of allometry is that these factors should affect both trait size and body size. Theories as to how allometric slopes evolve must therefore consider selection on organ size relative to body size, rather than organ size alone. Consequently, future studies should explore how selection acts on genital size in males of varying sizes. Our prediction is that, if there is selection for hypoallometry, the strength and direction of selection on genital size will depend on the size of the male the genital is attached to.

CHAPTER 3:

Female preference and the hypoallometry of male genitalia in *Drosophila*

Introduction

Morphological diversity is one of the primary manifestations of biological variation. Responses to selection in morphology are observable expressions of the many intricate pathways and systems that produce a functional organism. A fundamental property of morphological diversity in living organisms is variation in size (McMahon and Bonner 1983, Brown and West 2000). The range of sizes across taxa extends over many orders of magnitude, from single cell organisms up to conglomerations of many trillions of cells in the largest aquatic and terrestrial organisms (Schmidt-Nielsen 1984). A shared property across all organisms is that each must be able to counteract the physical and physiological limitations of their specific habitats. Organisms must also maintain functionality within the set of traits that defines them, subsets of which often act in a coordinated fashion with one another (Wagner 1996, Klingenberg and Marugan-Lobon 2013). To a large degree, the study of morphological diversification can be summarized by the relative size among traits that combine to form an organism (Bonduriansky and Day 2003).

Scaling relationships, or how traits are related in size, have been the focus of research on the evolution of morphology for the past century. The term allometry was first used to describe how differences in growth rates between traits could result in abnormal scaling relationships (Huxley 1932). Allometry now typically refers to the proportional change in size of one trait in relation to another trait, or often in relation to a measure of overall body size (Gould 1966). Implicit to this definition of allometry is covariation between the two traits. Historically, the most common method of quantifying scaling relationships has

been through the use of the allometric equation, $y = b*x^{\alpha}$, where x and y are the sizes of two given traits (Huxley and Teissier 1936). When log-transformed, the equation becomes the linear relationship $\log(y) = \alpha * \log(x) + \log(b)$ with a slope of α, called the allometric coefficient, and an allometric intercept of log(b). Log transformation of trait values is done to linearize the relationship between the two traits, quantifying variation of the scaling relationship in the two linear parameters of slope, α, and intercept, log(b) (Huxley and Teissier 1936, Gould 1966). Log transformation also allows for trait comparison independent of scale between traits (Kerkhoff and Enquist 2009, Shingleton 2012). Traits that maintain proportion to one another, that is a 1:1 ratio, have an allometric slope of 1 and are called isometric. Traits that have slopes < 1 or > 1, referred to as hypo- and hyperallometric respectively, have disproportionate size changes in relation to one another. Hypoallometric traits maintain a consistent size across a range of body sizes, meaning smaller individuals have proportionally larger traits and larger individuals have proportionally smaller traits. Hyperallometric traits are those that are proportionally larger in large individuals and proportionally smaller in small individuals.

Concurrent with the study of how traits relate to one another in size, the mechanisms that control size continue to be a burgeoning area of research (Shingleton 2010, Emlen et al. 2012, Koyama et al. 2013, Lavine et al. 2015). The regulation of size is controlled on at least two levels: systemically (affecting all tissues in an organism), and organ-autonomously (affecting tissues independently)(Brogiolo et al. 2001, Emlen and Allen 2003, Shingleton et al. 2007, Tang et al. 2011). For traits to have different final sizes within a single organism, there must be trait-specific regulation, or response, in the

common size determining mechanisms that affect all tissues. A primary mechanism of size control includes the pathways that transcribe nutritional inputs from an organism's environment to growth signals that are conveyed to developing tissues (Oldham and Hafen 2003, Grewal 2009, Nijhout et al. 2013). The insulin/insulin-growth factor (IIS) pathway works in concert with several other signaling pathways (e.g. ecdysone/juvenile hormone (Mirth and Shingleton 2012), sex determining pathway (Kijimoto et al. 2012, Gotoh et al. 2014) to control how traits respond to environmental variation. It has been shown that the unusual scaling relationships in some species, such as hyperallometry of horns in male rhinocerous beetles and the hypoallometry of male genitalia in *Drosophila melanogaster* can be correlated with either increased or decreased sensitivity to signaling of the IIS pathway (Tang et al. 2011, Emlen et al. 2012). While nutrition is only one of several mechanisms through with final trait size is controlled (Shingleton 2010) it is of paramount importance in the regulation of coordinated trait development within organisms and across taxa.

Traits related to sex have experienced a particularly high level of interest in evolutionary research. This is in part due to the role sexual characteristics play in charismatic sexual selection processes and speciation (Kraaijeveld et al. 2011) but is also a direct representation of how unusual sexual characteristics tend to scale in relation to the rest of the body. Most traits scale at or near the 1:1 ratio of isometry, but sexual traits often deviate from that pattern (Harvey and Pagel 1991, Green 1992, Bonduriansky 2007, Eberhard 2009). Across the arthropods, many taxa exhibit hypoallometric male genitalia, meaning small males have proportionally larger genitalia and larger individuals

have proportionally smaller genitalia (Eberhard 2009). During mating, male genitalia are often physically required to interact with the female reproductive organs meaning she has direct contact with the male and can assess the male based on his genitalia before fertilization (Eberhard 1996). If genitalia are important for mating success, why are male genitalia hypoallometric? Traditionally there have been four hypotheses proposed to account for the observed morphology of male genitalia:

1. *Lock-and-key hypothesis*. Male and female genitalia must be a particular size to physically fit together. Individuals with genitals that deviate from this size are physically unable to copulate and will be selected against (Dufour 1936).
2. *Pleiotropy*. Genital morphology is selectively neutral but genital size and shape are invariant due to stabilizing selection on a pleiotropic trait or traits (Mayr 1963).
3. *Sexual selection*. Genital size indicates male quality and females have a preference for males with extreme genital size. Under specific conditions this can theoretically produce hypoallometry (Eberhard 1985, Bonduriansky and Day 2003).
4. *One-size-fits-all*. The male genital size that stimulates the broadest range of female genitalia has the highess fitness. Intermediate-sized male genitalia is expected to maximize compatibility with females, and is therefore the optimum genital size (Eberhard et al. 1998).

Male genitalia have been widely recognized as some of the most diverse morphological structures of animals since before Darwin (Hosken and Stockley 2004). Based on the incredible variation in genital structures, Dufour (1844) proposed the lock-and-key hypothesis, genital structures act as a mechanism of species-specific recognition, to

account for the divergence of this trait. His hypothesis stated that the male genitalia were a ‘key’ to the female ‘lock’ and only two individuals of the same species would be able to physically achieve sperm transfer. The effect of the lock-and-key hypothesis on genital evolution is generally considered to be weak if present at all, but remains unfalsified as a contributing factor to diversification of genitalia (Eberhard 1985, Shapiro and Porter 1989, Mutanen et al. 2006).

The pleiotropy hypothesis states that the diversity of genital morphology is a result of selection on pleiotropic traits (Mayr 1963). The pleiotropy hypothesis was later refined to account for the unique prediction of hyper-variability in genitalia and not in other traits that may also be exposed to pleiotropic selection (Arnold 1973). Pleiotropic effects in the genitalia were predicted to cause more rapid evolution than other traits because as one sex developed a modification of genital morphology due to pleiotropy, the genitalia of the other sex would be expected to adapt to match the new morphology of the first sex. Thus, there would be a positive feedback of morphological changes in the genitalia resulting in increased levels of diversification. Additional predictions for the pleiotropy hypothesis include an increase in genetic variability of genital traits and strong condition dependency of the genitalia because they are selectively neutral (Arnqvist 1997). Empirical tests for the effect of pleiotropy on genital evolution have proven difficult, although pleiotropic effects on genitalia may be possible (Arnqvist and Thornhill 1998). In general, the effect of pleiotropy on genital morphology is also thought to be weak. Additionally, when compared to somatic traits the male genitalia of *Drosophila* are insensitive to changes in nutrition (Tang et al. 2011) and to systemic genetic factors

(Dreyer and Shingleton 2011), both of which are predicted to be equal to somatic trait levels under pleiotropy, suggesting pleiotropy may have no significant effect on genital morphology in some organisms.

The hypotheses for genital divergence that have received much more attention are the sexual selection and one-size-fits-all hypotheses. Both of these hypotheses involve a preference by females for particular male genital sizes. Sexual selection for genital evolution includes four mechanisms: Fisherian selection, good genes, sperm competition and sexual conflict (Hosken and Stockley 2004). Fisherian selection and good genes both predict directional selection for larger genital sizes as direct indicators of male quality (Eberhard 1985). Sperm competition occurs when sperm from multiple males competes to fertilize eggs inside the female reproductive tract (Simmons 2001). Sexual conflict occurs when the two sexes struggle to maximize their own fitness, with males typically adapting to remove conspecific sperm and females adapting to retain sperm from the most fit males (Arnqvist and Rowe 2005).

The one-size-fits-all hypothesis states that intermediate male genital sizes are favored because they are compatible with the most typical female genital size (Eberhard et al. 1998). Female and male genitalia physically interact, therefore females may choose males based on the ability of the male genitalia to physically stimulate her reproductive tract. As a result of selection based on a physical stimulation rather than a visual inspection of male traits by females to assess male condition, intermediate sized genitalia could be the preferred size (Eberhard et al. 1998). Ultimately, the one-size-fits-

all hypothesis of genital evolution is a specific form of sexual selection where females are basing their preference for male genital size on tactile feedback and not on the size of the male trait as an indicator of the male's condition. Because it is necessary for the female to physically interact with the male genitalia, the female would be selecting for fertilization of progeny based on male phenotype. The ability of females to bias fertilization success of males they mated with is called cryptic female choice (Birkhead and Pizzari 2002). There are a number of mechanisms by which female *Drosophila* may be able to 'select' male sperm including sperm dumping of undesired sperm, differential sperm storage and differential abortion of embryos (Eberhard 1996, Birkhead 1998, Snook 2005).

An additional caveat to the evolution of genital morphology is that the majority of genital traits measured in arthropods, and in some vertebrate taxa as well, exhibit hypoallometry (Eberhard et al. 2009). One of the predictions of the one-size-fits-all hypothesis is that selection for male genitalia is for the intermediate size, i.e. stabilizing selection. Hypoallometry is thought to be a possible outcome of stabilizing selection on one of two covarying traits (Eberhard et al. 1998, Hosken et al. 2005). Sexual selection, in contrast, has traditionally been considered to exclusively produce hyperallometric scaling relationships (Green 1992, Kodric-Brown et al. 2006), but this concept has come under increasing challenge (Bonduriansky 2007). Specifically, Bertin and Fairbairn (2007) identified genital traits in water striders that are known to be under directional selection as hypoallmetric. A hypoallometric scaling relationship is a theoretical outcome of directional selection on a trait with viability costs (Bonduriansky and Day

2003) or when the strength of selection is stronger at smaller body sizes (Eberhard et al. 2009), but to best test which form of selection is acting on male genitalia *in vivo*, variation in male genital size alone would be required to see which size genitalia females actually prefer.

Here, the genetic toolkit of *Drosophila melanogaster* was used to engineer male flies with different sized genitalia while protecting the proportionality of somatic traits. The males with variation in genital size were then exposed to females in three mating regimes (single male, direct male-male competition, indirect male-male competition) to identify how selection is acting on the male genitalia.

Methods

Allometric Engineering

All transgenes were first isogenized by backcrossing insertions used into the wild-type *Samarkand* (*Sam*) line of *Drosophila melanogaster* for five generations. Male flies of different genital sizes were made using the UAS-GAL4 system to regulate gene expression in specific tissues (Duffy 2002). To limit expression of genes of interest to the genital tissues only, a GAL4-driver was used (*P{W6 Poxn-GAL4}14-1-7*) which is located on the third chromosome and drove expression in the male genital tissues that develop into the posterior lobe of the genital arch and in some neuronal tissues (Boll and Noll 2002) (kind gift of JP Masly). The *Poxn* driver was paired with a number of transgenes to manipulate IIS in the developing tissues to create ‘large’, ‘wild-type’ or ‘small’ genital size classes of males. Large-genital males were made using a

constitutively active version of the Inuslin Receptor (InR), *UAS-InR.CA*, genocopying increased nutrition in the target tissues (*Poxn > Inr.CA)*. Wild-type-genital control males were generated using a green florescent protein, *UAS-GFP.565T* (*Poxn>GFP*) that had no obvious additional phenotypic effects but did control for the effect of elevated transcription in the target tissues (*Poxn>GFP*). Small-genital males were made using a constitutively active version of the forkhead box transcription factor FOXO*, UAS-dFOXO-3X (f3-9)*, that genocopies starvation (*Poxn>UAS-FOXO-3x)*. All females used in mating assays were wild-type (*Poxn>GFP).*

To determine which male mated with the female in direct and indirect competition assays, each experimental male used was marked with a ubiquitously expressed GFP (*UAS-UbiGFP*, Bloomington, IN). *UbiGFP* is expressed in all tissues of the male making the entire body fluoresce when excited by a 405 nm Class II laser. Sperm from a male labeled with *UbiGFP* will also fluoresce, as will the bodies of any offspring fertilized by the labeled sperm. The *UbiGFP* insertion was on the X-chromosome, hence, only female offspring were characterized by GFP expression.

While the *Poxn* driver was expressed primarily in the developing genital tissues, there was some expression in non-target tissues including neuronal cells (Boll and Noll 2002). Preliminary mating assay trials determined a significant effect on the courtship latency of male flies expressing *FOXO* with the *Poxn* driver (Figure 3.1), therefore, a GAL80 with neuron-specific expression in the *Elav* domain (Koushika et al. 1996) was added to the experimental flies to competitively inhibit transcription of any GAL4 in those tissues.

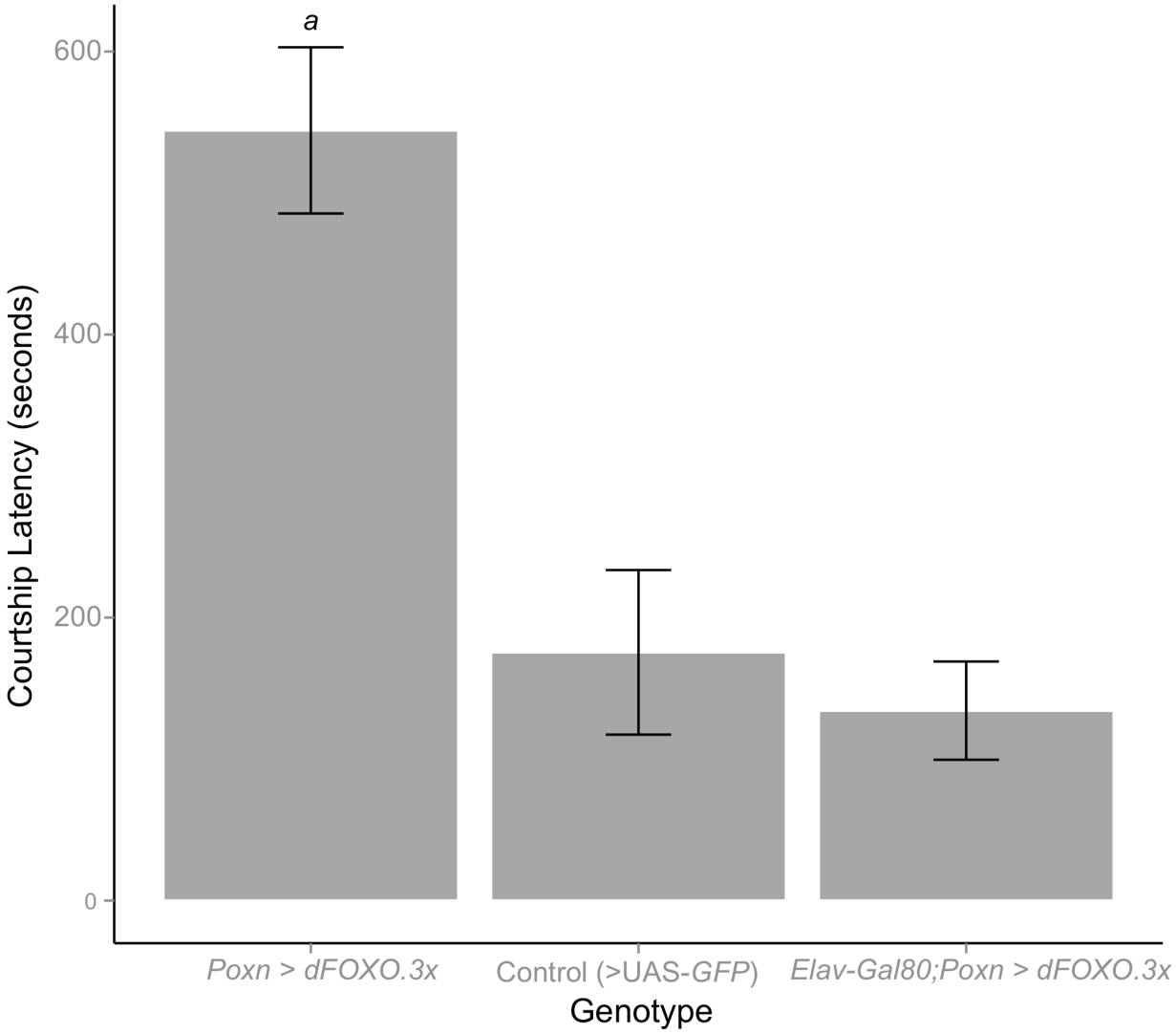

Figure 3.1. *Elav-GAL80* expressed with *Poxn* rescues wild-type mating behavior. Courtship latency of IIS manipulated flies (*Poxn > dFOXO.3x)* had significantly longer courtship latency than control male flies (*Poxn > UAS-GFP*). Coexpression of an *Elav-GAL80* that prevented expression of GAL4 in neuronal tissues recovered wild-type courtship latency (*Elav-GAL80:Poxn > FOXO.3x*). The symbol (a) denotes significance (Tukey's HSD $p < 0.0001$).

The *Elav-GAL80* was the kind gift of Christen Mirth. Subsequent preliminary trials confirmed that the simultaneous presence of *Poxn* and the GAL80 rescued wild-type courtship behavior (Figure 3.1). To make the final experimental flies, the *Elav-GAL80* was introgressed into all experimental flies (large: *Elav*-GAL80;*Poxn* > *Inr.del*, wild-type: *Elav-GAL80*;*Poxn* > *UAS-GFP*, small: *Elav-GAL80*;*Poxn* > *UAS-FOXO-3x*).

