THE DEVELOPMENTAL AND MOLECULAR-GENETIC MECHANISMS OF SEXUAL SIZE AND SHAPE DIMORPHISM IN *DROSOPHILA MELANOGASTER*

By

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ABSTRACT

THE DEVELOPMENTAL AND MOLECULAR-GENETIC MECHANISMS OF SEXUAL SIZE AND SHAPE DIMORPHISM IN *DROSOPHILA MELANOGASTER*

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Much of the morphological diversity in nature is due to the sexual dimorphism in both size and shape. While sexual size dimorphism (SSD) is exceedingly common and can be rather extreme, size differences between the sexes are often quite mild. The fact that males and females typically share most of a genome raises important questions about the ubiquity of SSD, namely about how males and females achieve such different phenotypes given a mostly shared genome. Historically, explanations to explain this trend have focused more heavily on evolutionary mechanisms than proximate and/or genetic mechanisms.

Using the fruit fly, *Drosophila melanogaster*, as a model organism I explore the developmental, physiological, and genetic mechanisms that regulate both sexual size and shape dimorphism. Like most insects, Drosophila females are larger than males and differ in many key morphological features (such as shape). I will describe a series of experiments aimed to successively uncover the mechanistic underpinnings of sexually dimorphic growth that regulate sex-specific size and shape.

To investigate the mechanisms of SSD, I first compared the relative contributions of proximate, developmental mechanisms to size in both males and females. Previous research has also implicated critical size, a hormone-mediated

physiological checkpoint, in helping to regulate overall body size in holometabolous insects. Here I demonstrated that, while males and females had equal larval growth durations, females reached their critical size at a larger weight and also grew faster in their final larval instar than males. The resulting SSD was further attenuated by an increase in female weight loss in the period intervening the achievement of peak larval mass and pupariation. Next, I demonstrated that mutants of the IIS pathway (specifically *InR*) completely eliminated SSD. Since condition-dependence is a common explanation for the evolution of SSD, the next step was to investigate the effects of nutrition on SSD. After demonstrating a negative relationship between SSD and nutritional quality, I investigated the effects of several candidate genetic pathways, including nutrient-sensitive growth pathways, on whole body SSD. I then investigated the sex-limited effects of mutants in similar pathways on sexual size and shape dimorphism where effects are unknown. Finally, I examined the ability of my own candidate pathways to influence both sexual size dimorphism (SSD) and sexual shape dimorphism (SShD).

Collectively these studies bring our understanding of the proximate mechanisms that regulate SSD and SShD to a new and more profound level. While much is known of the selective pressures that generate sexual dimorphism, we know very little about the specific genetic targets of these pressures, and how these targets may facilitate or hamper the evolution of sexual dimorphism. My study goes some way to filling this conspicuous gap.

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…To you, Darby, I owe everything

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Introduction

Introduction to Sexual Dimorphism

One of the quintessential themes of modern biology has centered on describing natural variation and answering the question of how such variation came to be. Indeed, this theme has remained prevalent since the early days of modern biology and has made a profound impact on how we study biology today. It was precisely the study of natural variation that lead both Charles Darwin and Alfred Russel Wallace to conclude that heritable variation associated with increased fitness causes some populations to produce more offspring than others.

Alongside his theory of descent with modification due to natural selection, Darwin also proposed the mechanism of sexual selection. Sexual selection, he reasoned, could be used to explain the variation between the sexes seen in many sexually reproducing species. Trait variation between sexes is known as sexual dimorphism and can affect virtually any trait.

Perhaps the most conspicuous trait an animal can possess is body size. It is often the first characteristic we notice when looking at unfamiliar species. After all, the difference between a housecat and a tiger is largely its size; mistaking one for the other could be catastrophic. While the difference in size between males and females is not commonly so stark, it is often still quite obvious. In the fruit fly, *Drosophila melanogaster*, females not only differ in key morphological features, but are also ~15% larger than their male counterparts (Testa et al. 2013). Total body size can be one of the simplest and most

intuitive ways that a species can vary by sex. Sex-specific variation in body size is called Sexual Size Dimorphism (SSD).

Differences in genome composition between sex

While scientists Thomas Hunt Morgan and Theodor Boveri were debating which part of the cell contributed inheritance to their offspring (cytoplasmic elements versus the nucleus, respectively), it was Nettie Stevens and Edmund Beecher Wilson that first demonstrated that males and females differed by the existence of a sex-specific chromosome (Stevens 1905; Wilson 1905). By independently demonstrating in numerous species that females had paired (XX) chromosomal contributions to their gametes and males had unpaired (XY or X0), Stevens and Wilson revealed that sex was “inherited” by the existence of a sex-specific chromosomal pattern (Gilbert 1987). The discovery of sex chromosomes in 1905 revolutionized the way biologists view the genetics of sex and has allowed us to further elaborate on the mechanisms that generate sexual dimorphisms.

Overall, males and females largely share the same genome. In other words, it can be said that there is typically a low genomic sexual dimorphism within a given species (Mank 2009). When two sexes differ by a unique chromosome, they are referred to as heterogametic. Regardless of how different these sex chromosome may be, however, they still only occupy a relatively small portion of the entire genome (Mank 2009). Other forms of sex determination exist, nonetheless, that make genomic differences between heterogametic sexes seem comparatively small or large. In polygenic sex determining

systems, sex determination can be caused by epistatic interactions between multiple genes and alleles (Moore & Roberts 2013), whereas few, if any, genetic differences exist between species whose sex is controlled environmentally (Devlin & Nagahama 2002). Any differences in gene expression therefore, often have more to do with the differential *regulation* of genes rather than *possession* of genes.

Heterogamety is an important source of genetic dimorphism between males and females. The familiar form is one in which females possess two X chromosomes and males possess an X and a Y. The XX/XY system, while common, is not the only genetic sex determination system. Most birds and lizards use ZW/ZZ as their sex chromosomes, but in this system the females are the heterogametic sex and males are homogametic (Mank 2009). Haplo-diploid species are those in which all fertilized eggs develop as females and unfertilized haploid eggs develop as males (Heimpel & de Boer 2008). Recent models suggest that rapid transitions between types of heterogamety are possible under certain conditions (Van Doorn & Kirkpatrick 2010), which may explain the staggering variation in the variety of sex chromosome types. Truly, there is a surprising amount of variation in sex chromosomes and sex-determination itself (Mank 2009; Adkins-Regan & Reeve 2014).

The genome as an impediment to Sexual Dimorphism

That males and females can be so divergent in phenotype and still essentially share most of a genome is astounding. Any individual species' ability to evolve sex-specific differences in size should be hindered when both sexes share the same set of genetic

instructions for generating body size. Despite the fact that males and females of a given species have different sex chromosomes, most genes reside on autosomal chromosomes and the alleles are therefore not segregating preferentially to either sex. Recent studies have even demonstrated the incomplete sex-specific inheritance of certain developmental traits responsible for generating SSD; traits like increased growth rate that define female-specific size are inherited by both males and females (Stillwell & Davidowitz 2010a).

If sexual dimorphism is expected to evolve given specific selective pressures (see below), it is constrained to work within the bounds of a species' genetic architecture. A given genome must be able to evolve sex-specific phenotypes. Males and females therefore have a reduced ability to pass on sex-specific beneficial alleles to offspring of the same sex; i.e. mothers and fathers transmit alleles to both daughters and sons. Selection therefore cannot always respond to sexual conflict, whether it occurs over different traits (interlocus sexual conflict) or over different alleles of the same gene influencing the same trait (intralocus sexual conflict; see discussion below).

This has been clearly demonstrated from studies of both artificial selection and experimental evolution. For example, simply selecting for larger female body size will not necessarily increase SSD, because males also share the same genes that caused females to grow larger in the first place (specific examples discussed below). The resulting selection experiment would be expected to only increase absolute size, rather than dimorphism in size (Teuschl et al. 2007; Lande 1980), though in some cases SSD

may actually evolve as a correlated response (Reeve & Fairbairn 1996). Even simultaneously selecting for smaller male size and larger female size at the same time should theoretically not be enough to overcome the genetic impediments, but it often is. Indeed, unless males and females have identical genomes, their shared genome will only slow their evolution towards sex-specific trait optima.

Because males and females typically share most of their genome, they cannot always respond individually to selection pressures, especially when males and females differ in their optima for a given trait (e.g. size), both conditions cannot be satisfied; this is called intra-locus sexual conflict. Sexually antagonistic selection may cause gene frequencies within populations to fluctuate until the conflict is resolved (Cox & Calsbeek 2009), the degree to which this occurs depends on the magnitude of the inter-sex genetic correlation. Furthermore, when one sex is experimentally allowed to “win” this genetic tug-of-war, the consequences are usually detrimental to the other (Prasad et al. 2007; Holland & Rice 1999; Rice et al. 2006). By using females that carried a pair of X-chromosomes fused at the centromere and a Y-chromosome, the authors were able to reduce the sex-specific information passed on in the female lineage; females would pass on one pair of fused X-chromosomes to their daughters and a Y-chromosome to their sons, while male Drosophila would be the ones to pass on their X-chromosome to their sons. This process effectively allowed genes on the X-chromosome to evolve based on selection pressures on the male and only the male, resulting in the evolution of male-specific fitness optima at the female’s expense.

Attempts to increase/decrease SSD by artificial selection are often accompanied by a correlated response in the opposite sex (Teuschl et al. 2007). These results are particularly important, because they illustrate a key concept in understanding the evolution of SSD: the genome cannot respond to selection on one sex completely independently of the other. This phenomenon has been observed in natural systems as well (Karubian & Swaddle 2001).The authors demonstrated that selection on one sex for increased or decreased body size tended to have a fully correlated response in female body size with very little countervailing selection. In order to circumvent the issues with sexual conflict predicted in previous models (Lande 1980), Reeve and Fairbairn (1996) used a more complicated selection regime. Instead of just selecting males or females with the most desirable trait value, Reeve and Fairbairn selected half-sib groups. This was achieved by allowing one male to mate with multiple females, whose offspring would all be half-siblings. The unit of selection was therefore a group of half-siblings, rather than by individual. Only by selecting on SSD within these families, rather than of unrelated individuals, the authors finally able to artificially select on SSD. Their response was strong and consistent with their respective selection regimes.

Natural variation in SSD (despite shared genome)

What is most shocking is that despite the potential constraint of both sexes sharing the majority of their genome (and transmitting alleles to both sons and daughters), SSD is exceedingly common within the animal kingdom. In fact, we see this pattern across all animal groups (Fairbairn 1997a). The magnitude and direction of dimorphism, however, is often consistent among closely related groups (Fairbairn 1997a). For example,

among mammals and some groups of birds, males are the larger of the two sexes. Among mammals, up to 40% of species display a moderate to extreme male biased SSD of 20% or more (Lindenfors et al. 2002). In extreme cases, such as with the elephant seal, males can be up to 8 times larger than females (Fairbairn et al. 2007). The levels of SSD reached by male-biased species pales in comparison to the level achieved by species with female-biased dimorphism. When females are the larger sex, this size dimorphism can be so extreme that females may be thousands of times larger than their male counterparts (Fairbairn et al. 2007). Some species, like the blanket octopus, have large (~2m long) females that are between 10,000-20,000 times larger than their nearly-microscopic male counterparts (Norman et al. 2002). Others still, like the anglerfish, are only hundreds of times larger than males, but upon mating, the male becomes absorbed into the female until it becomes merely a resource for gametes (Pietsch 2005). Extreme examples like these demonstrate that whatever the genetic correlation between sexes for these species, for such sexual dimorphism to evolve either selection must win, or the correlation be broken down.

Such examples of extreme dimorphism are rare; SSD is typically of smaller magnitude, with most species attaining between 10% and 20% dimorphism. It is this milder, more typical variety of SSD that is most broadly applicable, because it is the norm for sexually reproducing (invertebrate) animals (Stillwell et al. 2010). Ostensibly, these are the examples that showcase the difficulty of overcoming the genetic correlation between sexes.

Males and females don't diverge in size randomly from one another. A pattern known as Rensch's rule has attempted to explain much of the observed variation in SSD. In his 1950 paper, Rensch posited that SSD tends to increase with increasing body size when the male is the larger sex, and decrease with increasing body size when the female is the larger sex (Fairbairn 1997a). While the mechanisms for Rensch's rule are as of yet unknown, it would appear that it is much more difficult to increase female size relative to male size than it is the other way around. Unfortunately, there are many examples citing the failures of this rule (Blanckenhorn et al. 2007; Blanckenhorn et al. 2006; Webb & Freckleton 2007; De Lisle & Rowe 2013), thus it should only remain a curious trend.

Ultimate mechanisms of Sexual (Size) Dimorphism

Ultimately, sexual dimorphism arises due to the fact that males and females usually differ genetically (Dawkins & Krebs 1979; Mank 2009) and also contribute unequally to their gametes (Trivers 1972; Bateman 1948; De Lisle & Rowe 2015; Fairbairn 2005; Mank 2009; Bell 1978). While males and female investment in reproductive efforts is generally equal, females spend more energy on a small number of gametes, whereas males spend less energy on more gametes (plus the energy spent on finding and attracting mates). This phenomenon is called anisogamy. As each sex becomes adapted to its role as a large (female) or small (male) gamete contributor, specializations may arise that lead to the separation and definition of each sex, both physiologically and ecologically. Anisogamy, therefore, may create sexual conflict where males and females have differing fitness optima at the cost of one another.

Sexual selection and sexual conflict

Sexual conflict and sexual selection are commonly misinterpreted phenomena and often painted as opposing forces. The original definition of sexual selection dates back to Darwin (1871), where he described the advantages one sex may have over another with respect to competition over mates and fertilization. More recent definitions expanded the definition to include the differences in reproduction due to individual trait variation that affects success in competition over mates and fertilization (Andersson 1994; Kokko & Jennions 2014). While the definition of sexual conflict is more nuanced, it differs from sexual selection in that it only exists when sex A might gain a (cost-free) advantage over sex B at a selective cost to B (Kokko & Jennions 2014). Sexual conflict can therefore exist in any sexually reproducing species where selection may unequally favor one sex (Matsuda & Abrams 1999) and differs from sexual selection, precisely because it considers not only the effect of selection on one sex, but also the costs on the other. Regardless, both phenomena are expected to influence the evolution of SSD (Blanckenhorn 2005; Abbott et al. 2010; Abouheif & Fairbairn 1997).

Since females may invest heavily in offspring production, they often cannot afford to mate with low quality males. However, males of many species produce many “cheap” gametes, thus lowering some of the reproductive costs (although finding mates may remain costly). Intersexual selection occurs when one sex chooses its mate with a nonrandom bias or set of criteria. Typically, this translates to females choosing between males of varying phenotype to mate with, which increases the fitness of some trait values and decreases others. Trait values that are more desirable to females are

usually associated with greater fitness (Moller et al. 1999), although this is not necessary (Prokop et al. 2012). There are many competing hypotheses to explain how females choose traits—sexy sons (Weatherhead & Robertson 1979), good genes (Moller et al. 1999), Fischerian 'runaway' (Lande 1981), sensory bias (Fuller et al. 2005), etc…—but the common theme is that intersexual selection involves one mate's preference for traits in the other (Andersson & Simmons 2006; Andersson 1994).

Alternatively, limited access to females may cause another form of sexual selection, forcing males to compete against one another. In some cases, such as the horned Onthophagus beetles (Emlen 1997 and sources within), amphipods (Birkhead & Clarkson 1980), elephant seals (Mesnick & Le Boeuf 1991), baboons (Alberts et al. 1996), access to females is often guarded by stronger males. Intrasexual selection is frequently cited as the cause of male-biased SSD, particularly within male-biased bird and mammal species. Larger males are more likely to outcompete smaller males for access to females. This then imposes further selection for increased male size (Bininda-Edmonds & Gittleman 2000; Serrano-Meneses et al. 2007; Székely et al. 2004). It is not always, however, in the female's best interest to increase body size when male size increases (Lindenfors 2002). In cases of male-biased SSD, larger female size can potentially slow down and increase the expenditure of reproduction (Lindenfors et al. 2007).

As stated previously, a fitness advantage for one sex may negatively affect the fitness of the opposite sex, creating sexual conflict. Stark examples of sexual conflict (that are

also under sexual selection) include examples such as: traumatic insemination (Stutt & Siva-Jothy 2001), corkscrew and reverse-corkscrew duck genitals (Brennan et al. 2010), sexual cannibalism (Arnqvist & Henriksson 1997; Birkhead et al. 1988) but sexual conflict need not always be so dramatic.

One good example of sexual conflict over body size involves the sperm plug used by *Drosophila* males. Once mated with a female, a male leaves behind a sperm plug whose function is twofold. First, it is a mechanical barrier to prevent competitor sperm from entering the female (Chapman 2001). Second, the plug releases hormones into the female, reducing her propensity to mate with other males (Chapman 2001; Chapman et al. 1995). Unfortunately, the hormones released by the sperm plug are also toxic to the female (Chapman et al. 1995; Pitnick & García-González 2002). All of these effects scale with body size, making larger males more effective at preventing smaller males from mating with the same female, but also reducing the lifespan of their mates (Pitnick & García-González 2002).

However, if sexual conflict were to be experimentally reduced, selection will bias both sexes toward the same optimum, regardless of selection applied to either sex (Abbott et al. 2010; Holland & Rice 1999; Pitnick et al. 2001; Simmons & García-González 2008). In their study, Abbott et al. (2010) removed conflict by crossing an evolving population of males to a static population of females that was unable to respond to selection (based on Rice 1996). Since recombination does not happen in males and all chromosomes were tracked with phenotypic markers, the authors were able to

effectively tie female fitness to the male genome. In the face of several artificial selection regimes, both male and female wings (that are typically dimorphic; detailed below) responded by shifting toward the male's optimum wing shape for each regime.

Fecundity selection

It has long been accepted that fecundity selection, at least in invertebrates, can be a major driver of SSD (Andersson 1994; Darwin 1874). The idea is simple: larger females can generate more eggs and/or supply more nutrition to them, which is certainly true for some species (Preziosi et al. 1996). In such cases, if large male size is an impediment to mating, small male size may also be selected for (Moya-Laraño et al. 2002; Serrano-Meneses et al. 2007). In an important study, Reeve and Fairbairn (1999) demonstrated that selection for increased fecundity could, in fact, increase SSD; selecting for decreased fecundity, however, had no significant effect. Some sources dispute the fact that fecundity selection alone is sufficient to alter SSD, given that female body size does not always correlate with increased fecundity and increased body size frequently comes at a cost of increased energy requirements (Shine 1988). Still, most sources agree that fecundity selection often plays a large role (Reeve & Fairbairn 1999; Preziosi et al. 1996; Stillwell et al. 2010; Head 1995).

Countervailing Selection

Not all selection acts to drive population means in one specific direction. Instead, countervailing selection is a type of selection may prevent phenotypes from becoming too extreme. While other selective forces may be pulling sexual phenotypes apart,

opposing selection may be keeping it from going too far (Schluter et al. 1991). Physiological and environmental niche constraints may bind sexes to differing optima, limiting their ability to respond to other forms of selection (Cox & Calsbeek 2010; Blanckenhorn 2011; Schluter et al. 1991). In some cases, males and females can occupy separate ecological niches, incurring unique selective pressures on one another (Pearson et al. 2006; Herrel et al. 1999). Furthermore, viability selection may select against large body sizes in one or both sexes due to the time and energy constraints it takes for an organism to grow larger in the first place (Blanckenhorn, 2011 and sources within). Given that countervailing selection is by definition an opposition to selection (e.g. fecundity selection), other forms of selection are still more likely to cause increases in SSD, rather than simply preventing it from becoming too extreme.

Proximate mechanisms of Sexual Dimorphism

Fortunately, biologists have made great strides in understanding the genetic mechanisms that govern variation in sex-specific phenotypic differences in phenotype can be explained with genetic mechanisms. Perhaps the most obvious regulator of genetic differences between males and females is the sex chromosome, their primary source of genetic divergence (for many species). While it is the only place that different genes can be found between the sexes, sex-specific gene expression (and its potential influence on SSD) often has less to do with the genes of the sex chromosomes, and more to do with the instructions they carry for regulating shared autosomal genes (Fagegaltier et al. 2014; Barmina et al. 2005; Fear et al. 2015). For instance, males and

females differ in in the expression of many genes, yet the Y chromosome of male Drosophila largely only carries instructions for sperm motility (Brosseau 1960; Carvalho 2002).

The initial steps in sex determination in Drosophila is regulated by the ratio of sex:autosomal chromosomes. Both numerator (number of X chromosomes) and denominator (number of autosomes) elements work together to determine the expression of the first component of the pathway: sex-lethal (*Sxl*) (Cline 1993; Salz & Erickson 2010; Fear et al. 2015; Cline & Meyer 1996). Alternative splicing then plays a large role in determining how genes are expressed in males and females. Starting with *Sxl* in the sex determination pathway, nearly every subsequent gene has male and female isoforms, including: transformer (*tra*), transformer2 (*tra-2*), double-sex (*dsx*), intersex (*ix*), and fruitless (*fru*) (see Figure 1.1). Even though *Sxl* is on the X-chromosome (Maine et al. 1985), downstream genes such as *tra* (McKeown et al. 1987) and *dsx* (Baker & Wolfner 1988) are found on the autosomes. Genes like *tra* follow the pattern of *Sxl*, where in males, an inactive splicing variant is generated. Progress down the pathway proceeds, where the presence of *Sxl* promotes expression of female active *tra*, which promotes female-specific isoforms of further downstream elements (Cline 1984; Fear et al. 2015; Salz & Erickson 2010). Absence of a functional *Sxl* leads to production of the inactive male form of the *tra* transcript, further activating male-specific splicing of downstream elements. Evidence for the effect of *tra* can be seen in flies with mutant *tra* genes. Mutant adult females are nearly indistinguishable from males: the transformation is near complete (Sturtevant 1945).

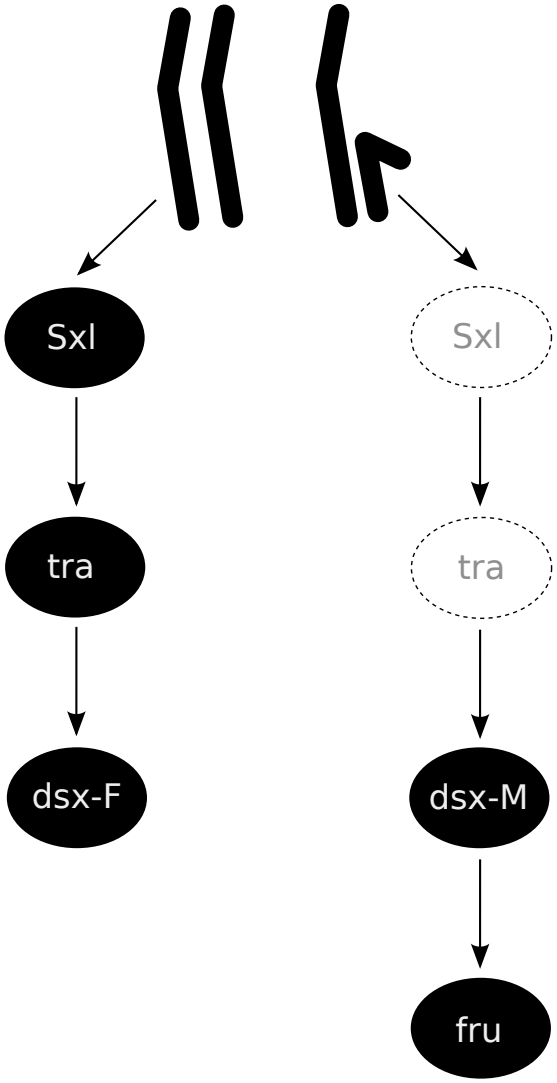

Figure 1.1. The Sex Determination Pathway in *Drosophila melanogaster*. Unlike most gene expression pathway diagrams, expression within this pathway is mediated primarily by sex-specific splicing patterns. The resulting male or female isoforms, displayed in the pathway above, ultimately depend on the expression of Sxl. In females, Sxl activity causes the female isoform of tra to be expressed, leading to female development. In males, the absence of Sxl leads to the production of the (non-functional) male isoform of tra, leading to male development.

After sex determination, the mechanisms of sex-specific regulation of genes and pathways become somewhat less clear. One of the best examples we have involves sex-specific control of pigmentation in *Drosophila*, where *dsx* is known to activate sex-specific coloration through cis-regulatory element binding. For example, males have a characteristically dark posterior abdomen, while females are typically much lighter. This sexual pigmentation dimorphism is mediated by alternatively spliced *dsx* products (Kopp et al. 2000). The male variant, *dsxM*, represses the pigment repressor bric-a-brac (*bab*) in the posterior abdomen, allowing homeotic gene *Abdominal-B* (*Abd-B*) to control pigmentation. Conversely, the female variant, *dsxF*, promotes *bab* expression and ultimately represses pigmentation. Examples like this paint a clear picture of the capacity of the sex determination pathway to regulate sex-specific phenotypes.

Dearth of knowledge on proximate mechanisms of SSD

While our understanding of the evolution of SSD and the development of many sexually dimorphic characteristics is relatively well studied, there is a dearth of knowledge on the proximate mechanisms that generate SSD.

Almost 15 years ago, Badyaev (2002) described the types of developmental mechanisms required to generate SSD. His developmental model was simple. Badyaev posited that SSD could be achieved if either sex deviates in initial body size, growth rate, and/or growth duration. Models like Badyaev's may sound intuitive, but the mechanisms governing each developmental characteristic are often complex. A more

complete understanding of the individual developmental mechanisms is therefore required to better understand how SSD is regulated.

Proximate mechanisms of overall body size and SSD

While initial size was originally interpreted as size at birth/hatching, this measure is somewhat more complicated for organisms with multiple discrete life stages. In Drosophila, initial egg size is controlled by the mother (Azevedo et al. 1997) and is assumed invariant with respect to sex. Because Drosophila is holometabolous, though, each stage of life has its own initial body size. Offspring are hatched as eggs, experience three larval molts, pupariate, then metamorphose into adults. Several of these stages are discrete enough to argue for their consideration as “initial body size” proxies. Given this view, it is critical to investigate the initial size, growth rate, and growth duration dimorphisms within each stage of life. If we can pinpoint at which stage and by which mechanism dimorphism occurs, we can more precisely describe the ontogeny of SSD.

Many discrete life stages within Drosophila are governed by hormone-mediated developmental events. One very important developmental-timing event is the point at which larvae commit to pupariation, referred to as the critical size (or critical weight) (Davidowitz et al. 2004; Chown & Gaston 2010; Nijhout 2003; Davidowitz et al. 2003). During development, once a larva achieves a specific size, it triggers an irreversible hormone cascade that terminates in pupariation. This cascade is putatively controlled

primarily by the timing of juvenile hormone (JH), prothoracitropic hormone (PTTH), and ecdysone release within the prothoracic gland (Orme & Leevers 2005; Yamanaka et al. 2013). Since larvae that never reach this critical size (due to starvation) will delay pupariation—often indefinitely—this is an excellent proxy for adult body size. Critical size is therefore a reasonable proxy for the size at which adult body development begins.

Like *Drosophila*, the hawkmoth *Manduca sexta* is a holometabolous insect with female-biased SSD, but unlike *Drosophila*, we know a great deal more about the development of its SSD (Stillwell & Davidowitz 2010a; Stillwell et al. 2012; Davidowitz et al. 2003). The rate of growth is not sexually dimorphic, regardless of environmental conditions. Males and females differ initially by their critical size, with females reaching a larger value. Females grow for a longer duration between critical size and peak larval mass than males do (Stillwell & Davidowitz 2010b). Sex-specific differences are attenuated in both low nutrition and low temperature environments. In these high stress environments, it is usually the male whose trait values decrease sharply relative to females. (Stillwell & Davidowitz 2010a; Stillwell & Davidowitz 2010b)

Knowing the development of SSD in a species like *Manduca* is certainly helpful, but in order to truly understand how it is regulated, we must be able to describe the development of SSD in a model species with excellent genetic tools. *Drosophila melanogaster* is a premier system for studying genetics and arguably has more available genetic tools than any other metazoan (Duffy 2002; Nicholson et al. 2008;

Blair 2003; Ashburner 1989). Furthermore, its relatively short generation time, high fecundity, low maintenance rearing, and visibly complex phenotypes make for an extraordinary model organism. Importantly it displays a great deal of sexual dimorphism including sex-specific structures like the sex comb and anal plate as well as females being on average 15% larger in overall size (Testa et al. 2013; Huey et al. 2006; David et al. 2003). The final differences in size are in large part due to differences in the overall cell size of the epidermal cells (Azevedo et al. 2002; Partridge et al. 1994).

In chapter 2, I will elucidate the proximate developmental mechanisms of SSD to provide insight into the potential underlying genetic mechanisms. Theoretically, sex-specific differences in the above mechanisms may be responsible for generating SSD, which may arise at any or all of the above physiological checkpoints. In chapter 2, I investigated SSD at each of these checkpoints and found that females diverge from males initially upon reaching their critical size. Females wait until larger to commit to pupariation. Females were also found to exhibit a faster growth rate, until reaching a peak size, after which females also exhibited a faster rate of weight loss. This pre-pupal weight loss was shown to account for a significant reduction in SSD between peak larval mass and pupariation (which did not significantly alter SSD).

Genetic mechanisms of size control and SSD

Originally borrowed from yeast, the Gal4-UAS system has been called the “Swiss army knife” of fly biology. It allows for the precise spatial and temporal control of the

manipulation of gene expression (Duffy 2002). Features such as increased transposition rate, maternal germline expression, precise inducibility of expression, refined mosaic analysis, and targeted RNAi knockdowns to mention just a few (Duffy 2002). Basically, the Gal4-UAS system works by crossing flies bearing a UAS-GeneX with those bearing GeneY-Gal4. Once combined, expression of GeneX is turned on in the expression pattern of GeneY (to a first approximation). If a UAS-GeneX-RNAi construct is used, this then knocks down the transcript abundance of GeneX in the expression domain of GeneY. This Gal4-UAS system can be used to efficiently tease apart differences in growth rate, growth duration, and initial size by controlling expression in candidate gene pathways (that are known to modulate these growth parameters). RNAi knockdowns can test whether candidates are necessary for the development of SSD, while over-expressions can test whether the increased expression of candidate genes is sufficient to alter SSD.

Knowing the proximate, physiological and developmental mechanisms of SSD is a great start to understanding the development of SSD; however, there still remains the mystery of how these differences are being regulated genetically to modulate development in sex-specific ways. Very few studies have investigated the direct genetic contribution of specific genes and pathways to SSD. While many studies have investigated heritability and genetic architecture of SSD (Stillwell & Davidowitz 2010a; Bonduriansky & Rowe 2016), few if any directly address the specific genetic contributors to SSD. Moreover, none (except Rideout et al, 2015) have arguably been able to establish a causal relationship between a certain gene/pathway and SSD.

One way to search for candidate genetic pathways is to observe the conditions associated with the loss of SSD. We know from certain studies that environmental factors play a role in regulating SSD (Stillwell & Davidowitz 2010a; Blanckenhorn et al. 2007; Stillwell et al. 2010). This link is strong enough that it has lead researchers to posit that perhaps the evolution of SSD itself is linked to condition dependence (Bonduriansky 2007b; Johnstone et al. 2009; Stillwell et al. 2010). Mechanisms responsible for generating a plastic response to an environmental factor like temperature or nutrition are thought to acquire sex-specific expression patterns to resolve conflicting selective pressures on body size. If this were the case, mechanisms that cause SSD to fluctuate with the environment could be the very same genes that cause differences in male and female body size. We have many examples that suggest sexual dimorphism is condition dependent with respect to temperature (Azevedo et al. 2002; Stillwell et al. 2010) and nutrition (Stillwell et al. 2010; Emlen et al. 2012; Gotoh et al. 2014; Koyama et al. 2013; Moczek & Kijimoto 2014). Given these examples, the best place to start searching or the genes that generate SSD are those that also control nutrient-sensitive growth.