Fly Assays

All experimental flies were sorted while in the pupa stage as male or female and transferred to individual 2mL microcentrifuge tubes for social isolation. It is necessary to maintain social isolation until exposure to conspecifics for flies being used in behavioral assays because flies are known to modify behaviors in response to the presence of conspecifics (Siegel and Hall 1979, Gailey et al. 1982). Females are unreceptive to mating for the first 48 h after eclosion, therefore all females used in mating experiments were between 2 and 6 d old (O'Dell 2003). Males were also between 2 and 6 d old.

Flies were reared under a 12 h : 12 h light : dark circadian rhythm, at a constant temperature of 25°C. Each mating assay consisted of 18 – 35 mm x 10 mm mating chambers (Corning) with one 35mm disk of filter paper (Whatman) wetted with 70 μm of distilled water (Appendix A1). For the single male regime, each mating pair consisted of one female and one male. Six males of each genital size class were randomly assigned one of the mating chambers and were mouth-aspirated from their individual tubes into the mating chamber, with a female from her individual tube. Once the mating pair was in the mating chamber, the mating chamber was placed into a climate-controlled box

(24.8°C ± 0.5°C, 55% relative humidity ± 1.5%) and all activity was video recorded for the duration of the experiment using a Sony Vixia HG21 video camera. The addition of all flies took approximately 15 min from the time the first mating chamber was placed in the climate-controlled box until the last mating chamber was in position. All experiments were started within 1hr of the set 'daylight' time, and were allowed to run for 60 min from the time the last mating chamber was placed into the climate-control box. If flies were observed to be mating after 60 min, the assay continued until there were no flies mating. After the trial, males were immediately stored in 75% EtOH with their puparial case. Females were placed in individual tubes of cornmeal/molasses food and allowed to oviposit for 2 - 24 h periods, and were then placed in 75% EtOH with their puparial case. All adults that ecolosed from mating regime trials were counted and scored for *UbiGFP* two weeks after their parental experiment date.

For the direct competition regime, two males of different genital size class were randomly assigned a mating chamber and mouth-aspirated with a female from their individual tubes into the mating chamber to create a full mating trial of 18 chambers. After each direct competition trial, males were identified by *UbiGFP* and placed in 75% EtOH with their puparial case. Females were placed in individual tubes of cornmeal/molasses food and allowed to oviposit for 2 - 24 h periods, and were then placed in 75% EtOH with their puparial case.

For the indirect competition regime, six males of each genital size class were randomly assigned a mating chamber and mouth-aspirated with a female from their individual

tubes into the mating chamber to create a full mating trial of 18 chambers. After the first trial, males were placed in 75% EtOH with their puparial case. Females were placed in individual tubes of cornmeal/molasses food and allowed to oviposit for 2 - 24 h periods. Once the females had laid eggs for 48 h, six males of a different genital size class than the first male were mouth-aspirated with a female into the mating chamber to create a second full mating trial of 18 chambers. After the second trial, males were placed in 75% EtOH with their puparial case and females were placed into a second set of cornmeal/molasses food vials to oviposit for an additional 2 – 24 h periods. Once the second egg lay period was complete, females were placed in 75% EtOH with their puparial cases.

Four behavioral parameters were scored using the video recording of each mating trial: courtship latency, courtship duration, copulation latency and copulation duration. Courtship latency was recorded as the length of time from the male(s) first being introduced to the female until any of the typical courtship behaviors was exhibited towards the female (O'Dell 2003). Copulation latency was the length of time from the male(s) first introduced to the female until copulation was initiated. Courtship duration was the time from initial introduction of male(s) to female until copulation was initiated, or as long as the male(s) continued to court the female if no successful copulation occurred. Copulation duration was the time from the initiation of copulation until the male and female separated. These parameters could only be measured in the single male and the indirect male-male competition assays because there was no visible way to differentiate between the two males of direct male-male competition.

In each of the three mating regimes (single male, direct male-male competition, indirect male-male competition) 216 males were exposed to females for an overall total of 648 male flies. In the single male assays, 150 male flies were dissected, 50 from each of the three genital size classes (‘large’, ‘wild-type’ or ‘small’). Males in the direct competition assays were dissected if at least one of the pair successfully mated with the female (n = 50). Males in the indirect competition assays were only dissected if both males successfully mated with the female (n = 48). For the direct and indirect regimes, only males that successfully copulated were included in the analysis because female preference for genital size based on *UbiGFP* expression could only be made if a male sired offspring.

Morphology

Three traits were measured for each male fly: puparial case area, wing area and area of the posterior lobe of the genital arch. Two organs were dissected from each male fly: the wing and the posterior lobe of the genital arch. Organs were mounted in dimethyl hydantoin formaldehyde for imaging. Organ measurements were taken as area for the wing and the posterior lobe of the genital arch using a Leica DM6000B compound microscope and Retiga 200R digital camera. Puparial case area was also measured as an indication of overall body size. The size of the puparial case is directly proportional to the size of the adult given that all growth for holometabolous insects is determined by the nutritional intake of the larva (Chiang and Hodson 1950). All images were analyzed using the program ImagePro. For an estimation of developmental

stability, the amount of variation in trait size due to stochastic developmental errors, fluctuating asymmetry (FA) was calculated for each organ of interest. The wing and posterior lobe of the genital arch from both sides of each fly were dissected and measured three times to correct for measurement error, and the analysis of FA was done using the methods of Palmer and Strobeck (Palmer and Strobeck 2003).

Analysis

Static Allometry

The allometric coefficient for the static allometry was calculated using log-transformed trait measurements. These data were used to calculate the variance-covariance matrix for traits among lines, and the first eigenvector from this matrix was extracted using the *svd* function in the base package of R (R-Development-Core-Team 2014). The allometric coefficient is reflected by the loadings of the first eigenvector. Isometry occurs when all loadings of the vector equal $1/\sqrt{n}$ where *n* is the number of traits measured. Multiplying the loadings by $\sqrt{n}$ gives the bivariate allometric coefficient for each trait against a measure of overall body size (Klingenberg 1996). We used a random-variable bootstrap method to generate 95 percent confidence intervals for the allometric coefficients for each trait (Shingleton et al. 2009).

Mating Assay – Behavioral Traits

To compare male mating behavior across groups, and to identify any confounding effects of the IIS manipulations, data were fit to a linear mixed model with the mating behavior as the response variable, genotype, relative humidity and

temperature as fixed effects and mating position (1-18) and rearing vial as random factors. Possible mixed models were generated using maximum likelihood estimates and compared using log likelihood ratio tests. The final model was selected as the simplest model where the addition of a factor did not significantly improve the fit of the model. Individuals were only included in behavioral trait analysis if the trait was applicable (e.g. copulation duration was only analyzed for flies that actually copulated). Tukey's Honest Significant Difference (HSD) tests were completed for each mating regime to compare the behaviors between genital size classes within selection regimes.

Mating Assay – Female Preference

To calculate female preference for males of different genital sizes the probability of a female successfully mating, and subsequently fertilizing eggs, was calculated. Probability of mating was estimated using logistic regression on the bivariate data of success or failure for mating or fertilization compared to genital size, wing size and puparium size. Likelihood ratio tests were used to compare the regression model parameters for significance. Parameters were only included if they significantly improved the fit of the model. For each mating regime, males were only included in the test for fertilization probability if they had mated with the female.

Mating Assay – Reproductive Success

Reproductive success, measured here as the number of offspring each male sired, was compared across the genital size classes for each mating regime using Ordinary Least Squares (OLS) regression. Linear regression was also completed using

major axis and standard major axis methods with no significant slopes estimated for any method (data not shown). Only males that were confirmed to have produced offspring were included in the analysis. Tukey's HSD tests were completed for each mating regime to compare reproductive success between genital size classes within selection regimes.

Developmental Stability

To calculate FA for the wing and posterior lobe of the genital arch the FA10b index was used, which corrects for measurement error (Palmer and Strobeck 2003). To calculate FA10b the repeated measurement of each trait were fit to the following model:

$$Y = u + S + I + SI + e$$

where *u* is the intercept term, *S* is the effect of body side, left or right (fixed factor), *I* is the effect of the individual (random factor), *SI* is the interaction between individual and side and *e* is measurement error. I used the *lmer* function in the *lme4* package in R to estimate the variance components for *SI* (σ^2_{SI}), which is used to calculate FA10b:

$$FA10b = 0.798\sqrt{2\sigma^2_{SI}}$$

The *MCMCglmm* function in the *MCMCglmm* package in R (Hadfield 2010) was used to generate values of 95 percent support for each trait's level of fluctuating asymmetry. A prior equal to the variation in wing size measurements was used to generate parameter

estimates that were compared the results using a non-informative prior. There was no significant difference in parameter estimates. All traits were tested for antisymmetry and directional asymmetry by assaying the distribution of trait size on the right (R) and left (L) side of an individual. For antisymmetry we tested the (R – L) distribution for normality and for directional asymmetry we compared the mean of the signed (R – L) to zero.

Results

Allometric Engineering

The absolute size of the genitalia varied by genital size class, but not the size of the wings or body size (puparial case area) (Figure 3.2). Within each size class there was no significant difference in absolute size of either the wing or genitalia between the genotypes labeled with the *UbiGFP* marker and those without (Tukey HSD $p > 0.05$), therefore, data were pooled across size classes for the analysis of the mating assays. Among size classes, there were significant differences between the small males and both the large and wild-type males ($\bar{x}_{\text{large}} = 8.11\ \mu m^2$, $\bar{x}_{\text{wild-type}} = 8.10\ \mu m^2$, $\bar{x}_{\text{Small}} = 7.56 \mu m^2$,Tukey's HSD $p < 0.001$) but not between the large and wild-type genital sizes (Tukey's HSD $p > 0.05$) (Figure 3.2). Additionally, there was no significant difference in size of the wing or puparial case size across any of the genital size classes (Tukey's HSD $p > 0.05$).

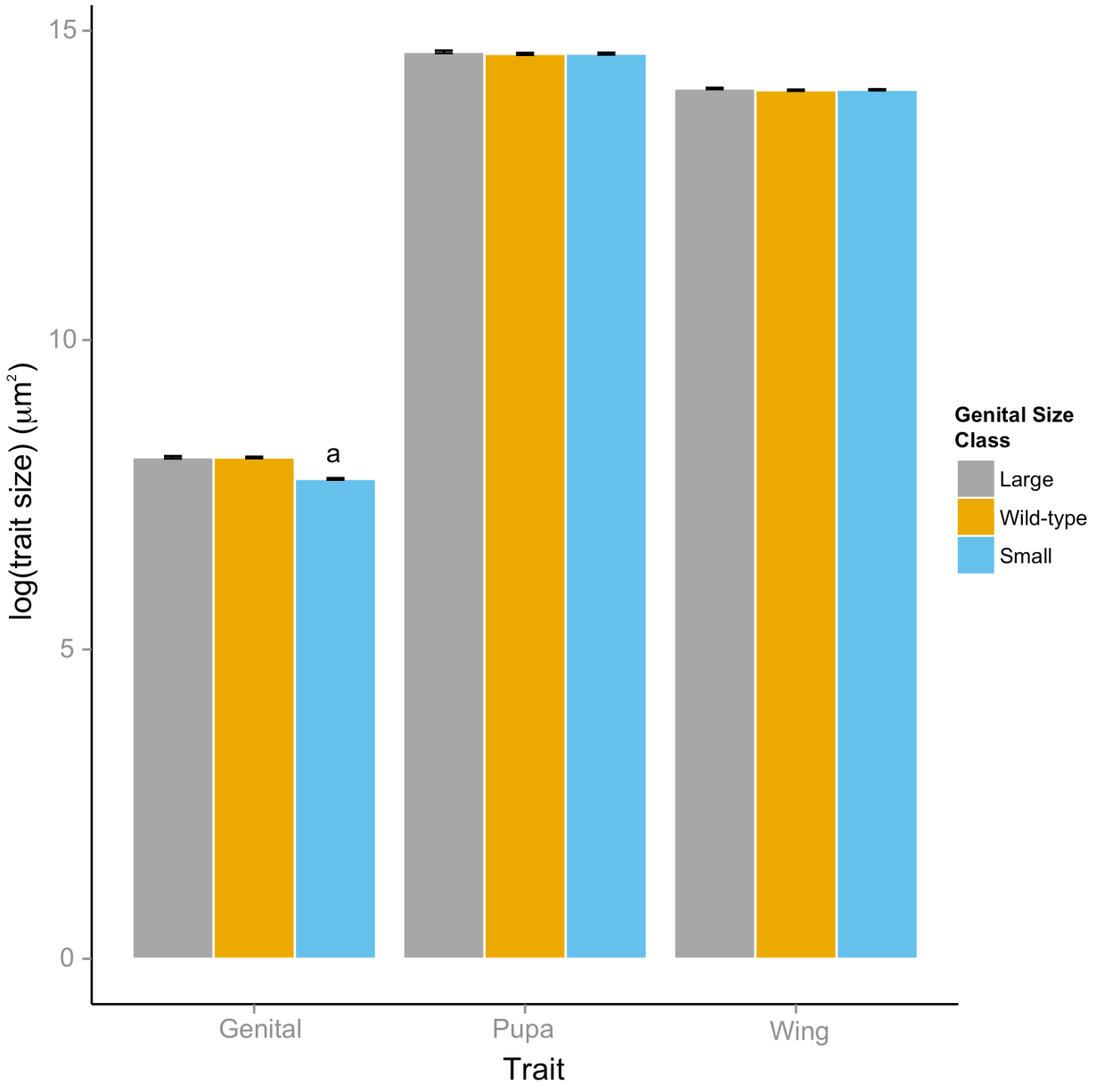

Figure 3.2. IIS perturbation using *Poxn* changes size of the genitalia only. Size of the posterior lobe of the genital arch was significantly smaller than the large or wild-type genital size classes (Tukey HSD < 0.001, represented by symbol 'a'). There were no significant differences in size of the wings or puparial between the genital size classes (Tukey HSD > 0.05). Data for genital size classes were pooled by the IIS transgene present in the fly (large: *InR.CA*, wild-type: *UAS-GFP*, small: *dFOXO.3x*) because there was no difference in size between flies with or without the *Ubi-GFP* (Tukey HSD > 0.05). Error bars represent SEM.

Static Allometry

Multivariate allometric coefficients were distinct between the two traits (wing and genitalia) but not within trait by genotype (Figure A2). Genitalia across the three genotypes had a hypoallometric coefficient ($\bar{x}\ \alpha$ =0.512) confirming what has been shown in other studies. The wings were slightly hyperallometric ($\bar{x}\ \alpha$ = 1.32).

Mating Assay – Behavioral Traits

Male *Drosophila* mating behaviors were analyzed across the single male and indirect competition mating regimes using a linear mixed-model. Mating behavior not measured in the direct competition assays because males were indistinguishable during the mating trial. There were no significant differences for the early stages of courtship in either the single male or indirect competition regimes between genotypes (Appendix, Table A1, Tukey HSD < 0.05). Differences in behavior for mating latency and mating duration were attributed to variation in factors beyond our control, as there were no differences in the initiation of courtship.

Mating Assay – Female Preference

Female flies preferred mating with wild-type-sized genitalia in all mating regimes. Successful and failed copulations and fertilizations by males were coded as a 1 or 0, respectively, and were analyzed using logistic regression for each mating regime.

Females were more likely to copulate as genital size increased in the single male regime ($p < 0.05$ based on 123 matings, Figure 3.3) and more likely to fertilize eggs

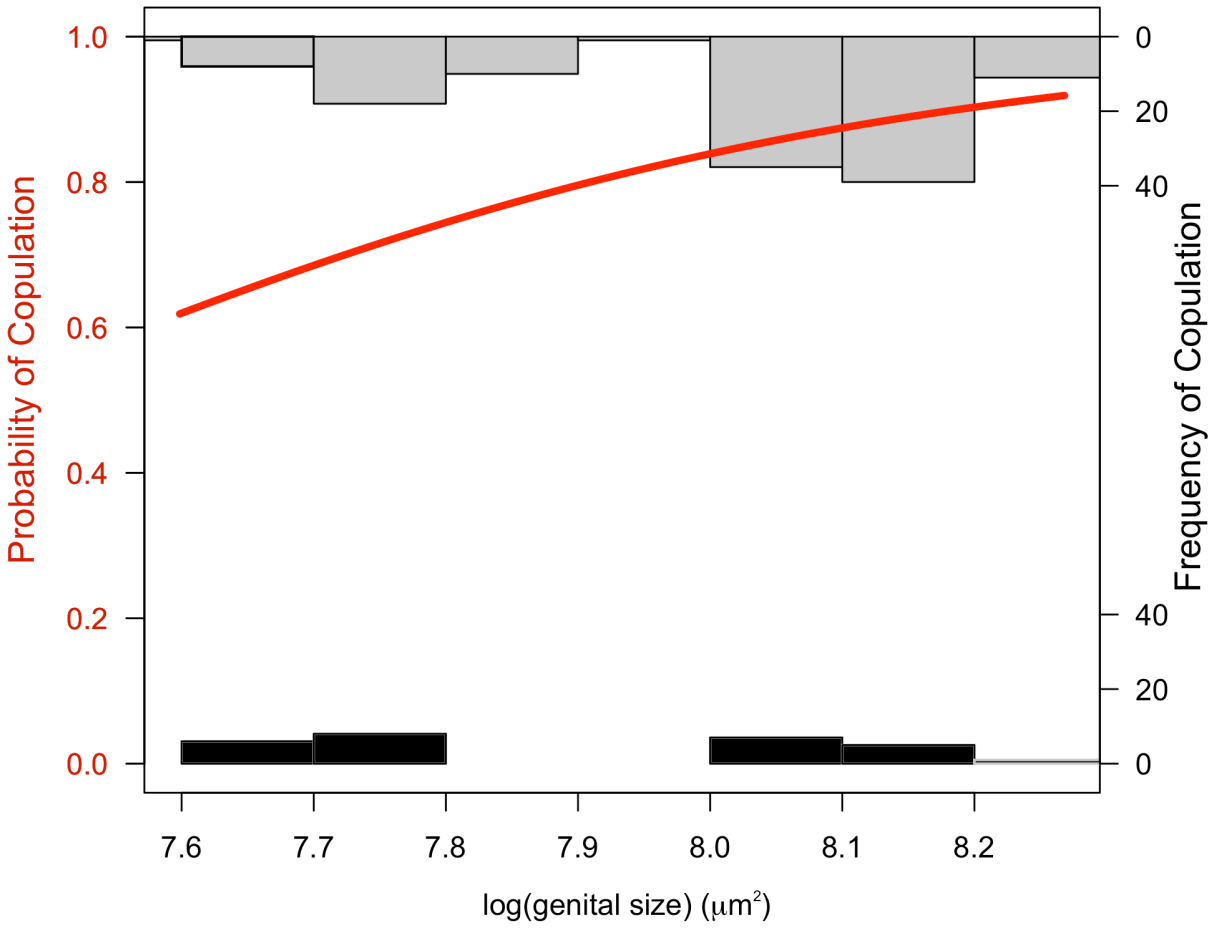

Figure 3.3. Males with larger genitalia are more likely to mate in single male regime. Logistic regression of male mating success (probability = 1.0) or failure (probability = 0) by log-transformed genital size of males. Females were more likely to mate with males that had larger genitalia (p = 0.009). Males with small genitalia were able to successfully mate with females, but did so at a lower frequency than males with larger genital sizes. Histogram bars in grey (top) show frequency of successfully copulation and black bars (bottom) show frequency of failure to copulate by genital size. Line represents logistic regression probability curve.