One well-studied growth pathway, the Insulin/Insulin-like Signaling (IIS) pathway has already been implicated in regulating overall growth, and potentially modulating SSD. Mutants of the IIS pathway, such as Insulin-Receptor, *InR* (Testa et al. 2013), and *Foxo* (Carreira et al. 2011) eliminate or reduce SSD. The resulting reduction in body size for IIS mutants is typically said to phenocopy starvation conditions by forcing cells to act as

though there are fewer nutrients available than are truly available. Unfortunately, it is unknown whether there are sex-specific differences in *InR* knockdowns and overexpressions. That the IIS pathway is necessary for SSD is evidence that IIS and possibly other nutrient sensitive growth pathways may be involved in positively and negatively regulating SSD.

To determine causation within a given pathway, one must be able to determine three important factors: necessity, sufficiency, and proximity. Necessity refers to the dependence of a trait on a particular gene. If a trait is affected by the absence of a gene, it is said to be necessary for that trait's expression. In this way, InR has been shown to be necessary for SSD. What is unknown is whether overexpression of InR is able to increase the value of SSD (i.e. whether it is sufficient). Even if genes are shown to be necessary and sufficient for a trait, however, expression must be somehow linked to the spatial or temporal region of that trait to truly be a candidate.

In Drosophila, nutrient-sensitive growth can be regulated through several different signaling pathways. In response to circulating nutrients, Drosophila Insulin-like Peptides (dILPs) and insulin-like growth factors are produced primarily in the brain by the medial neurosecretory cells (but also in the fat body and imaginal discs) (Edgar 2006). Insulin signaling then begins at the transmembrane insulin-receptor, InR. Upon binding to a ligand, e.g. dILPs, InR begins a signaling cascade that ends with the repression of a growth repressor, Foxo (see Figure 1.2). Many other pathways, such as TOR and Ras, are more straightforward (Figure 1.2). For example, TOR signaling responds to

circulating levels of amino acids, which derepresses *RHEB* by repressing the negative growth regulator *TSC1/TSC2* (Edgar 2006). TOR signaling then proceeds by activating a positive growth regulator, S-6Kinase (S6K). Since Ras was the only component of the Ras pathway that we considered, it will not be discussed here.

Another candidate growth pathway we tested was the Hh signaling pathway (Figure 1.3). In 2005, Horabin demonstrated the ability of *Sxl* to affect levels of full length *Ci* and also wing imaginal disc size. Their hypothesis was that female wings grow larger because of endogenous levels of *Sxl* increasing activation of the Hh pathway. Normally Hh ligands bind to the transmembrane receptor patched (*ptc*), which activates growth by derepressing the positive growth regulator smoothened (*smo*). In the presence of *Sxl*, which exists in females and not males, nuclear entry of full length *Ci* is increased, speeding up growth (Horabin 2005).

In Chapter 3, I examined the genetic mechanisms responsible for generating SSD. Here, I tested the necessity and sufficiency of genes in several candidate pathways to affect whole body SSD (see figures 1.1, 1.2, and 1.3). The resulting data add support to recent findings (Rideout et al. 2015) with regard to the control of SSD in the sex determination pathway (Figure 1.1), and also contribute a novel candidate gene capable of affecting SSD. Furthermore, we test the necessity and sufficiency of other growth pathways to generate SSD, including the following: nutrient sensitive growth pathways (Figure 1.2) and the Hh pathway (Figure 1.3). Our findings suggest caution when attempting to uncover the genetic basis of traits. While preliminary data suggested that

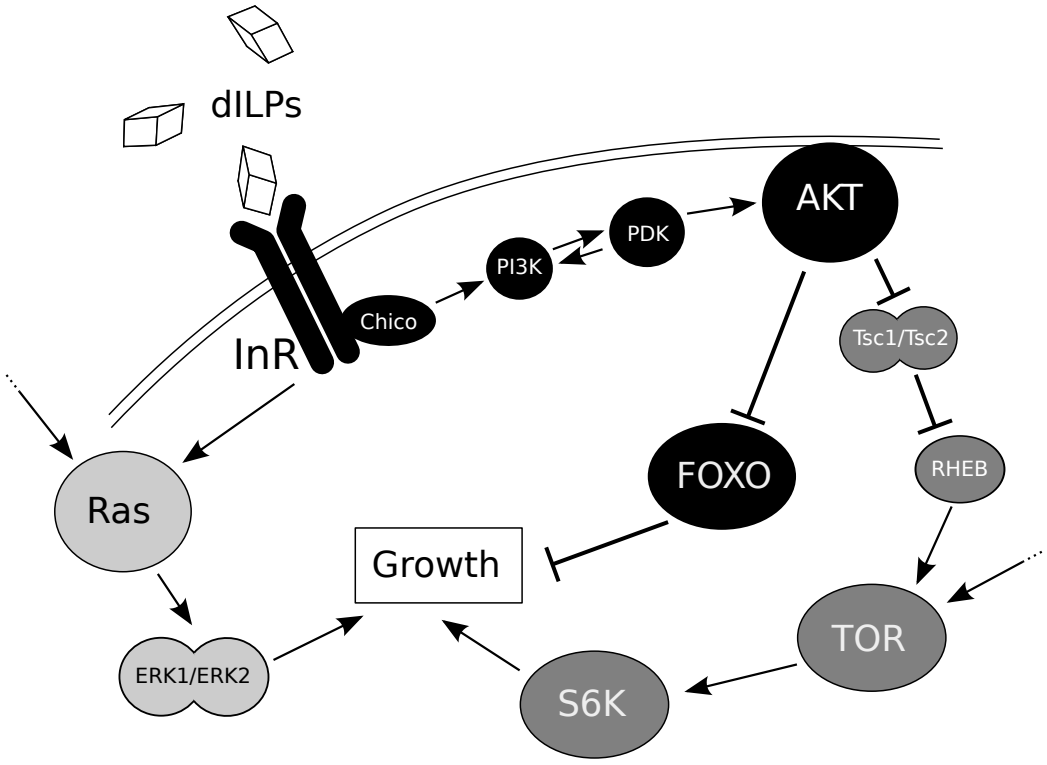

Figure 1.2. Nutrient-Sensitive Growth Pathways in *Drosophila melanogaster*. Represented are three candidate pathways for controlling SSD in Drosophila, including: (black) Insulin and Insulin-like Signaling (IIS) pathway, (medium gray) TOR signaling pathway, and (light gray) the Ras signaling pathway. Arrows represent positive interactions between two components and bars represent negative interactions (repression).

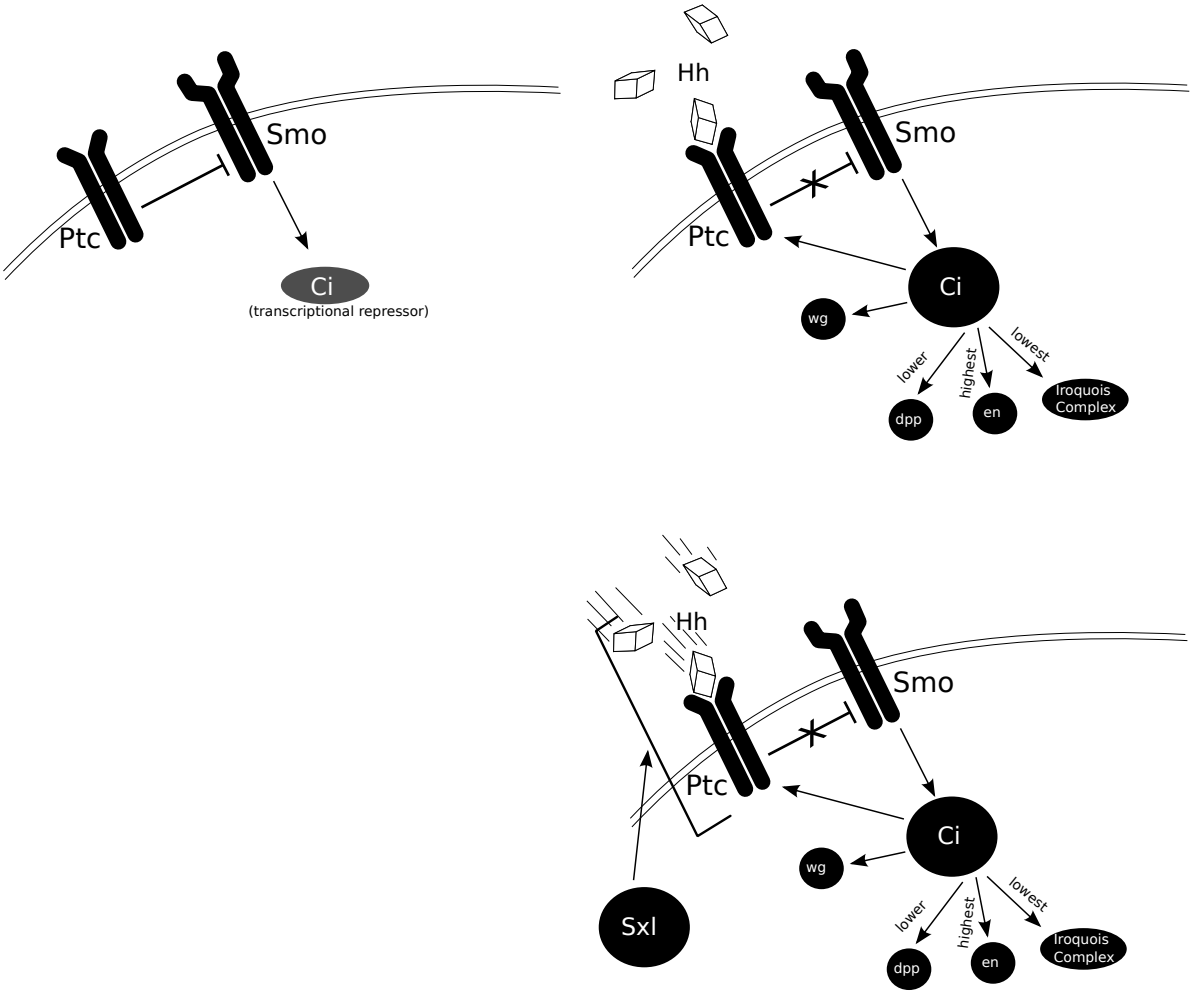

Figure 1.3. Hh signaling pathway in *Drosophila melanogaster*. This pathway was put forth by Horabin (2005) as a hypothesis for the regulation of SSD in Drosophila. In the absence of the Hh ligand, a short and repressor version of the transcription factor, Ci, is produced since patched (ptc) is inhibiting smoothened (smo). In the presence of Hh, ptc de-represses smo, allowing for the expression of the full length version of Ci, which upregulates growth. Horabin (2005) demonstrated that, in the presence of endogenous Sxl protein, this reaction is catalyzed, increasing growth in females.

IIS pathway was our best candidate pathway, our experiments yielded conflicting results. Finally, we reveal an unanticipated effect of ubiquitous over-expression of nutrient-sensitive growth

How are size and shape related? A Primer on Shape Analysis

Since most of the natural variation visible in organisms is related to size and/or shape, it is unthinkable that we know so little about how it is regulated with respect to sex, especially on the genetic level. Much of the literature investigates the size or shape control in sexually dimorphic structures—e.g. beetle horns, (Kijimoto & Moczek 2016; D.J. Emlen et al. 2005), Drosophilid sex combs (Barmina & Kopp 2007) and anal plate (Glassford et al. 2015), *C. elegans* tail phenotype (Suzuki et al. 1999), queen bee phenotype (Kamakura 2011)—but many more have explored size and shape dimorphism in homologous structures exhibited by both sexes (Gidaszewski et al. 2009; Abbott et al. 2010; Cheng & Kuntner 2015; David et al. 2003; Stillwell & Davidowitz 2010a).

While the regulation of either size or shape is relatively well-studied (Nijhout 2008; Oldham et al. 2000; Edgar 2006; Weber 2005; Gidaszewski et al. 2009; Day & Lawrence 2000), much less is known about what individual genes/pathways contribute to both size and shape. Furthermore, the extent to which size and shape controlling genes overlap in function is poorly understood. Some studies, such as Carreira et al. 2011, are beginning to uncover sex-specific differences in how size and shape are

regulated. In their study, the authors used an unbiased random screen to identify mutations that differed in sexual size and shape dimorphism. They showed that, of the mutations used, males were more likely than females to respond with changes in wing size only. Conversely, roughly two thirds of mutations caused an effect on wing shape in only one sex, suggesting that most mutations have sex-specific effects. While this is certainly an important contribution to the field, its scope is too broad to really elucidate how certain pathways contribute to sexual size dimorphism (SSD) or sexual shape dimorphism (SShD).

Because sexually dimorphic traits are often multi-dimensional, studying characteristics that are more quantifiable than size would allow for a more complete understanding of the underlying sexual dimorphism. One such sexually dimorphic, multi-dimensional trait is shape.

One important question in shape analysis is “What makes size so inherently easy to quantify while shape is so complicated?” Shape change can be thought of as a relative change in size of a given trait. If you change the size of the anterior portion of a wing, for example, you change the entire shape of the organ. Similarly, by changing the size of the wing, relative to the body size, the shape of the whole organism changes as well.

The study of scaling relationships (allometry) have been central to the study of body size variation. D’Arcy Thompson was one of the first scientists to outline a thorough approach to the study of allometry in his 1917 book, *On Growth and Form* (Thompson

1917). Thompson illustrated the differences in relative size of organs to overall body size by using visual deformation grids. The most famous example demonstrates the transformation from the porcupine fish, *Diodon,* to the sunfish, *Mola*. The deformation grid allows us to see that major differences occur only in the relative size of structures between these organisms. This is an example of an early method of describing allometric “shape” changes. Unfortunately, Thompson’s illustrations are merely qualitative in nature. How then do we quantify differences in shape between organisms?

Early crude methods focused on using linear measurements between predetermined points. Usually measurements were taken for variables such as length and width. In his 1932 book, *Problems of Relative Growth*, Huxley described how one part of a structure might change with respect to another, e.g. wing vs body size (Huxley 1932). The allometric scaling can certainly change the overall shape of an organism, but unfortunately measures like these are still not good estimates of shape. Other crude methods use ratios of univariate measurements to quantify “shape.” This method, however, is often severely flawed, because two entirely different starting shapes may yield the same results (e.g. a diamond and an oval). Obviously these methods are not sufficient to capture shape variation in any real sense.

Outline analysis was yet another promising method to describe changes in shape. Elliptical Fourier Analysis, a common example, works by estimating the minimum number of ellipses required to mimic the shape. Again, this process is flawed, because it only uses the outline of the specimen and does not take any biologically meaningful

traits into account. Because of this, outline-based approaches can be used to directly compare the shape of a human scapula, a potato chip, and a chocolate chip cookie without regard for actual homology (Zelditch et al. 1995).

To date, landmark-based approaches are our best method for presenting a clear picture of shape differences between two specimens. Landmarks are placed on a 2- or 3-dimensional representation of the specimen with X, Y (and sometimes Z) Cartesian coordinates. Where the landmarks are placed is somewhat arbitrary, but there are several rules that must be followed. All landmarks must be homologous between specimens and be as unambiguous as possible. Early methods for analysis focused on quantifying the variation of one point to adjacent points, such that a “truss” was measured (Bookstein 1985). These methods were soon replaced by more sophisticated methods that described shape as the residual variation left over after location, rotation, and scale effects were removed. Calculating shape variation now allows for all specimens to be standardized to a common baseline. Bookstein’s shape coordinates allowed for a common baseline, but since these points are arbitrary, they can certainly influence shape inferences made on their behalf.

Least-squares superimposition, i.e. Ordinary Procrustes Analysis (OPA) resolves most of the issues of early landmark-based techniques. This technique uses the centroid as the seat of translation, rotation based on optimal fit of landmarks between configurations, and centroid size for ensuring consistent scale. Generalized procrustes

analysis (GPA) goes a step further and repeats this superimposition of OPA in an iterative fashion to minimize variation due to translation, rotation, and scale.

In Chapter 4, I begin to dissect the contributions of specific genes/pathways to the generation of shape and size dimorphism. I use a previously published data set to investigate the sex-limited effects of mutants on size and shape dimorphism. The primary use of this Chapter was to describe and develop the methods used in the following chapter. Both chapters utilize landmark-based geometric morphometrics to quantify the size and shape differences between wild-type fly wings and those of treatment groups. Here I demonstrate the sex-limited effects of genes within two growth pathways (EGFR and TGF-ß), where effects on SSD and SShD are previously unknown.

Growth pathways can conceivably influence both size and shape of developing organisms, yet little attention has been paid to their combined efforts. In Chapter 5, I test explicit hypotheses about the ability of candidate pathways with known effects on whole body SSD to influence SSD and SShD in the wing. Similar genes were found to be necessary for whole body and wing-specific SSD, and many more found to be sufficient to increase SSD. Surprisingly, data from this chapter conflict with the literature in a few key ways, but nonetheless suggest important ways that our candidate pathways influence differences in shape and size.

Collectively these studies bring our understanding of the proximate mechanisms that regulate SSD and SShD to a new and more profound level. While much is known of the selective pressures that generate sexual dimorphism, we know very little about the specific genetic targets of these pressures, and how these targets may facilitate or hamper the evolution of sexual dimorphism. My study goes some way to filling this conspicuous gap. Only by quantifying the effects of genes on all aspects of trait variation can we fully appreciate a gene's effect on the sexual dimorphism of a trait. Understanding the proximate developmental and genetic mechanisms of sexual dimorphism can allow us to truly appreciate the ability of a shared genome to generate "endless forms most beautiful and most wonderful [that] have been, and are being, evolved."

CHAPTER 2:

Testa, N. D., Ghosh, S. M. & Shingleton, A. W. Sex-Specific Weight Loss Mediates Sexual Size Dimorphism in *Drosophila melanogaster*. PLoS One 8, e58936 (2013).

Introduction

Sexual Size Dimorphism (SSD), the difference in body size between males and females, is an extraordinarily widespread and conspicuous phenomenon in the animal kingdom (Fairbairn 1997a). This condition is extremely variable and evolutionarily labile. For example, male southern elephant seals can weigh seven times that of a female (Bininda-Edmonds & Gittleman 2000), while female blanket octopuses can weight 10,000-20,000 times their male counterparts (Norman et al. 2002). The degree of SSD in insects is generally less extreme, however. Consistent with most invertebrates, the female is often the larger sex among insects, a pattern seen in approximately 88% of insect species (Stillwell & Davidowitz 2010b). Despite the ubiquity of SSD, however, very little is known of the underlying developmental mechanisms that generate it or how these mechanisms evolve.

In general, final body size is regulated by a combination of three developmental factors: initial body size (size at hatching/birth), growth rate, and growth duration (Badyaev 2002; Blanckenhorn et al. 2007). Changing any of these individually or in combination results in an alteration of adult body size and may underlie size differences between males and females. Nevertheless, the molecular-genetic and physiological regulators of initial size, growth rate, and growth duration are poorly understood except in a very few organisms. One such organism for which these mechanisms are known, is the fruit fly *Drosophila melanogaster,* which like most insects, shows marked SSD between female and male body size (Stillwell & Davidowitz 2010b; Blanckenhorn et al. 2007). The

extensive research on growth regulation in *Drosophila* and its readily apparent SSD make these animals an ideal model to more deeply elucidate the proximate mechanisms that regulate SSD.

Drosophila are typical holometabolous insects: they begin life as worm-like larvae, molting through three larval instars before undergoing complete metamorphosis as a pupa and eventually eclosing into their adult form (Nijhout 1981). Adult flies, like all arthropods, have a stiff exoskeleton, meaning they cannot grow. Larval body size upon termination of growth, therefore, ostensibly determines adult body size.

In *Drosophila,* the timing of metamorphosis is regulated by a larva reaching a size checkpoint called critical size (or critical weight) early in its final larval instar. Attainment of critical size is associated with initiation of a hormonal cascade that ends in metamorphosis. There is, however, temporal separation between the attainment of critical size and the subsequent rise in the ecdysteroid titer that causes the larva to stop feeding and ends body growth. This delay provides a final period of growth for the larvae, called the Terminal Growth Period (TGP), during which *Drosophila* larvae can more than triple their mass (Shingleton et al. 2008; Stieper et al. 2008). Body size in *Drosophila* is therefore regulated by the critical size, plus the amount of growth achieved during the TGP (Shingleton et al. 2008; Shingleton et al. 2007; Davidowitz et al. 2003; Nijhout et al. 2006; D'Amico et al. 2001), or more formally:

$$\text{Final Body Size} = \text{Critical Size} + (\text{Growth Rate} * \text{TGP})$$

where Critical Size is the weight at which larvae commit to pupariation, TGP is the time between critical size and cessation of growth, and Growth Rate refers to the rate of growth within the TGP. SSD in *Drosophila* is therefore a consequence of sex-specific differences in one or all of these parameters.

Research over the last twenty years has begun to establish the developmental mechanisms that regulate critical size, growth rate and the duration of the TGP (Mirth et al. 2005; Caldwell et al. 2005; Nijhout & Williams 1974; Shingleton 2005). The goal of this study is to determine the proximate mechanisms responsible for SSD in *Drosophila melanogaster*. To test the hypothesis that sex-specific differences in a combination of developmental events underlies SSD, we measured critical size, growth rate, and growth duration for the developing larvae and pupae. Identifying how these developmental parameters differ between male and female flies therefore allows us to begin to link the observed SSD to the endocrine, and ultimately molecular-genetic, mechanisms that regulate growth and development.

Materials and Methods

Fly Strains and Maintenance

All flies were derived from an isogenic stock of Samarkand (SAM) *Drosophila melanogaster*. Ubi-GFP ($y^{1}w^{67c23}$P{Ubi-GFP.D}ID-1) flies were obtained from Bloomington Stock Center and back crossed into a SAM background for five

generations to eliminate background effects. Flies were raised on standard cornmeal-molasses medium at 25°C on a 24 hour light cycle.

Critical Size

Mid-third instar larvae that weighed between 0.3 to 2.3mg were placed into individual tubes and starved. Time to pupariation (TTP) was recorded on an individual basis and critical size was calculated as the weight at which starvation no longer delayed pupariation [see Stieper et al., 2008 for additional details]. Flies that survived to the late pupal period were sexed using the presence or absence of sex combs. For those pupae that died before the presence or absence of sex-combs could be scored, sex was determined by presence or absence of the Y-chromosome-specific Ppr-y gene using PCR and gel electrophoresis. DNA from un-sexable pupae extracted using a Qiagen DNA extraction kit and PCR was conducted under standard conditions. Optimal annealing temperature for PCR primers (below) was found to be 58ºC.

Forward: 5' TGT GTT GAT GAC CGT GAC GCC A 3'

Reverse: 5' CGA GTC GCA ATT GTG TCT TCT CGC 3'

Growth rate

Eggs were laid in six-hour cohorts from which larvae were sampled every six hours and developmental stage and mass were recorded. Larval sex was determined by using presence or absence of a paternally inherited X-chromosome marked with a constituently active GFP. Sex was recorded based on presence or absence of GFP, to detect females and males respectively. Pupae were staged into four-hour cohorts at

pupation and massed every 12 hours. Timing of pupariation was determined by using SAM flies laid in six-hour cohorts. Starting at 94 hours, we recorded pupariation state for individual larvae. Pupal sex was determined retrospectively by presence or absence of sex combs.

Statistical Analysis

All statistical analyses were conducted using R statistical software (version 2.14.1). Critical size was calculated using the methods described in Stieper et al. (2008). To assess the probability of observed sex-specific differences in critical size, we used a permutation test with one thousand replicates to generate a null distribution of the difference in critical size between males and females. The same test was also applied to determine differences in time to pupariation from the critical size data. Growth rate was calculated using a linear regression of log-transformed weight against time, while interactions with sex were tested using an Analysis of Covariance (ANCOVA). We calculated TGP by subtracting the time at which critical size is attained from the time at which larval weight no longer significantly increases, for each sex. We applied the values for critical size to the growth curve to determine the timing of critical size and used multiple comparisons analysis (Hsu's MCB) to determine the age at which there is no longer any significant increase in mass for each sex. Since this approach does not allow us to calculate 95% confidence intervals for the timing of growth cessation, confidence intervals for the duration of the TGP were predicted using those for critical size alone. In all larval cultures, we noticed that some larvae stopped growing prematurely and subsequently failed to pupariate. In order to avoid including these

abnormal larvae in our growth calculations, any larvae whose weight was below critical size by the time the rest of the population had stopped growing were excluded from the analysis. Values for the timing of developmental stages were determined by logistic regression of developmental stage against age. Finally, all SSD indices were calculated as per the 1992 Lovich and Gibbons paper (Lovich & Gibbons 1992; Smith 1999), such that SSD = (F/M)-1, where F is female weight and M is male weight.

Results

There are three potential mechanisms by which SSD can be generated in *Drosophila*, namely, sex-specific differences in critical size, TGP, and growth rate. We found that male larvae have a significantly smaller critical size than females (permutation test, P = 0.008) (Figure 2.1a). This does not, however, wholly explain the adult SSD. Females also grow more rapidly than males during their TGP (ANOVA, P = 0.0084) (Figure 2.1b), although their TGPs are approximately the same duration (17.5 and 16 hours, respectively). The nature of our data does not allow us to test this statistically, however, males have a significantly longer time to pupariation from critical size than females (permutation test, P = 0.01), which is a proxy for the TGP. Finally, the timing of both larval and pupal molts as well as eclosion timing do not differ significantly between sexes (logistic regression; molt to 2nd instar, P = 0.5330*;* molt to 3rd instar, P = 0.8282; pupal molt, P = 0.7432; eclosion, P = 0.9628). (Figure 2.1d)

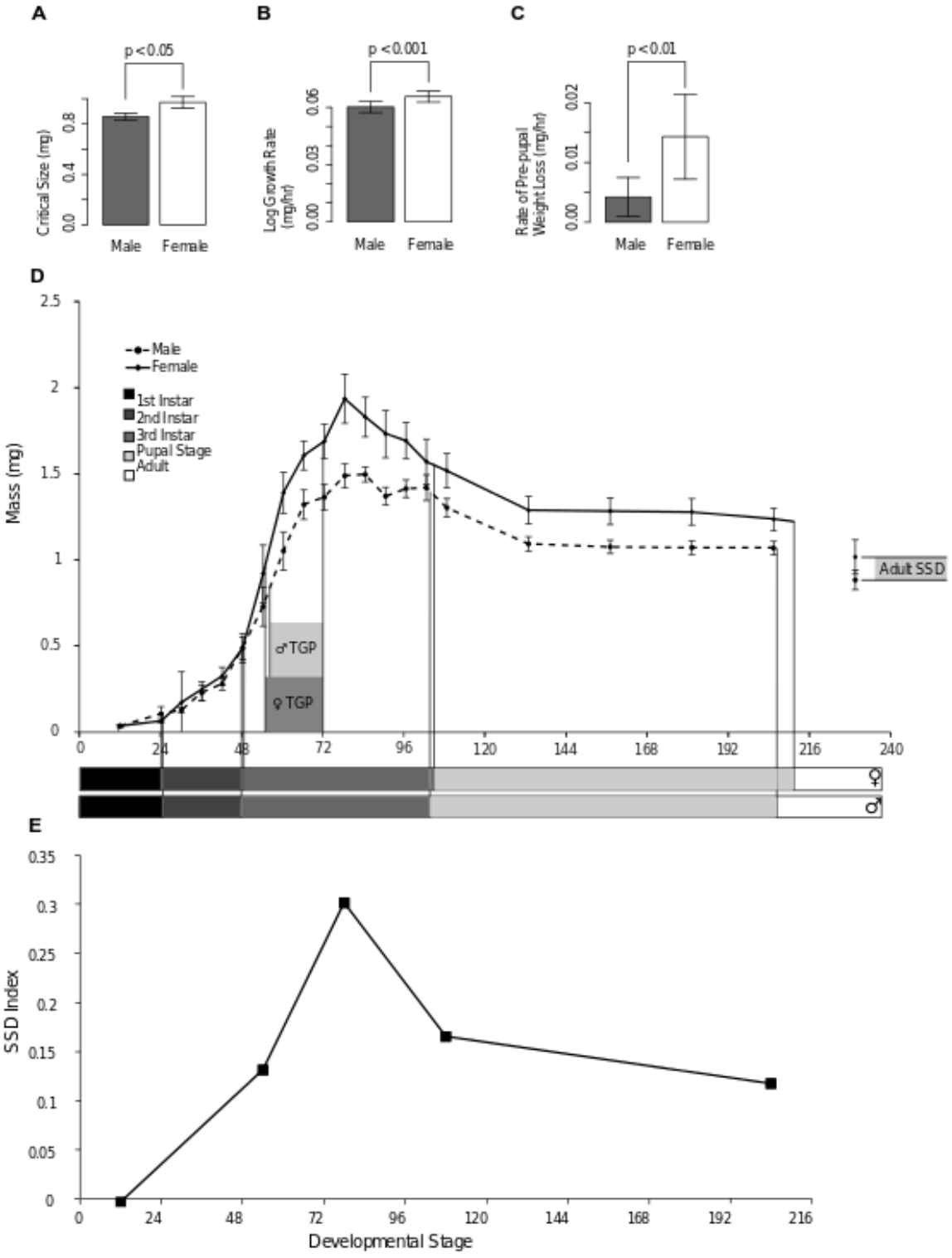

Figure 2.1. Complete growth profile by sex for *Drosophila melanogaster*. Factors shown to contribute to SSD include (a) critical size, (b) growth rate, (c) and pre-pupal weight loss and are reflected in the sex-specific growth curve (d). The SSD at specific

Figure 2.1. (cont'd)

developmental events (hatching, critical size, peak larval mass, pupariation and eclosion) illustrates the changes in SSD throughout development (e). Error bars show 95% confidence intervals.

Surprisingly, SSD at peak larval mass is twice that of the uneclosed adult fly: females were 30% larger than males at peak larval mass and 12% larger than males by end of pupal development (Figure 2.1e). To determine why this difference in SSD exists, we measured pre-pupal weight loss, weight lost in the period intervening peak larval mass and pupariation, and pupal weight loss, weight lost during the pupal stage. The female rate of weight loss during the larval stage is significantly greater than the male rate (ANCOVA, $P = 0.0116$) (Figure 2.1c), whereas there was no significant difference in pupal weight loss (ANCOVA, $P = 0.6078$).

Discussion

Consistent with the female biased dimorphism in insects, female *Drosophila* adults are significantly larger than their male counterparts. Our data indicate that this sexual size dimorphism arises because females 1) initiate metamorphosis at a larger size than males, that is they have a larger critical size, and 2) grow faster than males in the terminal growth period between critical size and the cessation of larval growth. Surprisingly, however, the resulting SSD at the peak of larval mass is subsequently reduced before metamorphosis because females lose more mass during the pre-pupal period. Additionally, our data show that the timing of larval molts and pupation are nearly identical in males and females, and that the duration of growth is not different between the sexes. Males do, however, eclose slightly earlier than females.

There is a paucity of data concerning the patterns of growth that generate SSD in other insects (Blanckenhorn et al. 2007; Stillwell & Davidowitz 2010a; Molleman et al. 2011; Fischer & Fiedler 2001). Perhaps the best study has been in the tobacco hornworm, *Manduca sexta*, where critical size and the duration of the TGP (called the ICG in *M. sexta*) are important mechanisms contributing to SSD at the cessation of larval growth (Stillwell & Davidowitz 2010a). Additional studies indicate that SSD in other Lepidopterans accumulates during development primarily due to females adding more instars than males (Esperk et al. 2007). This is consistent with females having a longer TGP/ICG. In contrast, a previous study indicates that SSD among Drosophilidae is a consequence of sex-specific differences in growth rate and this is supported by our study (Blanckenhorn et al. 2007). Different insect species therefore appear to generate SSD using different developmental mechanisms. It is possible, however, that differences in SSD for both *Drosophila* and *Manduca* are a consequence of the differing environments in which each was reared.

The observation that SSD is influenced strongly by the loss of mass between the cessation of growth and pupation is a novel one, although post-eclosion weight loss has been implicated in regulating SSD in Lepidopterans (Molleman et al. 2011; Fischer & Fiedler 2001). To a certain extent, mass loss after a larva has stopped feeding is an inevitable consequence of ongoing metabolic and developmental activity. What is not clear is why females lose more mass than males; it seems counterintuitive for females to accrue mass only to lose it. One hypothesis is that selection for larger female size targets a systemic increase in growth rate, both of the body as a whole, but also of the

imaginal discs, the precursors of adult organs. Importantly, growth and development of the imaginal discs continues after the cessation of feeding (Freeman 1997; Garcia-Bellido & Merriam 1971), relying on stored nutrients to proceed (Slaidina et al. 2009; Okamoto et al. 2009). Thus, we might expect that larger females with larger organs will utilize more of these stored nutrients during post-feeding imaginal disc growth. Consequently, both the increase in the body's growth rate before cessation of feeding and the increase in weight loss after the cessation reflect the same mechanisms of elevated growth rate for increased body size in females.

There are a number of pathways that control growth rate, which include IIS, TOR, MAPK, and HIF-signaling pathways (Britton et al. 2002; Wullschleger et al. 2006; Liu et al. 2006; Seger & Krebs 1995; Shingleton et al. 2005). Of these, the insulin signaling pathway has been demonstrated to have an important role in regulating final body size (Ikeya et al. 2002; Chen et al. 1996). This pathway regulates the rate of cell growth and proliferation in response to insulin-like peptides that are released in a nutrient dependent manner by the brain and other tissue around the body (Shingleton et al. 2005; Edgar 2006; Emlen et al. 2012). Ostensibly, therefore, insulin signaling regulates growth and final body size with respect to developmental nutrition. However, data from *Drosophila* and other animals suggest that differences in insulin signaling may account for body size variation among different populations (Fabian et al. 2012; De Jong & Bochdanovits 2003; Sutter & Bustamante 2007), suggesting that it may be a more general regulator of size. An intriguing hypothesis therefore, is that female *Drosophila* are larger than males because elevated levels of insulin signaling increases growth rate.

The insulin signaling hypothesis was first proposed to explain SSD in *Manduca sexta* (Stillwell et al. 2010), however, evidence suggests that it may be important in regulating SSD in *Drosophila* as well. Support for this hypothesis comes from the observation that SSD is eliminated in flies mutant for the insulin receptor (*InR*) (Figure 2.2), indicating that insulin signaling is necessary to generate size differences between males and females. However, the fact that there is no SSD in *InR* mutants suggests that insulin signaling is also regulating other mechanisms that generate SSD, specifically difference in critical size.

Regardless of the function of pre-pupal weight loss, our understanding of how body size is regulated in *Drosophila melanogaster* needs to be extended. Pre-pupal weight loss should now be viewed as an additional variable for calculating final body size, such that:

Final Body Size = Critical Size + (Growth Rate * TGP) - Weight Loss.

In conclusion, our data suggest that the mechanisms regulating critical size and growth rate are responsible for generating SSD in *Drosophila melanogaster*. Our understanding of the underlying molecular-genetic mechanisms that regulate these processes indicate that these studies can be extended to generate a deeper understanding of the development of SSD.

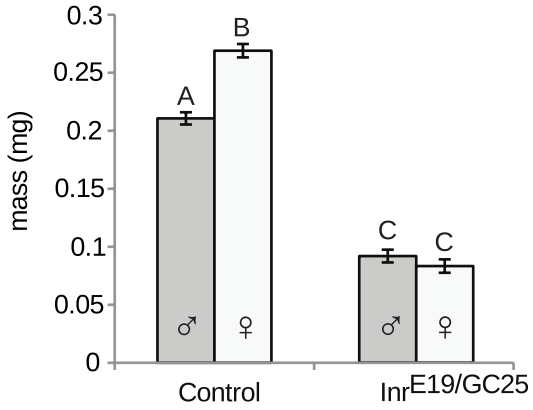

Figure 2.2. SSD is lost in insulin-signaling mutants. The dry mass of male and female adult InrE19/InrGC25 and wild-type (InrE19/TM3) control flies reared at low density at 24˚C. Columns with different letters are significantly different (Tukey HSD at P<0.05). Error bars are standard errors.

CHAPTER 3:

The Genetic Regulation of Whole Body Sexual Size Dimorphism in *Drosophila melanogaster*

Introduction

One of the most striking differences between male and female fruit flies is that of size. Sexual size dimorphism (SSD) is nearly omnipresent among sexually reproducing organisms, only ever completely vanishing in truly monogamous species (Székely et al. 2007). *Drosophila melanogaster* is no exception to the ubiquity of SSD. While some species may display flashy and extreme SSD, Drosophila's is of the much more common variety: moderate (Huey et al. 2006). Males and females differ in body size by only about 15-20%, depending on the measure of SSD (Testa et al. 2013). Given how typical their SSD is, studies on *Drosophila* are well suited to making more general statements about SSD than those with more dramatic dimorphisms.

Drosophila are holometabolous insects, which means they experience several discrete life stages. Once their eggs hatch, Drosophila larvae cycle through three instars (stages) before pupariating, metamorphosing, and eclosing as adults. Recent evidence demonstrates that SSD arises in the final larval instar because females have a greater critical size and growth rate than males (Testa et al. 2013). The increased growth rate in females causes them to gain more mass than males until peak larval weight, then lose more before pupariation.

The reason for this pre-pupal weight loss is still speculative; however, current hypotheses suggest that differences in energy used during the pre-pupal wandering stage, where larvae leave the food in search of suitable pupation locations, is to blame.

If this is the case, it appears that, for an equivalent time spent eating and fasting, females gain more mass while feeding and lose more when not. SSD should therefore be dependent on nutritional state.

We know that body size is condition-dependent (Ghosh et al. 2013), specifically with respect to environmental nutrition (Britton et al. 2002), but whether or not this phenomenon is sex-specific is unknown. Determining to what extent SSD is nutrient-dependent, will allow us to confirm a suite of candidate pathways responsible for generating SSD.

One nutrient-sensitive pathway, the Insulin/Insulin-like Signaling (IIS) pathway has already been implicated in regulating SSD. One study demonstrates that mutants of the Insulin-Receptor (*InR*) completely lack SSD (Testa et al. 2013). The resulting reduction in body size for IIS mutants phenocopies starvation by forcing cells to act as though there are fewer nutrients available than there really are. Evidence shows that this appears to be the case (Britton et al. 2002; Marshall et al. 2012), but sex-specific differences in other growth-related genes are still unknown. Ultimately, *InR* controls growth by repressing the protein product of the negative growth regulator, the forkhead transcription factor Foxo. Further research has demonstrated the ability of *Foxo* overexpression in the wing to decrease size in a sex-specific manner (Carreira et al. 2009). The dependence on Foxo and the requirement for an active IIS pathway is necessary for SSD is evidence that IIS and possibly other nutrient sensitive growth pathways may be necessary for regulating SSD.

Unfortunately it is unknown whether IIS activity is *sufficient* to positively regulate SSD. Research in Drosophila demonstrates the ability of IIS overexpression to increase organ size (Brogiolo et al. 2001; Aron et al. 2010). It is conceivable that an increase in IIS expression may be met with a sex-specific response in growth, especially if females are more sensitive to levels of IIS. If increase in IIS expression is sufficient to increase SSD, then a causative link may be established between IIS and SSD. One link between the sex determination and IIS pathways was proposed by Rideout, Narsaiya, & Grewal (2015), where mutation of a negative regulator of the IIS pathway, *PTEN*, was found to rescue female body size in artificially masculinized crosses (via *tra* knockdown). We hypothesize that it is growth-related pathways such as IIS that are regulating sex-specific differences in nutrient-sensitive growth.

The IIS pathway is not the only nutrient-sensitive growth pathway, however (Gokhale & Shingleton 2015). Neither is it the only pathway previously implicated in sex-specific growth patterns. We know very little about the effect of many of these other pathways responsible for controlling growth in Drosophila. We suspect that perturbations of other nutrient-sensitive growth pathways will also result in changes to SSD. Alternate candidate pathways include the following:

TOR signaling pathway- This pathway interacts with the IIS pathway in regulating growth. One major difference is that, rather than responding indirectly to levels of nutrition (through insulin-like peptides, dILPs), TOR signaling responds to cellular levels

of amino acids. According to a recent paper, the downstream element S6-Kinase (*S6K*) is responsible for the drastic increase in SSD found in honeybee queens (Kamakura 2011). He further demonstrated that it has similar body size effects in Drosophila, though they did not specifically investigate its effect on SSD.

Ras pathway- While this pathway was originally described for its role in cancer biology, it has long since been known to control bodily growth through regulating the rate of cell division (Shingleton et al. 2007). In Drosophila, Ras has also been shown to control body size through the release of the molting hormone, ecdysone, via the prothoracic gland (Caldwell et al. 2005).

Hh signaling pathway- Recent evidence suggests that this pathway is involved in the regulation of sexually dimorphic growth rate of wing imaginal discs (Horabin 2005). The proposed mechanism is that circulating proteins used in the sex-determination pathway catalyze growth regulation in this pathway. Further research has implicated the Hh signaling pathway in generating sexually dimorphic appendage size in Onthacophagus beetles, through regulating IIS activity (Kijimoto & Moczek 2016).

Sex determination pathway (SDP)- This pathway is ultimately responsible for the regulation of SSD. What is unclear though, is where along this pathway SSD is actuated. Recent evidence suggests the gene transformer (*tra*) is involved (Rideout et al. 2015), but it remains unclear whether additional elements of the pathway are as well (Cline 1984).

We propose that the above pathways are involved in regulating SSD. We already know that some (IIS and SDP) are necessary for SSD, but which pathways are sufficient to increase SSD are as of yet unknown. Specifically, our hypothesis for nutrient-sensitive growth pathways (IIS, TOR, Ras) is that females are larger than males due to an increased sensitivity to pathway activity. If any candidate genes within these pathways are, in fact, involved in SSD regulation, then genetic manipulations should reveal their effects. Specifically, if a pathway is a positive regulator of SSD, then increasing signaling through the pathway by increasing expression of its constituent genes, should increase SSD. Conversely, if a pathway is a negative regulator of SSD, the reverse should be true. Other candidate pathways would be expected to yield similar, positive correlations with SSD if they are responsible. Fortunately, the genetic tools available in Drosophila allow for the very precise control of gene expression. By using the GeneSwitch Gal4-UAS system, we were able to titrate gene expression to explore the effects of increasing or decreasing signaling through different pathways on SSD. Our results suggest that suppressing signaling through several pathways can suppress SSD, but that it is difficult to increase SSD.

Materials and Methods

Fly strains and Maintenance

All fly lines were chosen based on their ability to overexpress or knockdown specific predetermined candidate genes. With few exceptions, all flies were obtained from the Bloomington Stock Center. See table 3.1 for complete stock list. Flies were maintained

Table 3.1. Fly Stocks used in Chapters 3 and 5. Table includes response element (gene), direction of manipulation, genetic background into which each gene is introgressed, Bloomington Stock center ID (where applicable), and full stock genotype.

gene	direction	genetic background	Stock #	Stock
Akt	overexpression	Sam	8191	*y[1] w[1118]; P{w[+mC]=UAS-Akt1.Exel}2*
Akt	knockdown	y,v	31701	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM04007}attP 2*
Ci	overexpession	Sam	32570	*w[*]; P{w[+mC]=UAS-ci.HA.wt}3*
Ci	knockdown	y,v	28984	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01715}attP 2*
dsx	F overexpression	Sam	44223	*y[1] w[*]; P{w[+mC]=UAS-dsx.F}24-3*
dsx	M overexpression	Sam	44224	*y[1] w[*]; P{w[+mC]=UAS-dsx.M}2*
dsx	knockdown	y,v	26716	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02256}attP 2*
FOXO	overexpression	Sam	Shingl eton Lab	*y, w: +:UAS-dFOXO-wt (f19-5 II)*
FOXO	knockdown	y,v	27656	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02734}attP 2*
fru	overexpression	Sam	17551	*y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}fru[EY09 280]*
fru	knockdown	y,v	31593	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01182}attP 2*

Table 3.1. (cont'd)

InR	overexpression	Sam	8262	*y[1] w[1118]; P{w[+mC]=UAS-InR.Exel}2*
InR	knockdown	y,v	35251	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00139}attP 2*
Ptc	overexpression	Sam	5817	*w[*]; P{w[+mC]=UAS-ptc.J}B1*
Ptc	knockdown	y,v	28795	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03223}attP 2*
Ras	overexpression	Sam	4847	*w[1118]; P{w[+mC]=UAS-Ras85D.V12}TL1*
Ras	knockdown	y,v	31653	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01445}attP 2*
S6K	knockdown	y,v	41702	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02267}attP2*
S6K	overexpression	Sam	6910	*w[1118]; P{w[+mC]=UAS-S6k.M}2/CyO*
Sxl	overexpression	Sam	17354	*y1 w67c23 P{EPgy2}SxlEY06108 SxlEY06108*
Sxl	knockdown	y,v	34393	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00609}attP2*
Tor	overexpression (dominant negative)	Sam	7013	*y[1] w[*]; P{w[+mC]=UAS-Tor.TED}II*
Tor	overexpression	Sam	7012	*y[1] w[*] P{ry[+t7.2]=hsFLP}1; P{w[+mC]=UAS-Tor.WT}III*
Tor	knockdown	y,v	35316	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00222}attP 2*
tra	F overexpression	Sam	4590	*w[1118]; P{w[+mC]=UAS-tra.F}20J7*
tra	knockdown	y,v	28512	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03132}attP 2*

on standard cornmeal-molasses food medium at 24˚C on a 12/12 hours light/dark schedule.

Each overexpression line was introgressed into the common wild-type background, Samarkand (Sam), for seven generations to avoid background effects. We performed introgressions by backcrossing red-eyed females (carrying the insertion element) to males of the Sam background. Since RNAi knockdown lines utilized TRiP insertional elements—all of which are already in a common background, “yellow, vermillion” (y,v), and lack readily available identifiers—we did not introgress these into Sam.

Nutritional SSD Assay

For this assay, all flies used were from the Sam genetic background. Food was prepared ahead of time for four separate treatment groups: 100, 10, 5, and 2% food. Each group consisted of a specific percentage of standard cornmeal-molasses medium mixed with a 2% agar solution. All food treatments yielded food of similar volume and consistency, but varying greatly in nutrition (see Stieper et al., 2008). For each experimental group, we allowed 8-10 flies of each sex to oviposit on standard apple juice-agar plates for 12 hours. After oviposition, eggs were washed and distributed to experimental food vials. All surviving pupae were collected and placed in individual 1.5ml Eppendorf tubes along with a square of ideally-moist Kimwipe. Twelve hours after eclosion, flies were harvested with 70% ethanol, and then allowed to dry overnight. Individual masses were recorded for flies on a Mettler Toledo XPE26 Microbalance.

Determining candidate genes by pathway

Positive vs Negative growth regulators – Within our candidate pathways, genes can either positively or negatively affect growth through their interactions. Any individual component can either be a positive regulator (one that increases expression of the pathway and increases growth), or a negative regulator (one that represses activity of the pathway and reduces growth). Most of the genes within our candidate pathways are positive regulators, meaning that gene expression and phenotype are expected to positively covary. For negative regulators, the covariance between gene expression and phenotype is reversed (negative). In the case where genes are negative growth regulators, their expected effects will be explicitly made clear, otherwise it can be assumed that a gene is a positive growth regulator.

IIS pathway – To increase IIS pathway activity we chose to overexpress *InR* and Akt and knock down expression of *Foxo* with RNAi. *Foxo* is a negative growth regulator, and so reducing its expression is theoretically equivalent to increasing expression of positive growth regulators. *InR* is the first component in this pathway, and is a positive growth regulator, so an increase in *InR* also increases *Akt* and deactivating *Foxo* protein activity. Likewise, an increase in *Akt* will also inactivate *Foxo*. To decrease IIS activity, we did the opposite by knocking down *InR* and *Akt* with RNAi and overexpressing *Foxo*. Overexpression of *Foxo* will lead to growth suppression, the same result expected by knocking down *InR* and *Akt*. See Figure 1.2 for a summary pathway diagram.

TOR pathway – To increase TOR pathway activity we chose to overexpress the positive growth regulators *Tor* and *S6K*. Since *Tor* is upstream of *S6K*, an increase in *Tor* should cause a corresponding increase in *S6K* protein activity. To decrease TOR activity, we knocked down expression of *Tor* and *S6K* with targeted RNAi. See Figure 1.2 for a summary pathway diagram.

Ras pathway – To increase Ras pathway activity we chose to overexpress the positive growth regulator *Ras*. To decrease Ras activity, we knocked down expression of *Ras* with targeted RNAi. See Figure 1.2 for a summary pathway diagram.

Hh pathway – To increase Hh pathway activity we chose to increase expression of both *Sxl* and full length *Ci* and knock down expression of the negative growth regulator *ptc*. Normally, *ptc* inhibits the transcription of full length *Ci*. By knocking down *ptc* with a targeted RNAi, we will increase the positive growth regulator *Ci*. Increasing *Sxl* will catalyze this process, further increasing activity. To decrease Hh activity, we knocked down *Ci* and *Sxl* and overexpressed *ptc*. See Figure 1.3 for a summary pathway diagram.

SD pathway – To increase female-specific expression of the SD pathway we chose to overexpress the female isoforms of *Sxl*, *tra*, and *dsx*. Each component is expected to feminize males, while leaving females unaffected. To increase male-specific expression of the SD pathway we chose to knock down genes *Sxl*, *tra*, and *dsx* and also overexpress the *dsx* male isoform. Since male isoforms of each of these genes (except

dsx) are essentially non-functional, these crosses were expected to masculinize females while leaving males unaffected. See Figure 1.1 for a summary pathway diagram.

The above experiments would allow us to overexpress or knockdown individual components of each candidate pathway. Unfortunately, this method does not allow us to control the level to which we are actually affecting activity. In some cases, a full knockdown or overexpression is expected to be lethal. Only by explicitly controlling the level of expression in each pathway could we confidently conclude the effect each gene is having (without inducing lethality).

Titrating gene expression

Each cross was initiated by crossing virgin *Actin5c-GeneSwitch-Gal4* females with males of each genetic treatment. The way the GeneSwitch system works is very similar to the traditional Gal4/UAS system in Drosophila with one important caveat: the GeneSwitch driver is condition-dependent and will only activate transcription once bound to a specific ligand, mifepristone (RU486). Because RU486 is synthetic progesterone, and progesterone is a mammal-specific hormone, the addition of RU486 to the Drosophila diet is believed to be biologically inert. Since RU486 additively activates the GeneSwitch-Gal4, the amount of RU486 ingested by each larva corresponds to activation level of each response gene.

Overexpression and knockdown lines were carefully chosen to affect only the gene of interest in each cross. The goal of each was to demonstrate the necessity of a gene for SSD (knockdown crosses) and also the sufficiency of a gene to increase SSD (overexpression crosses). Overexpression lines were chosen to drive increased expression of the common, wild-type allele of each gene. Similarly, knockdown lines were chosen to dampen the expression of genes with a targeted gene-specific RNAi. In this way, overexpression crosses would increase the production of target genes, while knockdowns would cause a decrease in final gene product.

Titration of gene expression was achieved through the combined use of the *Actin5c-GeneSwitch-Gal4* driver and introduction of a synthetic hormone, RU486, into the food ingested by larvae. Each gene-treatment contained each of the following sub-treatments: high dose RU486 (1.25μM), low dose RU486 (0.75 μM), ethanol solvent without RU486, or no addition to food.

Ultimately, the concentrations of RU486 doses were based on a literature search for appropriate levels (Nicholson et al. 2008; Shen et al. 2009), which were further refined by preliminary experiments. To determine the ideal concentrations of RU486 that would be strong enough to drive expression without being lethal, we crossed flies bearing *Actin5c-GeneSwitch-Gal4* with those bearing a copy of *vg*-RNAi, reared them on varying doses of RU486, and assessed the resulting wing phenotypes. Since the vestigial gene (*vg*) is necessary for proper wing development, its absence (caused by driving expression of *vg*-targeted RNA interference) causes a distinct ablation of the

wings; fortunately, this wing ablation is dose-dependent. High doses of RU486 were therefore expected to completely ablate adult wings, while the wings of low dose flies were expected to show only partial ablation. As expected, low doses of RU486 in *vg*-RNAi crosses typically yielded flies with wings of moderate *vg* phenotype, while high doses usually caused a much more severe phenotype (though, penetrance was variable).

Experimental Food Preparation

All food used in our experiments was based on standard cornmeal-molasses food medium with extra yeast added to the surface to stimulate mating and oviposition. Food was refrigerated in small batches until needed, then melted down and, once cool enough, mixed with the proper dose of RU486 solution. Since RU486 is ethanol-soluble, we prepared solution for each batch based on experimental group, varying the amount of RU486 and ethanol to maintain a constant volume across groups. We then used an immersion blender to thoroughly mix the food to ensure an even distribution of RU486 solution, before pouring a standard amount of food into each vial.

Experimental Fly Crosses

All crosses were maintained at 24C with a 12/12 hour light/dark schedule. For all treatments, we crossed four virgin female Actin5c-GeneSwitch-Gal4 flies with four male flies of the appropriate treatment group. Parental flies were then allowed to oviposit on the RU486 food medium, allowing their progeny to be raised completely on the respective experimental food. All vials were monitored for egg density to ensure that no

vials were under-crowded (20 eggs) or over-crowded (50 eggs). Once pupae began to sclerotize (red eyes and black wings visible through pupal case), they were collected and placed into individual 1.5ml Eppendorf tubes along with a damp square of kimwipe; kimwipe squares were sufficiently damp to keep flies hydrated until they were old enough to collect and store in ethanol, but not so damp that flies would drown. Crosses typically yielded between 15-30 adult males and females, but varied by treatment. Those that produced inadequate numbers of males or females were omitted (e.g. weak *Foxo* knockdown). Once each adult fly had completely sclerotized, based on the presence of a fully formed adult wing, we filled each vial with ~1ml of 70% ethanol to preserve each fly along with its own pupal case. Body size estimates were based on pupal area, which has been shown to be an ideal proxy for overall body size (unpublished data; Stillwell, Dworkin, Shingleton, & Frankino, 2011). Pupal area was calculated by imaging dry pupal cases using the methods in (Stillwell et al. 2011; Tang et al. 2011).

Analysis of SSD

SSD was calculated based on pupal size using the same index as in Chapter 2.

The direction of effect of each gene's effect was assessed by means of multiple statistical models. In all cases, each treatment was assigned a directional value corresponding to the expected level of gene expression, such that a high dose of RNAi was assigned -2, low dose of RNAi was assigned a -1, control flies were assigned 0, low dose overexpression was assigned +1, and high dose overexpression was assigned

+2. Using these values, we were able to calculate the additive effects of gene expression as it was either added or subtracted from the control. The specific models we used (in R) were as follows:

PupalSize ~ Sex + ExpressionLevel + Sex:ExpressionLevel

This model was run twice: once as a linear model with ExpressionLevel as a numerical variable (which assumes a linear relationship between expression level and pupal size) and once with it as a categorical (which does not assume linearity or rank order). Significance values were collected from both versions under the following scenarios: standard model, where additive effects of expression from lowest to highest expression levels (using all values), and the perturbation models, where absolute change from control (using the absolute value of all values, such that a -1 was coded as +1) was used to assess the similarity of effect in either direction.

Significance values were calculated for differences between one treatment at a time to and each control group separately. Fisher's summation of *P*-values was used to summarize the effect of a given gene manipulation on each line in the control pool. For example, the InR strong knockdown treatment was compared to each control, yielding a significance value for each; the resulting list of significance values were then summarized using Fisher's summation, yielding the likelihood that InR strong knockdown was different from the control group.

All statistical analyses were conducted using R statistical software (version 3.2.2). Calculation of pupal area was conducted using the custom macro used in Tang et al.

(2011). All error bars are 95% confidence intervals generated by non-parametric bootstraps.

Results

Nutrient Sensitivity of SSD

As expected, reduced nutrition caused an elimination of SSD (Figure 3.1). Individual t-tests demonstrate highly significant SSD for 100% ($P < 0.001$) and 10% ($P < 0.05$), but no significant differences at 5% ($P > 0.5$) or 2% ($P > 0.4$) nutrition. Additionally, linear models ($P < 0.01$), analysis of variance ($P < 0.01$), and analysis of covariance models ($P < 0.01$) show that food quality has a major effect on SSD.

Each genetic cross was carefully constructed to either increase or decrease the activity of each candidate pathway. We increased pathway activity by driving expression of positive regulator of each pathway or by knocking down expression of negative regulators of each pathway, using RNAi. Conversely, we decreased pathway activity by knocking down expression of positive regulators using RNAi, or by driving expression of negative regulators (see Figure 3.2 for example and Table 3.2 for confidence intervals).

Titrating gene expression: IIS pathway

For the IIS pathway, *Inr* and *Akt* are positive regulators of pathway activity, while *Foxo* is a negative regulator of activity. Previous research indicates that suppression of IIS reduces SSD (Böhni et al. 1999; Testa et al. 2013; Rideout et al. 2015), and, consistent

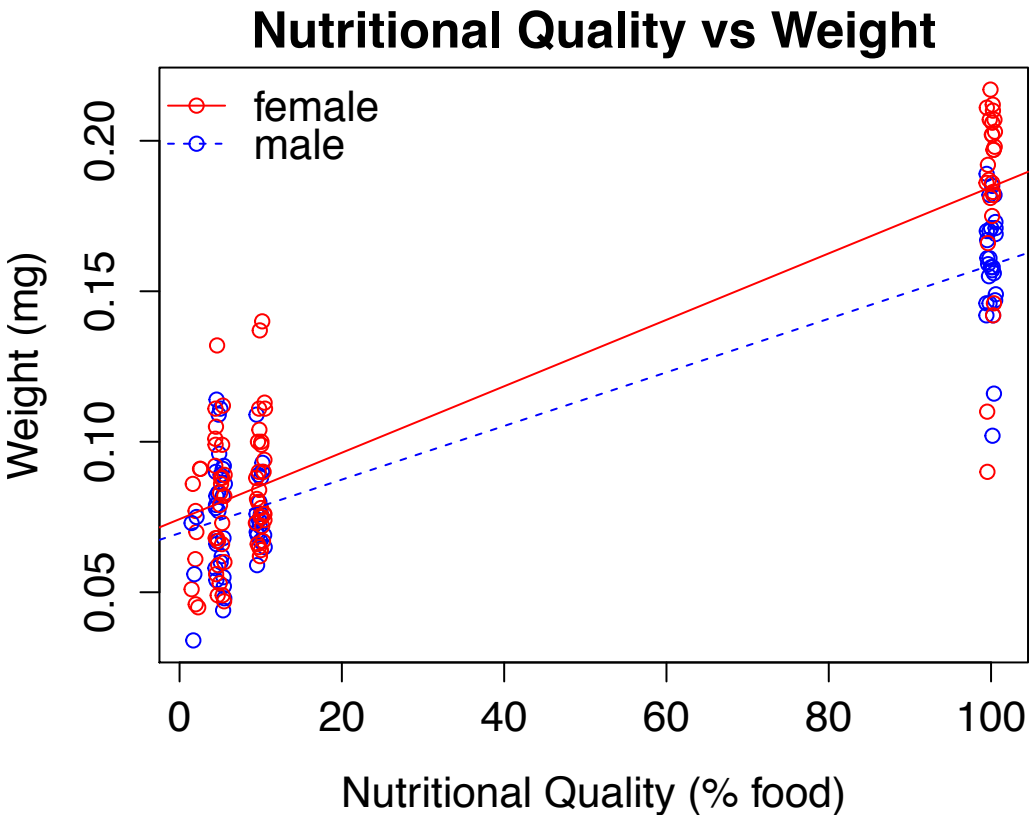

Figure 3.1. Nutritional Quality vs Weight for both male (blue) and female (red) Sam flies. Lines show regression estimates for each sex. As nutritional quality decreases, so does the difference between male and female body size. Note, once nutritional quality is reduced below 10%, SSD is effectively eliminated ($P < 0.05$).

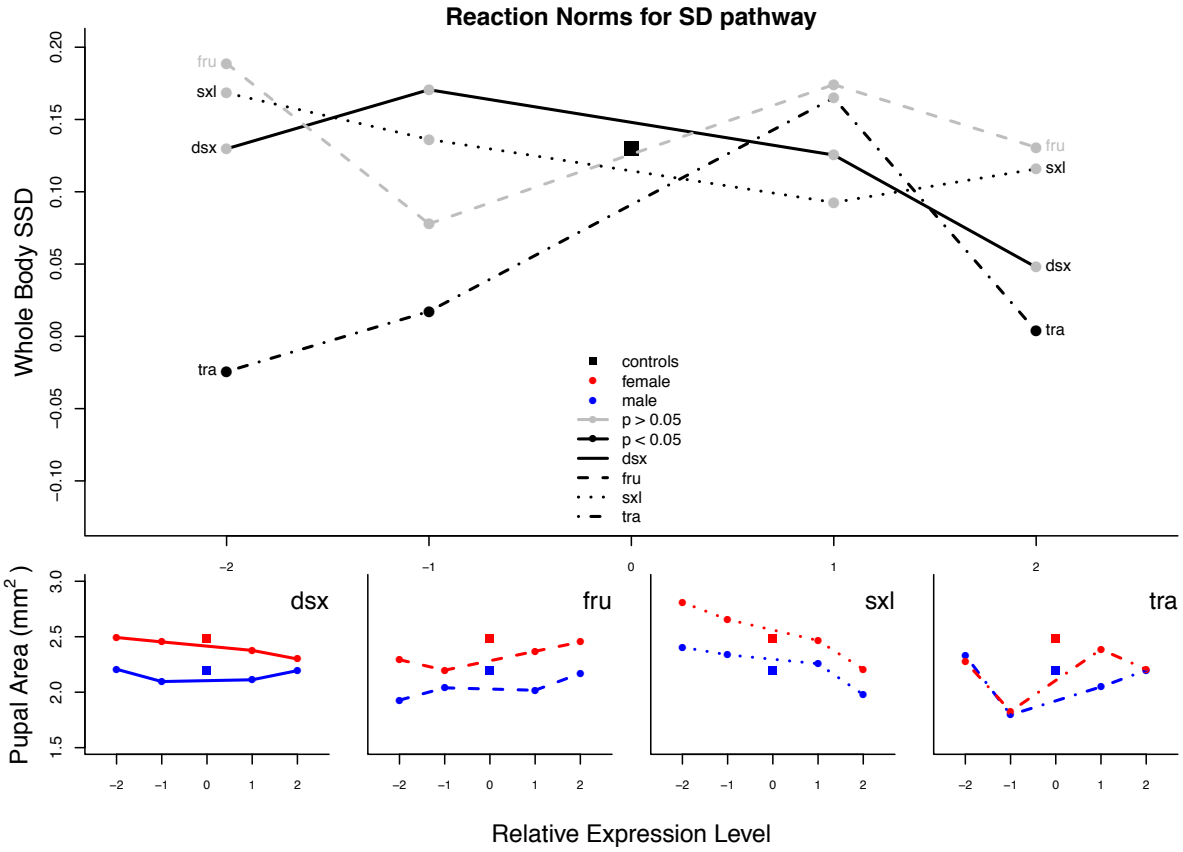

Figure 3.2. Reaction norm for SSD (above) and body size in males and females (below) in sex determination (SD) pathway manipulations. Genetic manipulations reveal that as expression of *Sxl* and *dsx* increase, SSD is reduced. For *Sxl*, this is by disproportionately affecting female body size, but in *dsx* this is due to increased *dsx* expression causing both sexes to develop intersex body sizes. In nearly all treatments, *tra* eliminates body size.