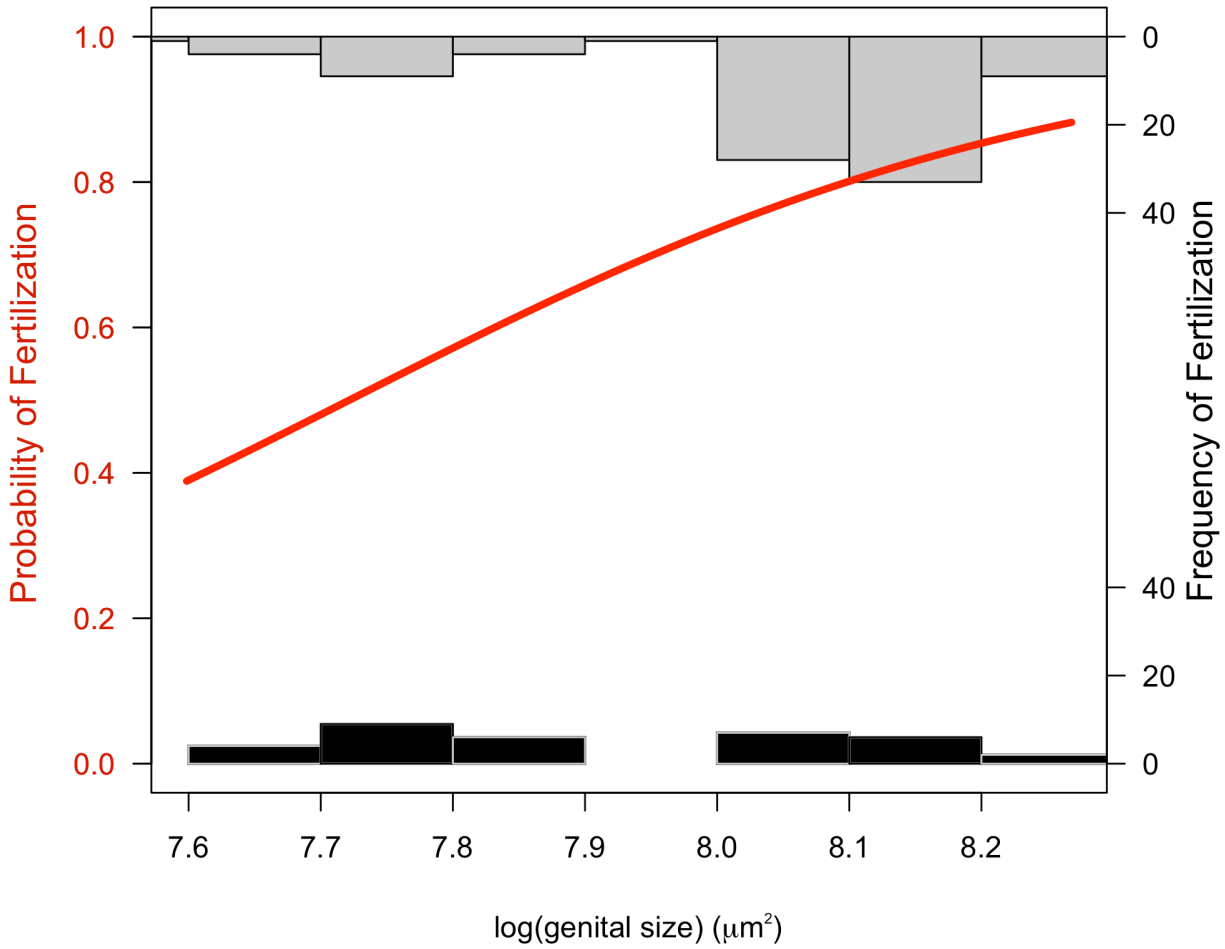

Figure 3.4. Males with larger genitalia are more likely to sire offspring in single mating regime. Logistic regression of a female fertilizing (probability = 1.0) or not fertilizing (probability = 0) eggs by log-transformed genital size. Females were more likely to fertilize eggs using sperm from males with larger genitalia (p = 0.001). Females fertilized eggs using sperm form males with small genitalia, but at a lower frequency than sperm from males with larger genital sizes. Histogram bars in grey (top) show frequency of successful fertilization and black bars (bottom) show frequency of failure to fertilize by genital size. Line represents logistic regression probability curve.

using sperm from males with larger genitalia ($p < 0.05$ based on N = 3435 progeny from 90 female, Figure 3.4). In the direct competition regime, females were more likely to fertilize eggs using sperm from males with larger genitalia ($p < 0.05$ based on N = 505 progeny from 25 females that could be assigned a mate, Figure 3.5). Only males that sired offspring were included in the analysis as the identity of the male the female chose could only be determined by the presence/absence of *GFP* in offspring. Therefore, the probability of fertilization was 1.0 for all males recorded in the direct competition regime.

In the indirect regime only male pairs that both successfully mated with the female were included in the analysis. The competition of male sperm, and the cryptic female choice of which male's sperm to use, could only be assessed if a female had mated with both males she was exposed to. Therefore, the probability of mating was 1.0 for all males in the indirect competition regime. Females were more likely to fertilize offspring using sperm from males with larger genitalia ($p = 0.031$ based on N = 333 progeny that could be assigned sired from 23 females , Figure 3.6). Females in the indirect competition had mated twice and therefore had sperm from two males available in their seminal receptacle and spermathecae. There was no evidence of sperm precedence as evidenced by no significant effect of the interaction between male genotype and mating order on the number of progeny sired (all pairwise comparisons $p > 0.05$ Tukey's HSD).

For the mating regimes that measured male mating probability, males of all genital sizes were physically able to mate with the female, but did so at lower frequencies than males with larger genitalia (Figures 3.3, 3.5). For each of the mating regimes, a linear

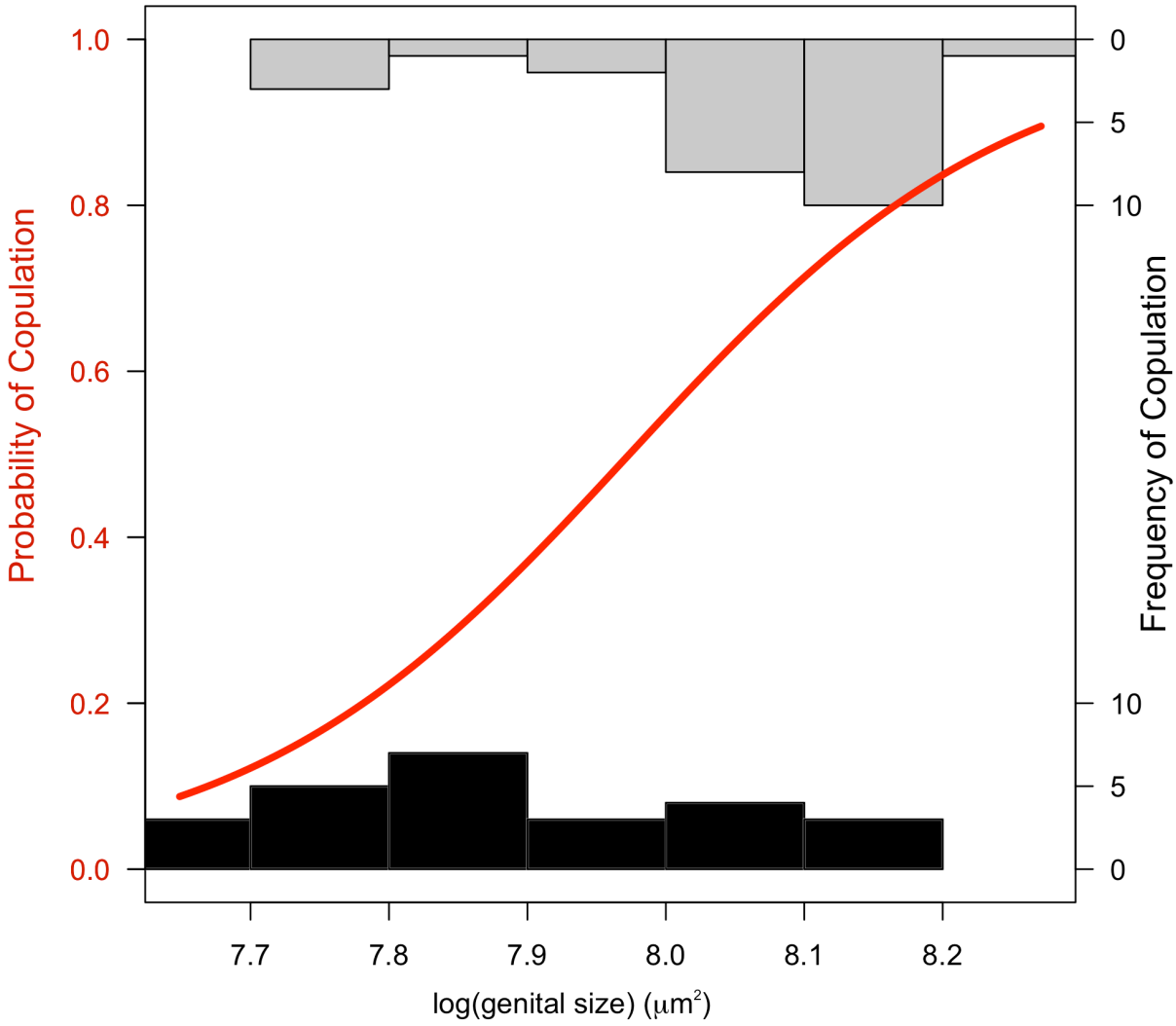

Figure 3.5. Males with larger genitalia were more likely to mate in the direct competition regime. Logistic regression of a male mating success (probability = 1.0) or failure (probability = 0) by log-transformed genital siz. Females were more likely to mate with males that had larger genitalia (p = 0.002). Males with small genitalia were able to successfully mate with females, but did so at a much lower frequency than males with larger genital sizes. Histogram bars in grey (top) show frequency of successful copulation and black bars (bottom) show frequency of failure to copulate by genital size. Line represents logistic regression probability curve.

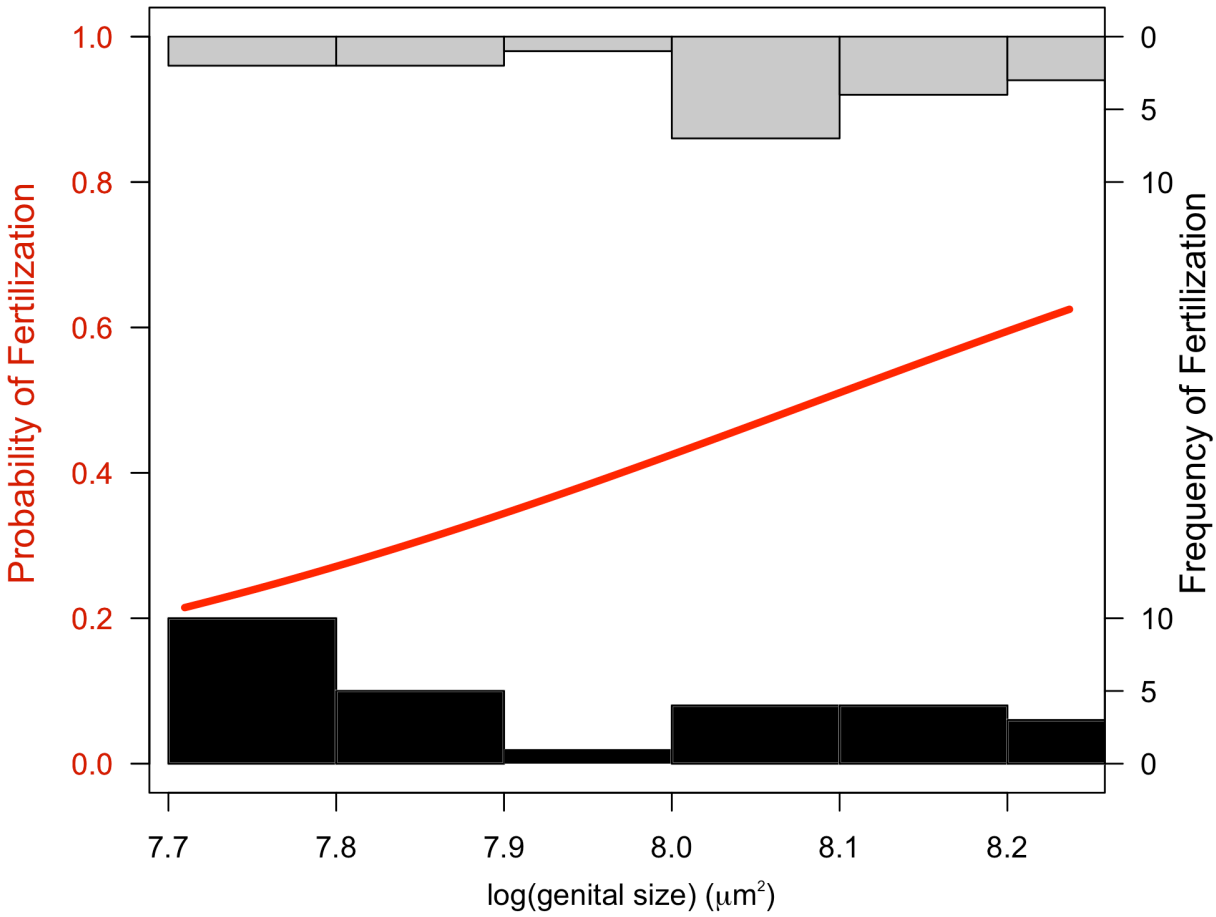

Figure 3.6. Males with larger genitalia were more likely to sire progeny in the indirect competition regime. Logistic regression of female fertilizing (probability = 1.0) or not fertilizing (probability = 0) eggs by log-transformed absolute genital size. There was a significant positive relationship between genital size and fertilization probability (p = 0.031). Sperm from males with small genitalia was used to fertilize eggs, but at a much lower frequency than sperm from males with larger genital sizes. Histogram bars in gray (top) show frequency of fertilization and black bars (bottom) show frequency of failure to fertilize by genital size. Line represents logistic regression probability curve.

regression was used to compare genital size and the total number of progeny a male sired if he mated and his sperm was used to fertilize eggs. Across each regime, there was no significant relationship or correlation between number of offspring and log-transformed male genital size (p_{single} = 0.094, p_{direct} = 0.537, $p_{indirect}$ = 0.608)(Appendix, Table A2).

Developmental Stability

Fluctuating asymmetry (FA) was calculated for all males that were exposed to females in any of the mating contexts. FA for both the wing and genital arch were estimated. Males were compared by genotype within a mating regime. There was a substantial difference in FA values between the wings and the genitalia in each of the mating regimes, with the FA much higher in the genitalia than in the wings (Figure 3.7). There was no significant difference in FA across the three genotypes within a trait indicating females were likely unable to derive any information on a male based on the FA of his genitalia. Because there was no significant difference across the size classes of males in the single male regime, males were pooled based on if they were selected by the female in direct competition or not, and by if the male produced offspring or not in the indirect competition regime. There was no difference between the FA of males that were selected by females vs. those that were not (Figure 3.8) or of males that sired offspring vs. those that did not (Figure 3.9).

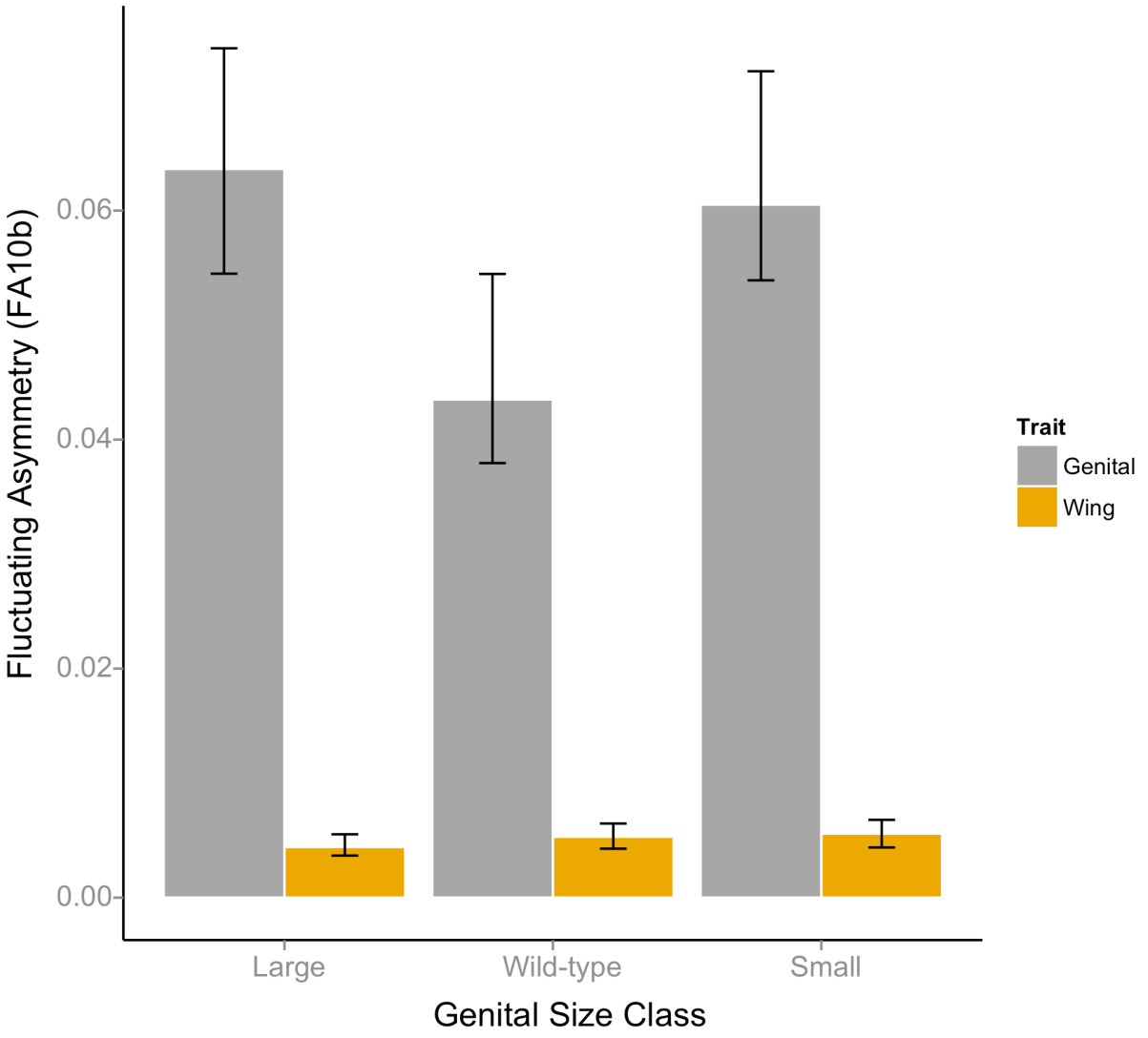

Figure 3.7. Fluctuating asymmetry (FA) is higher in genital traits than the wings in the single male regime. There was no difference in FA values by trait across the genital size classes, but there was a difference between the traits. FA was calculated using the FA10b value which accounts for measurement error in log-transformed traits. Error bars are 95% confidence intervals.