Table 3.2. Estimates for SSD and corresponding confidence intervals for all treatment crosses. Treatment is given in the form of "gene to manipulate" _ "direction and magnitude of manipulation," such that "akt_-2" corresponds to a strong knockdown of akt and "akt_1" corresponds to a weak overexpression of akt. 95% confidence intervals were generated using non-parametric bootstraps.

Treatment	SSD	low 95% CI	high 95% CI
control_pool	0.13015	0.02281	0.22959
akt_-2	0.10785	0.05377	0.15818
akt_-1	0.07974	0.02204	0.14277
akt_1	0.10844	0.01089	0.21374
akt_2	0.09142	0.00802	0.17537
ci_-2	0.0865	-0.01663	0.20296
ci_-1	0.02358	-0.04963	0.09193
ci_1	0.16113	0.0862	0.24504
ci_2	0.14584	0.07303	0.22845
dsx_-2	0.12973	0.02077	0.27605
dsx_-1	0.17058	0.08808	0.25583
dsx_1	0.12544	0.00947	0.2596
dsx_2	0.0478	-0.04057	0.14703
foxo_-2	0.13883	-0.00397	0.28284
foxo_1	0.04405	-0.02863	0.11647
foxo_2	0.10219	0.04705	0.1548
fru_-2	0.18884	0.09059	0.29949
fru_-1	0.07781	-0.01746	0.17407
fru_1	0.17414	0.08885	0.2624
fru_2	0.13071	0.03415	0.23956
inr_-2	0.10107	0.03982	0.16093
inr_-1	0.03014	-0.02886	0.0796
inr_1	0.09294	-0.12688	0.34509
inr_2	0.08778	-0.15454	0.35885
ptc_-2	0.11948	0.06427	0.167
ptc_-1	0.09009	0.02287	0.15809
ptc_1	0.06426	0.01348	0.11772
ptc_2	0.13618	0.06047	0.21706

Table 3.2. (cont'd)

ras_-2	0.17031	0.1041	0.24263
ras_-1	0.12479	0.05399	0.20805
ras_1	-0.10125	-0.24716	0.01256
ras_2	0.10593	0.02712	0.17878
s6k_-2	0.01559	-0.05578	0.09016
s6k_-1	0.15234	0.01497	0.34868
s6k_1	0.1048	-0.05056	0.28696
s6k_2	0.05893	-0.00787	0.12934
sxl_-2	0.16837	0.09873	0.24015
sxl_-1	0.13627	0.06474	0.19776
sxl_1	0.09259	0.03229	0.15113
sxl_2	0.11579	0.00312	0.23073
tor_-2	0.14399	0.09261	0.20266
tor_-1	0.13997	0.06834	0.20438
tor_1	0.08637	0.01393	0.16531
tor_2	0.05565	-0.00298	0.11705
torted_1	0.1914	0.12913	0.26932
torted_2	0.18598	0.09591	0.28152
tra_-2	-0.02437	-0.10875	0.05601
tra_-1	0.01724	-0.14088	0.15869
tra_1	0.16489	0.10024	0.23537
tra_2	0.00358	-0.08119	0.08748

with these studies, we found that weak knockdown of *InR* also reduced SSD (Figure 3.3, weak knockdown; $P = 0.05$). However, an increase in *InR* expression did not increase or decrease SSD. Further, changes in the expression of both *Akt* and *Foxo* had no effect on SSD or body size (Figure 3.3).

Titrating gene expression: TOR pathway

In the TOR signaling pathway, both *Tor* and *S6K* are positive growth regulators and therefore were expected to behave as such. Instead, we found that the *Tor* has a negative effect on SSD with increasing expression ($P < 0.001$; Figure 3.4). Conversely, the downstream element, *S6K*, exhibits a significant reduction in SSD in strong knockdowns ($P < 0.001$). Strong knockdowns reduce SSD primarily through impacting female size relative to male size. Knock down of *Tor* and overexpression of *S6K* has no effect on body size or SSD.

Titrating gene expression: Ras pathway

While both male and female body size tend to increase with Ras expression, Ras exhibits a negative effect on SSD with increasing expression ($P < 0.01$; Figure 3.5). Specifically, it is the weak ($P < 0.001$) overexpression line of Ras that causes the most marked reduction in SSD. Thus, as Ras activity increases body size increases, but female size reacts inconsistently, thus SSD actually decreases.

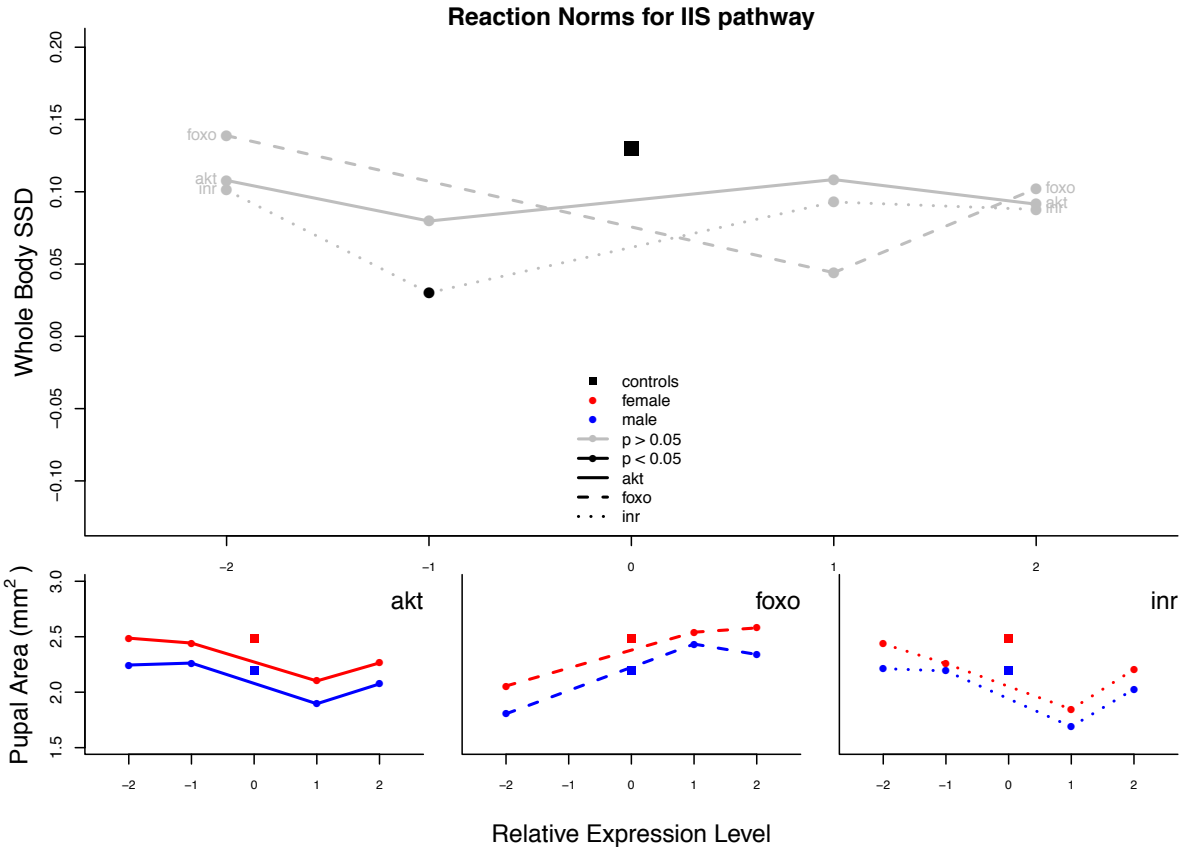

Figure 3.3. Reaction norm for SSD (above) and body size in males and females (below) in Insulin and Insulin-like Signaling (IIS) pathway manipulations. Of all possible treatment groups, only the weak knockdown of InR significantly reduced SSD. In general, SSD was relatively consistent across expression levels, despite body size for males and females being more labile.

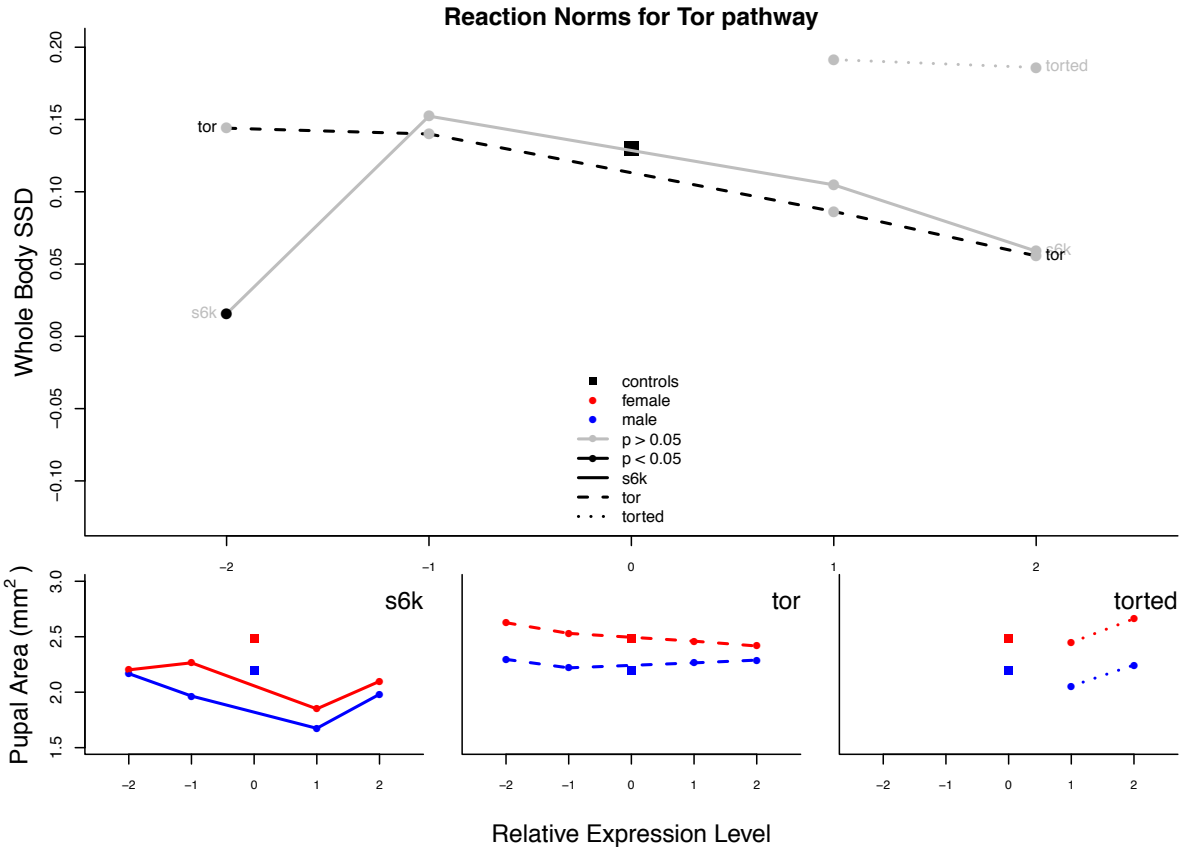

Figure 3.4. Reaction norm for SSD (above) and body size in males and females (below) in TOR signaling pathway manipulations. Tor demonstrates a negative relationship with SSD; as Tor expression increases, SSD decreases due to a reduction in female relative to male body size. S6K demonstrates a different pattern, where strong knockdowns eliminate SSD. Since Tor^{TED} is a dominant negative allele of Tor, its sign is effectively flipped. Its effects are consistent with, but stronger than, those of Tor manipulations.

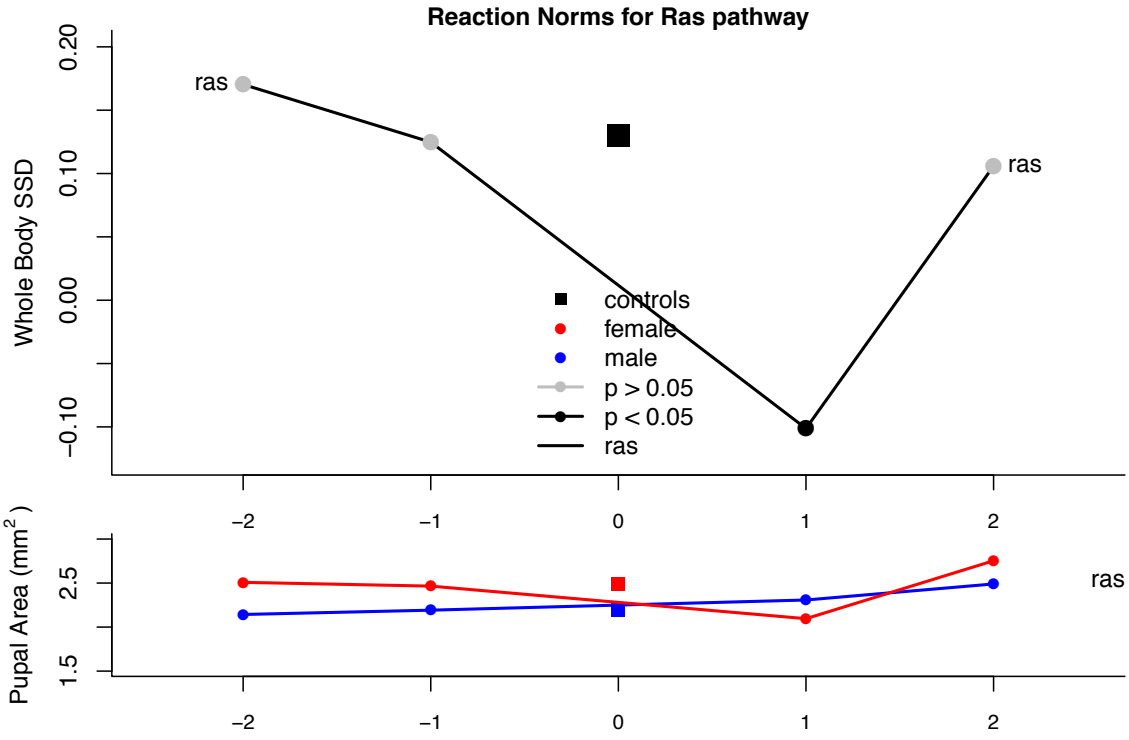

Figure 3.5. Reaction norm for SSD (above) and body size in males and females (below) in Ras signaling pathway manipulations. As Ras expression increases, SSD is reduced. Surprisingly, weak overexpression causes a reversal of SSD through an exaggerated female reduction in body size.

Titrating gene expression: Hh pathway

For the Hh signaling pathway, we expected similar results to the nutrient-sensitive growth pathways. Normally, *ptc* inhibits smoothened (*smo*) activity, which is a positive regulator of full-length *Ci*. Once *Hh* ligands bind to *ptc*, it derepresses *smo* and full-length *Ci* is expressed. Since *ptc* is a negative growth regulator, knockdowns are expected to increase SSD and overexpressions to decrease SSD. We do observe the trend that *ptc* decreases overall body size, but SSD remains unaffected (Figure 3.6). The other genes, *Ci* and *Sxl*, were then expected to function as positive growth regulators. Indeed, with *Ci*, we see a strong positive relationship with SSD. *Ci* strong-knockdowns reduce and weak-knockdowns eliminate ($P < 0.001$) SSD, which then increases with additional overexpression. The results of *Sxl* are described below.

Titrating gene expression: Sex determination pathway (SDP)

Finally, the sex determination pathway crosses were set up in such a way that the resulting offspring should be either feminized or masculinized, depending on the cross. The way that the SDP works is fundamentally different than growth-regulating pathways; rather than components activating and/or repressing downstream genes, each element of the SDP alternatively splices the protein products of downstream elements. Because knockdowns in female-specific isoforms would cause male-specific versions to be spliced, knockdowns were expected to masculinize females. Conversely, overexpressions of female-specific isoforms were expected to feminize males, by driving expression of the female-specific isoforms. In both cases, we expect to see a reduction in SSD.

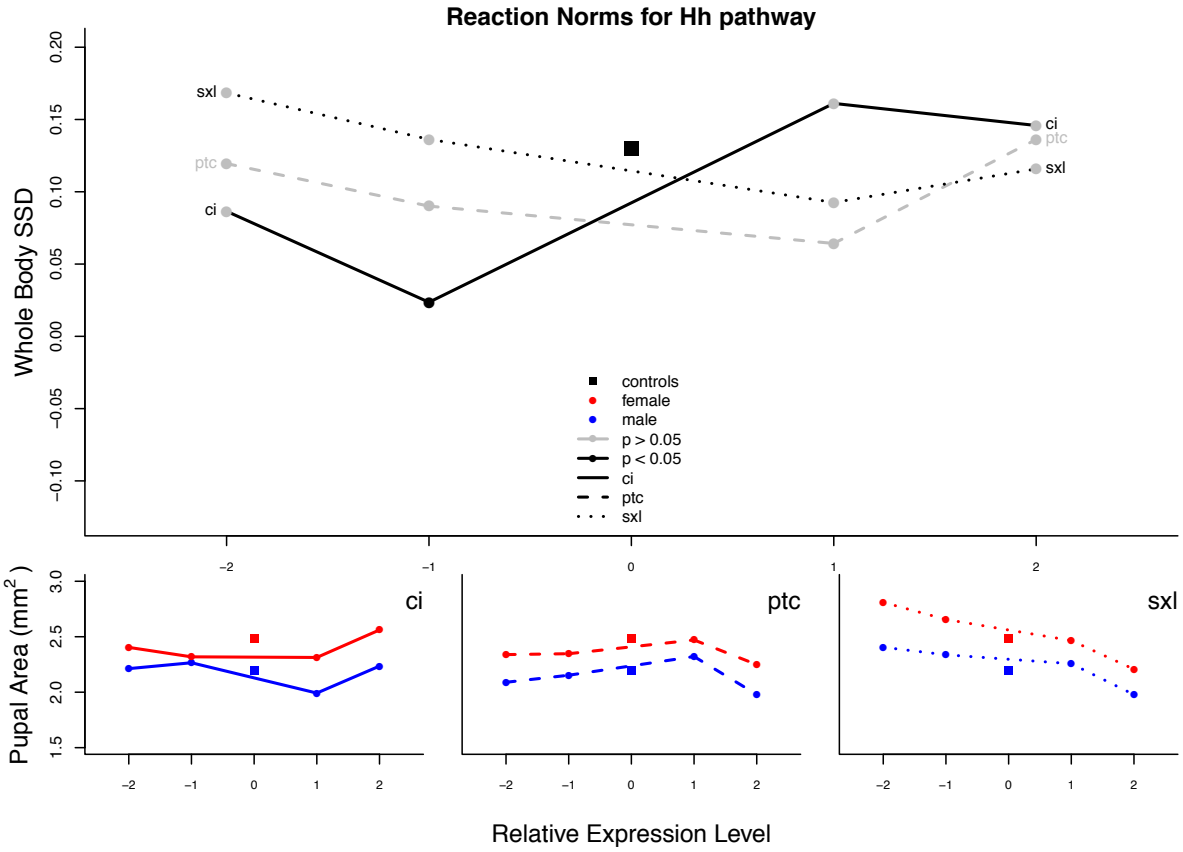

Figure 3.6. Reaction norm for SSD (above) and body size in males and females (below) in Hedgehog (Hh) signaling pathway manipulations. Within individual treatment groups, SSD is only significantly affected in weak *Ci* knockdowns where SSD is eliminated. Regardless, there is a positive significant trend between *Ci* expression and SSD. This trend is countered by a lack of significance in *ptc* and a significant negative trend in *Sxl* expression.

At the top of the SDP is *Sxl*, whose name properly suggests that loss-of-function and constituently expressing mutants of the gene are lethal for both females and males, respectively. It was expected that females would respond to Sxl knockdowns by masculinizing (decreasing) their size, leaving males unaffected. Ubiquitous overexpression and knockdowns in the larval stage, however, were not lethal, but yielded counterintuitive results. While expression of *Sxl* is normally only found in females, we found that when its expression was increased, *Sxl* reduced and masculinized female body size, displaying a fully additive negative relationship with SSD. Starting with strong knockdowns (that increase SSD; $P < 0.02$), as *Sxl* expression increases, body size and SSD decrease. Males appear to be relatively resistant to perturbations in *Sxl* activity; it is fluctuations in the female body size that cause these changes in SSD.

Furthermore, both downstream elements *tra* and *dsx* also have reducing effects on SSD (Figure 3.2). Statistical models suggest that as *tra* increases, so does SSD, but this is likely due to the weak overexpression being the only treatment failing to eliminate SSD. In the perturbation statistical models—the version of our models where absolute expression, rather than overall expression, is taken into account—we see a highly significant trend of perturbations causing reduction in SSD ($P < 0.0001$). Strong perturbations (-2 or 2) also eliminated SSD ($P < 0.0001$). As expected, *tra* is both necessary and sufficient to alter SSD in every case except for weak overexpression. Essentially, as *tra* increases or decreases, we expected SSD to be lost by either

feminizing males or masculinizing female flies, respectively. Certainly, females are masculinized in *tra* knockdown crosses, but they are also masculinized in strong overexpression crosses as well. *Dsx* also has a directional effect, where SSD increases with decreasing *dsx* activity. In spite of this, no individual doses had a significant effect in *dsx* crosses. Since *fru* is ostensibly only expressed in the nervous system and only affects male behavior, it was not expected to contribute to SSD. As expected, *fru* had no significant effects on SSD, though overall body size appears to fluctuate somewhat with dose.

Discussion

Effect of Nutrition and Nutrient-Dependent Pathways on SSD

Our results support the hypothesis that SSD is nutrition-dependent. As food quality decreases below 10%, SSD is eliminated. These data are consistent with predictions made by Bonduriansky (2007) about the condition-dependence of SSD with respect to nutrition. Clearly the mechanisms of SSD are linked to those of nutrient-sensitive pathways. Because our low quality food treatments are ostensibly phenocopies of nutrient-sensitive growth pathway mutants (Testa et al. 2013), we can confidently conclude that SSD is regulated (at least in part) by nutritional responses.

Our primary hypothesis was that, since IIS was a nutrient-sensitive pathway, IIS would be both necessary and sufficient for SSD. IIS reduction experiments verify that IIS signaling is necessary for SSD, but unfortunately our initial hypothesis that IIS activity is sufficient to increase SSD cannot be supported. Recent evidence suggests that IIS

activity may be responsible for differences in SSD (Rideout et al. 2015). Flies with a mutation of the negative IIS regulator, *PTEN*, were found to rescue female body size in artificially masculinized crosses (via *tra* knockdown), placing IIS downstream of *tra* activity. When individual components of the IIS pathway are overexpressed throughout the body in both sexes, however, a similar trend was certainly not observed. The reason for this discrepancy is as of yet unclear. Notably, overexpression in the IIS pathway causes neither an increase nor decrease in SSD. This is important to note for all of our data. Regardless of the effect of over- or under-expression on body size, it is strictly the difference in male and female body sizes and the direction of effect that is of most relevance to this study. Thus, even when overexpression causes growth reduction, this reduction is not sex-specific.

In the TOR pathway, *Tor* shows a significant trend of decreasing SSD with increasing expression. The strong knockdown of *S6K* supports this claim: *S6K* is necessary for SSD, but when overexpressed ubiquitously, it also appears to decrease SSD (though not significantly so; $P = 0.06$). Our evidence from *S6K* knockdowns are consistent with previous data for overall body size, where the sex-specific effects were previously unknown (Montagne et al. 1999). The reason for the discrepancy between *Tor* and *S6K* data is unclear. Given that *S6K* knockdown reduces female size and *Tor* overexpression increases male size, it is plausible that sex-specific expression of TOR signaling, rather than sex-specific sensitivity to it, is regulating SSD. Further data are needed to assess this hypothesis.

For Ras signaling, our only candidate gene was *Ras* itself. *Ras* demonstrates a similar relationship to TOR signaling in that increased expression caused a reduction in SSD. This is strongest in the weak overexpression lines, where SSD is reversed, due to females losing considerably more size than males (Figure 3.5).

Our data support the claim that nutrient-sensitive growth pathways (defined as IIS, TOR, and Ras) tend to be necessary for SSD. Individual components of IIS and TOR signaling both demonstrate their necessity for SSD. If SSD is indeed nutrient-sensitive, these results are to be expected. Surprisingly, our ubiquitous knockdowns do not appear to function quite the same way that traditional mutants do. For example, Testa et al. (2013) demonstrated that IIS mutants eliminated SSD, but only the weak knockdown of *InR* had a significant effect on SSD in our crosses. Since it is still unknown how mutation of other growth regulation pathways affects SSD, we cannot fully interpret our results until more data are collected, though we suspect the results will be similar to *InR*.

In general, it appears to be much more difficult to increase SSD than it is to decrease it. An alternative hypothesis to "SSD is influenced by sex-specific differences in sensitivity to signaling pathways" could be that for signaling systems to produce SSD, there must be a very specific balance of inputs; any other general state of signaling (within reasonable limits) leads to the "default" of sex-invariant sizing. In this case, it is possible that our genetic manipulations have simply disrupted normal developmental processes leading to reduction in the ability to produce sex-specific phenotypes, rather than direct

manipulation of SSD. Perhaps a less directed set of candidate genes might yield more unbiased results. Finally, given how high the environmental and background variation tend to be in populations (as seen in Chapter 4), we may need to rethink how we approach these sorts of questions in future studies.

Our data generally support Takahashi and Blanckenhorn's (2015) claim that deficiencies in most genes tend to reduce rather than increase SSD. Of the 15 candidate genes, no individual treatment was sufficient to increase SSD (while the linear statistical model found that increasing *Ci* expression increased SSD, no individual treatments were significant. We believe that this effect is a statistical artifact of knockdowns reducing SSD, while overexpessions had no effect). Individual dosage effects revealed that, in the case that a gene caused a significant effect, that effect was always to decrease SSD. It seems likely then, that directional effects on SSD are caused more by the uniform reduction of SSD on one end of the dosage spectrum (knockdown or overexpression) than they are by reduction on one end and increase on the other.

Overexpression of nutrient-sensing growth pathways reduces body size

One of the most surprising results of these experiments is the fact that nutrient-sensitive growth pathway (IIS, TOR, Ras) overexpression crosses tended to decrease overall body size. This is surprising, because previous research has shown that overexpression of individual components of the IIS pathway, such as *InR*, *Akt,* and/or *PI3K*, has the capacity to increase organ size when overexpressed in one organ (Aron et al. 2010; Gokhale et al. 2016). Indeed, these findings are somewhat of a novelty in the

Drosophila literature; no study to date has investigated the effects of ubiquitous overexpression of multiple growth control pathways. Our driver, Actin5c-GeneSwitch, causes expression of UAS response elements everywhere in the body that Actin, a critical cytoskeletal element of most cells, is expressed (therefore it is referred to as ubiquitous). One such study did find that a ubiquitous overexpression of *InR* caused a reduction in growth rate, presumably in both sexes (Wong et al. 2014), but seemingly no further examples exist. Our data suggest that overexpression in nutrient-sensitive growth pathways is more nuanced that simply overexpressing activity in a single organ.

One explanation for this phenomenon could be that ubiquitously driving expression of each gene may be activating negative feedback loops in their respective pathways. Prolonged exposure to insulin is known to cause insulin-resistance in mice, effectively deactivating the IIS pathway (Manning 2004; Um et al. 2004; Rui et al. 2001). According to Rui et al. (2001), serine (Ser^{307}) phosphorylation at the insulin receptor substrate (IRS) is a common mechanism to counter-regulate insulin signaling in scenarios such as overstimulation. These negative feedback loops are also associated with other nutrient-sensitive growth pathways, including TOR (Manning 2004; Shah et al. 2004; Um et al. 2004) and Ras (Dougherty et al. 2005; Macrae et al. 2005). If negative feedback loops are indeed being activated in overexpression lines, then insulin resistance is a likely cause for the anomalous decreased body size in overexpression lines.

Another explanation could be that tumor suppressor genes are repressing the increased growth caused by our overexpression crosses. Tumor suppressors are genes known to regulate overgrowth by regulating cell cycle arrest and apoptotic cell death within many different nutrient-sensitive growth pathways. Since the goal of our overexpression crosses was to increase growth, and the primary function of tumor-suppressor genes is to stop over-growth, it is possible that tumor suppressor genes may be responsible for the reduction in body size in our overexpression crosses. Relevant examples within the IIS, TOR, and Ras signaling pathways, respectively, include: PTEN (Di Cristofano & Pandolfi 2000), TSC1/TSC2 complex (Garami et al. 2003), and RASSF1 (Armesilla et al. 2004). It is therefore plausible that tumor suppressor activity is suppressing growth ubiquitously in response to sustained activation of nutrient-sensitive growth pathways.

Even if overexpressing genes within nutrient-sensitive growth pathways causes a reduction in size, our data still do not conform to our expectations of how growth pathway mutants function. In *InR* mutants, SSD is eliminated. We do not see a similar reduction in SSD for overexpression crosses even though body size is often reduced below that of RNAi knockdowns. One would expect that in the above scenarios SSD would decrease as though that pathway were knocked down. Instead, what we see is a reduction in body size with SSD behaving more-or-less independently of body size.

Perhaps the most plausible explanation is that, growth suppression (either by insulin resistance or tumor suppressor) might be physiologically different in males and females rather than acting as a *sex-uniform* suppression. In other words, males and females

may be responding *independently* not only to growth regulators (those that facilitate growth), but also to growth mediators (those that moderate growth). Indeed, Testa et al. (2013) demonstrates that there is already precedence for this explanation as males and females both gain and lose mass at differing rates. This explanation would account for both the reduction in body size and maintenance of SSD in growth pathway overexpression crosses.

Understanding the mechanisms that cause our observed body size reduction is both interesting and important, but well beyond the scope of this study. While this discovery is significant, its impact is no less diminished if we do not have a molecular explanation for this effect. Further experiments are certainly required to test these hypotheses. By individually testing the hypotheses above, we may gain some insight into the molecular regulation of this phenomenon.

Alternative interpretations of data

However interesting the above results may be, it is important to consider that the effects observed in these experiments may have alternative or non-biological explanations. Since we have no molecular data about how these perturbations are influencing the activity of each signaling pathway, we must be cautious in interpreting our results. Potentially, changes in transcript abundance may be insufficient to substantially alter activity for some of these genes. However, for some of these genes, previous evidence has shown that genetic manipulations like this have been shown to work (Aron et al.

2010; Dworkin et al. 2011; Rideout et al. 2015).

It is possible that strong knockdowns and over-expressions are simply generating conditions that are outside of normal pathway functioning and thus “breaking” the established systems. If this is the case, we might expect to see a stronger effect in the weak manipulations than the strong ones, because the weak manipulations are the only ones affecting pathway activity as expected. In some way, we know that these manipulations are definitely physiologically altering the phenotype (see Table 5.1). Additionally, we see that most of the pupal sizes also occupy a similar range to those generated in Tang et al. (2011), where *Foxo* expression was over-expressed and knocked down.

There are also other, non-biological, explanations for certain trends in our data. First, we know that sample size can have large effects on analysis. Particularly, in the case of *Ras* weak over-expression, we know that sample sizes are lower than average and that a couple of low female outliers are pulling down the mean, causing female size to be lower than male. In instances like this, the trends we see in *Ras* overexpression may be more likely due to statistical artifacts rather than true biological effects. Additional data would confirm that this effect is, in fact, due to sampling error and low sample size.

Even though the progesterone mimic, RU486, is not found in insects, it could potentially be having some effect on dimorphism. We did control for these effects, but since our

analyses involved analyzing the effects of each treatment against a pool of controls, these effects may not specifically be explicitly dealt with.

Hh signaling and its link to the SDP

One of the stronger competing hypotheses to explain the mechanism of SSD was the Hh-Sxl hypothesis put forward by Horabin (2005). The authors demonstrated that ectopic *Sxl* protein was sufficient to cause overgrowth in female wing imaginal discs, but not in males. This sex-specific overgrowth is supposedly caused by an increase in full length *Ci* (Horabin 2005). While our results indicate a statistical trend for increasing *Ci* expression causing a corresponding increase in SSD, it is unclear if our experimental over-expression of full length *Ci* is leading to the activator Ci protein. Ogden et al. (2004) suggests that the full length activator version of full length Ci may not be produced in the absence of Hh signaling ligand. In this case, we may not be able to make strong statements about the overexpression of Ci in our experiments. Regardless, there is no evidence for any other factor within the proposed Hh signaling pathway, including the proposed link between the Hh pathway and *Sxl* of the sex determination pathway. This might be due to the fact that we used a ubiquitous driver, rather than an organ-specific driver. While female wings are likely to be in the presence of greater amounts of *Sxl* protein, so is the rest of the body. Indeed, it appears that ubiquitous expression of *Sxl* in females has the exact opposite effect of organ-specific overexpression of *Sxl*. Therefore, we suspect that the Hh-Sxl pathway is unlikely to be regulating whole body SSD.

At what level in the SDP are sex-specific body sizes determined?

The earliest accounts of *tra* demonstrated by loss of function mutants for *tra* had no effect on male development, while females were found to be transformed into males that were wild-type in every respect except for body (and genital) size (Sturtevant 1945). For nearly 70 years the conventional wisdom was that SSD was controlled at some level in the SDP *other* than *tra* (Sturtevant 1945; Baker & Ridge 1980; Cline & Meyer 1996; Salz & Erickson 2010; Cline 1984; Horabin 2005) . More recent literature has challenged the notion that *tra* is unable to control SSD (Rideout et al. 2015). Our data support the recent literature in determining that *tra* is the component of the sex determination pathway most active in regulating SSD. Indeed, it is *tra* that shows the greatest amount of support for regulating SSD. While *dsx* and *Sxl* also demonstrate that they also decrease SSD with increasing expression, they are not necessary for SSD.

As male and female isoforms of *dsx* are expressed, SSD is lost. The response of SSD is nearly identical regarding male vs female isoforms; both produce equally intersex flies (with respect to size), thus we reported both together. These results would be expected if *dsx* were positively regulating SSD. Indeed, while many studies have investigated the phenotypic effects of dsx expression (Shirangi et al. 2006; Erdman & Burtis 1993; Kijimoto et al. 2012; Burtis & Baker 1989; Waterbury et al. 2000; Williams et al. 2008), few have examined its effects on size (Kijimoto et al. 2012). Our data suggest that correct sex-specific expression of *dsx* is required to produce correct male and female sizes; when either sex-specific isoform is inappropriately expressed, SSD is lost.

Interestingly, a lack of *dsx* is expected to masculinize female body size. Since it does not, *dsx* cannot be necessary for SSD.

Upstream of both *dsx* and *tra* is *Sxl*, which, according to some studies (Horabin 2005; Salz & Erickson 2010), should be sufficient to increase SSD. In fact, our data directly contradict these claims. As *Sxl* increases, not only does SSD decrease (and vice versa), but whole body size decreases as well. This effect appears to be the result of greater female sensitivity to *Sxl*, where decreased levels produce larger females and increased levels smaller females.

As discussed in Carreira et al. (2009), body size regulation is far too complex to be accounted for by few genes of large effect. Both Carreira et al. (2009) and Takahashi et al. (2015) demonstrate that it is much more common for genetic perturbations to decrease SSD. Our data are less consistent with “sensitivity” hypothesis proposed by Takahashi et al., where it would be expected that both sexes respond unequally to genetic perturbations. Regardless, our data certainly support the view that body size regulation is immensely complex.

CHAPTER 4:

Testa, N. D. & Dworkin, I. The sex-limited effects of mutations in the EGFR and TGF-β signaling pathways on shape and size sexual dimorphism and allometry in the Drosophila wing. Dev. Genes Evol. (2016). doi:10.1007/s00427-016-0534-7

Introduction

In spite of our wealth of knowledge about the natural world, biologists continue to be fascinated by the prevalence of sexual dimorphism. Where sexual dimorphism is often found, it is most often subtle, despite important exceptions of sex-limited characteristics (Bonduriansky & Day 2003), or traits that are highly exaggerated in one sex, but not the other (Lavine et al. 2015). This is particularly evident for morphological traits that demonstrate sexual size (SSD) or sexual shape (SShD) dimorphism (Kijimoto et al. 2012). Within evolutionary biology, explanations for sexual dimorphism have focused on a number of mechanisms that are likely responsible for the origin and maintenance of sexual dimorphism (Reeve & Fairbairn 2001; Allen et al. 2011; Bonduriansky & Chenoweth 2009; Mank 2009; Cox & Calsbeek 2010; Hedrick & Temeles 1989; Shine 1989; Fairbairn & Blanckenhorn 2007) including sexual conflict, differences among the sexes in the variance of reproductive success leading to sexual selection (Fairbairn 2005), and sex specific aspects of natural selection (Preziosi & Fairbairn 2000; Ferguson & Fairbairn 2000). Despite this, our understanding of the genetic mechanisms that contribute to variation in sexual shape and size dimorphism is still lacking (Mank 2009; Blanckenhorn et al. 2007; Fairbairn & Roff 2006; Fairbairn 1990).

There is considerable experimental evidence demonstrating that patterns of SSD and SShD can be altered by influencing the condition of individuals (Bonduriansky & Chenoweth 2009; Bonduriansky 2007b). There has unfortunately been less success on directly experimentally evolving consistent changes SSD or SShD, with some notable

exceptions where dimorphism evolved in response to selection on fecundity (Reeve & Fairbairn 1999) or due to experimental manipulation in the degree of sexual conflict (Prasad et al. 2007). There are even fewer instances where experimental evolution has been able to alter existing size/shape (allometry) relationships (Bolstad et al. 2015).

Despite previous difficulties with directly selecting for SSD or SShD, we still find evidence for genetic variation in SSD within a number of species (David et al. 2003; Merila et al. 2011). Several studies have utilized induced mutations (Carreira et al. 2011) or defined genomic deletions to examine patterns of SSD (Takahashi & Blanckenhorn 2015). They find that, in general, mutations tend to attenuate differences in SSD and sexual developmental timing difference. Interestingly, while ~50% of the random insertion mutations influenced size and shape, only half of those were consistent between males and females, suggesting considerable sex limitation of the mutational effects (Carreira et al. 2011).

With respect to the influence of mutations on sexual dimorphism, one important consideration is whether the mutations themselves are directly influencing aspects of sexual dimorphism. Alternatively, mutations may be influencing size and shape of the organism, but are modulated in a sex-limiting fashion. Arguably, it is difficult to distinguish between these possibilities, although for the purposes of this study, we consider a mutation to be modulated by the influence of sex if it influences size or shape as well as having an additional influence on sex (i.e. a sex-by-genotype interaction).

The extent to which such mutations influence SSD and SShD remains poorly understood.

To address these questions, we examined the influence of characterized induced mutations that influence two signaling pathways important for wing development, Epidermal Growth Factor Receptor (EGFR), and Transforming Growth Factor - β, (TGF-β). The Drosophila wing is an excellent model for the study of SSD and SShD. First, as it is a premiere model system for the study of development, and as such a great deal is known and understood about the mechanisms governing overall growth and patterning (García-Bellido et al. 1994; Weinkove et al. 1999; Day & Lawrence 2000; Weatherbee et al. 1998). Additionally, *Drosophila melanogaster* and closely related species have a strong pattern of sexual size dimorphism for many traits (and overall body size), with wing size demonstrating some of the greatest degree of overall dimorphism (Testa et al. 2013; Abbott et al. 2010; Gidaszewski et al. 2009). There is extensive variation for size and shape within and between Drosophila species, and for the extent of SSD and SShD as well (Gidaszewski et al. 2009). Importantly, the mutational target size for wing shape (Weber 2005) is high (~15% of the genome), thus providing plenty of opportunity for mutations to influencing shape, and potentially those modulated by sex.

In this study we utilize a previously published data set that examine the influence of 42 mutations in the EGFR and TGF-β signaling pathways when examined in a heterozygous state. We re-analyze this data set to examine the extent to which the mutations have sex-limited phenotypic effects that influence SSD or SShD. Furthermore

we examine how patterns of allometric variation between size and shape are altered by both sex and wild type genetic background of the mutations. Despite most mutations having substantial phenotypic effects on either size, shape or both, only a small subset of them appear to have their effects modulated by sex, with respect to both direction and magnitude of effects. Furthermore, we demonstrate that the allometric relationship between size and shape is only subtly influenced by sex and genetic background for these alleles. We discuss these results within the context of sex-limited effects of mutations and their influence on SSD and SShD, and how to interpret allometric relationships between size and shape in Drosophila.

Materials and Methods

Provenance of Samples

The data used for this study was originally published by (Dworkin & Gibson 2006). We compared wings from flies across several treatment groups, including: sex, wild type genetic background (Oregon-R and Samarkand), progenitor line and genotype (mutant vs. wild type allele). Fifty different p-element insertion lines, each marked with w+, were introgressed into two common wild type backgrounds (Samarkand and Oregon-R), were used along with their respective controls. All wing data, in the form of landmarks, were collected from digital images, as detailed in Dworkin and Gibson (2006). For a more detailed description on the source of these strains and the experimental design, please refer to Dworkin and Gibson (2006).

Insertional mutations were selected from the Bloomington Stock Center and subsequently introgressed into two wild-type lab strains, Samarkand (Sam) and Oregon-R (Ore). Introgressions were performed by repeated backcrossing of females bearing the insertion to males of Sam and Ore-R. Females from replicate vials within each generation were pooled for the subsequent generation of backcrossing. Since both backgrounds contain a copy of the mini-white transgene, eye color for all flies lacking p-elements was white. Selection was therefore based entirely on the presence of the eye color marker, precluding unwitting selection for wing phenotypes. While the introgression procedure (14 generations of backcrossing) should make the genome of the mutant largely identical to that of the isogenic wild types, some allelic variation in linkage disequilibrium with the insertional element may remain. All experimental comparisons of mutant individuals were therefore made with wild-type siblings from a given cross and should share any remaining segregating alleles unlinked to the p-element. We separated mutants and their wild-type siblings by their corresponding mutant “line” number (supplementary Table 1) to avoid these and potential “vial effects”. All crosses were performed using standard media, in a 25°C incubator on a 12/12-hr light/dark cycle.

Two vials for each line were set up carefully to result in low to moderate larval density. The temperature of the incubator was monitored cautiously for fluctuations, and vial position was randomized daily to reduce any edge effects. After eclosion and sclerotization, flies from each cross were then separated into mutant and wild type individuals—those with and without the p-element-induced mutations, respectively—

based on eye color and stored in 70% ethanol. A single wing from each fly was dissected and mounted in glycerol (see supplementary table 1B for sample sizes). Images of the wings were captured using a SPOT camera mounted on a Nikon Eclipse microscope. Landmarks (as shown in Figure 1) were digitized using tpsDig (v. 1.39, Rohlf 2003) software.

Our analysis necessitated that there be flies from each representative treatment group; those lines with flies missing (e.g. from one background or sex) were left out of the analysis. Of the original 50, 42 lines were ultimately used.

Analysis of Sexual Size Dimorphism (SSD)

Centroid size (i.e., the square root of the sum of the squared distances from each landmark to the centroid of the configuration) was used as the size variable in our analyses. Individual size values for male and female within each line and background were taken from the coefficients of a linear model where centroid size was modeled as a function of genotype, sex and their interaction.

SSD was then calculated based on a common index, wherein the dimorphism is represented as the proportion of female size to male size (Lovich & Gibbons 1992; Smith 1999):

$$SSD = \frac{Size_F}{Size_M} - 1$$

The resulting index represents the relative size difference between males and females where 0 indicates a complete lack of dimorphism and 1 indicates that females are 100% larger than males. Negative values represent male-biased dimorphism.

Analysis of Sexual Shape Dimorphism (SShD)

Generalized Procrustes Analysis (GPA) was used to super-impose landmark configurations after correcting for position and scaling each configuration by its centroid size. This procedure removes non-shape variation from the data—size, orientation and position. From the nine two dimensional landmarks, we are left with 14 dimensions of variation, and thus applied a Principal Components Analysis (PCA) to the Procrustes coordinates (i.e., the shape coordinates after GPA) and the first 14 PC scores were used as shape variables in subsequent shape analyses.

Two different shape scores were used in this study: one to examine sexual shape dimorphism and one to assess the strength of the allometric relationship of shape on size. First, SShD was estimated using the tangent approximation for Procrustes distance (i.e. Euclidian distance) between the average of male and female wing shape for a given treatment. Additionally, we calculated shape scores from the multivariate regression of shape onto size based on Drake & Klingenberg (2008). Specifically we projected the observed shape data onto the (unit) vector of regression coefficients from the aforementioned multivariate regression. We used these shape scores and regressed them onto centroid size to approximate allometric relationships. Confidence

intervals for SSD and SShD as well as allometric coefficients were generated with random non-parametric bootstraps, using 1000 iterations.

All significance testing for the analyses involving shape data was done with Randomized Residual Permutation Procedure (RRPP) as implemented in the *geomorph* library in R (Collyer et al. 2015). This method differs from the analyses in the original paper in two important ways. First, the linear model is based upon Procrustes distances, and second the resampling procedure more easily enables inferences within nested models (Collyer et al. 2015) with interaction terms. Specifically, this approach samples (without replacement), the residuals from the "simple" model under comparison, adding these to fitted values, and refitting under the "complex" model. We used the following models to assess the difference in shape dimorphism for each line and wild type background:

Model1: Shape ~ Sex + Genotype
Model2: Shape ~ Sex + Genotype + Sex:Genotype

We then performed such analysis for increasing degrees of interactions for the influence of sex, genotype, genetic background and size (for models of shape variables).

SShD was calculated with one of two methods: the advanced.procD.lm() function in the *geomorph* package (v.2.1.8) in R (v. 3.2.2) and standard Euclidean distances among treatment groups using the lm() function; both approaches yielded equivalent results. To

evaluate the mean shape difference caused by sex, we used linear models based upon Procrustes distance (with RRPP) to compare models where sex is and is not a predictor of shape using the procD.lm and advanced.procD.lm functions in *geomorph*. These analyses were randomized (by individual) and repeated 1000 times per treatment group to assess whether the magnitude of effect was greater than expected by chance.

Despite having separate and independent "control" (wild type) lineages for each cross (to control for any potential vial effects or residual segregating variation), we utilized a sequential Bonferroni correction to maintain our "experiment-wide" nominal alpha of 0.05. Given the large number of comparisons being made, it is likely that this will yield extremely conservative results, and we expect this underestimates the number of mutations that influence sexual dimorphism or mutational effects of allometry of shape on size.

Vector Correlations

While the above linear model assesses the magnitude of the effects, for shape it is also important to examine the direction of effects. Specifically, whether the mutations influenced the direction of SShD. To examine this, the vector of SShD was calculated within each genotypic group (wild type VS. mutant). We then estimated the vector correlation between the vectors of SShD for the wild type and mutant as follows:

$$r_{vc} = \frac{|SShD_{wt} \cdot SShD_{mt}|}{||SShD_{wt}|| \times ||SShD_{mt}||}$$

where the SShD for each genotype is equal to difference between the female and male vectors within each genotype. We used the absolute value of the numerator to avoid

arbitrary sign changes. The denominator consists of the product of the length (norm) of each vector. As with a Pearson correlation coefficient, a value of 0 corresponds to no correlation, while a value of 1 means that each vector is pointing in the same direction (even if they differ in magnitude). Approximate 95% confidence intervals were generated using a non-parametric bootstrap of the data for each line (The alpha used for the 95% CIs were not adjusted for the number of mutant alleles tested).

Statistical Analysis

All statistical analyses were conducted using R statistical software (version 3.2.2). Significance testing (specifically those involving RRPP) was conducted using functions within the geomorph package (v. 2.1.8) and with custom functions. All error bars are 95% Confidence intervals generated by non-parametric bootstraps. All scripts including custom functions are available on github (https://github.com/DworkinLab/TestaDworkin2016DGE).

Results

Different wild type strains vary for Sexual Size Dimorphism (SSD) and Sexual Shape Dimorphism (SShD) in wing morphology, for both direction and magnitude.

As each mutation was repeatedly backcrossed into two distinct wild type strains—Oregon-R (Ore) and Samarkand (Sam)—we first examined patterns of sexual size and shape dimorphism between these two strains.

We observed considerable, and highly significant, differences in both SSD and magnitude of SShD between the wild type strains (Figure 4.1A). Further, with respect to the vector of SShD, both wild-type backgrounds were somewhat divergent (Figure 4.1B). The computed vector correlation for SShD between both backgrounds falls within the same range as those calculated for SShD by genotype (0.937, 95% CI 0.92, -0.95), suggesting only subtle changes in direction. Additionally, the allometric relationship between shape and size differs between the two wild type backgrounds. While Ore has a stronger overall slope than Sam, the magnitude of both males and females slopes are reversed by background; for females, shape has stronger association with size relative to males in Ore (F 0.113, 95% CI 0.105, 0.122; M 0.099, 95% CI 0.091, 0.107), whereas the opposite is true for Sam (F 0.105, 95% CI 0.097, 0.113; M 0.120, 95% CI 0.112, 0.129). These differences in size, shape and allometry are all significant based on the randomized resampling permutation procedure (see methods).

Despite tight control of experimental variables (food, temperature) we observed a surprising amount of residual environmental variation for SSD and SShD among each replicate of the two wild type lineages. In the design of the experiment, where for each mutation, within each background, wild-type controls were generated from the cross that shared the environment (vials) with their otherwise co-isogenic mutant sibling. As all of these offspring across the vials are genetically co-isogenic, and only differ in the subtle aspects of rearing environment across vials, this allows us to assess some aspects of how environmental variation influences SSD and SShD. As shown in Figure 4.1A, in

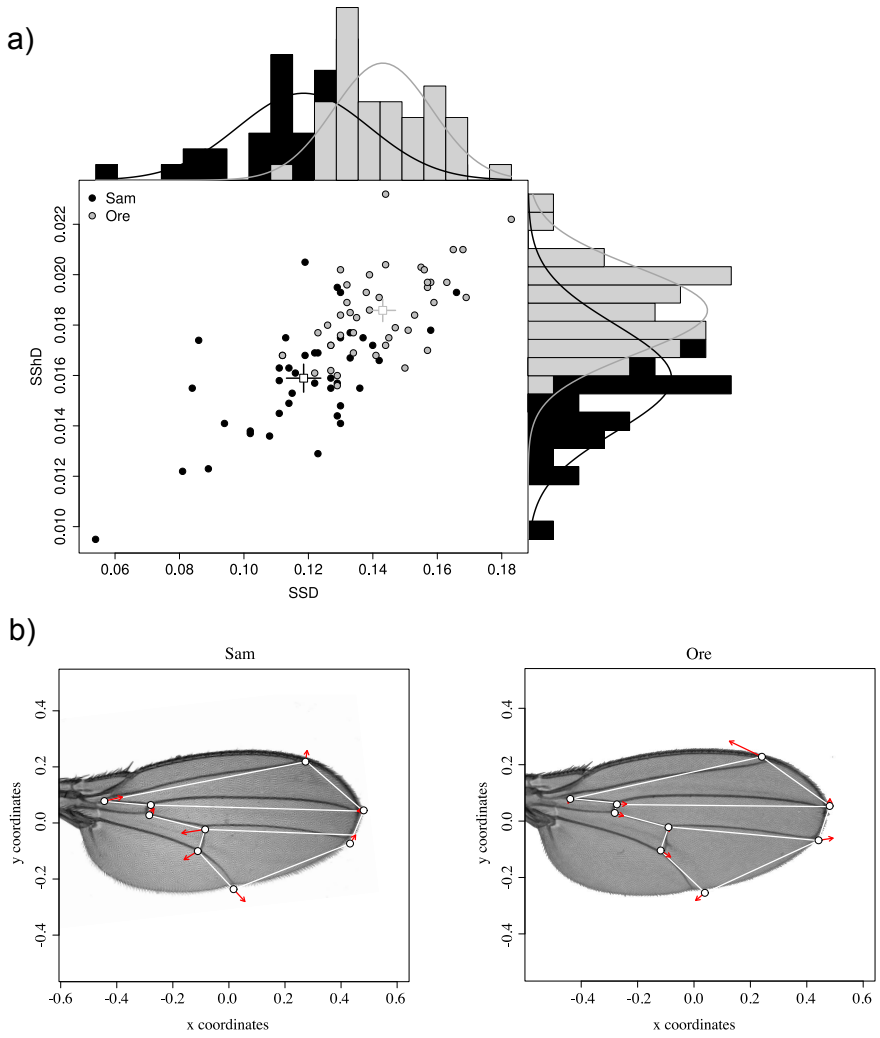

Figure 4.1. Natural variation in SSD and SShD for two wild type strains. A) SSD and SShD in both wild type background strains are represented three ways: scatterplot (center), SSD histogram with density curve (x-axis, top) and SShD histogram with density curve (y-axis, right). Data points represent mean value for wild-type siblings of each heterozygous mutant cross from a given vial. The Samarkand wild type background has a wider range of SSD, encompassing the low end of the spectrum, whereas Oregon-R tends to be more consistently large in SSD. B) Average direction of SShD in a typical Samarkand (left) and Oregon-R (right) wild type wing. Landmark coordinates are mapped onto a typical wing to demonstrate shape. Arrows represent the vector of shape change (magnified 5x) from female to male wing shapes.

addition to differences between the two wild type strains for SSD and SShD, there is also variation around the mean estimates for each. Since each data point in Figure 4.1A corresponds to each mutant's wild-type siblings from a given cross, these points largely reflect variation among "vial" effects. Indeed, models based on Procrustes distance suggest that there are significant vial effects (P = 0.009) and vial by sex (P = 0.001) even within the background control populations, which are largely attributable to micro-environmental variation. This is somewhat surprising as external sources of variation such as food (all from a common batch) and rearing temperature (all vials reared in a common incubator, with daily rotation of vials to minimize edge effects) were highly controlled in the experiment. This suggests that the magnitude of SSD and SShD for wing form is influenced by subtle environmental changes, suggesting that high levels of replication to control for these factors is generally necessary.

Despite many mutations having substantial effects on overall shape, a relatively small number influence SSD and SShD.

As demonstrated in the original study (Dworkin & Gibson 2006) and confirmed here, the vast majority of mutations have a significant influence on shape when measured in the heterozygous state (supplementary table 1). Of the subset of 42 mutations used in the current study (from the original 50), all but 10 had a significant effect for genotype (most surviving even a conservative Bonferroni correction) using the Residual permutation (Collyer et al. 2015). Of those 10, most had significant genotype-by-background effects, consistent with the earlier study (despite a different underlying inferential approach).

Despite this, only 18 of the mutations showed evidence for “significant” sex-limited genotypic effects (based on the sex-by-genotype effects), of which 2 survived sequential Bonferonni correction. Additionally, another 12 show evidence for significant effects of sex-by-genotype in combination with other factors in the model (size and/or background). Only one of these 12 survived correction for multiple comparisons. While inferences based on significance alone is quite limited (see below), these results suggest that only a small subset of mutations appear to have sex-limited influences on shape (Table 4.1).

To understand these results more fully, we next focused on the magnitudes of SShD and the SSD index, using non-parametric bootstraps to generate confidence intervals on our estimates. We performed the analyses separately for each wild type genetic background given that they can differ for both magnitude and direction of SShD. As shown in Figure 4.2, while several mutants show significant effects for either SSD, SShD or both in one or both of the backgrounds, the magnitudes of these effects are small, especially considering the relatively large amount of environmental variation in SSD and SShD observed within strains (Figure 4.1A). Interestingly, while only a few mutations showed evidence for an overall effect on size, these tend to have sex-limited effects (Figure 4.2).

In addition to examining the magnitude of effects, we also examined the direction of effects, and whether the mutations substantially changed the direction of SShD relative to their co-isogenic wild type. As shown in Figure 4.3, the mutations examined in this

Table 4.1. Summary table of significant effects by background among mutants. All significant values are taken from Figures 1-4. In the case of vector correlations, 80% was chosen arbitrarily to represent only a small subset of mutants of large effect.

Mutant	Allele	Pathway	Δ SSD	Δ SShD	Δ Vector Correlation	Δ Allometry
aos	W11	Egfr				
omb	md653	TGF-β	Ore	Sam, Ore	Sam, Ore	
cv-2	225-3	TGF-β				
GAP1	mip-w[+]	Egfr				
ksr	J5E2	Egfr				Sam
dad	J1E4	TGF-β	Ore	Ore		
drk	k02401	Egfr		Ore		
bs/DSRF	k07909	Egfr		Ore		Sam
s	k09530	Egfr				Sam
spi	s3547	Egfr				
mad	k00237	TGF-β				Ore
ed	k01102	Egfr				Sam
tsh	A3-2-66	TGF-β	Sam			
cos	k16101	Hh				
tkv	k19713	TGF-β				
babo	k16912	TGF-β/Hh				
trl	S2325	TGF-β				
rho-AP	BG00314	?	Sam		Sam	
pka-C1	BG02142	Hh				
sbb	BG01610	TGF-β				
psq	kg00811	Egfr	Ore			Ore
osa	kg03117	Chromatin Remodeling			Sam	
rasGAP	kg02382	Egfr				
pnt	kg04968	Egfr	Ore	Ore	Ore	

Table 4.1. (cont'd)

drk	k02401	Egfr				
cbl	kg03080	Egfr				
mam	kg02641	N/Egfr				
rho-6	kg05638	Egfr		Ore	Sam, Ore	
dpp	kg04600	TGF-β			Ore	Sam
pka-C3	kg00222	Hh			Sam	Sam
p38b	kg01337	TGF-β/Egfr				
tkv	kg01923	TGF-β				
wmd	kg07581	Unknown				
mad	kg00581	TGF-β				
ast	kg07563	Egfr				
dpp	kg08191	TGF-β				
rho1	kg01774	Egfr?				Sam
sax	kg07525	TGF-β				
*sax**	sax4	TGF-β	Sam	Sam	Sam	
egfr	k05115	Egfr		Sam, Ore	Ore	Sam
src42A	kg02515	Egfr				Ore
rho/stet	kg07115	Egfr				

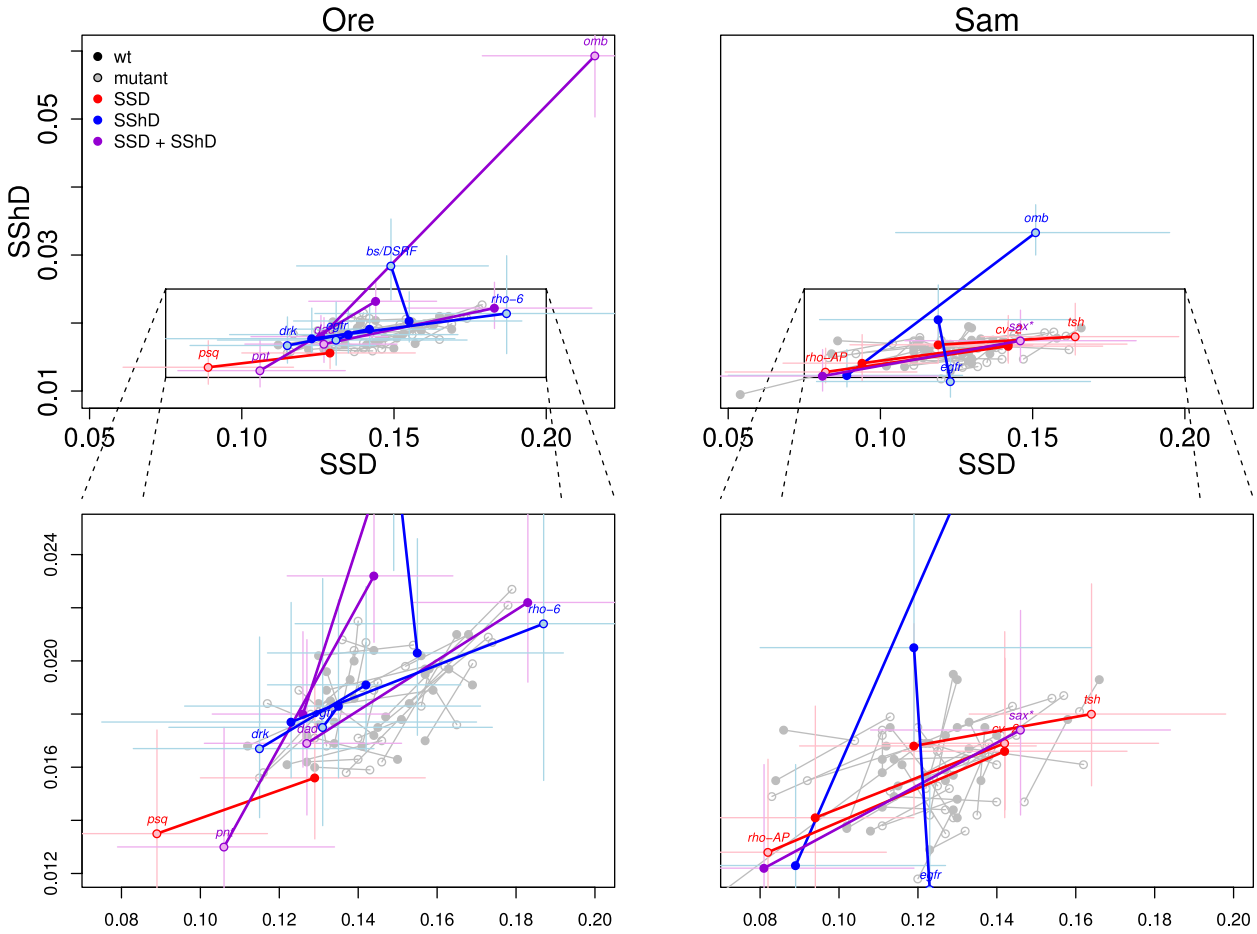

Figure 4.2. Magnitude of SSD and SShD for 42 mutants in Oregon-R (left) and Samarkand (right) wild type backgrounds. The effect of each mutant is mapped out in a size-and-shape dimorphism space. Genotypic means for each mutant are indicated by point style and connected by a solid line. SSD is plotted on each x-axis for all plots and SShD is displayed on the y-axis. The plots above display the entire range of variation observed, while those below display only the area with the highest density of points. Lines with significant sex-by-genotype effects are highlighted as follows: effect on both size and shape, shape only and size only. Only significant genes (after sequential Bonferroni correction) from the linear models are colored. Few mutations in this study alter sexual dimorphism of size or shape. In addition, the effect of mutations also appears to be highly background dependent, as only two lines, *Omb* and *Egfr*, were consistent in both backgrounds. Error bars are 95% confidence intervals (unadjusted alpha). All gene names are displayed lower-case, regardless of dominance.