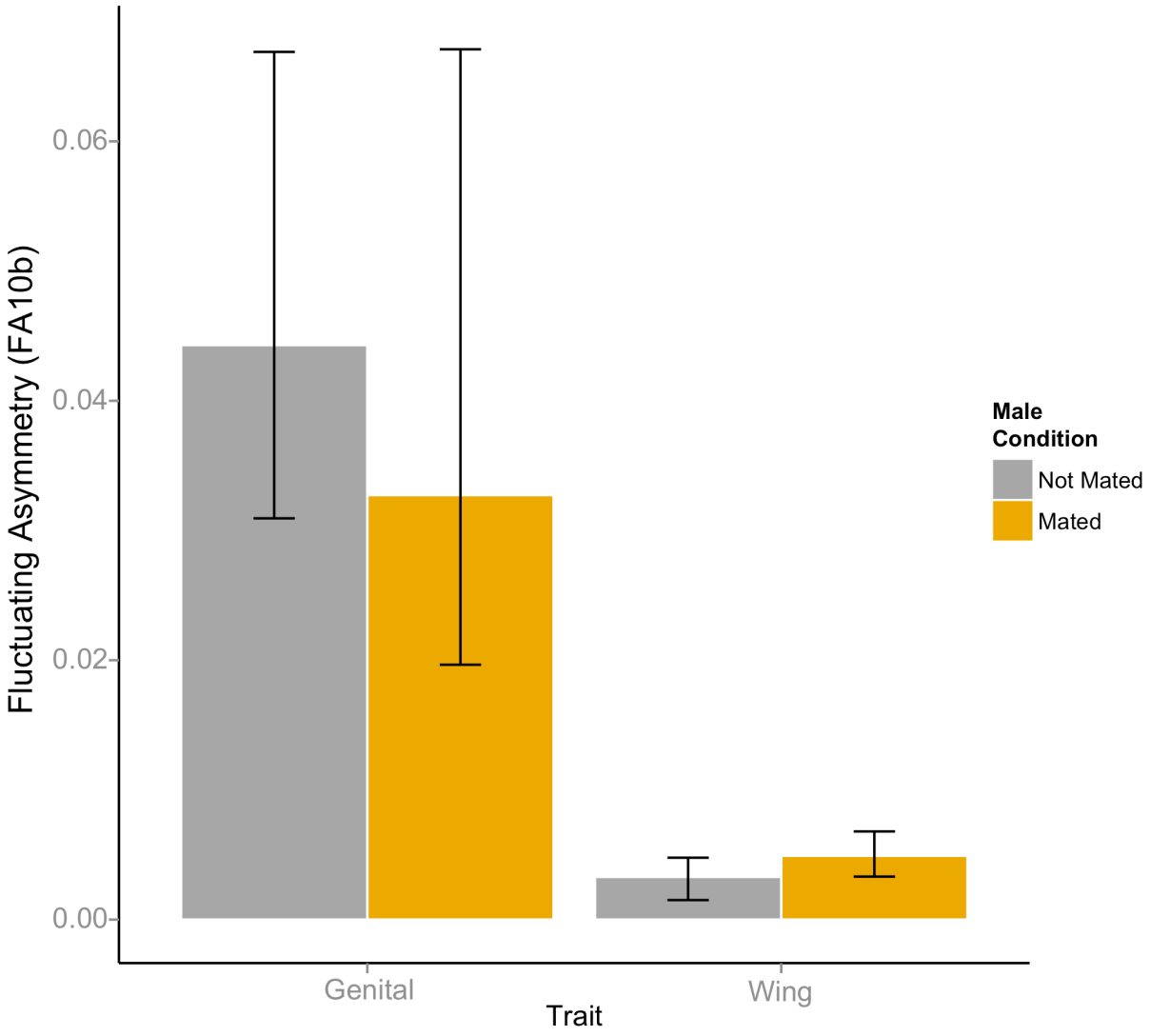

Figure 3.8. No difference in FA between flies that mated and did not mate in the direct competition regime. Error bars are 95% confidence intervals.

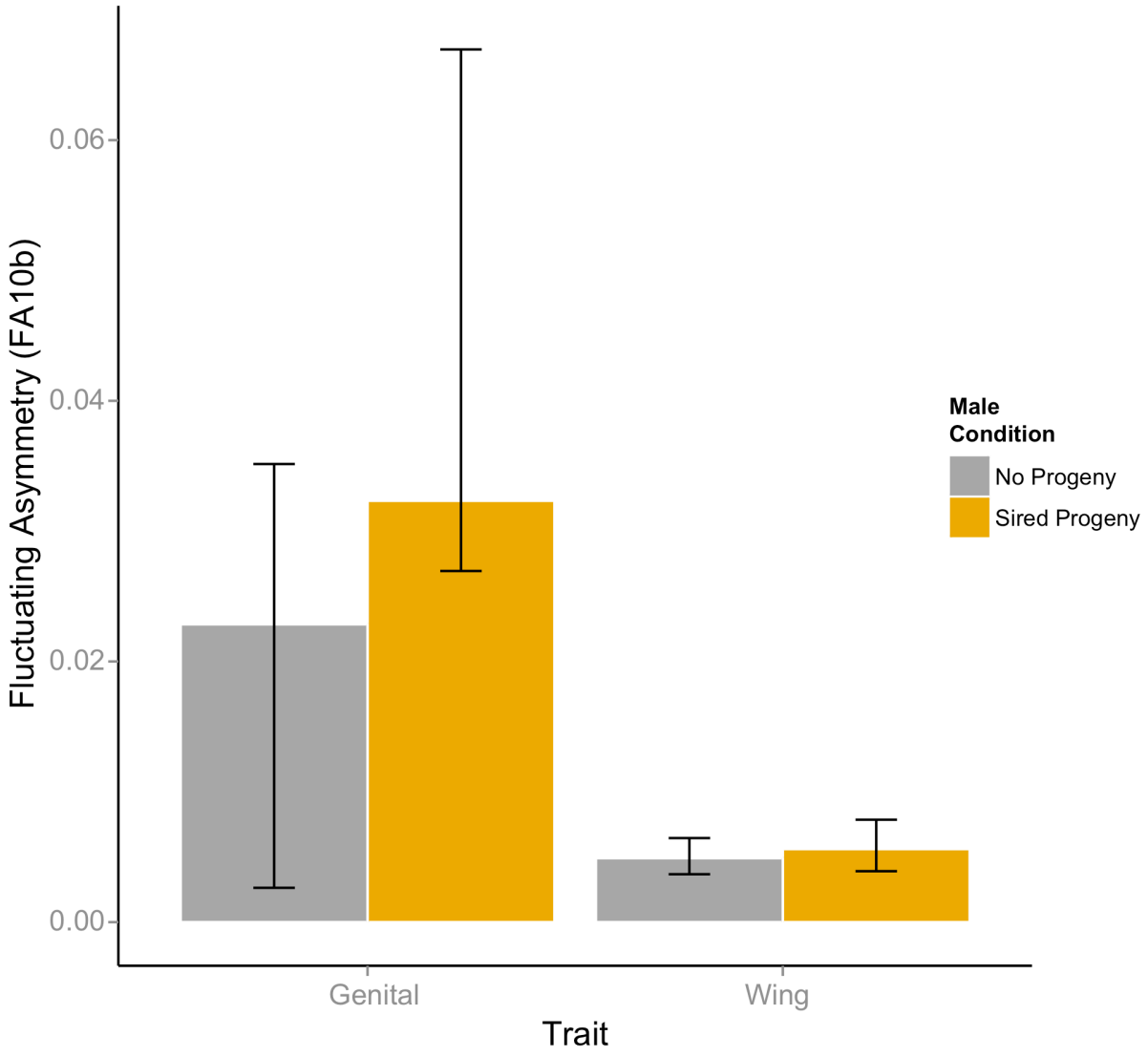

Figure 3.9. No difference in FA between flies that sired progeny and those that did not in the indirect competition regime. Error bars are 95% confidence intervals.

Discussion

Female *Drosophila melanogaster* preferred males with larger genital sizes than those with smaller genital sizes. Using the genetic tools available for *D. melanogaster* the only detectable trait that varied in size across the treatment groups were the genitalia, eliminating any confounding effect of covariation in other traits. This was especially important with regards to the wings as they are known to play a critical role in the courtship display of males (Spieth 1974).

Female *Drosophila* have ample opportunity to prevent a male from forcing copulation (Spieth 1995, Lasbleiz et al. 2006 2006). That a female can physically assess a male and his genitalia prior to mating with him indicates that genital size may be an important factor in female mate choice. The significance of genital morphology is further highlighted by the increased levels of diversity in the primary sexual traits across taxa (Eberhard 1985, Hosken and Stockley 2004). It is well documented that male genitalia across the phylum Arthropoda, and beyond, exhibit hypoallometry of their genitalia in relation to overall body size (Eberhard 2009). The hypoallometry of male genitalia is also commonly attributed to stabilizing selection acting on the genitalia (Eberhard et al. 2009). The ‘one-size-fits-all’ hypothesis states that the intermediate size genitalia would be selected because they are compatible with the larges number of potential female mates (Eberhard et al. 1998). Theoretically, stabilizing selection on one of two correlated traits could result in a hypoallometric relationship where one trait has very little variation in size across a range of sizes in the associated trait. However, the

paucity of empirical data supporting this hypothesis has made it difficult to reach a definitive answer.

Using a range of metrics, the preference of female *Drosophila* for genital size was directly addressed. Females had a clear preference of copulating with males that had wild-type-sized genitalia when compared to males with smaller genitalia, both when the females had only one male to mate with and when they had a choice (Figures 3.3, 3.5). The increased likelihood of a female choosing to mate with a male that had larger genital size when exposed to only one male suggests preference for larger genital size may have a genetic correlation with larger genital size. Males with smaller genital sizes were able to successfully mate with females, suggesting that the lock-and-key hypothesis is inconsistent with the observed genital morphology of male *Drosophila*.

Female *Drosophila* are able to control mating via behavioral modifications during courtship (Lasbleiz et al. 2006) and can also control fertilization. There was likely a significant effect of cryptic female choice as females preferentially fertilized progeny using sperm from males with larger genital size. This outcome supports the hypothesis that some form of sexual selection is occurring on the male genitalia in *Drosophila* as cryptic female choice is one of the primary mechanisms of sexual selection (Eberhard 1996). There was no difference in the final number of progeny sired by a male across any of the mating regimes, however. Eggs are a costly investment for females, and the data suggests that once a female decides to copulate and fertilize eggs using sperm from a male, she no longer differentiates between male genital sizes.

Sexual selection acting on male genitalia could be based on an assessment of male quality based on their genitalia (Eberhard 1985). One measure females could use to determine male quality is an appraisal of developmental stability. Developmental stability refers to the ability of an organism to resist stochastic developmental errors resulting in small levels of variation in phenotype, particularly across planes of symmetry. Males that are of high quality would be expected to have very symmetrical traits due to increased levels of nutrition and general developmental robustness. While there are some traits that are known to be asymmetrical (e.g. antisymmetry of male fiddler crab claws), male *Drosophila* genitalia have no bias in their symmetry (Dreyer and Shingleton 2011). No evidence of was found to support the hypothesis that females use developmental stability of male genitalia to guide mate choice (Figures 3.7 – 3.9). There was no difference in FA between the different size classes or between males that mated or did not mate in the direct and indirect competition regimes.

The genitalia of male *Drosophila* are hypoallometric, but the type of selection acting on them is unclear. Specifically, there is evidence for sexual selection, but the distinction between directional or stabilizing selection is equivocal. The hypoallometry of male *Drosophila* genitalia has been shown to be a result of reduced sensitivity to IIS signaling (Tang et al. 2011). In this study, modification of the IIS pathway in the developing genital tissues was only able to decrease the size of male genitalia, not increase it (Figure 3.10). IIS signaling was depressed in the developing genitalia by expression of a constitutively active form of the negative growth regulator, *FOXO*, and enhanced by expression of a constitutively active form of the positive growth regulator, *InR*. The

unidirectional effect of IIS perturbation in the genitalia stands in contrast to the response of *Drosophila* wings which significantly increased and decreased in size with up- and

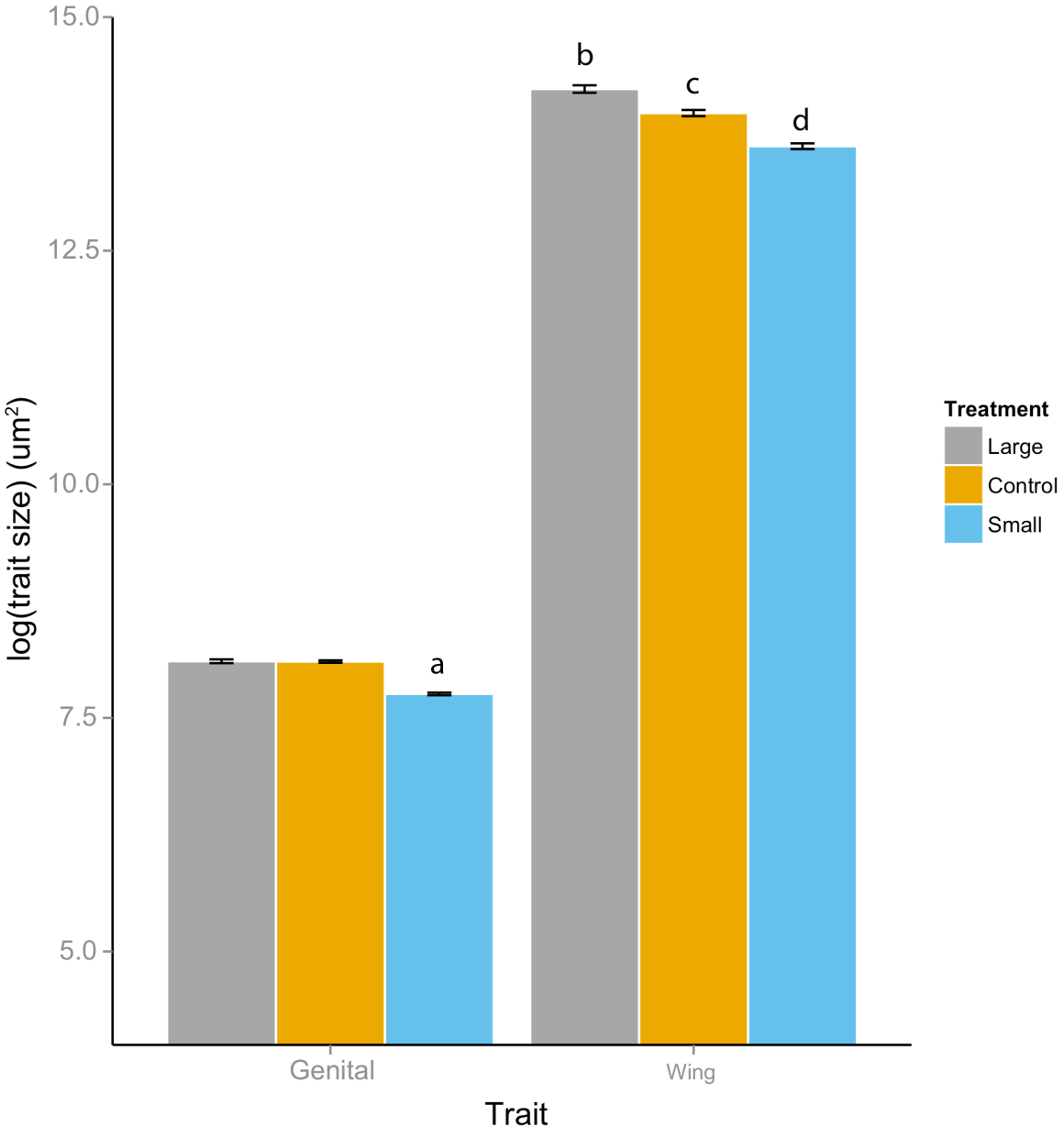

Figure 3.10. Response in genitalia and wings to IIS manipulation to make large and small traits. Wing significantly increased and decreased in size in response to up-regulating (large: *NP6333>InR.CA*) and down-regulating (small: *NP6333>InR.DN*^{25}) IIS in those tissues (Tukey's HSD < 0.05). Posterior lobe of the genital arch only decreased in size in response to down-regulating IIS (small: *Poxn>dFOXO.3x*) but did not increase in size when IIS was up-regulated (large: *Poxn>InR.CA*) suggesting the genitalia are insensitive to positive nutritional growth regulators or are at a maximum size in

response to nutrition. Error bars are 95% confidence intervals. Control flies were > *UAS-GFP* for both traits.

down-regulation of the same pathway via *InR* mutants (Figure 3.10). The lack of size increase in genitalia in response to an increase in perceived nutrition suggests that they may be insensitive to increases in InR or may be at a maximum size as determined by IIS signaling.