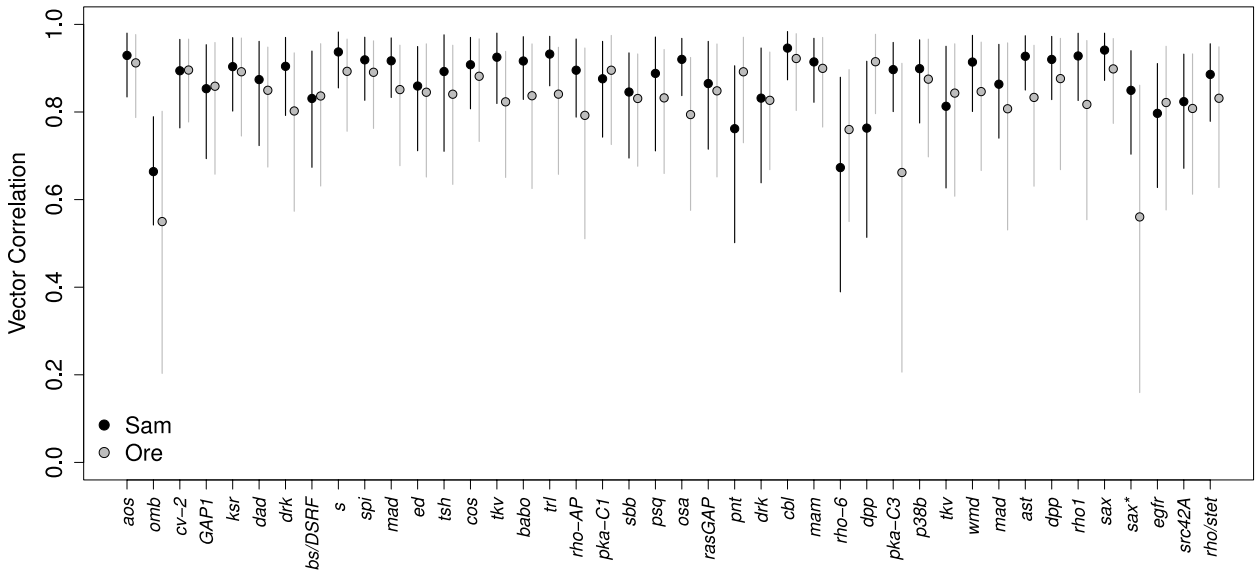

Figure 4.3. Vector correlations to assess similarity of direction for sexual shape dimorphism (mutant VS. wild type) by background. While genetic background appears to have little effect on the direction of SShD for most mutations, several stand out with more divergent directions of SShD. Those mutations with large background effects are also notable for their large effect on size and/or shape. Error bars are 95% confidence intervals (unadjusted alpha).

study generally do not substantially influence the direction of SShD, with several notable exceptions such as the mutation in the *Omb* gene, as well as more subtle effects from mutations such as *sax*, *pnt*, *drk* (among others). Even when the bootstrap confidence intervals do not approach 1, the estimated vector correlation are still generally greater than ~0.9, suggesting only modest changes in the direction of SShD.

Mutations do not substantially alter directions of SShD, nor patterns of allometry. One important aspect of assessing variation in shape, and in particular in situations where there is either (or both) SSD or SShD, is to account for the allometric effects of size on shape when computing the magnitude and direction of SShD. One important approach is to assume a common allometric relationship between size and shape across the sexes (after adjusting for mean differences in size and shape), and regressing out the effects of size. Then using either the residuals or predicted values of shape (after accounting for size) to compute an "allometry corrected" measure of SShD (Gidaszewski et al. 2009). To utilize such an approach requires that the assumption of a common allometric relationship be valid, as has been observed across Drosophila species for the wing shape and size relationship (Gidaszewski et al. 2009).

Prior to computing the allometry-corrected measure we examined this assumption among the mutations used in this study. Of the 42 independent mutations (with their independent controls), 13 had a significant interaction of sex-by-size on the influence of shape (with three surviving the sequential Bonferroni correction). Another eight of them had a sex-by-size interaction imbedded within a higher-order interaction term. Despite

this the overall magnitudes of effects and directions of allometric relationships appear to be highly similar, with a few important exceptions (Figure 4.4). Thus it is unclear whether using an allometry-free correction is warranted within the context of this study. It is worth noting that making the assumption of a shared allometric relationship, and computing the allometry-corrected measure of SShD did not substantially alter our findings (Supplementary Figure 4.1; Supplementary Table 4.2).

Discussion

While previously underappreciated, it is clear that mutations in genes in several growth factor pathways can act in a sex-specific manner. Of the 42 mutations analyzed, 12 had a significant sex-by-genotype interaction on size, shape or both (Figure 4.2). Only a few mutant alleles had the ability to affect the sexual dimorphism in allometry, the relationship between shape and size (Figure 4.4). Furthermore, nearly all of the mutants appear to act in a background-dependent manner, affecting shape or size in one genotype, but not the other (Figure 4.2).

Previous research has demonstrated the ability of growth pathways to respond to various perturbations, including: individual mutation (Palsson & Gibson 2004; Gao & Pan 2001; Tatar et al. 2001), genetic background (Chandler et al. 2013; Dworkin & Gibson 2006; Paaby & Rockman 2014) and environment (Ghosh et al. 2013; Shingleton et al. 2009; de Moed et al. 1997). Our results are unique in that they allow us to directly assess the effects of these perturbations on relative growth based on sex for both

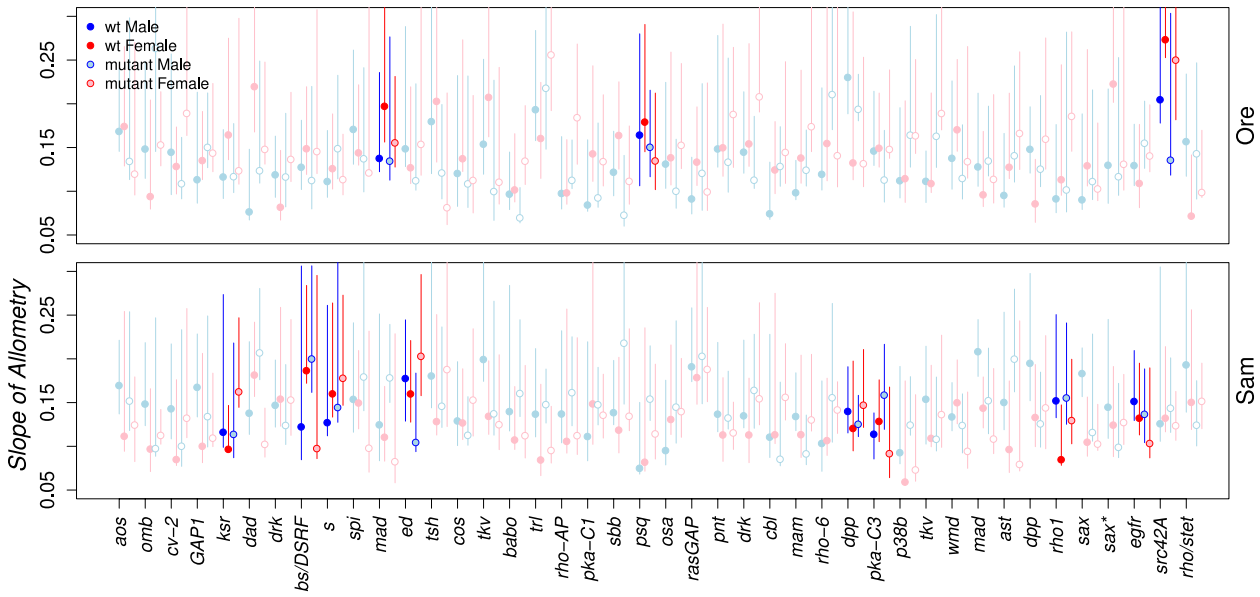

Figure 4.4. Variation in the magnitude of association between shape and size allometric coefficients among mutations in the Oregon-R (top) and Samarkand (bottom) wild type backgrounds. The “slope” of the allometric relationship for shape on size is displayed by sex and genotype. The magnitude of allometric effects appears to be relatively stable across strains, with few mutants substantially altering the wild type pattern of allometric co-variation. Individual lines whose mutants cause a significant sex-by-size interaction are represented dark in contrast to non-significant (faded) lines. Error bars are 95% confidence intervals (unadjusted alpha) for each individual treatment; significance is assessed based solely the interaction terms from the multivariate linear models.

direction and magnitude. Relative differences between male and female growth patterns due to these mutations are ultimately responsible for the generation of SSD and/or SShD.

The importance of multiple independent control lineages

As expected, different wild type strains vary in magnitude and direction of effects for SSD and SShD (Figure 4.1). The Oregon-R wild type background displays greater dimorphism in both size and shape compared to Sam. Implicit in our results is the understanding that genetic background itself has a profound effect on the underlying wild-type growth pathways and all of the downstream consequences this can have.

Somewhat more surprising is that both SSD and SShD appear quite environmentally sensitive (despite the genotypic effects being relatively insensitive based on our previous work). While great care was taken to reduce the effects of microclimactic variation, edge effects, nutritional variation and even genotypic variation, our results demonstrate that size and shape dimorphism remain highly variable (Figure 4.1).

There always remains the possibility that environmental variation does not entirely account for the wild-type variation observed. For each backcrossed line, a small amount of genetic information surrounding each p-element insertion site is unavoidable, especially during recombination in final cross with mutants and wild-types. This effect is somewhat unlikely, however, due to the fact that these recombination events are rare and affect only single measured individuals. Regardless, such a large amount of

variation in trait values within “isogenic” lines is unexpected. Most studies attribute any such variation within genetically (and environmentally) identical lines to stochastic variation in gene expression (Rea et al. 2005; Kirkwood et al. 2005; Raj & van Oudenaarden 2008). Such claims are, however, outside of the scope of our current study.

Rare sex-limited effects on wing form among mutations in EGFR and TGF-β signaling

While much is known about the development of wing size and shape (Shingleton et al. 2005; García-Bellido et al. 1994; Weinkove et al. 1999; Day & Lawrence 2000; Prober & Edgar 2000), comparatively little is known about the sex-specific effects of the genes involved (Horabin 2005; Abbott et al. 2010; Gidaszewski et al. 2009). While these mutations represent only a subset of the almost innumerable potential mutations within and among genes, they serve as a lens through which we can view the sex-limited effects of mutations. It is now clear that only a handful of genes associated with growth may be acting in a sex-dependent manner. Indeed, these results call for a further investigation of the formerly understudied sex-effects of growth pathways.

One such study confirms a link between many of the patterning mutations used in the current study and the development of SSD in the wing (Horabin 2005). In her 2005 paper, Horabin demonstrated that components of the sex-determination pathway (specifically, Sxl) were responsible for activating size-regulating genes within the Hedgehog signaling pathway. In fact, of the handful of genes to display sex-limited effects on SSD or SShD, a few were associated with this pathway, including: *Omb*, *dad*

and *Dpp* (Horabin 2005; Abu-Shaar & Mann 1998). This does not appear to be coincidence as these are the only mutants in this pathway that we utilized for this study. Since these mutants only represent a subset of those with sex-limiting effects, we cannot assign causality to this pathway. Instead, this demonstrates that sex-limiting effects of genes interact with more complexity than previously understood; no one pathway appears to be acting in a sex-dependent manner to generate shape/size.

Another candidate pathway involved in the generation of SSD is the Insulin and Insulin-like growth factor (IIS)/Target of Rapamycin (TOR) pathway. Evidence suggests that components of this pathway, such as *InR* (Testa et al. 2013; Shingleton et al. 2005) and foxo (Carreira et al. 2011) can contribute to SSD and/or SShD.

Further studies, such as Takahashi & Blanckenhorn (2015) have found that most mutations appear to decrease the SSD of wing form. Our data appear to yield an interesting trend for the direction of SSD based on genetic background. Ostensibly, growth-pathway mutants in the Ore wild type background tend to decrease SSD, whereas mutants that affect SSD in Sam tend to increase it. At this point it is impossible to say if this trend is biologically meaningful, but given that Ore has a greater underlying magnitude of SSD (and is already in conflict with Rensch's rule), these mutations may be interfering with genetic mechanisms influencing sexual dimorphism in the Ore background.

Our data is somewhat inconsistent with the findings of another previous mutation screen study, namely those of Carreira et al. (2011), wherein the authors found a much greater proportion of random insertion mutations appeared to have sex-specific effects on wing shape. The reasons for this are as of yet unclear, but may reflect methodology, magnitude of mutational effects used or that in the current study all mutations were limited to two signaling pathways. First, our methods allowed us to effectively tease apart the sex-limited interactions of sex for each genotype pair by plotting them in a size-shape space. Second, the authors used a different wild type genetic background than either that were used in this study (Canton-S). It is clear from this study and others (Dworkin & Gibson 2006; Chandler et al. 2013) that genetic background has an appreciable effect on gene function. At least part of the variation in the number of genes affecting wing SSD must necessarily be due to genetic background effects; however, genetic background effects cannot wholly account for the differences observed. Third, we cannot rule out the effects of dominance when discussing the effects of gene function. The genotype of flies in the study by Carreira et al. (2011) was homozygous for all mutants used. Their lines were chosen specifically for their non-lethal homozygous phenotype, whereas mutation used in our study were chosen irrespective of lethality. Because of this, our flies necessarily had to be heterozygous in order to avoid lethality associate with the homozygous phenotype. Perhaps not all loss-of-function mutants within our study were sufficient to alter the phenotype in a sex-limited manner. Finally, because our mutants were deliberately selected based on their association with wing shape morphogenesis, our results are not strictly comparable to those of Carreira et al. (2011).

Disentangling mutant-phenotype relationships

Our findings suggest that in most cases when a mutational analysis is performed to understand the genetic architecture of SSD or SShD, it is important to assess whether the mutation is only affecting SSD/SShD or whether it is instead demonstrating some degree of sex-biased influence. Many genes may therefore appear to alter SSD/SShD, but are instead only affected by sex as one of several variables of its expression. This may seem like an arbitrary distinction, but it is important if we are to fully understand the genetic underpinnings of complex phenotypes. Many mutants, such as those in the EGFR signaling pathway used here, are either lethal or at least partially ablate development of certain organs as homozygotes, indicating that these genes are necessary for the development of the organ itself. If heterozygotes have sex-specific effects on size or shape, we cannot necessarily conclude that this gene affects SSD or SShD, but rather that the gene is important for formation of an organ and has sex-dependent effects. Only in the case of genes such as *Maf1*, a gene that has been demonstrated to directly effect SSD in Drosophila (Rideout et al. 2012), can we conclude that said gene is affecting SSD and not simply acting in a sex-limited manner.

To fully understand the scope of SSD and SShD, one must precisely define what is meant by size and shape. While the definition of size is relatively straightforward to interpret, shape is somewhat more nuanced. For many organs, shape can essentially be broken down into the relative size of component parts of the larger structure (given that all aspects are homologous). For instance, during development in Drosophila there are multiple quadrants of the developing wing imaginal disc whose individual sections

may grow more or less in relation to the others, thus altering the “shape” of the wing. Mutant phenotypes may manifest as changes to large sections, such as a widening of the entire central portion of the wing (*Ptc*) or they may be subtler in effect, altering the placement of only a single crossvein (*cv-2*) (Dworkin & Gibson 2006). While these mutants may have local effects on size, such that they alter shape, what is less clear is whether these mutants are affecting size in a localized manner or the actual shape itself.

The effects of each pathway appear relatively consistent despite differences in genetic background. While mutations within the Egfr pathway tended to affect primarily SShD, those in the TGF-β pathway had a more mixed effect (more frequently affecting SSD). This pattern suggests that genetic background may only alter a mutation’s quantitative effect, rather than its qualitative effect.

Ultimately, our results demonstrate the importance of distinguishing between the relative contributions of each mutation to sexual dimorphism for shape, size or both. Of those mutants with sex-limited effects, even fewer exclusively affect either shape or size dimorphism (Figure 4.2). While some studies have been successful in artificially altering SSD of specific traits through selection (Bird & Schaffer 1972; Douglas J. Emlen et al. 2005; Reeve & Fairbairn 1996), it is unclear whether whole trait size or simply trait shape (e.g. length) has been altered. Our results demonstrate the need to exercise caution when discussing the effect of mutants on size or shape dimorphism.

Reassessing the assumption of common allometry

One important method for quantifying “shape” changes involves examining allometric relationships, specifically static allometry, which is the relationship among adult individuals between body size and organ size (Huxley 1932; Stern & Emlen 1999). In fact, one of the most obvious ways that males and females can differ is through differences in scaling relationships between body parts; these encompass some of the most obvious sources of variation in the natural world (Bonduriansky & Day 2003; Shingleton et al. 2009). By studying the relationship between two traits (e.g. body vs organ size), we can glean important information about the relative growth of traits and, therefore, the underlying mechanisms of differences in the growth of these traits. Consequently, allometry is an important tool for biologists to assess differences in size and shape dimorphism within (and across) a species. Our results support the claim for the importance of studying allometry by demonstrating that, while some mutants may have sex-limited effects on shape and/or size dimorphism (Figure 4.2), they do not necessarily affect the relationship between trait shape and size (Figure 4.4). Many mutants cause significant differences in sexual dimorphism of allometry, but do not necessarily alter SSD or SShD. These results may seem counterintuitive, but it is important to remember that, while changes in SSD or SShD may shift the direction of slope of allometry along one or more dimensions (in shape space), this does not necessarily alter the allometric slope itself (Frankino et al. 2005).

Since D'Arcy Thompson (1917) outlined his approach of how relative changes in body and organ size can be mapped out onto Cartesian coordinates to visualize relative

growth, the study of allometry and shape have been closely linked. Modern approaches use similar, albeit much more complicated methods to assess changes in relative landmark positions (Sanger et al. 2013; van der Linde & Houle 2009; Abbott et al. 2010). Ostensibly, one of the downfalls of shape analysis is that shape inherently carries information about its underlying relationship to size, despite the fact that geometric morphometric analyses partially separates it from shape (Gidaszewski et al. 2009; Mosimann 1970; Gould 1966; Nevill et al. 1995). More specifically, size itself is a measurement based on some aspect of shape. If size and shape do not scale isometrically (such that unit increase in size is accompanied by an equal increase in shape), then the underlying co-variation will be reflected in estimates of shape that are disproportionately affected by size (Mosimann 1970). This issue is implicit in the geometry of shapes themselves; as absolute size increases, surface area to volume ratios decrease (Gould 1966). This is particularly bad news for studies wishing to analyze induced changes in shape and size, because it means that the degree of independence between these two variables may be difficult to infer. However, by plotting size on shape and using the residuals from this model, Gidaszewski et al. (2009) were able to effectively eliminate the issue of non-independence with size and shape. These residuals represent the total variation in shape that is not due to allometric effects of size.

Allometric patterns of variation across sex and genotype are necessarily more complicated. While it is known that shape (and shape dimorphism) is strongly influenced by its relationship with size, it is not always clear that the assumption of a common

allometric relationship across sexes is met. Previous studies examining patterns of SSD and SShD (Gidaszewski et al. 2009) generally made the assumption of a common allometric relationship between males and females within each Drosophila species. This was despite their analysis suggesting that this assumption may not hold for all species. For the data we examined here, we could reject this assumption based on inferences based on statistical significance. Yet, it is clear that the magnitude of such differences were small, and allometric relationships were similar in most cases. Indeed, the allometric influence of size on shape appears to be largely consistent with respect to direction of effects, with a few notable exceptions (Figure 4.4). Regardless, we erred on the side of caution with this matter and decided to eschew analysis of SSD and SShD under assumption of common allometry. It is worth noting that the assumption of common allometry did not substantially alter the observed results (Supplementary Figure 4.1). As with other studies, we suggest that a rejection of this assumption simply based upon significance may not be optimal, and future work should determine what the consequences of making such assumptions might be for studies of sexual dimorphism and allometry.

Our results clearly demonstrate the effects of growth pathway mutants on SSD and SShD. Most notably, we cannot rule out the sex-specific effects of any genes involved in growth. Our results demonstrate the current lack of understanding of how growth-related genes interact with the sex of the individual. By visualizing the effects of each mutation within the framework of size/shape space we gain a previously unrealized understanding of the role each mutant plays in generating a sex's phenotype. While this

method is especially powerful for studying sexual dimorphism, its applications are not restricted to it. We therefore present this method as a means for dissecting the contributions of mutants to the development of size and shape.

CHAPTER 5:

The Contribution of Candidate Genes to sexual size and shape dimorphism

Introduction

In many species males and females can differ from one another in virtually all measurable traits including morphology, behavior, physiology, and life history. These differences are sometimes so distinct that individuals can be unambiguously assigned with minimal effort. Such qualitative distinctions between the sexes stem from the fact that males and females usually differ genetically (Dawkins & Krebs 1979; Mank 2009) and also contribute unequally to their gametes (Trivers 1972; Bateman 1948; De Lisle & Rowe 2015; Fairbairn 2005; Mank 2009). As each sex becomes adapted to its role as a large (female) or small (male) gamete contributor, specializations may arise that lead to the separation and definition of each sex.

Because both sexes still share the majority of a genome, most phenotypic differences between them must arise largely from sex-specific gene regulation rather than sex-specific genes. While in some (but not all) species, males and females differ genetically by their sex chromosomes, but these make up a relatively small portion of their respective genomes (Mank 2009). Despite a largely shared genome, this is not generally considered an impediment to prevent sexual dimorphism from evolving when there are sex-specific fitness optima. This is clear from the many examples of sexual dimorphism are exceedingly abundant in nature (Bininda-Edmonds & Gittleman 2000; Norman et al. 2002; Pietsch 2005; Badyaev 2002; Blanckenhorn et al. 2007; Huey et al. 2006). If males and females share most of a genome and sexual dimorphism is so

abundant, then phenotypic variation must therefore be due to *how* genes are being expressed, rather than *which genes* are being expressed.

Perhaps one of the most fundamental (in terms of its performance) phenotypic "traits" of an organism is its size. Like many other traits, size can be regulated to produce sex-specific means. Indeed, SSD has been the subject of possibly hundreds or thousands of studies, as well as many reviews (Lovich & Gibbons 1992; Allen et al. 2011; Fairbairn 1997b; Chown & Gaston 2010; Blanckenhorn 2005; Smith 1999; Badyaev 2002; Stillwell et al. 2010) and a dedicated edited volume (Fairbairn & Blanckenhorn 2007). However, most such studies are either descriptive with respect to SSD, or are studying them in the context of evolutionary, behavioral, or physiological questions. Few of these studies have investigated the proximate mechanisms that govern SSD (but see Cox et al. 2009; Tammaru et al. 2009; Zhao & Liu 2014; Esperk et al. 2007; Stillwell & Davidowitz 2010b). On the other hand, a substantial body of research has been dedicated to the study of size control, especially with respect to developmental genetics (Carreira et al. 2011; Oldham et al. 2000; Gokhale & Shingleton 2015; Mirth & Riddiford 2007; Nijhout 2003; Edgar 2006). In Chapter 3, I discussed in detail a number of growth-related candidate pathways and their effect on growth. We now know that both nutrients and nutrient-sensitive growth pathways are necessary for the proper development of sexual size dimorphism (SSD).

However, one must consider what is really meant by "size". After all, an organism is more than a sum of its parts. Importantly we can consider the relative sizes of different

organs in an individual, as these do not necessarily scale isometrically with one another, and may differ among the sexes (Bonduriansky 2007a; Kodric-Brown et al. 2006; Bonduriansky & Day 2003; Gould 1966). Thus the shape of an individual or shape of organs within the individual may be just as important to consider as overall size. While there have been many ways of defining shape in the biological literature (see chapters 1, 4 for some overview), a commonly accepted approach is to define shape as the residual variation of homologous structures once the effects of location, scale, and rotation have been removed. In this definition, shape itself is a single geometric representation of a multi-dimensional trait, allowing for relatively intuitive descriptors across biological groups. Sex-specific variation in shape is important, because it allows for the development of more complex phenotypes than size. Importantly, there is substantial evidence of SShD (Dworkin & Gibson 2006; Gidaszewski et al. 2009; Sanger et al. 2013; Pitchers et al. 2013; Cheng & Kuntner 2015; Shine 1989; Valenzuela et al. 2004; Collyer et al. 2015) including species that do not necessarily differ in SSD (Schwarzkopf 2005). In Chapter 4, I demonstrated the extent to which certain wild type and mutant strains of *Drosophila melanogaster* wings can vary by size and shape. Indeed, in fruit flies alone, we know that proper wing shape is essential for predator avoidance (DeNieu et al. 2014), adaptation to various environmental conditions (Pitchers et al. 2013; Trotta et al. 2010), and courtship (Menezes et al. 2013; Hoikkala et al. 1998; Abbott et al. 2010). Because of its strong influence on fitness and the fact that shape and size covary considerably within and between species (Mitteroecker et al. 2004; Klingenberg 2016; Testa & Dworkin 2016; Gidaszewski et al. 2009), a more

complete understanding of the relationship between SSD and SShD is necessary, in particular with regards to whether they share common mechanisms that regulate them.

In Chapter 3, I discussed in detail a number of growth-related candidate pathways and their effect on overall growth and SSD. We now have some idea of how these candidate genes function to regulate size (Horabin 2005; Rideout et al. 2015; Mirth & Riddiford 2007; Shingleton et al. 2005; Stocker et al. 2003; Caldwell et al. 2005) and some insights into whether they influence sexual size dimorphism (chapter 4, Böhni et al., 1999; Rideout et al., 2015). Chapter 4 demonstrated how some genes in growth-related signaling pathways (EGFR and TGF-ß) might vary with respect to influencing size and shape dimorphism. Chapter 3 suggests that TOR signaling may potentially be both necessary for and sufficient for whole-body SSD, but what we don't know is the relative contribution of these pathways to organ (wing) size and shape. While we understand a lot about these sex-specific interactions, we still cannot point to any specific gene as being both necessary nor sufficient for SShD, despite a small set of studies examining this (Testa & Dworkin 2016; Abbott et al. 2010; Carreira et al. 2011).

Sex-biased gene expression itself is largely attributed to the sex-specific splicing of common genes. For instance, the core of the sex determination pathway can be summarized by the sex-specific splicing of three major genes: sex-lethal (*Sxl*), transformer (*tra*), and double-sex (*dsx*) (Fear et al. 2015). As a result of the sex-specific sex:autosome ratio, an active (female) or inactive (male) *Sxl* protein will ultimately be produced. Since male *Sxl* protein is non-functional, it does not properly splice *tra*,

resulting in a male *tra* splice variant that is also inactive. Flies that are mutant at the *tra* locus have been demonstrated to develop as normal males (Sturtevant 1945). It is precisely this inactivity though, that allows for male-specific splicing of the active male isoform of *dsx*, which then helps to regulate the development of a suite of male-specific phenotypes including pigmentation (Williams et al. 2008), sex combs (Tanaka et al. 2011), and the anal lobe (Glassford et al. 2015). In this way, genes that exist in both sexes can be activated or deactivated spatially and temporally in order to produce sex-specific phenotypes.

While we have a fairly complex understanding of the effects of downstream components of the sex determination pathway (Fear et al. 2015), we know very little about the specific effects of those near the top. For instance, we know that genes like transformer (*tra*) act to determine male vs female form (Sturtevant 1945; McKeown et al. 1988; Verhulst et al. 2010; Belote & Baker 1982) for discrete traits, but what is still unknown is at what level sex-specific size and shape are determined. By shape, we refer not to the primary sexual characteristics, but rather to the secondary. Both sexes have wings, yet we know that males and females have substantial dimorphism for both wing size and shape (Testa & Dworkin 2016; Sonnenschein et al. 2015; Gidaszewski et al. 2009; Abbott et al. 2010). Recent evidence has suggested that SSD is mediated in part by *tra* (Rideout et al. 2015); however, it is unclear whether *tra* of other components of the sex determination pathway may also influence SShD.

To gain some insight into the relationship of the proximate mechanisms of SSD and SShD, I examined several growth pathways in tandem with the sex determination pathway to identify genes that modulate SSD, SShD, or both. We use the same candidate pathways discussed previously. We know that some pathways (IIS, Tor, and SDP) are necessary for SSD and some are sufficient (Tor), but how these pathways affect SSD and SShD in the wing are as of yet unknown. Specifically, my hypothesis is that SSD and SShD are being regulated by unequal sexual responses to growth pathway genes. If any candidate genes within these pathways are, in fact, involved in some combination of SSD or SShD regulation, then genetic manipulations should reveal their effects. Fortunately, the statistical tools available with geometric morphometrics will allow for the very precise quantification of size and shape. By quantifying SSD and SShD and examining their relationship to one another, we can investigate the contribution of each gene to SSD, SShD, or both. If SSD and SShD are indeed regulated by the same gene, *tra*, then we would expect these data to reflect that. Other candidate pathways would be expected to reveal similar patterns of SSD vs SShD if they are responsible. Our results suggest a general reluctance for candidate genes to decrease SSD or SShD, which differs considerably from previous studies (Takahashi & Blanckenhorn 2015; Carreira et al. 2009; Testa & Dworkin 2016). Regardless, our candidate pathways have demonstrated that control of SSD and SShD are only partially correlated to one another, and the results suggest that wing and overall body size SSD are potentially not mediated by similar mechanisms.

Materials and Methods

Fly strains and Maintenance

All strains were chosen based on their ability to over-express or knockdown specific predetermined candidate genes. With few exceptions, all flies were obtained from the Bloomington Stock Center. See Chapter 3 for the list of strains used. Driver lines consisted of Actin5c-GeneSwitch-Gal4 (see Chapter 3), A9-Gal4, and Bx-Gal4 (see discussion). Flies were maintained on standard cornmeal-molasses food medium at either 24˚C or 18˚C on a 12/12 hour light/dark cycle.

Each over-expression line was introgressed into the common wild-type background, Samarkand (Sam), for seven generations to avoid background effects. We performed introgressions by backcrossing red-eyed females (carrying the insertion element) to males of the Sam background. Since knockdown lines utilized TRiP insertional elements—all of which are already in a common background, “yellow, vermillion” (y,v)—we did not introgress these into Sam. While some of the TRiP insertion lines also are segregating the X-linked *scute* allele, this was not the case for any of the lines except *Sxl* and *InR*.

For the experiments discussed in this chapter, we used wings from the flies in Chapter 3 where gene manipulation occurred organism wide, in addition to a suite of new genetic crosses using the wing-specific Gal4 lines so that the gene expression was only modulated in the wing.

Modulating gene expression using the Gal4 sensitivity to temperature

The Gal4-UAS system is known to be temperature-sensitive (Duffy 2002), with increasing Gal4 activity at warmer temperatures. As such, I generated all crosses at two temperatures: a high temperature (24˚C) to drive high levels of expression and a low temperature (18.5˚C) to drive lower levels of expression. These temperature treatments were replicated for both *A9* and *Bx* drivers; since gene expression in GeneSwitch-crossed flies was already modulated, they were not included in the low-temperature replicate. Using this system, we were able to drive the overexpression and/or knockdown of genes that would otherwise have ablated the wings at higher temperatures.