The most parsimonious explanation for why *Drosophila* genital tissue would respond to a downstream IIS growth regulator that decreases size but not an upstream IIS growth regulator that increases size is that the trait is insensitive to changes in nutrient signaling or it can simply not get any larger through insulin-signaling. Directional selection is a likely type of selection that would result in a trait being as large as it can be in response to any input, environmental or genetic, but it is possible that strong stabilizing selection on a trait could isolate it from nutrient signaling as well. It appears that the genitalia of *Drosophila,* at least, are insensitive to an increase in InR due to a reduction in sensitivity to the negative growth regulator, FOXO (Tang et al. 2011). Even as InR levels increase in the genital tissues, if there is low or no FOXO remaining in the tissues to shut off, the tissues will not increase in size. There is at least one example of a hypoallometric genital trait that is the result of strong directional selection: the male genital structures of the water strider *Aquarius remigis* (Bertin and Fairbairn 2007). Directional selection has been proposed as a hypotheses for the hypoallometry of genitalia but only in the specific conditions of directional selection with viability increasing with body size but decreasing with trait size (Bonduriansky and Day 2003) or when selection is acting disproportionately on small males as opposed to large (Eberhard et al. 2009). More work needs to be conducted to investigate how choosey

females are being, and if females would preferentially copulate with and/or fertilize offspring with males that have absolute larger genitalia. Collectively, the results of these experiments suggest that the observed hypoallometry of male *Drosophila*, while perpetuated by a female preference for larger genital sizes compared to smaller genital sizes, are also the result of a more complicated underlying developmental size control structure.

APPENDIX

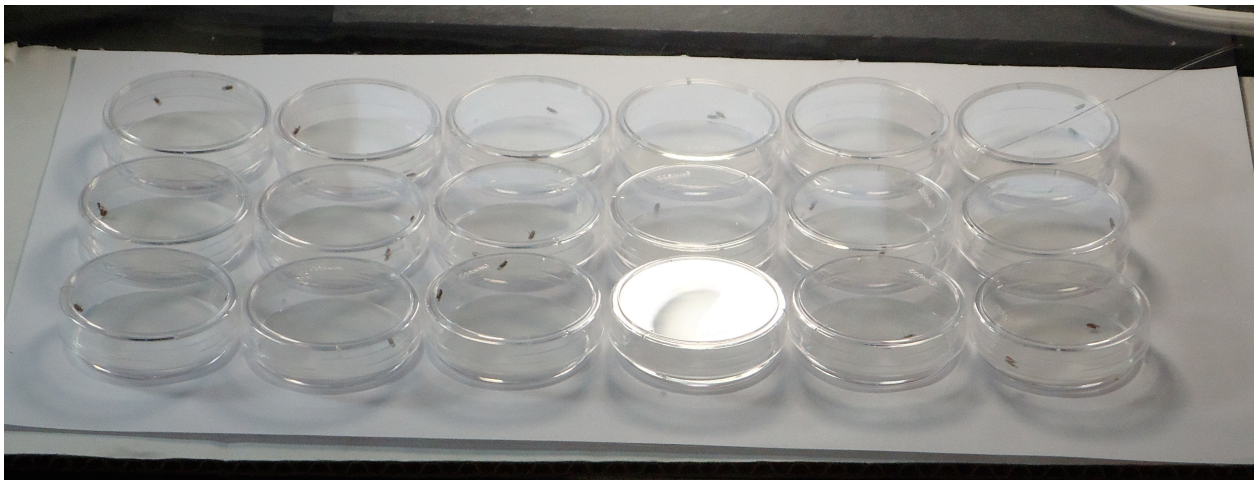

Figure A1. Complete mating assay consisting of 18 – 35mm x 10mm mating chambers.

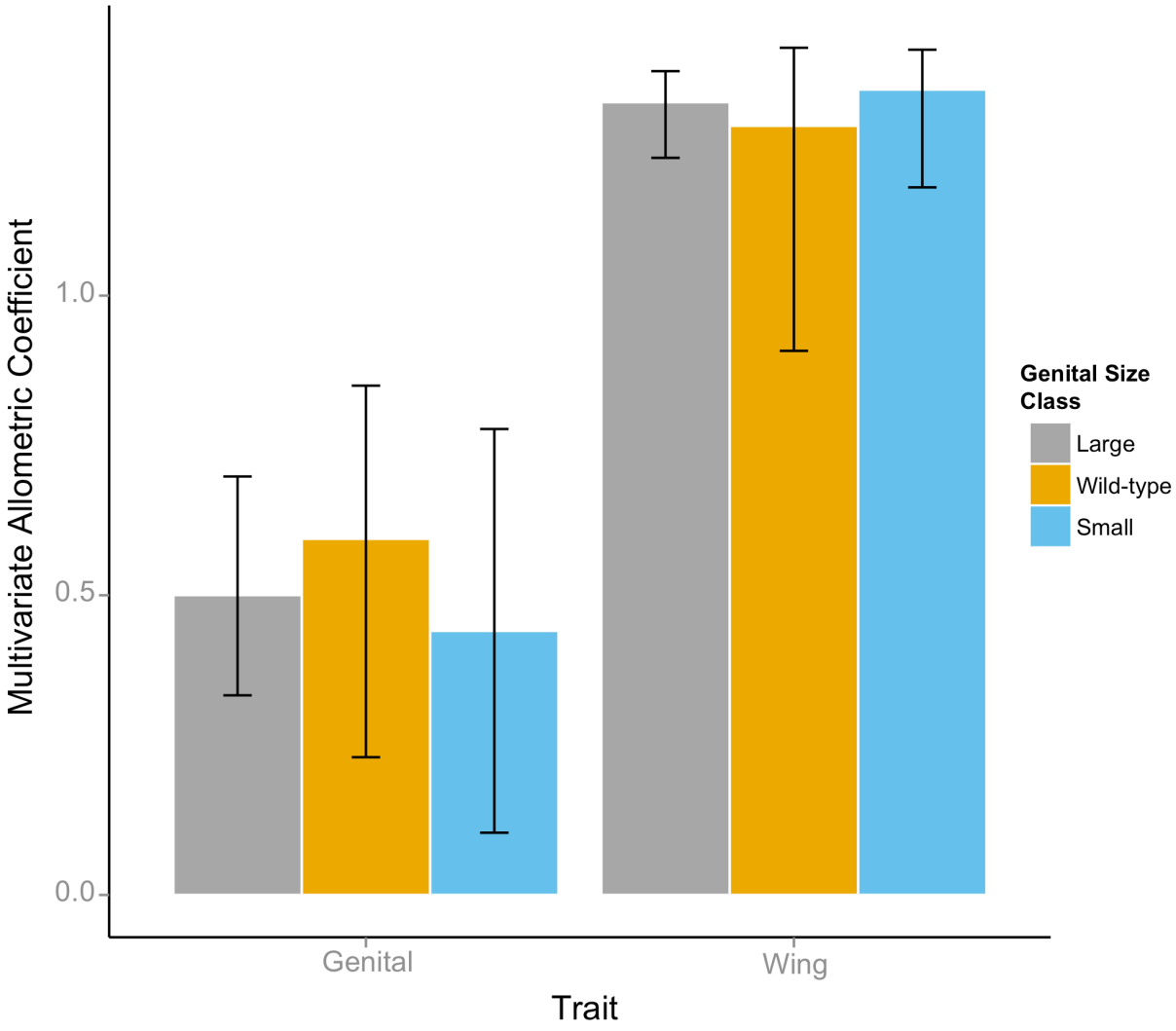

Figure A2. There was a difference in allometric coefficient between, but not within, traits. The allometric coefficient was lower in the genitalia thatn the wing, as expected. he allometric coefficients do not vary across size class, but are different between traits. Error bars are 95% bootstrap confidence intervals from 1000 replicates.

Table A1. Pairwise comparisions by genotype of mating behaviors. No significant difference between genotypes for early stages of courtship. Significant differences in behaviors are indicated by (*). Direct competition regime was not analyzed because males could not be differentiated in video recording of mating trial. All *p*-values calculated by Tukey's HSD tests.

	No Competition			Indirect Competition		
	UAS-GFP & InR.CA	*UAS-GFP & FOXO.3x*	*InR.CA & FOXO.3x*	*UAS-GFP & InR.CA*	*UAS-GFP & FOXO.3x*	*InR.CA & FOXO.3x*
Courtship Latency	0.644	0.908	0.389	0.439	0.843	0.781
Courtship Duration	0.526	0.996	0.513	0.206	0.438	0.193
Copulation Latency	0.090	0.895	0.043*	0.355	0.712	0.553
Copulation Duration	0.008*	0.071	0.766	0.587	0.802	0.899

Table A2. No relationship between the number of progeny sired and genital size. Linear regression of total number of offspring compared to genital size for each mating regime. No correlation or significant relationship between genital size and reproductive success across all males that sired offspring.

	Single Male	Direct Competition	Indirect Competition
OLS slope	15.0	-10.9	9.63
R^2	0.031	0.016	0.016
p-value	0.094	0.537	0.608

CHAPTER 4:

Trait development and the evolution of allometries *in silico*

Introduction

Morphological diversity is broadly characterized by variation in body proportion (Brown and West 2000). The variation in size among traits can largely be attributed to adaptations that are a response to the universal requirements for survival. All organisms must overcome the biological necessities such as securing nutrition, out-competing other organisms and reproducing while balancing the physical and physiological limitations of life on Earth. Natural selection has given rise to extreme biological diversity across orders of magnitude from the smallest to the largest organisms. In an effort to focus on tractable systems of study on the incredible range of size variation, scientists have developed methods to describe patterns of phenotypic divergence. One approach that has received great interest over the past century is the study of scaling relationships between biological traits (Gayon 2000). Research on the relationships between correlated traits in animals often compares some measure of body size to a correlated trait, for example, metabolic rate (White and Seymour 2005) exaggerated secondary sexual characteristics (Emlen and Nijhout 2000, Kodric-Brown et al. 2006, Eberhard et al. 2009, Nijhout and German 2012), and brain size (Lande 1979). The correlation between traits within and between species serves as a way to measure morphological variation quantitatively, which can help address the question of why we observe the phenotypic diversity that we do.

Research on scaling relationships focuses on how the relative size of traits varies within and among species (Schmidt-Nielsen 1984, Brown and West 2000).The differences between many closely related taxa can be summarized by the scaling relationships

between different traits (Cheverud 1982). In the class insecta, for example, diversity in relative size among the fixed set of characteristic insect features (three pairs of legs, head, thorax, abdomen, two pairs of wings) represents their wide-ranging morphological differences. Applying this principle to other groups of organisms, it is readily apparent how important body proportion is to quantifying morphological diversification.

Traditionally, body proportion has been described using the scaling relationship between two morphological traits, or between trait size and body size. How traits scale in relation to one another is commonly referred to as an allometry (Cock 1966, Gould 1966). The comparison of scaling between traits can be done at the level of taxanomic groups (evolutionary allometry), within a species at different developmental stages (ontogenetic allometry) and within a species at the same developmental stage (static allometry) (Cheverud 1982). Static allometries can be further subdivided based on the causes of size variation. Variation due to genetic factors generate genetic static allometries while variation due to environmental factors, such as nutrition or temperature during development, generate environmental static allometries (Shingleton et al. 2007). Traditionally, the scaling relationships of static allometries are modeled mathematically using the allometric equation, $y = bx^{\alpha}$ where y and x are measurements of morphological traits (Huxley and Teissier 1936). When log-transformed, the allometric equation produces a linear relationship of $log(y) = \alpha * log(x) + log(b)$ with a slope of α, the allometric coefficient, and an allometric intercept term, $log(b)$ (Gould 1966, Klingeberg 1998). Changes in body proportion are easily described by changes in the slope, α, and the allometric intercept, $log(b)$, resulting in widespread application of the

allometric equation to myriad examples of scaling relationships including those in vertebrates (Maina et al. 1989, Dixon 1993, Lemaitre et al. 2014) invertebrates (Eberhard 2009, Arendt and Fairbairn 2012) and plants (Niklas 1994, Chave et al. 2005).

Complementing the extensive empirical work on allometry, there have been many hypotheses of how allometries are expected to change in response to selection (Green 1992, Eberhard et al. 1998, Bonduriansky and Day 2003, Kodric-Brown et al. 2006, Bertin and Fairbairn 2007, Nijhout and German 2012, Pelabon et al. 2014). However, these hypotheses have been unable to provide a unifying framework to explain the breadth of empirical research. For example, the hypoallometry of male genitalia in arthropods is typically believed to be the result of stabilizing selection on intermediate genital size (Eberhard et al. 1998, Eberhard 2009), but it has been shown that genital traits under strong directional selection also exhibit hypoallometry (Bertin and Fairbairn 2007). In practice, it has also proved difficult to alter the slope of an allometry through artificial selection on the allometric coefficient alone to test the theories (Egset et al. 2012). The difficulty of connecting predictions to observed patterns may result from hidden assumptions about the structure of population-level scaling relationships and suggests the need for an updated framework for allometry evolution.

Scaling relationships are the result of covariation between traits in response to factors that regulate trait growth (Stern and Emlen 1999, Shingleton et al. 2008). Therefore, incorporating our understanding of these mechanisms into predictions of allometry

evolution is a necessity. Some of the most influential pathways controlling the development of trait size are nutrient-sensing pathways (Brogiolo et al. 2001, Oldham and Hafen 2003). Coordination of growth in response to nutritional access during development has been well studied in holometabolous insects with a focus on the highly-conserved insulin/insulin-growth factor signaling pathway (IIS) (Claeys et al. 2002, Dupont and Holzenberger 2003). In IIS signaling, the level of nutrition that is taken in from the environment determines the amount of insulin-like peptides (ILPs) produced, which initiates a signaling cascade that ultimately controls the size of individual organs and overall body size of organisms (Mirth and Riddiford 2007, Mirth and Shingleton 2012, Nijhout et al. 2013). This means that individuals with high levels of ILPs will typically have large body/trait sizes whereas individuals with lower ILP levels will exhibit smaller body/trait sizes. The two are not necessarily linked, however. The relative sensitivity among traits to the ILPs present in a developing organism are one determinant of relative size between traits, and consequently, of an individual's static allometry (Emlen et al. 2006, Shingleton 2012). This relationship between ILPs and allometry has been supported for both hyperallometric and hypoallometric traits. In male rhinoceros beetles for example, exaggerated traits have been shown to be more insulin-sensitive than other body structures (Emlen et al. 2012). Similar research in *Drosophila* has demonstrated that the hypoallometry of male genitalia is due to relative insensitivity to circulating *Drosophila* Insulin-Like Peptides (dILPs) in the developing genital tissues resulting in adult genitalia that remain a consistent size across a range of nutritional levels (Tang et al. 2011).

Empirical and theoretical work has focused on population-level scaling relationships. However, a key insight is that population-level static allometries (populationSA) reflect responses at the individual-level to the genetic and environmental factors that regulate trait size. Individual-level responses are the output of a fixed system (the individual's genotype) responding to the environmental factors that affect trait size. If there is genetic variation among individuals in their responses to the environment, each individual has an unexpressed genotype-specific scaling relationship of all possible trait sizes that individual could posses if reared in different environments (Figure 4.1). Because each individual develops in a single environment, only one of the possible genotype-specific scaling relationships is ever expressed, but the collection of possible scaling relationships represents a hidden individual-level static allometry (individualSA). As will become clear, the patterns of individualSA in a population are extremely important in the prediction of allometry evolution.

Here we present a simple stochastic mathematical model of population-level covariation between two traits using current understanding of the developmental mechanisms underlying trait size within individuals (Stern and Emlen 1999, Shingleton et al. 2008, Nijhout and German 2012, Nijhout et al. 2013). The model uses the fundamental developmental parameters listed above to generate a population of individuals, each with a unique individualSA. These theoretical populations can then be subject to different forms of selection to determine how the populations as a whole, and the developmental parameters within each individual, evolve. Establishing the how of

proportional trait evolution will be a tremendous step forward in understanding the broader question of the origins of morphological diversity.

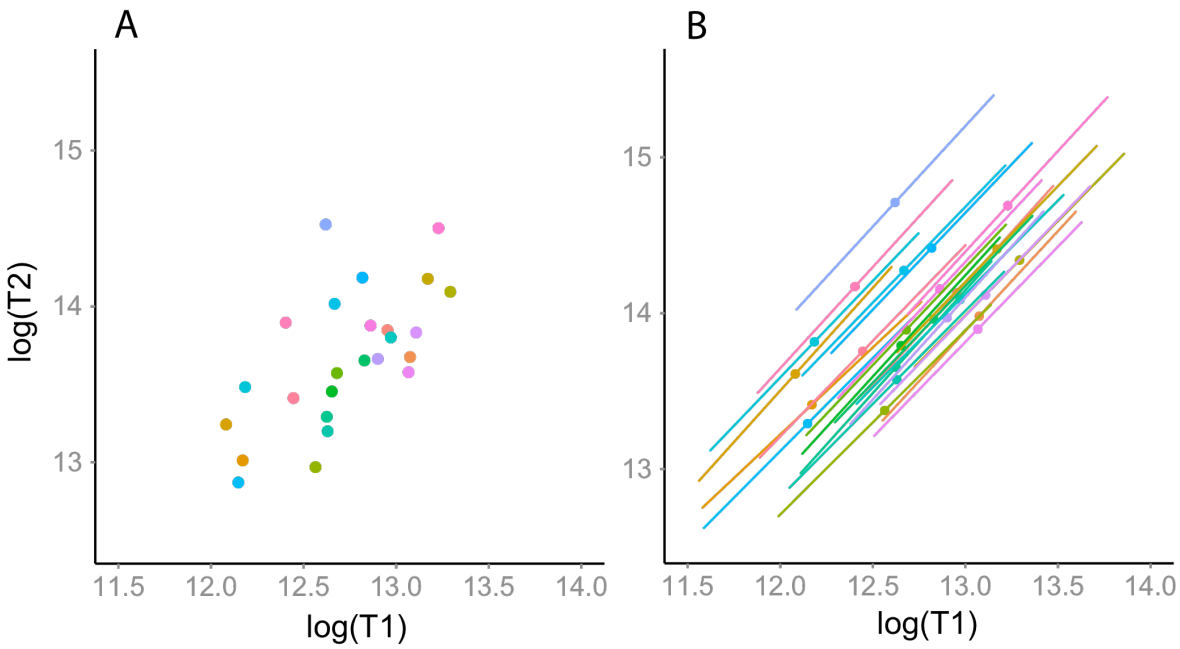

Figure 4.1. Population static allometry and the individual static allometry. (A) A hypothetical populationSA demonstrating the relationship between covarying traits T1 and T2 comprised of 25 individuals, represented by unique colors. (B) Individuals express only one phenotype (colored circle) of the possible individualSA possible for their genotype (colored lines) based on the environmental conditions they are exposed to during development.

Methods

Developmental Basis

Broadly, there are at least two classes of size regulation mechanisms in developing tissues: those related to systemic factors, and organ autonomous factors (Stern and Emlen 1999, Emlen and Allen 2003, Shingleton et al. 2007, Tang et al. 2011). Systemic factors are expressed throughout the entire developing organism. Examples of systemic factors include circulating hormones, developmental temperature and nutrition levels. There are also positive and negative regulatory responses to the systemic growth factors. The organ-specific response controls how sensitive organs are to the systemic growth factor. Because the organ-specific response of multiple organs is to a single systemic signal, it follows that the degree of covariation between traits will be dependent on the ratio of their responses to the shared systemic factor.

The second class of growth regulators are the organ-autonomous growth factors. These factors are intrinsic to each developing tissue and act independently of any external influences. Organ-autonomous factors include the following: specific signals that determine growth rate and duration in individual tissues, patterning genes, and tissue-specific expression of genes such as *chico* or *Akt* in developing organs (Bohni et al. 1999, Verdu et al. 1999)

Model

The model of morphological trait development described here was built around growth as a geometric process. All morphological traits start as a single cell that undergoes

many rounds of division to give rise to a final adult trait. Therefore, a modified geometric growth model was used:

$$T = pe^{rt} \qquad [1]$$

where T is the final trait value, p is the starting number of cells, here set to one, e is the exponential constant, r is the growth rate and t is time. Time in the model is standardized to 1 as the model is a simulation of the effects of changing growth rate, while the growth duration of two tissues within a single organism is assumed to be equivalent. Thus, the modified geometric growth equation used in the model is:

$$T = e^{r} \qquad [2]$$

where T is the final trait value, e is the exponential constant and r is the growth rate that is the result of the developmental parameters controlling trait size.

The mathematical model is constructed by combining the geometric nature of morphology with the factors listed above; systemic factors, organ-specific sensitivity to the systemic factors and organ-autonomous factors. The three factors are included in the model as: G, the systemic growth factor that affects all tissues, k, the organ-specific response to the systemic growth factor and i, the organ-autonomous growth factor for traits. Substituting these growth parameters into equation 2 gives the equation for generic trait growth, where growth rate is determined by the multiplicative interaction of the systemic growth factor and the tissue specific response with the additive interaction of the tissue autonomous growth factor:

$$T = e^{Gk+i} \qquad [3]$$

To produce two traits, T_1 and T_2, that have covariation in size, trait specific parameters are substituted into equation 3 to generate an equation for each trait:

$$T_1 = e^{Gk_1 + i_1} \quad [4]$$
$$T_2 = e^{Gk_2 + i_2} \quad [5]$$

Equations 4 and 5 can be log-transformed to create linear equations for the size of each trait:

$$\log(T_1) = Gk_1 + i_1 \quad [6]$$
$$\log(T_2) = Gk_2 + i_2 \quad [7]$$

and the relationship between the two traits can be summarized as:

$$\log(T_2) = k_2/k_1 * \log(T_1) - k_2/k_1 * i_1 + i_2 \quad [8]$$

Equation 8 demonstrates that the slope of the scaling relationship between T_1 and T_2 is equivalent to the value of k_2/k_1, the interaction of the trait specific responses to the systemic growth factor. Equation 8 also mathematically describes the range of possible genotype-specific scaling relationships, or the individualSA. As a result of the importance of the k_2/k_1 value, the degree to which any form of selection is able to target the k_2/k_1 ratio in individuals will likely determine how effective the selection will be in changing the populationSA.

To create a populationSA, unique parameter values for k_1, k_2, i_1, and i_2 were assigned to each individual. The final value for each parameter was the sum of a diallelic system of additive co-dominance for each parameter. Each individual has two values (alleles) for each parameter (k_{1a} and k_{1b}) that combine to form the total parameter value. Parameter values are all sampled from normal distributions with known means and standard

deviations, and from the same distributions within a parameter type (k_1, k_2, i_1, and i_2). The collection of parameter values defines a population of individuals that have no phenotype, but each have an individualSA. Individuals are then assigned a value of *G* which determines where on their individualSA their final phenotype will be expressed.

IndividualSA vs PopulationSA

An important aspect of the model is that different patterns of individualSA can generate the same populationSA. For example, when $\mu_G = 0$, the individualSA rotate around the bivariate mean of the populationSA (Figure 4.2, A, A'). When $\mu_G > 0$, the individualSA rotate around a point that is less than the bivariatie mean of the populationSA, even though the populationSA of the two populations is ostensibly identical (Figure 4.2 B, B'). When $\mu_G < 0$, the individualSA rotate around a point that is greater than the bivariate mean of the population SA, but the populationSA is indistinguishable from either of the other populationSA (Figure 4.2 C, C'). The distribution of individualSA patterns will be referred to as 'bowtie' ($\mu_G = 0$), 'speedometer' ($\mu_G > 0$) and 'broomstick' ($\mu_G < 0$), reflecting the general pattern of the individualSA in each (Figure 4.2, A', B', C').

Selection

Once an initial population of individuals was created, selection was carried out. Selection is defined in the model as a set number of individuals being removed from the population (truncation selection). Removing the same proportion of individuals for each type of selection allows for a direct comparison of the outcome of selection across generations and forms of selection. The type of selection performed was dependent on

what part of the distribution of trait values individuals were removed. After individuals and their associated parameter values were removed from the population, parameter values from surviving individuals were recombined to form new individuals and the population was returned to its original size by assigning new values of *G* for each new individual.

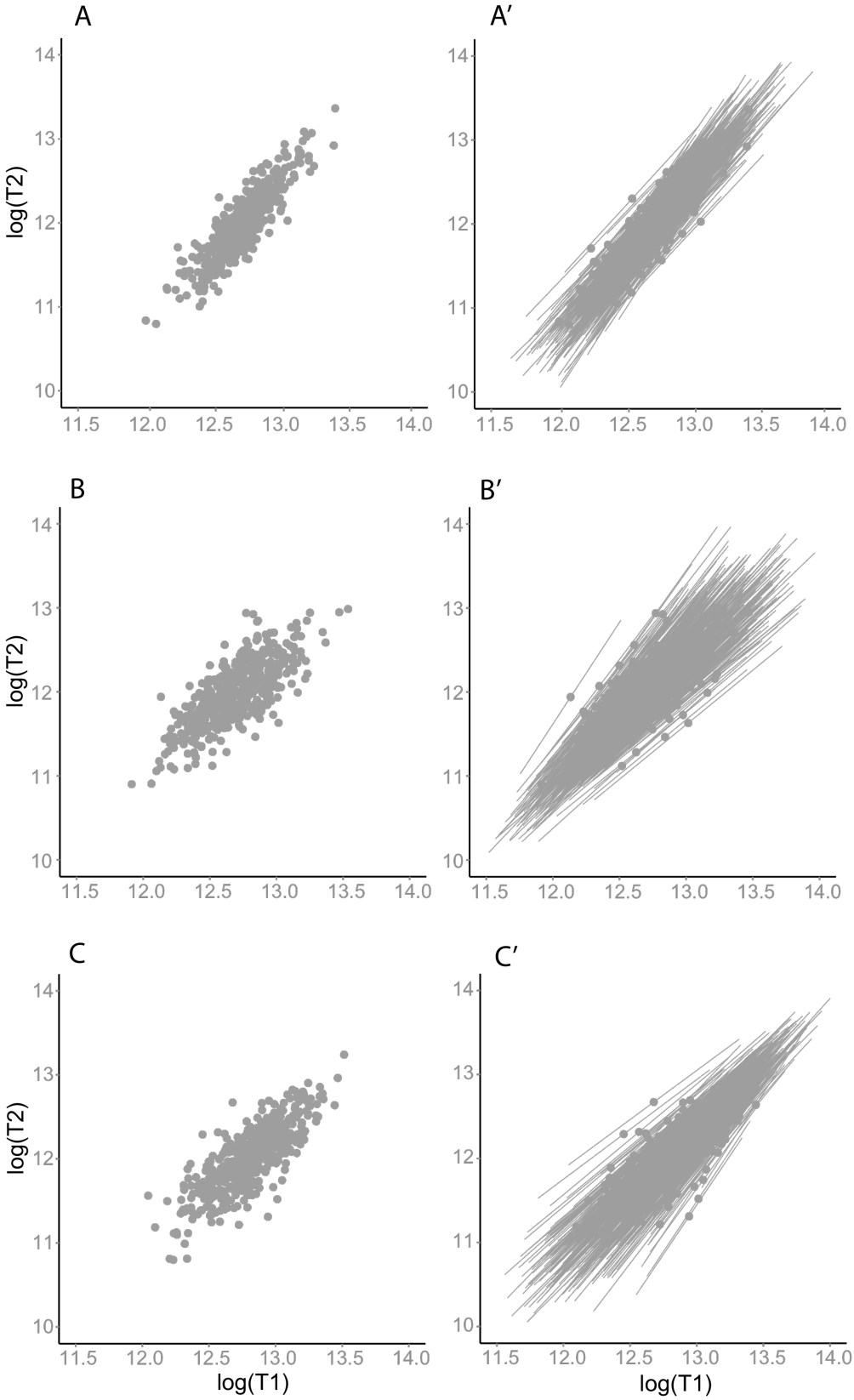

Figure 4.2.

Figure 4.2 (con't). Patterns of individualSA. (A, A') When $\mu_G = 0$, the populationSA appears normally distributed (A) and the individualSA have equal levels of variation as the size of T1 increases or decreases (A') creating a bowtie shaped pattern. (B, B') When $\mu_G > 0$, the populationSA appears similar in distribution to populations with $\mu_G = 0$, including similar slope and covariation between traits T1 and T2 (B), but the underlying individual-level static allometries exhibit more variation as the size of T1 increases (B') producing a speedometer shaped pattern. (C, C') When $\mu_G < 0$, the population-level static allometry has the same appearance and statistical properties as populations with $\mu_G > 0$ or $\mu_G = 0$, but the individual-level static allometries exhibit more variation as the size of T1 decreases (C') creating a broomstick shaped pattern.

Each pattern of individualSA (bowtie, speedometer and broomstick) was subjected to selection. The parameter values for each population type were selected to maintain population structure such that each population was essentially identical in terms of slope, intercept and trait variance. There were four selection regimes that each population type was exposed to were as follows:

1. Stabilizing selection – individuals were removed based on both the maximum and minimum extreme values of absolute T2 size (Figure 4.3, A),
2. Directional selection – individuals were removed from either the maximum (negative directional selection) or minimum (positive directional selection) values of absolute T2 size (Figure 4.3, B),
3. Proportional selection – individuals were removed based on the relative size of T2 being larger than T1 (negative proportional selection) or smaller than T1 (positive proportional selection) (Figure 4.3, C) and
4. Correlational selection – individuals were removed based on their having a large T1 and a proportionally small T2 or small T1 and proportionally large T2 values (positive correlational selection) or on their having large T1 and a proportionally large T2 or a small T2 and a proportionally small T2 (negative correlational selection) (Figure 4.3, D).

Population Structure

Population size for each trial was 1000 individuals, and each population was allowed to evolve for ten generations. A total of eleven populations were recorded, the initial population and the subsequent ten generations that were the result of selection.

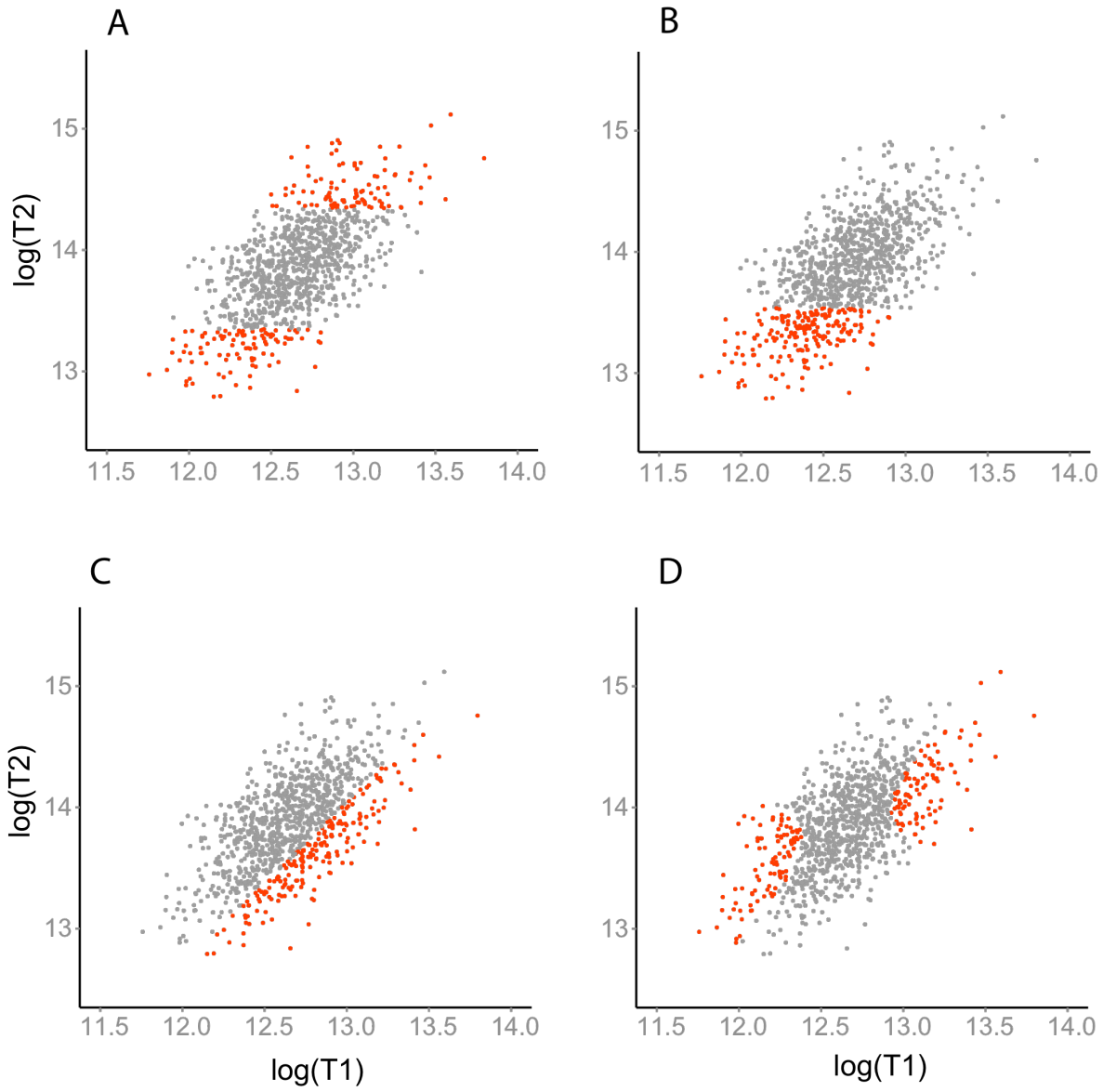

Figure 4.3. Four selection regimes: Stabilizing selection (A), Directional selection (B), Proportional selection (C) and Correlational selection (D). Grey points indicate selected individuals red points indicate individuals removed from the population prior to reproduction. The positive form of selection is shown in B-D with negative selection for those types removing individuals from the opposite orientation in the population.

For each generation, the mean parameter values across the total population were recorded as were the selection differential and selection intensity for each selection regime, and the allometric coefficient and intercept of the static allometry for each population. The allometric coefficient and intercept were calculated using ordinary least squares (OLS), major axis (MA) and standardized major axis (SMA) linear regression methods. Only the MA slope and intercept are included here as it represents the closest estimation of the mathematical allometric coefficient between the two traits, defined here as k_2/k_1 that is the interaction of the trait specific responses to the shared systemic growth factor, G. Selection was replicated 1000 times for each regime and population type to generate confidence intervals for the population parameter statistics. The founding populations for each replicate were generated using the same initial parameter distributions within each population type.

All of the computer modeling was implemented using R (R-Development-Core-Team 2014) and executed using the High Performance Computing Cluster at Michigan State University.

Results

Visually and statistically indistinguishable populationSA with different underlying individualSA responded differently to the selection regimes. The general structure of the populationSA (speedometer ($G > 0$), bowtie ($G = 0$), broomstick ($G < 0$)) was the same; there were no distinguishing characteristics that clearly delineate which pattern of individualSA was underlying a given populationSA (Figure 4.2 A-C). Within a population type, the positive and negative forms of each selection regime had nearly equal and

opposite effects on the allometric coefficient and intercept of the population. These results indicate that predictions of how an allometry will evolve cannot be made based on the populatoinSA alone.

Stabilizing and correlational selection were both weak forms of selection. Across all individualSA population types, stabilizing selection had only a minimal effect on the allometric coefficient and intercept of the populationSA (Figures 4.4 – 4.6: B). Correlational selection also had a weak effect on the allometric slope in each individualSA population type (Figures 4.4 – 4.6: E,E').

For selection types that had a large effect, the pattern of individualSA in the population determined how the allometry responded to selection. Both directional and proportional selection readily changed the allometric slope of the populationSA, but only in speedometer ($\mu_G > 0$) and broomstick ($\mu_G < 0$) populations (Figures 4.4 – 4.6: C/C', D/D'). Bowtie populations ($\mu_G = 0$) did not have large changes in allometric slope in response to any selection type (Figure 4.5).