It is worth noting that, while GeneSwitch crosses were expected to drive expression in all wing tissues, A9 and Bx are only known to drive expression in the dorsal pouch of the wing imaginal disc. Manipulations in the dorsal pouch will therefore not necessarily affect the ventral portion of the wing disc in an identical manner. In the case where the growth of wing imaginal discs are perturbed, particularly by nutrient-sensitive growth pathway manipulation, growth is coordinated within (and among) other developing organs (Gokhale et al. 2016; Stieper et al. 2008). Wing discs therefore respond to spatially restricted perturbations by coordinating aspects of wing growth. In this way, perturbations solely in the dorsal pouch are expected to affect both wing size and shape. However, it remains that for individual genes (whose effects are entirely cell autonomous) that the effects could be limited to the dorsal portion of the wing. However, such effects should generate a telltale phenotypic effect of “bending” or “delaminating”

of the adult wing (as one layer differs in size from the other), were rarely observed in these crosses (table 5.1). Furthermore, in many RNAi knockdowns, the whole wing (and not just the dorsal layer) was ablated.

Titrating gene expression with GeneSwitch

See Chapter 3 for full protocol.

Experimental Fly Crosses

All crosses were maintained at 24˚C or 18.5˚C with a 12/12 hour light/dark schedule. For all experimental treatments, we crossed four virgin females carrying a wing driver (both drivers are X-linked) with male flies of the appropriate treatment (i.e. target genes whose expression was to be modulated). We used the following three drivers to driver expression into the wing: A9, Beadex (Bx), and Actin5C-GeneSwitch (GeneSwitch). The first two, A9 and Bx, were wing-specific, only driving expression in the wing imaginal disc. The third driver, GeneSwitch, was a ubiquitous driver (see Chapter 3 for full details).

Parental flies were allowed to oviposit on the food medium at their respective temperature treatment, allowing their progeny to be raised completely in the proper temperature. All vials were monitored for egg density to ensure that no vials were under-crowded (20 eggs) or over-crowded (50 eggs). Once adults had exclosed and wings sclerotized (based on the presence of a fully formed adult wing), flies were collected and placed together into 1.5ml Eppendorf tubes for storage per treatment

Table 5.1. List of wings displaying aberrant phenotype and their relative frequencies.

driver	response	temperature (˚C)	aberrant phenotype	relative frequency
A9	Akt-RNAi	24	blistered	common
A9	Akt-RNAi	24	ablation	rare
A9	Ci	18	ablation	full
A9	Ci-RNAi	24	vein defects	common
A9	Ci-RNAi	24	blistered	rare
A9	fru	24	crumpled	rare
A9	InR	18	vein defects	uncommon
A9	InR	24	vein defects	common
A9	InR-RNAi	18	vein defects	common
A9	InR-RNAi	24	vein defects	uncommon
A9	Ptc-RNAi	24	vein defects	common
A9	Sxl-RNAi	18	vein defects	full (female)
A9	Tor[TED]	24	partial-full ablation	near full
A9	Tor[wt]	24	small wing	common (female)
A9	Tor[wt]	24	ablation	uncommon (female); full (male)
Bx	Akt	18	crossveinless	uncommon
Bx	Akt-RNAi	18	ablation	full
Bx	Ci-RNAi	18	vein defects	full
Bx	Ci-RNAi	18	blistered	uncommon
Bx	Ci-RNAi	24	blistered	common

Table 5.1. (cont'd)

Bx	dsx-RNAi	18	crossveinless	common
Bx	Foxo-RNAi	18	crossveinless	common
Bx	Foxo-RNAi	24	vein defects	common
Bx	Foxo-RNAi	24	crossveinless	uncommon
Bx	fru-RNAi	18	crossveinless	common
Bx	InR	18	vein defects	uncommon
Bx	InR	18	crossveinless	uncommon (female); near full (male)
Bx	InR-RNAi	18	crossveinless	uncommon
Bx	InR-RNAi	24	vein defects	common
Bx	InR-RNAi	24	crossveinless	uncommon
Bx	ptc	18	crossveinless	rare
Bx	ptc-RNAi	18	vein defects	full
Bx	ptc-RNAi	18 & 24	crossveinless	common
Bx	Ras	18	crossveinless	common
Bx	Ras-RNAi	18 & 24	blistered	common (female)
Bx	Ras-RNAi	18 & 24	partial-full ablation	full (male)
Bx	S6K	18 & 24	crossveinless	common
Bx	Sxl-RNAi	18	vein defects	full (female)
Bx	Tor-RNAi	18 & 24	crossveinless	common (female); full (male)
Bx	Tor[wt]	18	ablation/blistered	full (male)
Bx	tra.F	18	crossveinless	common
Bx	tra-RNAi	18	crossveinless	common

group. Each tube was filled ~1ml of 70% ethanol to preserve all flies. Adult flies were dissected in a phosphate buffered solution and mounted on slides in a solution of 70% glycerol and 30% PBS. Wings were taken from the right side of each fly for consistency and mounted on labeled slides.

Imaging and data acquisition

Once all wings were dissected, slides were then imaged using an Olympus BX51 microscope. Resulting wing images were cropped and resized for processing based on the software requirements of tpsDig (version 1.39; Rohlf 2003) and Wings (version 3.72; Houle et al. 2003). Using tpsDig, two reference landmarks were placed on each wing so that Wings could summarize each wing's shape with a series of splines. Wing spline data were then read into CPReader (version 1.12r; CPR 2015) and converted to a series of landmarks and sliding semi-landmarks. Since many treatments resulted in wings with aberrant crossvein phenotypes, crossvein landmarks were intentionally omitted from these analyses (Table 5.1). CPReader allowed us to calculate shape coordinates from the raw landmarks by using a Generalized Procrustes Analysis to remove the effects of rotation, while location, and scale are removed beforehand. Centroid size (i.e., the square root of the sum of the squared distances from each landmark to the centroid of the configuration) was then calculated and scaled based on original wing size (accounting for any changes due to cropping and resizing images for data acquisition).

Morphometric Analysis

While the scaling of the landmark configurations by their centroid size removes any isometric effects, the allometric component of size on shape remains. As has been shown previously (Gidaszewski et al. 2009; Pitchers et al. 2013; Debat et al. 2016) the allometric component of shape in Drosophila wings can be considerable and needs to be taken into account for studies of sexual dimorphism. In order to account for differences in allometric slopes within groups, we removed the effect of allometric (size vs shape) scaling on wing shapes for each. For each treatment group (separated by driver, response, and temperature), we fit a multivariate linear regression of shape on size and used the resulting shape residuals as our new shape coordinates. These values correspond to variation in shape that is ideally uncorrelated with variation in size. (Klingenberg, 2016; and references therein). Unless otherwise differentiated, all references to SShD will be of the allometry-corrected rather than uncorrected values.

Analysis of SSD and SShD

Both SSD and SShD were estimated and statistical inferences made using the same approaches as detailed in Chapter 4.

All statistical analyses were conducted using R statistical software (version 3.2.2). Significance testing (specifically those utilizing RPPP) was conducted using functions with the *geomorph* package (v. 2.1.8) and with custom functions (see chapter 4). All error bars are 95% confidence intervals generated by non-parametric bootstraps.

Specific models differed slightly from Chapter 4; we used the following models to assess the difference in shape dimorphism for each driver, response, temperature, and dose (where “genotype” corresponds to control vs treatment):

Model 1: shape~sex + genotype

Model 2: shape~sex + genotype + sex:genotype

Results

Of the candidate gene pathways tested, few yielded significant differences in SShD; most either increased or decreased SSD (Figure 5.4). Surprisingly, and inconsistent with the previous literature, none of the crosses resulted in a decrease of SShD. Several genes did modulate SShD. When *Sxl* (A9 and Bx crosses at 18˚C; 24˚C were unviable), *InR* (Bx at 18˚C and GeneSwitch at 24˚C crosses), and *Ci* (A9 and GeneSwitch crosses at 24˚C) are knocked down, SShD increases. In all cases, increased SShD appears to be due to disproportionate response in female shape vs male shape (observation not quantified; Figure 5.1). For genes like *Sxl* and *InR*, knockdowns are usually accompanied by a decrease in SSD and increase in SShD. Since *Ci* knockdowns only consistently affect SShD and not SSD, it is a much better candidate for necessity of SShD.

Obviously, the effect of each treatment on size and shape in males and females was not always equal, suggesting sex-limited effects. Figure 5.3 demonstrates how male and female size (top) and shape (bottom) covary with one another for sex determination

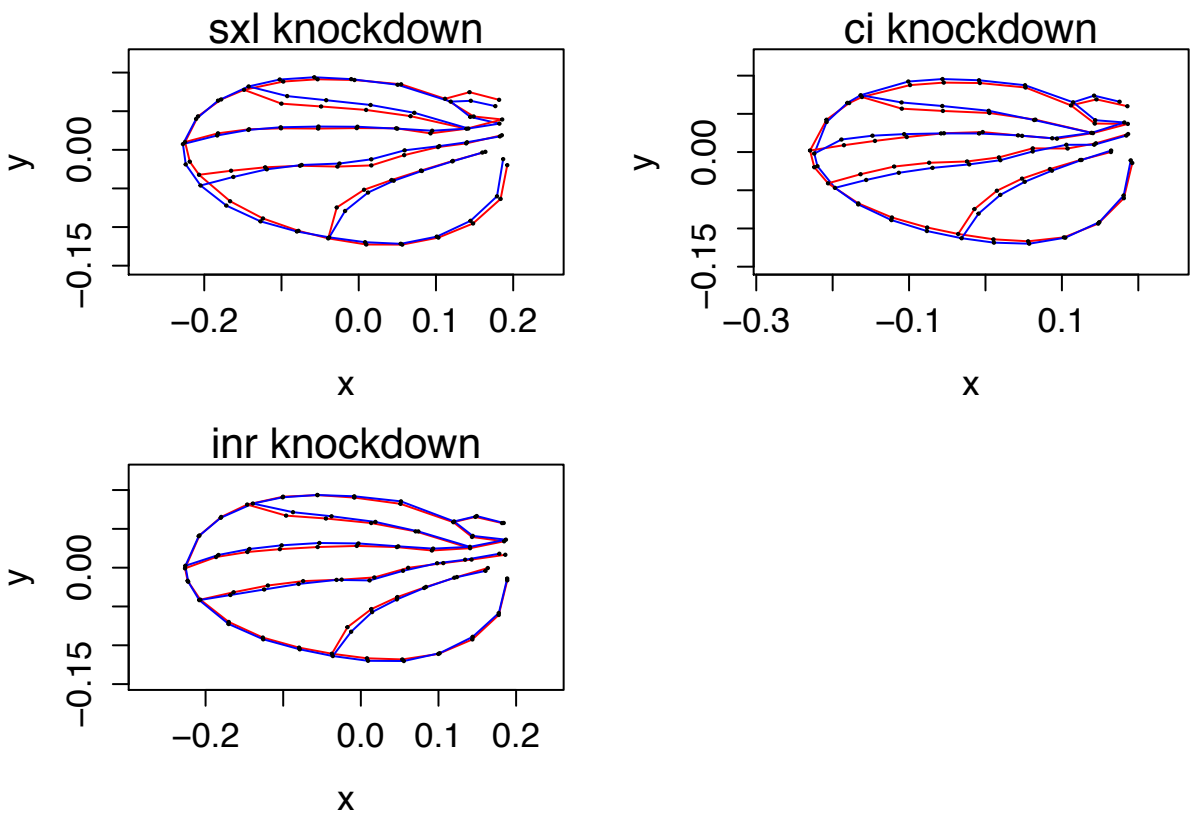

Figure 5.1. Sexual Shape Dimorphism in knockdowns that caused an increase in SShD. Average direction of SShD in a Sxl knockdown (top left), Ci knockdown (top right), and InR knockdown (bottom left) wings. Landmark coordinates are connected to display typical wing veins and outlines to demonstrate shape. Colors represent both female (red) and male (blue) shapes (magnified 1x) from female to male wing shapes. Knockdowns that increase SShD tend to do so by causing an exaggerated female response to shape vs males.

pathway treatments. The resulting male/female scaling relationships were not always isometric, thus we corrected SShD values to remove the effects of allometry (see methods for details).

After correcting SShD values for allometric scaling effects, we noticed a negative trend between SSD and SShD (Figure 5.2). Before correction, however, this trend did not exist, suggesting that allometry-free values of SShD may be an artifact of rescaling the size/shape relationship. Since overall size and SSD are uncorrelated, their responses to SShD and allometry-corrected SShD tend to covary similarly. Figure 5.2 allows us to visualize the all such relationships between all treatments in the context of SSD, SShD, Allometry-free SShD, and Size (due to unequal scaling relationships; Figure 5.3).

Of those crosses that had an effect on SSD, most perturbations tended to increase SSD rather than decrease it (Figure 5.4). A total of 20 treatments significantly increased SSD, while only 11 significantly reduced it. We also found a driver-specific bias in those crosses that increased SSD. Seven of the overexpression crosses significantly increased SSD, but only two of these were non-Bx mediated. Results from knockdown crosses were even more dramatic. In fact, nine of the 11 total knockdowns that increased SSD were driven by Bx-Gal4. Because Bx-Gal4 is X-linked and also acts as a hypomorph, these results were not unexpected (see discussion for rationale).

Within the sex determination pathway, only *Sxl* and *tra* stand out. *Sxl* knockdowns produce flies with much smaller SSD and larger SShD, whereas overexpression of the

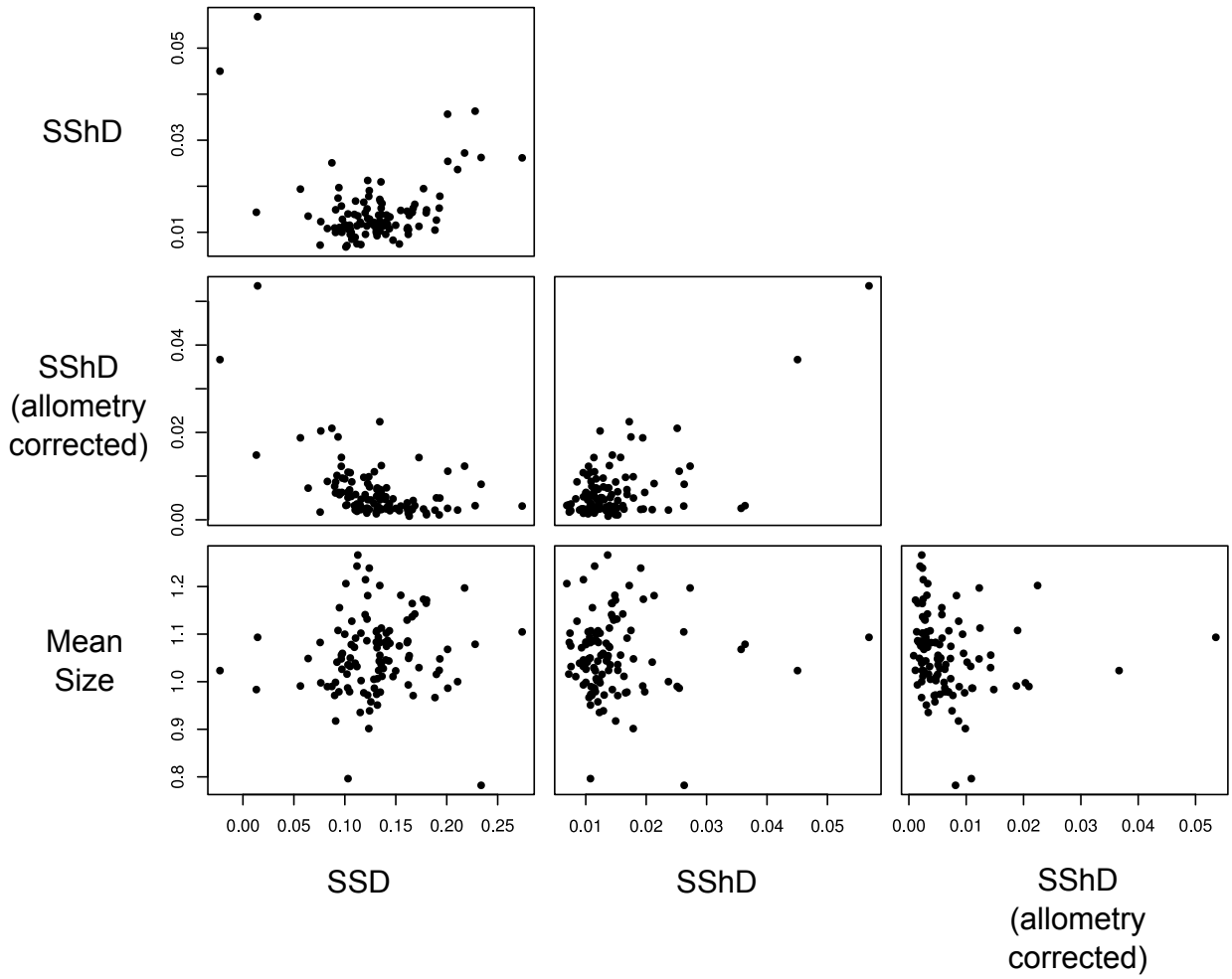

Figure 5.2. Pairwise comparision between SSD (first row/column), unmodified SShD (second row/column), allometry corrected SShD (third row/column), and Mean Size for males and females (fourth row/column). In allometry-corrected plots, there tends to be a negative relationship between SShD and both SSD and Mean Size. Uncorrected SShD does not share similar trends, but remains somewhat uncorrelated.

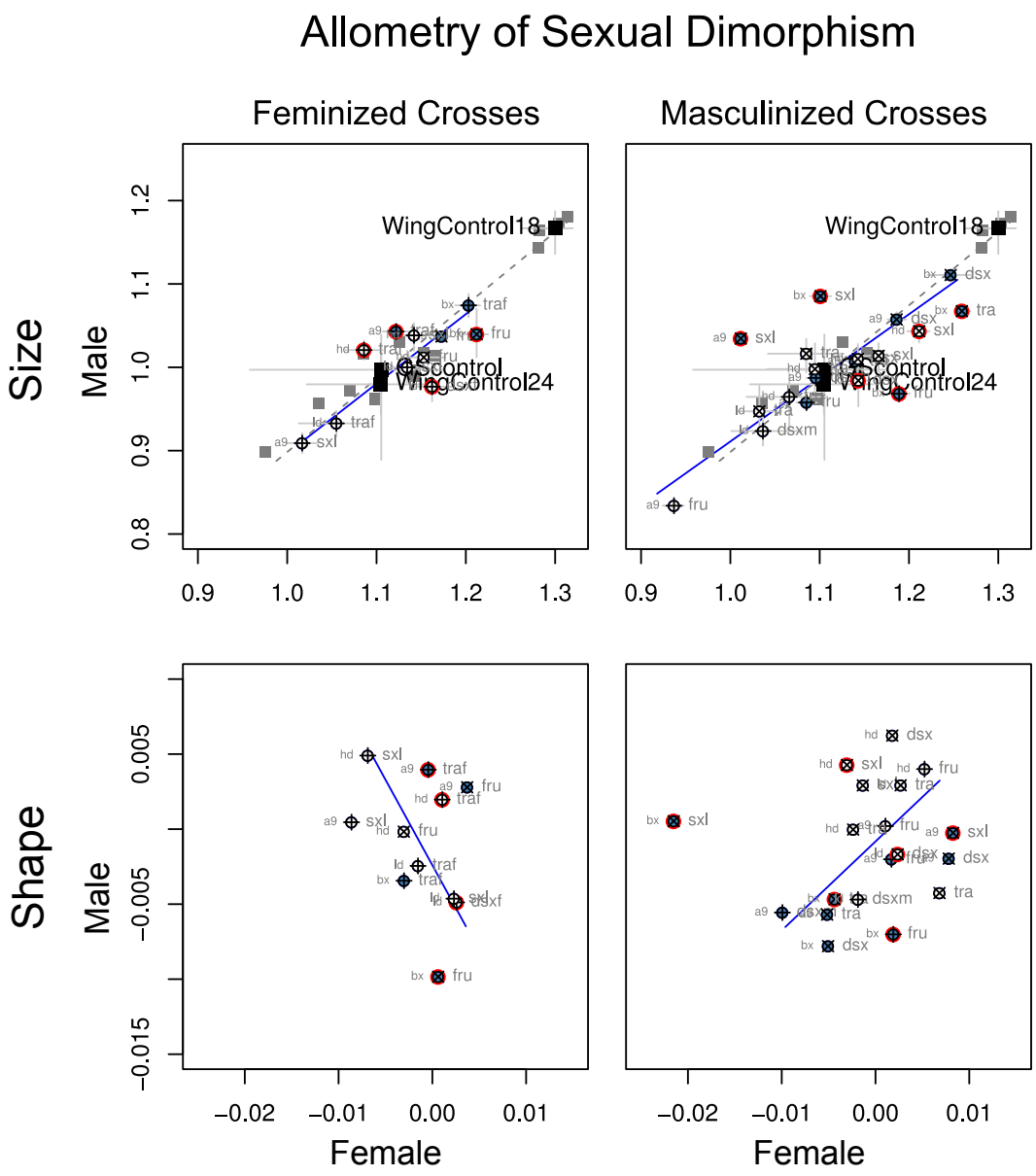

Figure 5.3. The allometry of SSD (above) and SShD (below) for feminized (left) and masculinized (right) crosses. Female scores are plotted on the x-axis, while male scores are on the y-axis. Centroid size was used as the size variable for SSD plots. Shape scores generated using methods outlined in Drake and Klingenberg (2008) were used to represent male and female shape. We recognize that there are issues with estimates of shape calculated this way (that they correlate with size); however, this method remains a valuable tool for summarizing overall shape. Red point outlines represent significant outliers from the Standardized Major-Axis Regression (sma), whose line is represented as blue. A dotted gray line in the SSD plots corresponds to the control group sma line. *dsx* was included in "feminized" crosses arbitrarily due to the

Figure 5.3. (cont'd)

fact that other sex determination pathway knockdowns were expected to cause masculinization, despite the fact that *dsx* knockdown was expected to affect both males and females.

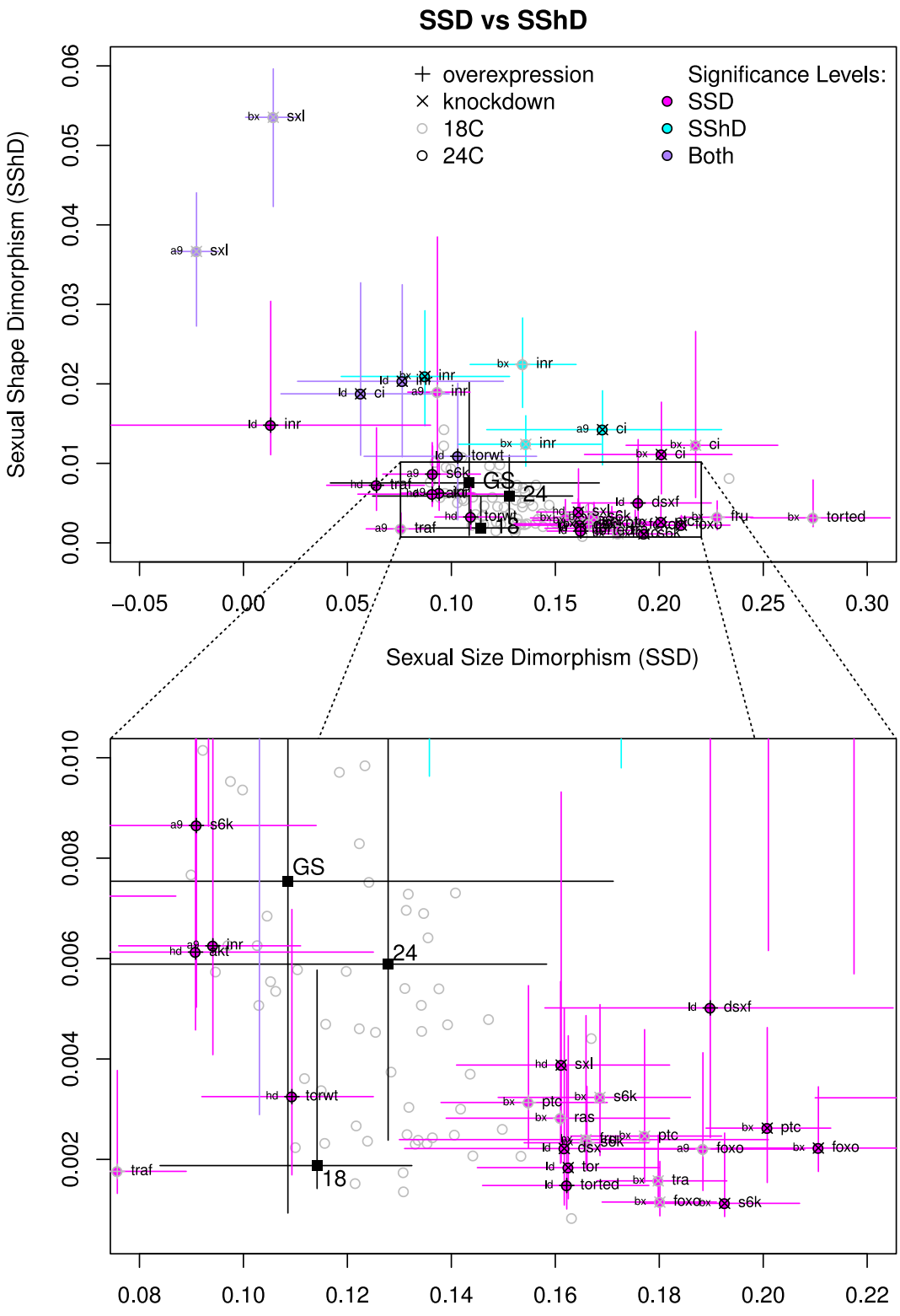

Figure 5.4. Magnitude of SSD and SShD for knockdown and overexpression crosses. The effect of each manipulation is mapped out in a size-and-shape dimorphism space. Genotypic means for each treatment type are indicated by point

Figure 5.4. (cont'd)

style (knockdown vs overexpression) and color (temperature). SSD is plotted on each x-axis for both plots and SShD is displayed on the y-axis. The plots above display the entire range of variation observed, while those below display only the area with the highest density of points. Lines with significant sex-by-genotype effects are highlighted as follows: effect on both size and shape, shape only and size only. Only significant genes (after sequential Bonferroni correction) from the linear models are colored. Few mutations in this study reduce sexual dimorphism of size or shape. Error bars are 95% confidence intervals (unadjusted alpha). All gene names are displayed lower-case, regardless of dominance.

female isoform tra.*F* causes reduction of SSD, but no effect on SShD. Data for *tra* knockdowns was less consistent for wing-specific expression (A9-Gal4 over-expression caused reduction in SSD, but Bx-Gal4 knockdown caused increase in SSD. This is interpreted as a Bx-specific effect, since GeneSwitch-Gal4 over-expression was consistent with A9-Gal4).

These data are further supported by vector correlations between control and treatment wings. Vector correlations for SShD in *Sxl* were consistently below 60%, and those for tra even lower (Figure 5.5). Others treatments with consistently low vector correlations (and SSD vs SShD scores) include the following: *Ci*, *InR*, *ptc*. Occasionally other treatments—such as *akt*, *Foxo*, *Tor*, *S6K*, and *fru*—yielded low vector correlations in overexpression lines.

Discussion

Our data suggest low sensitivity for genetic perturbations to decrease SSD or SShD, counter to previously observed effects. Of the crosses that showed significant effects, somewhat fewer caused a reduction in SSD and none caused reduction of SShD. These data conflict with results from Testa and Dworkin (2016; V P Carreira et al., 2009; Takahashi & Blanckenhorn, 2015), wherein genetic perturbations appear to show no preference for increasing or decreasing SSD.

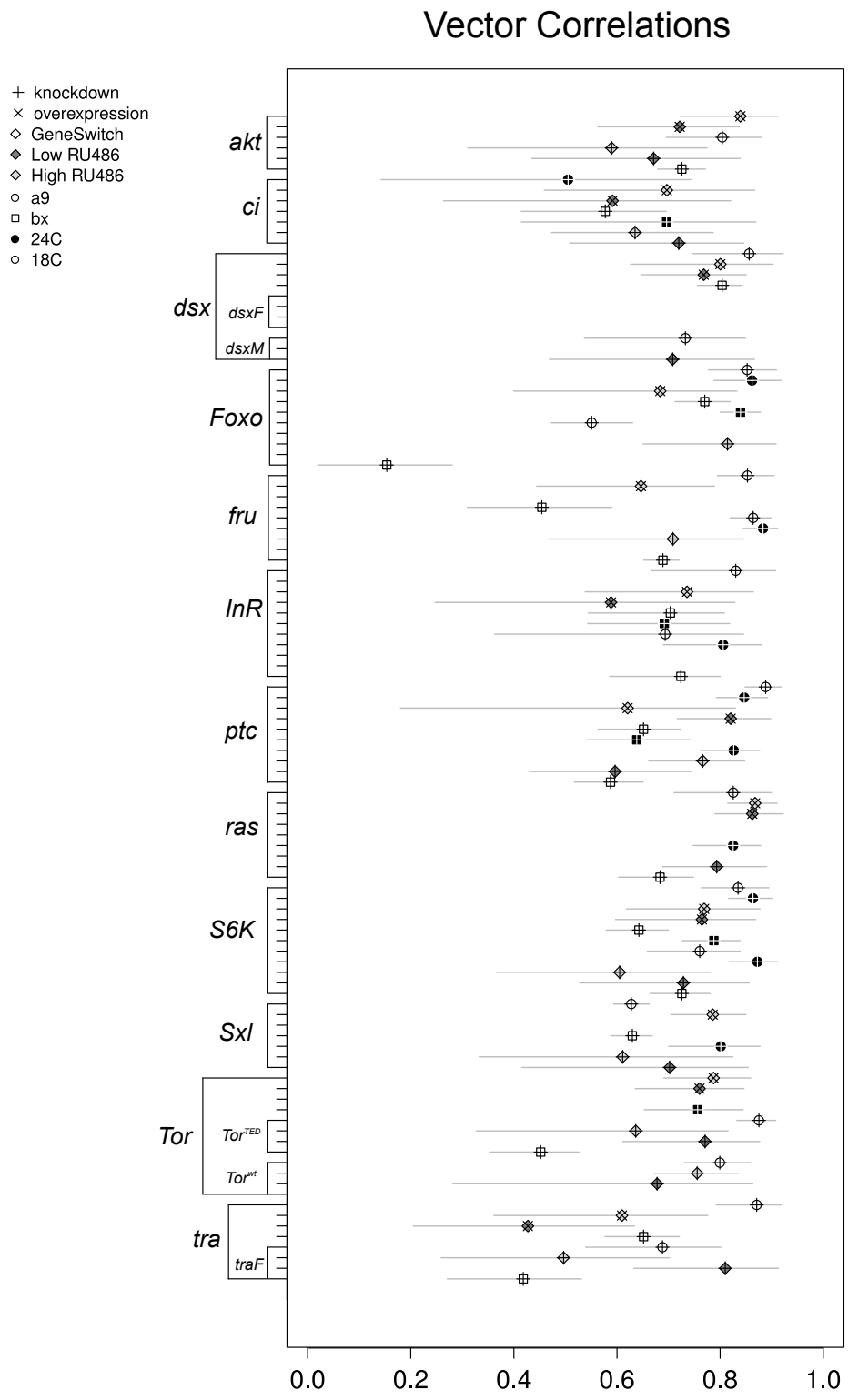

Figure 5.5. Vector correlations to assess similarity of direction for sexual shape dimorphism (treatment vs. control). While temperature appears to have little effect

Figure 5.5. (cont'd)

on the direction of SShD for most mutations, several stand out with more divergent directions of SShD. Those manipulations with significant effects on SShD in previous figures are also notable for their large effect on vector correlation. Lower values correspond to lower shape correlation, thus a larger overall effect. Error bars are 95% confidence intervals (unadjusted alpha).