Figures 4.7 – 4.9 display an initial and final population for all population and selection types and how the populationSA changed after 10 generations of selection. For each population type, positive and negative proportional selection increased the correlation between T1 and T2, while the remaining selection regimes largely preserved the initial trait correlations (Figure 4.7-4.9: D,D')

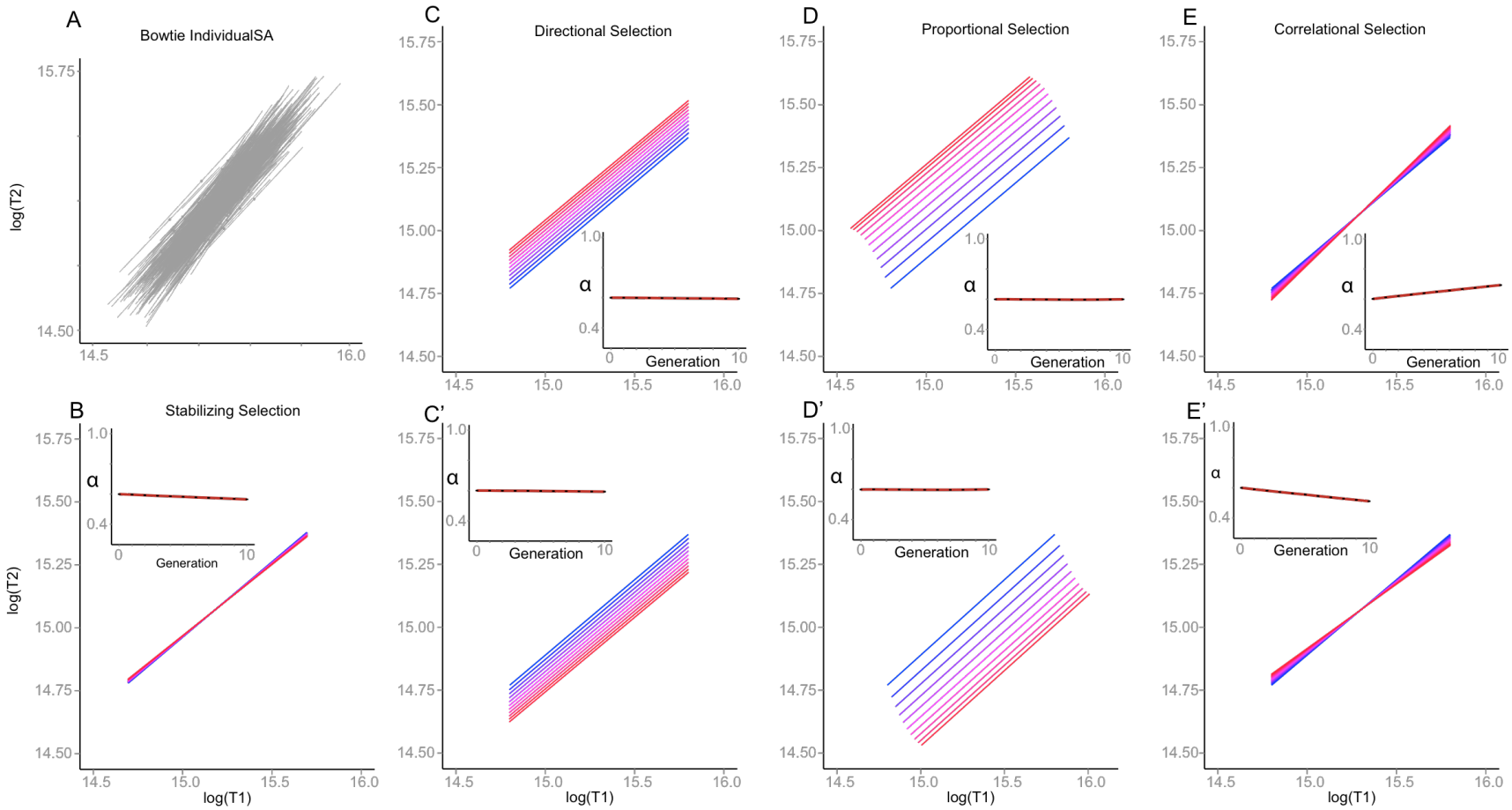

Figure 4.4. Evolution of populationSA for ten generations of selection on bowtie populations ($\mu_G = 0$). Inset plots display the change in the allometric coefficient (α) through time (generation). (A) The individualSA pattern when $\mu_G = 0$. Generations 0 – 10 represented by colored lines blue – red. The effect on the populationSA from stabilizing selection (B), positive and negative directional selection (C,C'), positive and negative proportional selection (D,D') and positive and negative correlational selection (E,E').

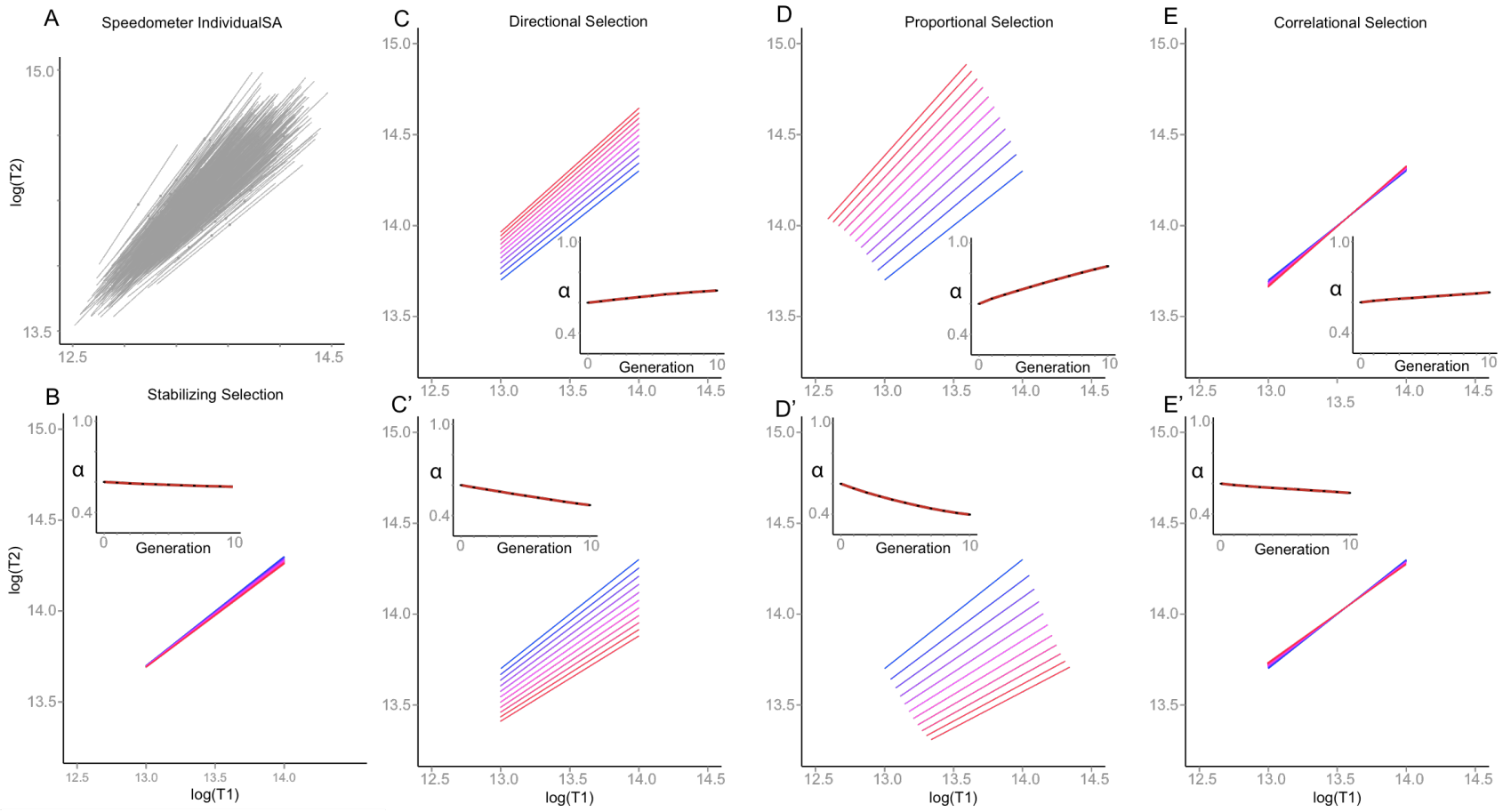

Figure 4.5. Evolution of populationSA for ten generations of selection on speedometer populations ($\mu_G > 0$). Inset plots display the change in the allometric coefficient (α) through time (generation). (A) The individualSA pattern when $\mu_G > 0$. Generations 0 – 10 represented by colored lines blue – red. The effect on the populationSA from stabilizing selection (B), positive and negative directional selection (C,C'), positive and negative proportional selection (D,D') and positive and negative correlational selection (E,E').

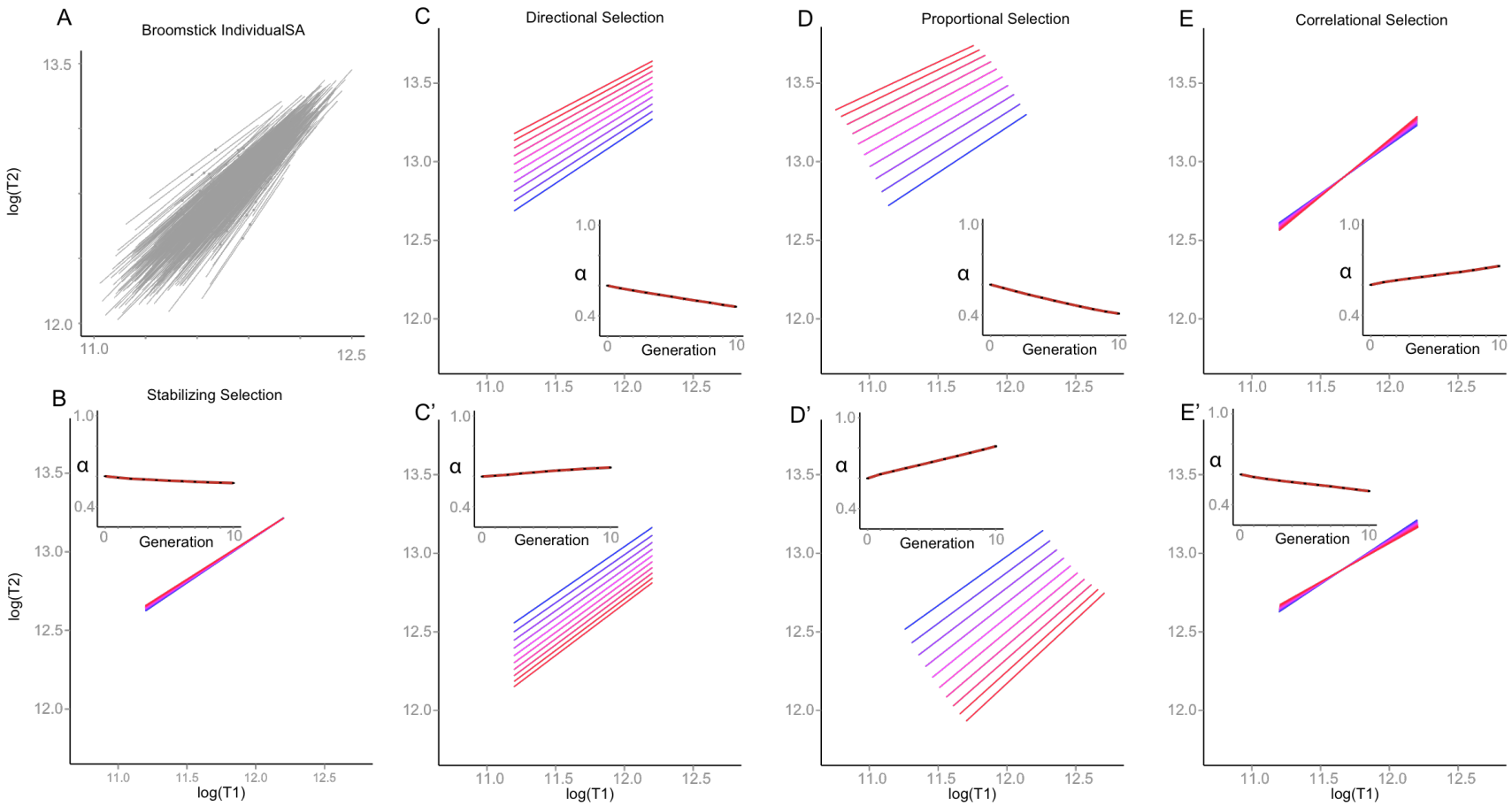

Figure 4.6. Evolution of populationSA for ten generations of selection on broomstick populations ($\mu_G < 0$). Inset plots display the change in the allometric coefficient (α) through time (generation). (A) The individualSA pattern when $\mu_G < 0$. Generations 0 – 10 represented by colored lines blue – red. The effect on the populationSA from stabilizing selection (B), positive and negative directional selection (C,C'), positive and negative proportional selection (D,D') and positive and negative correlational selection (E,E').

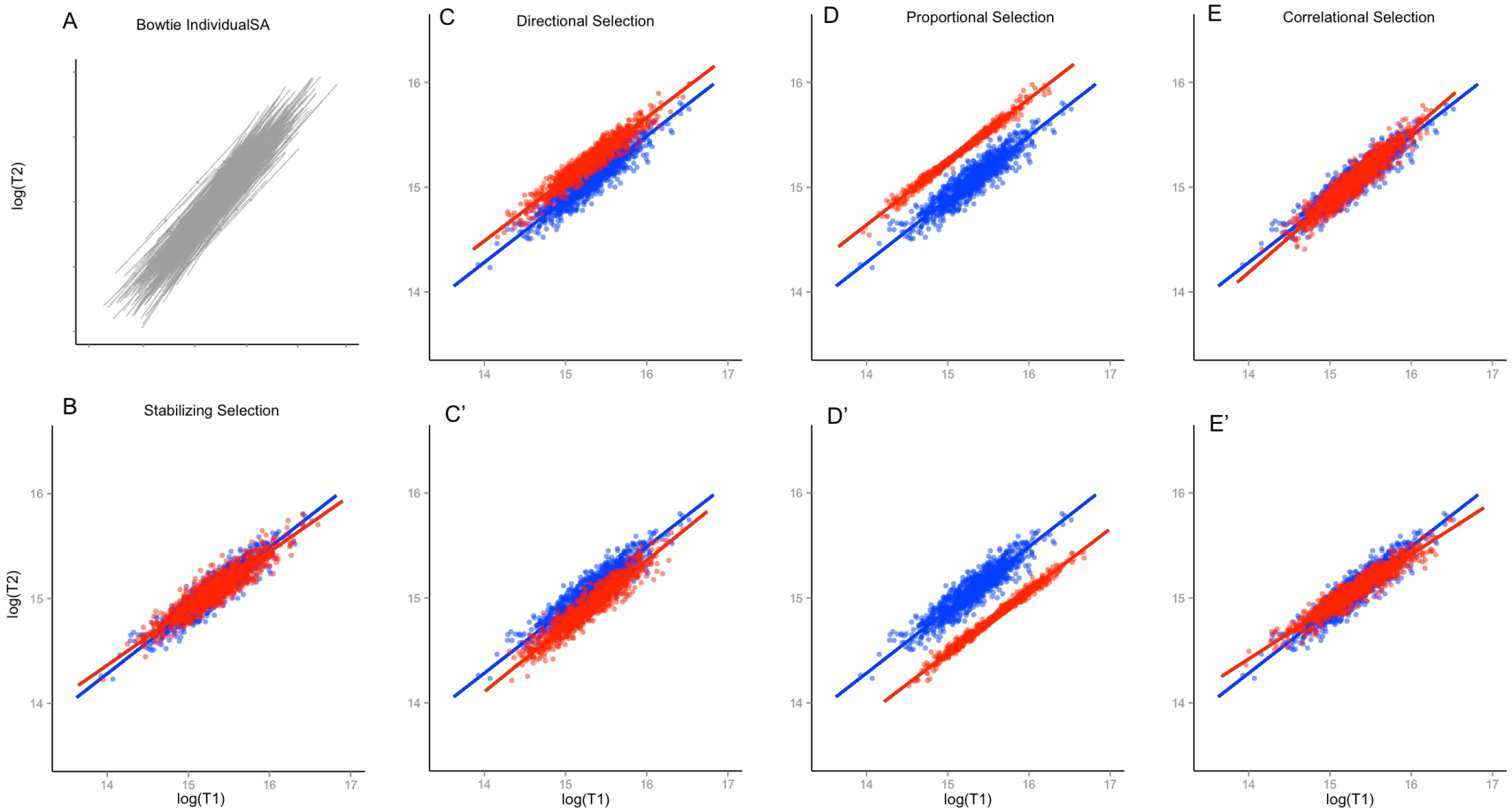

Figure 4.7. Initial (blue) and final (red) bowtie populations ($\mu_G = 0$) after 10 generations of selection. All lines are of the corresponding colored populationSA calculated using major axis regression. Represented are stabilizing selection (B) positive and negative directional selection (C, C') positive and negative proportional selection (D, D'), positive and negative correlational selection (E, E').

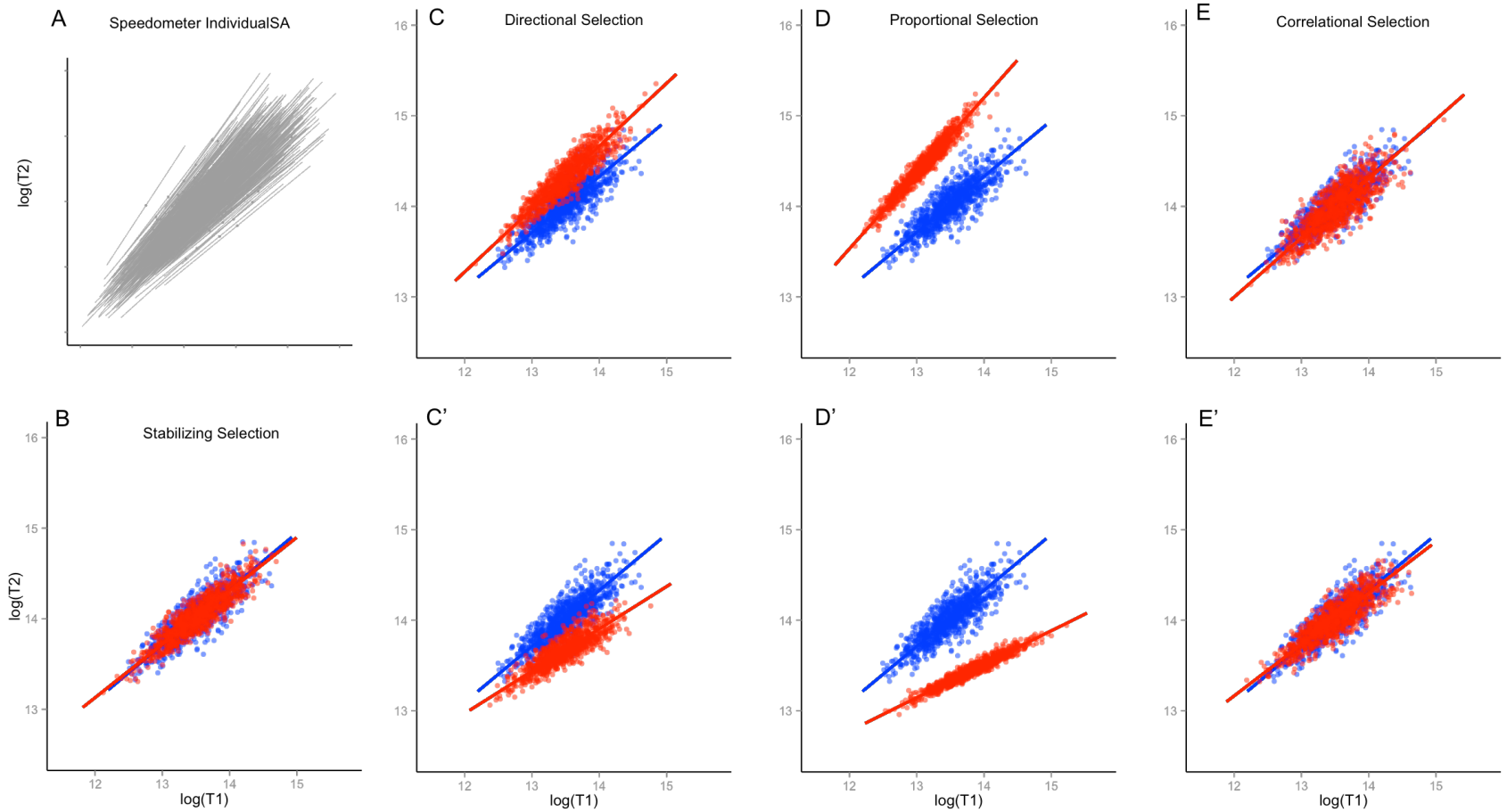

Figure 4.8. Initial (blue) and final (red) speedometer populations ($\mu_G > 0$) after 10 generations of selection. All lines are of the corresponding colored populationSA calculated using major axis regression. Represented are stabilizing selection (B) positive and negative directional selection (C, C') positive and negative proportional selection (D, D'), positive and negative correlational selection (E, E').

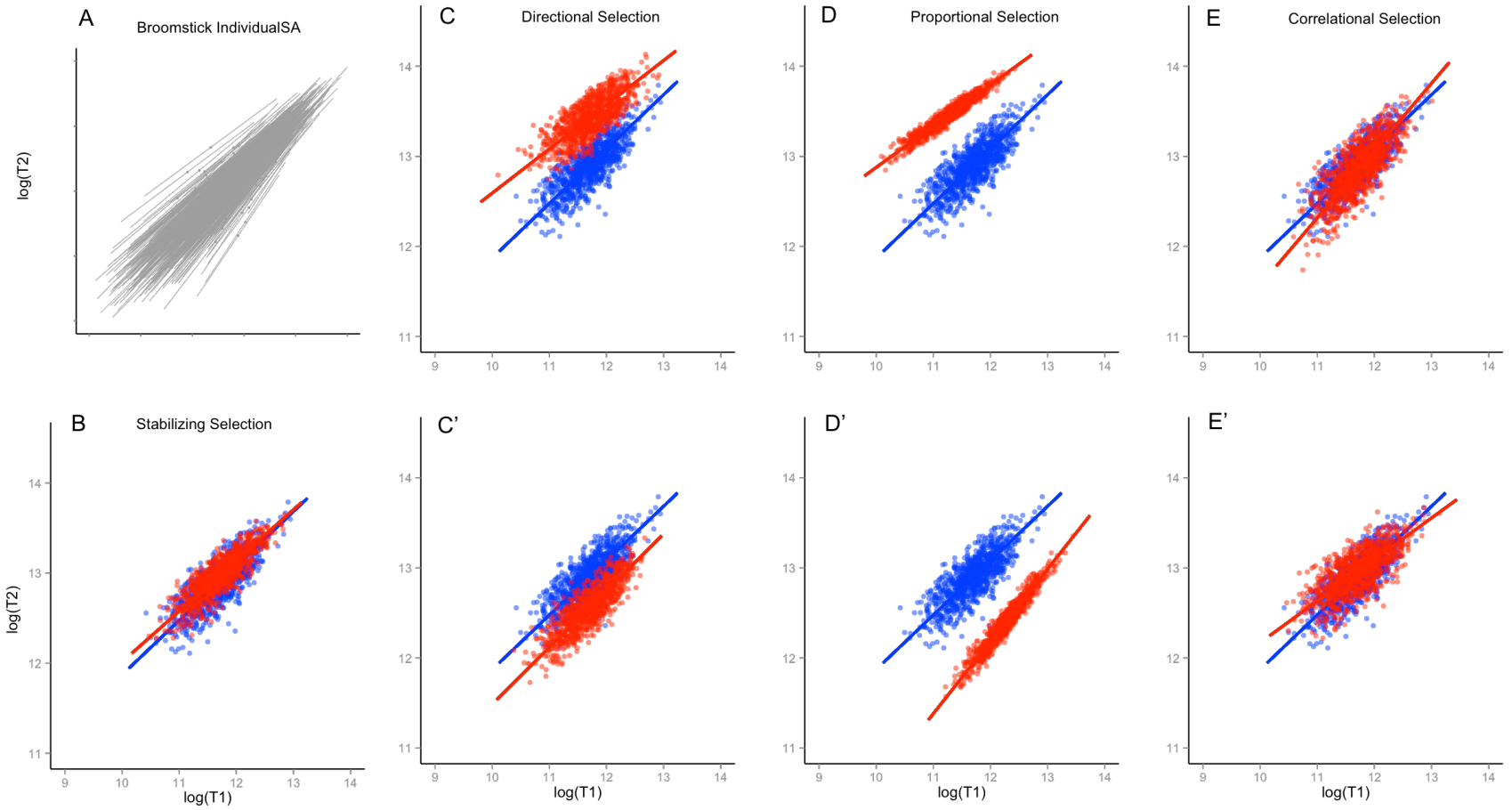

Figure 4.9. Initial (blue) and final (red) broomstick populations ($\mu_G < 0$) after 10 generations of selection. All lines are of the corresponding colored populationSA calculated using major axis regression. Represented are stabilizing selection (B) positive and negative directional selection (C, C') positive and negative proportional selection (D, D'), positive and negative correlational selection (E, E').

Discussion

How scaling relationships evolve is largely dependent on the underlying pattern of individualSA in a population. The individualSA patterns are determined by the μ_G. *G* represents the environmental condition input as a systemic growth factor, able to enhance and repress growth. The value of μ_G represents the degree of control between positive and negative growth regulators acting on individuals. In populations with a $\mu_G > 0$, positive growth factors are primarily regulating growth, i.e., growth is being enhanced and not suppressed. Alternatively, in populations where $\mu_G < 0$, negative growth factors are primarily regulating growth and trait growth is being suppressed and not enhanced. When $\mu_G = 0$, there is a balance between the control of positive and negative growth regulators. While we do not know what specific regulators are acting on tissues in this manner, some systemic positive and negative regulators of growth in the IIS pathway have been indentified. InR is a positive regulator of growth that responds to increased levels of nutrition (ILPs) by initiating a signaling cascade and stimulating growth (Chen et al. 1996, Goberdhan and Wilson 2002). A known negative regulator of growth is the forkhead transcription factor, FOXO, which represses growth in response to low levels of nutrition (Kramer et al. 2003). Genetic variation for a trait's relative sensitivity to nutrient signals such as InR and FOXO are thought to contribute to the evolution of trait-specific disproportionate growth (Lavine et al. 2015). An additional consideration for the outcome of selection is that while individuals were selected based on their realized allometry, their complete individualSA was also being selected. Therefore, possible outcomes of selection were dependent on much more than the observed populationSA.

Stabilizing selection is often invoked as the ultimate cause of hypoallometric traits in nature, particularly of the male genitalia in arthropods (Eberhard et al. 1998, Eberhard 2009). This prediction is a possible outcome of selection that restricts phenotypic variation in one trait when compared to a covarying trait. The model does not support this hypothesis as stabilizing selection had the weakest effect across the three population types, regardless of the underlying pattern of individualSA (Figures 4.4 – 4.6, D). What combination(s) of selection and population type did predict a decrease in allometric coefficient? Both directional and proportional selection had the potential to decrease the allometric coefficient. In speedometer populations ($\mu_G > 0$), negative directional and proportional selection decreased the allometric coefficient, while in broomstick populations ($\mu_G < 0$), positive directional and proportional selection decreased the allometric coefficient. Therefore, one mechanism predicted to result in hypoallometric relationships is positive directional selection on a trait that is controlled by a negative growth factor.

Is there evidence for positive directional selection generating hypoallometry in organisms? Bertin and Fairbairn (2007) describe genital traits that are known to be under strong positive directional selection in water striders scaling hypoallometrically to body size. Addtionallly, the hypoallometry of male genitalia in *Drosophila* has been shown to be the result of relative insensitivity to *FOXO* levels in the developing genital tissues (Tang et al. 2011). Based on the model, we know that positive directional selection in a broomstick population is predicted to result in hypoallometry. We also know that broomstick populations are the result of growth control by negative regulators

of growth, such as *FOXO.* Taken together, these two pieces of empirical evidence support the evolution of hypoallometry as presented by our model.

Our model also predicts that the weakest forms of selection in relation to the allometric scaling relationship are correlational and stabilizing selection (Figures 4.4 – 4.6). Artificial selection targeting the allometric slope of scaling relationships has proven difficult empirically (Egset et al. 2012), although given the type of artificial selection being performed, our model predicts a poor response. The type of selection used by Egset et al. (2012) most closely resembled correlational selection in our model, which we found to be a weak form of selection. Intriguingly, other instances of artificial selection targeting trait size rather that the slope of the scaling relationship resulted in changes in the allometric coefficient (Wilkinson 1993, Emlen 1996, Nijhout and German 2012). The ability of directional selection to change the allometric coefficients in these studies is also in congruence with our model in that the model predicts directional selection to change the slope of an allometry rapidly as compared to either stabilizing or correlational selection.

As shown in Figure 2 (A – C) the initial population types between the patterns of individualSA are nearly indistinguishable. How then are the underlying individualSA of a population to be indentified to for accurate predictions of how the population will respond to selection? To predict how allometries will evolve, it is clear that some understanding of the individualSA is necessary. One method of determining what the pattern of indivdualSA is would be rearing genetically identical individuals at various

environmental conditions to map out the static allometry for a single genotype empirically. Mapping of individualSA in this way is only theoretically possible in a select few model systems such as *Drosophila* where isogenic lines are available. For genetically distinct individuals, rearing a population under a series of tightly-controlled environmental conditions would create a distribution of genetic static allometries for those conditions. By comparing the variance of the sample distributions at each environmental condition, the type of individualSA underlying the populationSA could be estimated. The type of growth factor that is controlling trait growth is also an important component of the individualSA pattern. Elucidating how trait growth is actively being regulated in organisms should also help generate predictions of how traits will respond to a given form of selection.

The number of hypotheses for how allometries have evolved and are expected to evolve is a reflection of the level of interest in understanding the importance of relative trait size on morphological diversification. Our model predicted responses to selection based on the novel recognition that individuals in a population contribute the entire range of static allometries their genotype could express to a population, not just their realized phenotype. As a result, the patterns of variation in the hidden individualSA determine how a populationSA will evolve. The underlying individualSA pattern that is masked in the expression of the populationSA also explains how populations that appear to have the same scaling relationships respond differently to the same forms of selection. The effects of size regulation mechanisms such as IIS in determining how traits respond to environmental variation reiterates how important research on such

mechanisms of trait size control will continue to be for allometry research, and for the study of all morphological diversity.

CHAPTER 5:

Conclusion and future directions

Summary of Chapters

Chapter 1

- Morphological variation is largely the result of changes in the relative size of traits to one another
- Scaling relationships between traits, called allometries, are a measure of the co-variation between traits in response to factors regulating trait size.
- The field of allometry research is focused on describing the proportional size of traits across taxa (evolutionary allometries), within species at different developmental stages (ontogenetic allometries) and within species at the same developmental stage (static allometries).
- Sources of variation that contribute to changes in allometric relationships include genetic and environmental factors.
- How allometries evolve is an ongoing question and the inclusion of trait development in these predictions is an important step forward for allometry research.

Chapter 2

- Across many taxa, male genital size scales hypoallometrically to body size, meaning, the genitalia remain a constant size across a range of body sizes. The genitalia of male *Drosophila melanogaster* follow this pattern as well.
- The most frequent hypothesis to explain the hypoallometry of genitalia is stabilizing selection acting on genital size.

- Size variation may be the result of environmental variation, genetic variation and developmental instability (variation due to stochastic developmental perturbations within an individual).
- The genitalia of male *Drosophila* show relatively low responses to variation in environmental and genetic factors that affect all trait size. The genitalia do not show low levels of variation in genetic factors that act independently of other traits or of developmental instability.
- Increased levels of developmental instability of the genitalia in conjunction with decreased responses to genetic size control, suggest that stabilizing selection is not acting on the genitalia in *Drosophila*. If stabilizing selection were acting on genital size, low levels of response to organ autonomous size control mechanisms and of developmental instability would be expected.
- To determine how scaling relationships evolve, the focus needs to be on how the traits of interest interact with one another and not on one of the pair.

Chapter 3

- Testing for selection on genitalia *in vivo*, there must be necessary levels of size variation in the trait being studied.
- Using the genetic tools in *Drosophila melanogaster*, variation in genital size that extends beyond the norm was created by perturbations of the insulin/insulin growth factor pathway (IIS). Three genital size classes of males were engineered: small, wild-type and large, but only the small class of flies were significantly different in size.

- Males of different size classes were exposed to females in three mating contexts: no competition, direct male-male competition and indirect male-male competition.
- Females across all three mating contexts preferentially copulated with, and fertilized progeny from males that had larger genital sizes. Females did not differentiate between male genital size in the number of progeny produced.
- Despite being able to manufacture *Drosophila* with larger and smaller wings by increasing and decreasing IIS signaling in the developing wing tissues, genitalia only decreased in size.
- The lack of a response to an increase in IIS signal suggests that the genitalia may be less sensitive to increased nutrition or at a maximum size in response to nutritional variation.

Chapter 4

- To further explore allometry evolution, a mathematical model was created to incorporate trait development into the co-variation between two traits, T_1 and T_2, and then used to construct a population-level static allometry.
- The model used three classes of growth factors as parameters to create two traits: a systemic growth factor that affects all tissues (G), a trait-specific response to the systemic factor (k_1 and k_2) and a trait autonomous growth factor (i_1 and i_2).
- Parameter values to create each individual were randomly selected from given distributions to create individuals.

- Based on the parameter values that define an individual (k_1, k_2, i_1, i_2), each individual had a unique range of possible responses to a range of *G* values, called the individual static allometry (individualSA) of which only one point was actually expressed per individual.
- Three patterns of individualSA were identified based on the balance of regulatory control over trait size between positive and negative growth regulators.
- Populations of individuals with a T_1 and T_2 were generated and exposed to four selection regimes: direct, proportional, correlational and stabilizing selection.
- The effects of selection on a populationSA are largely dependent on the underlying pattern of the individualSA. To predict how allometries will evolve, the mechanisms controlling trait size, and consequently the individualSA, should be identified.

Conclusion

Genital Hypoallometry in Drosophila

The male genitalia in *Drosophila* are an ideal system in which to study the evolution of hypoallometry. Genetic manipulations, short generation times and the ability to run experiments in a controlled laboratory setting have each contributed a thorough test of the causes of hypoallometry in *Drosophila*. With over a decade of general support for stabilizing selection driving hypoalloetric scaling relationships (Eberhard et al. 1998 2009, Eberhard 2009) recent evidence has suggested that stabilizing selection may not be the de-facto cause (Bonduriansky and Day 2003). Results of experiments on the male genitalia of *Drosophila melanogaster* have also failed to support the

hypothesis of stabilizing selection. Male genitalia are more developmentally unstable than somatic traits and are equal response to variation in genetic factors that affect trait size as somatic traits (Dreyer and Shingleton 2011). Additionally, in Chapter 3 of this dissertation, female flies were demonstrated to prefer absolute larger genital sizes of males, both to copulate with and to fertilize progeny with. An inability to increase genital size in response to a simulated increase in nutrient signaling in the genitalia suggested genital tissues may be insensitive to positive growth factors in addition to being insensitive to negative growth regulators (Tang et al. 2011).

The evidence of these experiments remained equivocal as to the nature of the hypoallometry of the genitalia, but indicated that directional selection may be the ultimate cause rather than stabilizing selection. To further test this hypothesis, a mathematical model of population scaling relationship evolution was created. The results of the model simulations indicate that positive directional selection on a trait that is regulated primarily by a negative growth regulator will result in a hypoallometric trait. Collectively, the evidence presented here clearly points to positive directional selection as the ultimate cause of genital hypoallometry in male *Drosophila*.

Future Directions

The results of the mathematical model on the possible responses in allometries to different forms of selection outline a compelling new focus of allometry research. Effects of the hidden individual-level scaling relationships (how a genotype encoding many possible outcomes interacts with its environment to become a single phenotype)

present how much we have yet to learn about the interplay of trait regulation and morphological evolution. Specifically, the recognition that organisms are but one iteration of all possible ‘versions’ their genotype allows. Selection on a population of individuals, each with their own individualSA, is also selecting on the range of possible phenotypes each individual could have had. While this is not a revolutionary concept in and of itself, the degree to which the collective patterns of indivdualSA determine the evolution of covarying traits is a novel addition to what we currently know about allometry evolution. Future studies should focus on the identifying specific types of control mechanisms underlying a trait’s development. How trait size is regulated, be it predominantly through positive or negative control factors, determines the pattern of individualSA and subsequently how the population will respond to selection.

Research to identify growth control mechanisms is already rapidly expanding (Mirth and Shingleton 2012, Shingleton 2012, Nijhout et al. 2013, Lavine et al. 2015). While insects tend to be the study systems most amenable to research on growth, there are many as-yet unexplored opportunities to investigate morphological evolution. If the evolution of morphology is largely the evolution of allometry, it is a very exciting time to be invested in the study of scaling relationships.

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