For these experiments, we noticed a significant negative trend between SSD and SShD. Standardized Major Axis regressions reveal that as SSD increases, SShD decreases (Correlation = 0.20; $p < 0.0001$). While this trend holds true for SShD residuals (allometry-corrected values of shape), it is absent from other pairwise plots of shape on size (Figure 5.2). In this case, it may be a statistical artifact due to how the allometry correction may generate a spurious correlation for the effect of shape on size. Regardless, this trend is very curious from a biological standpoint. What this trend reveals is that perturbations of no genes increase or decrease both SSD and SShD at the same time. If this trend holds true on a species-level, it could mean that control of SSD and SShD is mutually exclusive.

The case for removing the assumption of common allometry

Not surprisingly, variation in shape is influenced by variation in size, both isometrically and allometrically. Simply put, shape often covaries with size. Some shape configurations may simply be associated with larger or smaller sizes, despite scaling by centroid size removing the isometric effect of size (Mitteroecker et al. 2004). In the case where shape can covary with more than one independent variable (e.g. when shape differs by sex and genotype), there may be more than one allometric slope along which shape can vary. For instance, if females had greater variation in size than males, then assuming a common allometric (shape vs size) slope would therefore not be valid. However as pointed out in Klingenberg (2016) a simple statement of sex:size interaction (and its significance) is insufficient, in particular with very large sample sizes. In such cases a very small (and biologically insignificant) alteration in the allometric relationship

may be statistically significant with large sample sizes. Thus it is important to examine the vector correlations for these allometric vectors.

With the data I have generated here, males and females do not covary isometrically across all of the experimental treatments (Figure 5.3). If the covariance between males and females for each group is different, then we cannot assume a common allometric slope for all groups (i.e. shape may not react similarly to changes in shape for every group). Given the wide variation in size of our data, we can no longer assume a common allometric slope for all data and must therefore correct for it. Fortunately, there are methods of reducing the allometric effect of size on shape between each treatment group (Gidaszewski et al. 2009).

In order to account for differences in allometric slopes within groups, we therefore removed the effect of allometric (size vs shape) scaling on wing shapes for each. The process of removing the effects of allometry involves fitting a multivariate linear regression of shape on size. The resulting line will display a “predicted” line of best fit, which represents the allometric component of shape on size. Movement along this line describes the change in shape as size varies. In order to remove that effect, we must use the shape (y-axis) residuals of this model. Ideally, residuals are supposed to be uncorrelated with the line of best fit, “i.e. allometry.” This must be done individually for each treatment group, so as to ensure we are not assuming any common allometries. The resulting values will correspond to the shape variation that is independent of size once the effect of shape on size has been accounted for, yielding the best possible

approximation to shape uncorrelated with size. (Klingenberg, 2016; and references therein)

Reasons for cautious interpretation

Because the insertion of driver and response p-elements into the genome is known to have phenotypic effects, we must cautiously interpret these data. In the case of Bx-Gal4, for instance, many more crosses yielded significant size effects than would be expected due to chance alone. This is likely due to the fact that Bx-Gal4 is an enhancer trap, meaning that the Bx-Gal4 driver itself is inserted into the native Bx locus. Since Bx-GAL4 is X-linked and acts as a recessive hypomorph (independent of its role as a GAL4) it was not unexpected to have different effects in males (who are hemizygous for the Bx-GAL4) and females (who are heterozygous for it). While much care went into generating proper crosses to control for the effects of p-element insertion, more controls would have been ideal. For example, we did not originally control for driver-specific effects of these crosses. We are currently generating the remaining necessary controls (and replication of key experimental treatments).

As mentioned earlier, A9-Gal4 and Bx-Gal4 are both wing-specific drivers, whose expression is concentrated more strongly in the dorsal than in the ventral pouch of the developing wing imaginal disc (Liang et al. 2014; Sun & Artavanis-Tsakonas 1997; Peterson & O'Connor 2013; Bejarano et al. 2012). A characteristic over or undergrowth of only the dorsal layer, rather than the whole wing, might be expected in crosses aimed to affect size. While such a warped or blistered phenotype was observed in a few

crosses, most crosses appeared to yield the intended phenotype for its respective cross (table 5.1). Whereas some studies using A9-Gal4 and Bx-Gal4 have found that occasional curling and “canoe-shaped” wings can occur (Liang et al. 2014), many still find that these drivers adequately influence the full adult wing (Nguyen et al. 1998; Sun & Artavanis-Tsakonas 1997; Jackson et al. 1997; Takeo et al. 2005; Bejarano et al. 2012). For A9 at least, the ability to produce full wing phenotypes is because expression is ubiquitous in the wing disc until the late third instar, where it is then restricted to the dorsal compartment (Haerry et al. 1998; Nguyen et al. 1998). Regardless, we now have reason to believe that this issue may affect our interpretation of the data. The effects we observe here are potentially those that we set out to measure; conversely, they could be reflecting a sex-specific sensitivity to these genetic manipulations instead.

For the purposes of this paper, we will focus on SSD and SShD as the sex-specific response to dorsal wing disc manipulation, rather than assuming a whole-organ response to genetic manipulation. This interpretation may explain why a reduction in SSD or SShD was so uncommon. A given genetic manipulation could be acting to reduce dimorphism, but if the ventral layer of the wing is unaffected then the overall wing shape is also expected to warp.

It is for these reasons that we will only discuss the results from candidate genes that had consistent effects across multiple treatments.

Consensus Among Crosses in Size/Shape Space:

In 2005, Horabin put forth the hypothesis that SSD was in part regulated by the Hh pathway. Central to this hypothesis is the condition that ectopic *Sxl* protein can upregulate Hh signaling, increasing expression of the activating form of the transcription factor *Ci*. Supposedly an increase in either *Sxl* or *Ci* should be sufficient to increase SSD (because females are sensitive to Sxl, while males are not). Conversely, Sxl and Ci should also be necessary to maintain SSD; in the absence of either, SSD should decrease. Our data do not support the claim that *Ci* and *Sxl* are sufficient and necessary for SSD when driven endogenously, but rather show that they are necessary for SShD (both) and that *Sxl* is necessary for SSD.

As for Horabin's (2005) hypothesis, our support is somewhat tenuous. We were only able to demonstrate that *Sxl* was necessary for SSD, which is consistent with data from other studies where *Sxl* knockdowns produced small female wings relative to males (Yan & Perrimon 2015). What our data do show, however, is that *Sxl* and *Ci* appear to be necessary for modulating sex-specific wing shape development. Ostensibly, *Sxl* could be activating the Hh signaling pathway in such a way as to increase the sex-specific difference in the shape of the wing. This explanation might explain overgrowth in wings exposed to ectopic *Sxl*. Obviously more data are needed to substantiate this claim. If true, we would expect overexpressions of *Ci* to rescue the phenotype of *Sxl* knockdowns. Unfortunately, given the nature of Sex-lethal being lethal when lost, these experiments may prove challenging.

While other components of the IIS pathway had little effect on SSD or SShD, *InR* knockdowns and overexpressions regularly yielded a decrease in SSD. These data are consistent with those in Chapter 3. Activation of IIS was not sufficient to increase whole body SSD (Chapter 3) or wing SSD, but our data suggest that it influences SShD. If this is the case, IIS activation not only controls proper male:female proportions in relative size, but likely also shape. This trend is noticeable before and after correction for allometry, though *Foxo* knockdowns tended to also influence SShD before allometric correction (data not shown). Differences in SShD attributed to *Foxo*, therefore, correlate with size.

Perhaps the most exciting find is that Tor has the ability to both increase and decrease SSD. Since Tor is a positive growth regulator, its function is to increase rather than decrease growth. The being said, increases in Tor appear to be increasing one sex's size (males) relative to the other (females). These data are also consistent with data from Chapter 3, where additively decreasing Tor activity yielded increased SSD (Figure 3.2.b). Furthermore, another element downstream of *Tor*, *S6K*, displayed weak evidence for controlling SSD. In Bx-Gal4 crosses, knockdowns at both temperatures increased SSD, whereas overexpression with A9-Gal4 decreased it. Since *S6K* does not show consistent support, we should remain skeptical, but given that it showed similar results at both temperatures and in two different drivers, it was worth mentioning.

In order to make a more compelling case for the TOR pathway's ability to regulate wing SSD, further experiments would be necessary. For instance, if overexpression of S6K

were able to rescue SSD in Tor knockdowns, a much stronger case for TOR pathway involvement could certainly be made. Until we measure actual expression levels of TOR signaling molecules during growth, however, we cannot be entirely certain that TOR is directly involved. Perhaps the most convincing piece of evidence would be to measure expression levels in males and females during the early third larval instar. This is crucial, because according to Chapter 1 it is precisely during this phase that SSD is generated (Testa et al. 2013).

The results from nutrient-sensitive growth pathway manipulations such as IIS/TOR are to be expected if sexually dimorphic traits such as shape and size are condition-dependent. In Chapter 3, I demonstrated that whole body SSD is nutrient dependent AND nutrient-sensitive growth pathway-dependent. Here, the data also suggest that not only is SSD condition dependent, but so is SShD. Given the propensity of sexually dimorphic traits for being condition dependent, this revelation is not very surprising (Andersson 1994; Price et al. 1993; Johnstone 1995). For condition dependence to evolve, a system should have sexually selected traits (e.g. shape and size) that are condition dependent while simultaneously having a large amount of variance for overall condition (Bonduriansky 2007b; Rowe & Houle 1996; Johnstone et al. 2009).

When are sex-specific shape and size determined?

Despite the vast literature on sex determination in *Drosophila*, we know comparatively little about how the sex determination pathway controls sex-specific phenotypes (Cline 1984; Kopp et al. 2000; DiBenedetto et al. 1987; Bopp et al. 1996; Baker & Belote 1983;

Salz & Erickson 2010; Younger-Shepherd et al. 1992; Bopp et al. 1993; Hodgkin 1989; Horabin 2005). We know that *Sxl* controls dosage compensation and, when females lacking *Sxl* or it is overexpressed in males, they die. Downstream of *Sxl*, is the *tra* gene. This gene is known from knockdown experiments to cause transformations from female to male (Cline 1984; Verhulst et al. 2010). It is unclear though, if *tra* is also causing a transformation from female to male size.

Our data suggest that proper functioning of *Sxl* and *tra* are necessary for SSD; no other downstream elements demonstrated such a strong necessity. In the case of *tra*, overexpression of the female-specific isoform causes a reduction in SSD. Additionally, knockdowns in *Sxl* were shown to cause an increase in SShD. We believe this is related to the fact that *Sxl* mutants are lethal for females. With decreasing levels of *Sxl*, females become not only smaller, but developmentally impaired. Since both *Sxl* and *InR* had such similar effects, we feel these data may add support to the claim made by Fear et al. (2015), where *InR* expression was correlated with *Sxl*. Further research is needed to confirm the relationship between *Sxl* and *InR*.

Somewhat surprisingly, SShD was never reduced in any of these crosses. If *tra* was in fact controlling SShD, a knockdown would be expected to reduce SShD. However unlikely, it is feasible that *tra* does not control secondary sexual characteristics, but only primary sexual characteristics. Further experimentation is needed to be certain.

Regardless, since no other components were capable of altering SSD, we can fairly confidently conclude that SSD is controlled at the level of *tra*. Since it is the farthest gene in the pathway to influence SSD, *tra* expression must be modulating sex-specific growth.

Wing SSD vs Whole Body SSD: Insights into Regulation of Allometry

Evidence from the literature suggests that the genetics of size and shape are often found to be uncorrelated (Gidaszewski et al. 2009; Gilchrist and Partridge 2001; Zimmerman et al. 2000). Because of this, it is not surprising that our results demonstrate few cases where both size and shape are affected and also show little support for similar regulation of organ and body size. That shape and size are genetically uncorrelated also has broader implications for the study of shape and size. First, size or shape cannot be used as proxies for one another, since they are uncorrelated. Second, selection may need to act specifically on either shape or size dimorphism in order to influence their evolution; selection on larger female relative to male body size is therefore not likely to alter aspects of shape dimorphism. Condition dependence in size and shape would then be more likely to evolve independently of one another.

Our data, when compared to Chapter 3's data, suggest that organ size and body size are controlled by very different mechanisms. While Chapter 3 argues that SSD cannot be increased with systemic gene overexpression, here the data paint a different picture. Our data confirm that overexpression of genes in specific tissues is sufficient to increase SSD and SShD. That ubiquitous expression fails to increase SSD suggests

that dimorphic trait values are generated not by systemic increases in specific genes/pathways, but rather by specific spatiotemporal effects.

Sex-specific isoforms of sex-determination genes are often generated through post-translational modification to the active protein. In addition, in the cases of *tra* and *dsx*, the mRNAs are alternatively spliced to produce proteins of varying activity. We know that sex-specific isoforms of proteins like these are already linked to cis-regulatory changes in sex-specific phenotypes. For example, male coloration is mediated in part by *dsx* and bric-a-brac (*bab*) expression in the thorax. Cis-regulatory mechanisms upstream of *bab* allow for male-specific isoform of *dsx* to drive expression of *bab*, increasing melanin content in the last few tergites (Williams et al. 2008).

Perhaps the most promising direction to search for the genetic mechanisms of SSD and SShD are in the cis-regulatory effects of these candidate genes, specifically those of the TOR pathway. Our data allow us to put forth the hypothesis that differences in TOR signaling are influencing SSD.

CHAPTER 6:

Conclusion and future directions

Summary of Chapters

Chapter 1

- Sexual Size Dimorphism (SSD), the difference in body size between males and females, is a common phenomenon.
- Males and females usually share most of their genomes, except for the sex chromosomes. Because both sexes largely share a genome, the evolution of SSD should theoretically be very difficult. Despite this, SSD is nearly ubiquitous among metazoans.
- SSD can ultimately evolve through one of three mechanisms: sexual selection, fecundity selection, or viability selection. While we also understand the proximate mechanisms of sex-specific gene expression, we do not yet understand the proximate mechanisms of SSD.
- Drosophila has specific developmental patterns for us to consider when teasing apart mechanisms. Drosophila are holometabolous and have discrete, hormonally controlled life stages. Critical size, the point at which starvation no longer delays pupariation, is a good proxy for us to study initial body size.
- We know a lot about the genetics of how growth is controlled in Drosophila, but we do not know how sex-specific growth is regulated to produce SSD
- Sexual Shape Dimorphism (SShD) is another important factor to consider when studying sexual dimorphism. Shape, however, is very difficult to quantify. Geometric morphometrics is currently our best method of quantifying shape.
- The genetics of SSD and SShD within organs is also relatively unknown. We have little idea how genes that contribute to growth affect either SSD vs SShD.

Chapter 2

- Females reach their critical size at a larger weight than males do.
- Females have a faster growth rate than males during their third larval instar.
- Both males and females hit a peak larval weight approximately halfway through their third instar, then begin to lose weight before pupariating. Females lose significantly more weight than males during this period.
- Males and females both lose weight during their pupal stages, but not in a sex-specific manner.
- Flies mutant for the Insulin Receptor (*InR*) completely lose SSD.

Chapter 3

- Flies reared on poor-quality diets lose SSD. SSD is therefore condition-dependent.
- When genes within nutrient-sensitive growth pathways are perturbed, SSD is often reduced or eliminated. Overexpression of growth pathway genes, however, causes an unexpected reduction in total body size for males and females; SSD is typically unaffected by this effect.
- IIS pathway genes are necessary for SSD, but not sufficient to increase it. Genes in the TOR pathway, however, demonstrate greater potential for regulating SSD.

- Sex-specific control of whole body SSD is controlled at the level of *tra* within the sex determination pathway. Downstream gene, *dsx*, is sufficient to decrease SSD.

Chapter 4

- Few mutants within EGFR and TGF-ß pathways affected SSD or SShD in a sex-specific manner. Only a handful of genes associated with growth act in a sex-dependent manner.
- Most mutations with sex-specific effects on SSD/SShD did so in a background-dependent manner. Many mutants only affected SSD or SShD within either Sam or Ore genetic backgrounds; very few were significant in both backgrounds.
- The response to mutation is also somewhat background-dependent. Mutations in Ore tended to reduce SSD, while mutants in Sam tended to increase SSD.
- It is important to distinguish between relative contributions of SSD vs SShD when analyzing the effects of candidate genes.

Chapter 5

- Most gene manipulations tended to increase SSD or SShD, rather than decrease. While some treatments reduced SSD, none reduce SShD.
- It is always important to investigate and assess the relationship between size and shape. In the event that size and shape covary differently across groups, the allometric effects of size on shape can and should be removed.
- SShD increases in perturbations of the Hh pathway. This is caused by exaggerated female response to genetic knockdowns of *Ci* and *Sxl*.

- Again, IIS pathway genes tend to be necessary for SSD, but not sufficient to increase it. TOR pathway genes, however, show additional support for controlling SSD.
- Sex-specific control of wing SSD is also controlled at the level of *tra* within the sex determination pathway.
- The upstream gene, *Sxl*, is necessary for SSD and proper SShD. *Sxl* knockdowns yielded some of the most dramatic sex-specific shape responses by females.
- Our data support the claim made by Fear et al. (2015) that *Sxl* expression is correlated with *InR*, due to the similar effects of both.
- Wing and body SSD are potentially controlled by different mechanisms.

Future directions to strengthen our arguments

The first way we intend to strengthen our argument would be to improve the quality and nature of our controls. Currently, our statistical estimates are conservative in such a way that only those crosses that are extremely different from the controls show significance. Specifically, this is because each treatment is compared to a pool of controls, which itself has a rather large variance. Additional controls would be sufficient to increase our power to detect significant effects of additional treatment groups. We are presently working on rearing up the proper controls to add to the analyses, which will be included in the eventual publication of these data.

Perhaps the most obvious way to further strengthen our arguments would be to specifically design more complicated crosses to detect each candidate's effect on SSD vs SShD. Currently, our data support the hypothesis that TOR signaling is involved in regulating SSD to some extent. If we could demonstrate that S6K overexpressions could rescue a Tor knockdown, then we would add a considerable level of support for the TOR activity hypothesis. Further crosses could refine and elucidate our understanding of how these pathways contribute to SSD and SShD.

An even greater amount of support would come from collecting gene expression data from some of the promising crosses in Chapters 3 and 5. By demonstrating the effect that our experiments are having on gene expression, we can be more certain that our experiments are having the intended effect. With these data, we could quantify the effect of each gene on SSD more quantitatively, rather than qualitatively. This will be an important piece of data that would be vital to our arguments. Additionally, and more importantly, we would be able to demonstrate that it is in fact increased TOR expression that is causing changes to SSD. Without this critical piece of data, we cannot be completely certain that TOR pathway genes are directly affecting SSD rather than indirectly influencing it.

APPENDIX

Table A1a. Significance Table (raw p values) for effects on wing shape where G= genotype, S= sex, B= background and Cs= (centroid) size

Mutant	Line	Allele	Pathway	G	G x S	G x B
aos	2513	W11	Egfr	0.001	0.671	0.14
omb	3045	md653	TGF-β	0.001	0.001	0.003
cv-2	6342	225-3	TGF-β	0.092	0.363	0.093
GAP1	6372	mip-w[+]	Egfr	0.001	0.034	0.001
ksr	10212	J5E2	Egfr	0.001	0.935	0.193
dad	10305	J1E4	TGF-β	0.001	0.054	0.593
drk	10372	k02401	Egfr	0.001	0.046	0.482
bs/DSRF	10413	k07909	Egfr	0.001	0.111	0.001
s	10418	k09530	Egfr	0.001	0.463	0.019
spi	10462	s3547	Egfr	0.001	0.518	0.003
mad	10474	k00237	TGF-β	0.001	0.56	0.001
ed	10490	k01102	Egfr	0.001	0.88	0.012
tsh	10842	A3-2-66	TGF-β	0.095	0.458	0.397
cos	11156	k16101	Hh	0.011	0.228	0.001
tkv	11191	k19713	TGF-β	0.001	0.121	0.001
babo	11207	k16912	TGF-β/Hh	0.054	0.428	0.047
trl	12088	S2325	TGF-β	0.001	0.136	0.315
rho-AP	12413	BG00314	?	0.523	0.006	0.005
pka-C1	12752	BG02142	Hh	0.225	0.946	0.356
sbb	12772	BG01610	TGF-β	0.001	0.203	0.038
psq	12916	kg00811	Egfr	0.011	0.38	0.223
osa	12945	kg03117	Chromatin Remodeling	0.001	0.188	0.036
rasGAP	13311	kg02382	Egfr	0.541	0.371	0.001
pnt	13535	kg04968	Egfr	0.002	0.063	0.021
drk	13943	k02401	Egfr	0.095	0.223	0.001
cbl	13944	kg03080	Egfr	0.003	0.459	0.018
mam	14189	kg02641	N/Egfr	0.001	0.908	0.577
rho-6	14208	kg05638	Egfr	0.809	0.35	0.814
dpp	14268	kg04600	TGF-β	0.006	0.898	0.022
pka-C3	14345	kg00222	Hh	0.177	0.045	0.035
p38b	14364	kg01337	TGF-β/Egfr	0.247	0.59	0.031
tkv	14403	kg01923	TGF-β	0.041	0.111	0.009
wmd	14541	kg07581	Unknown	0.093	0.264	0.04
mad	14578	kg00581	TGF-β	0.002	0.651	0.153

Table A1a. (cont'd)

ast	14638	kg07563	Egfr	0.014	0.972	0.074
dpp	14694	kg08191	TGF-β	0.002	0.822	0.074
rho1	14901	kg01774	Egfr?	0.082	0.059	0.353
sax	14920	kg07525	TGF-β	0.046	0.24	0.003
*sax**	5404	sax4	TGF-β	0.04	0.001	0.026
egfr	10385	k05115	Egfr	0.001	0.098	0.023
src42A	13751	kg02515	Egfr	0.001	0.057	0.023
rho/stet	14321	kg07115	Egfr	0.001	0.653	0.305

Table A1a. (cont'd)

Mutant	Line	Cs x G x S	Cs x G x B	G x S x B	Cs x G x S x B
aos	2513	0.863	0.827	0.324	0.093
omb	3045	0.004	0.001	0.001	0.086
cv-2	6342	0.023	0.822	0.027	0.677
GAP1	6372	0.674	0.881	0.405	0.428
ksr	10212	0.057	0.89	0.364	0.248
dad	10305	0.24	0.058	0.098	0.296
drk	10372	0.478	0.544	0.57	0.796
bs/DSRF	10413	0.157	0.163	0.437	0.165
s	10418	0.849	0.23	0.518	0.002
spi	10462	0.495	0.52	0.438	0.573
mad	10474	0.542	0.464	0.125	0.094
ed	10490	0.07	0.051	0.542	0.046
tsh	10842	0.947	0.775	0.165	0.262
cos	11156	0.267	0.096	0.288	0.36
tkv	11191	0.358	0.813	0.26	0.161
babo	11207	0.465	0.51	0.429	0.1
trl	12088	0.046	0.82	0.997	0.23
rho-AP	12413	0.983	0.815	0.196	0.098
pka-C1	12752	0.032	0.89	0.825	0.601
sbb	12772	0.323	0.647	0.02	0.539
psq	12916	0.979	0.732	0.03	0.01
osa	12945	0.876	0.373	0.043	0.123
rasGAP	13311	0.037	0.038	0.286	0.307
pnt	13535	0.173	0.752	0.504	0.609
drk	13943	0.514	0.22	0.107	0.222
cbl	13944	0.765	0.57	0.947	0.195
mam	14189	0.298	0.035	0.245	0.066
rho-6	14208	0.448	0.72	0.152	0.25
dpp	14268	0.285	0.058	0.912	0.025
pka-C3	14345	0.587	0.276	0.1	0.018
p38b	14364	0.566	0.906	0.771	0.902
tkv	14403	0.222	0.835	0.501	0.269
wmd	14541	0.651	0.157	0.039	0.378
mad	14578	0.815	0.199	0.216	0.753
ast	14638	0.211	0.934	0.666	0.781
dpp	14694	0.162	0.878	0.421	0.424

Table A1a. (cont'd)

rho1	14901	0.083	0.677	0.082	0.03
sax	14920	0.31	0.519	0.5	0.389
*sax**	5404	0.205	0.701	0.808	0.795
egfr	10385	0.12	0.112	0.077	0.316
src42A	13751	0.054	0.212	0.617	0.13
rho/stet	14321	0.064	0.558	0.102	0.327

Table A1b. Sample size of treatments, where M= male, F= female, w= wild-type, m= mutant, O= Oregon-R background, S= Samarkand background

Mutant	Line	M, w, O	F, w, O	M, w, S	F, w, S	M, m, O	F, m, O	M, m, S	F, m, S
aos	2513	21	20	21	20	20	20	20	22
omb	3045	19	18	20	20	12	19	19	20
cv-2	6342	10	11	20	20	10	10	20	20
GAP1	6372	19	18	20	19	18	19	20	20
ksr	10212	17	20	19	20	20	20	22	20
dad	10305	20	20	18	20	20	20	20	20
drk	10372	20	20	20	20	20	20	20	21
bs/DSRF	10413	19	19	20	20	20	19	20	20
s	10418	20	20	18	21	20	20	20	20
spi	10462	18	20	20	20	20	19	21	21
mad	10474	19	20	20	22	20	20	18	22
ed	10490	20	20	19	20	20	20	21	21
tsh	10842	19	21	20	20	20	19	22	19
cos	11156	19	20	19	20	22	19	20	18
tkv	11191	20	20	20	21	20	21	18	17
babo	11207	19	21	20	20	20	20	20	20
trl	12088	20	20	21	20	20	21	20	20
rho-AP	12413	20	20	20	20	20	19	20	19
pka-C1	12752	20	20	18	20	20	20	21	21
sbb	12772	20	20	20	19	20	22	20	21
psq	12916	21	20	21	20	20	20	20	21
osa	12945	31	30	20	20	30	24	20	20
rasGAP	13311	19	19	20	19	21	21	20	20
pnt	13535	20	20	20	19	20	19	20	20
drk	13943	20	19	20	21	16	17	21	21
cbl	13944	20	20	21	20	20	20	20	20
mam	14189	10	8	21	21	10	10	20	20
rho-6	14208	19	21	20	20	19	20	20	21
dpp	14268	21	20	19	20	20	18	22	18
pka-C3	14345	21	20	20	20	21	20	20	20
p38b	14364	10	10	20	20	10	6	20	20
tkv	14403	11	10	10	12	10	11	20	20

Table A1b. (cont'd)

wmd	14541	20	20	20	21	20	21	21	20
mad	14578	20	19	22	21	21	19	20	20
ast	14638	20	20	20	20	20	18	20	20
dpp	14694	20	19	19	20	20	20	20	20
rho1	14901	20	20	20	21	20	20	20	20
sax	14920	19	19	20	20	20	20	21	20
sax*	5404	20	21	18	19	20	20	20	20
egfr	10385	20	19	20	20	20	20	20	20
src42A	13751	19	20	19	20	19	20	19	20
rho/stet	14321	19	19	19	20	19	21	19	19

Table A2. Significance Table (raw p values) for effects on wing shape after the effects of allometry are removed where G= genotype, S= sex, B= background and Cs= (centroid) size.

Mutant	Line ID	G	G x S	G x B	Cs x G x S	Cs x G x B	G x S x B	Cs x G x S x B
aos	2513	0.098	0.221	0.001	0.664	0.31	0.236	0.625
omb	3045	0.001	0.072	0.001	0.6	0.311	0.002	0.264
cv-2	6342	0.066	0.215	0.171	0.494	0.412	0.619	0.208
GAP1	6372	0.002	0.235	0.001	0.075	0.012	0.177	0.299
ksr	10212	0.008	0.005	0.002	0.165	0.24	0.165	0.526
dad	10305	0.16	0.009	0.797	0.158	0.003	0.962	0.205
drk	10372	0.005	0.174	0.031	0.115	0.021	0.021	0.607
bs/DSRF	10413	0.001	0.126	0.001	0.204	0.021	0.005	0.427
s	10418	0.001	0.002	0.106	0.377	0.188	0.009	0.107
spi	10462	0.001	0.527	0.002	0.558	0.371	0.025	0.359
mad	10474	0.001	0.019	0.005	0.594	0.656	0.005	0.746
ed	10490	0.001	0.778	0.591	0.998	0.225	0.393	0.164
tsh	10842	0.008	0.216	0.002	0.837	0.828	0.051	0.988
cos	11156	0.001	0.019	0.037	0.025	0.03	0.036	0.375
tkv	11191	0.001	0.004	0.006	0.404	0.947	0.301	0.135
babo	11207	0.048	0.427	0.02	0.208	0.517	0.721	0.092
trl	12088	0.001	0.001	0.04	0.776	0.891	0.005	0.38
rho-AP	12413	0.075	0.444	0.017	0.805	0.69	0.194	0.009
pka-C1	12752	0.001	0.227	0.107	0.081	0.513	0.002	0.204
sbb	12772	0.001	0.537	0.004	0.703	0.001	0.396	0.069
psq	12916	0.004	0.283	0.143	0.677	0.127	0.048	0.823
osa	12945	0.001	0.255	0.02	0.699	0.492	0.728	0.368
rasGAP	13311	0.027	0.666	0.031	0.565	0.587	0.792	0.607
pnt	13535	0.016	0.199	0.111	0.439	0.199	0.659	0.293
drk	13943	0.002	0.005	0.001	0.414	0.905	0.081	0.051
cbl	13944	0.138	0.07	0.329	0.148	0.002	0.754	0.518
mam	14189	0.001	0.15	0.043	0.001	0.109	0.043	0.386
rho-6	14208	0.086	0.091	0.433	0.664	0.959	0.009	0.099
dpp	14268	0.017	0.322	0.08	0.798	0.109	0.871	0.822

Table A2. (cont'd)

pka-C3	14345	0.019	0.024	0.567	0.151	0.237	0.345	0.23
p38b	14364	0.003	0.498	0.171	0.542	0.001	0.466	0.6
tkv	14403	0.021	0.225	0.023	0.403	0.673	0.454	0.395
wmd	14541	0.028	0.118	0.435	0.248	0.599	0.001	0.172
mad	14578	0.001	0.038	0.026	0.639	0.456	0.357	0.11
ast	14638	0.078	0.091	0.002	0.995	0.317	0.007	0.132
dpp	14694	0.001	0.299	0.045	0.476	0.027	0.56	0.801
rho1	14901	0.221	0.362	0.436	0.366	0.329	0.394	0.244
sax	14920	0.002	0.394	0.38	0.758	0.272	0.135	0.338
*sax**	5404	0.007	0.007	0.047	0.442	0.929	0.299	0.845
egfr	10385	0.001	0.125	0.03	0.742	0.393	0.014	0.211
src42A	13751	0.031	0.37	0.002	0.384	0.195	0.411	0.586
rho/stet	14321	0.002	0.156	0.19	0.02	0.12	0.207	0.421

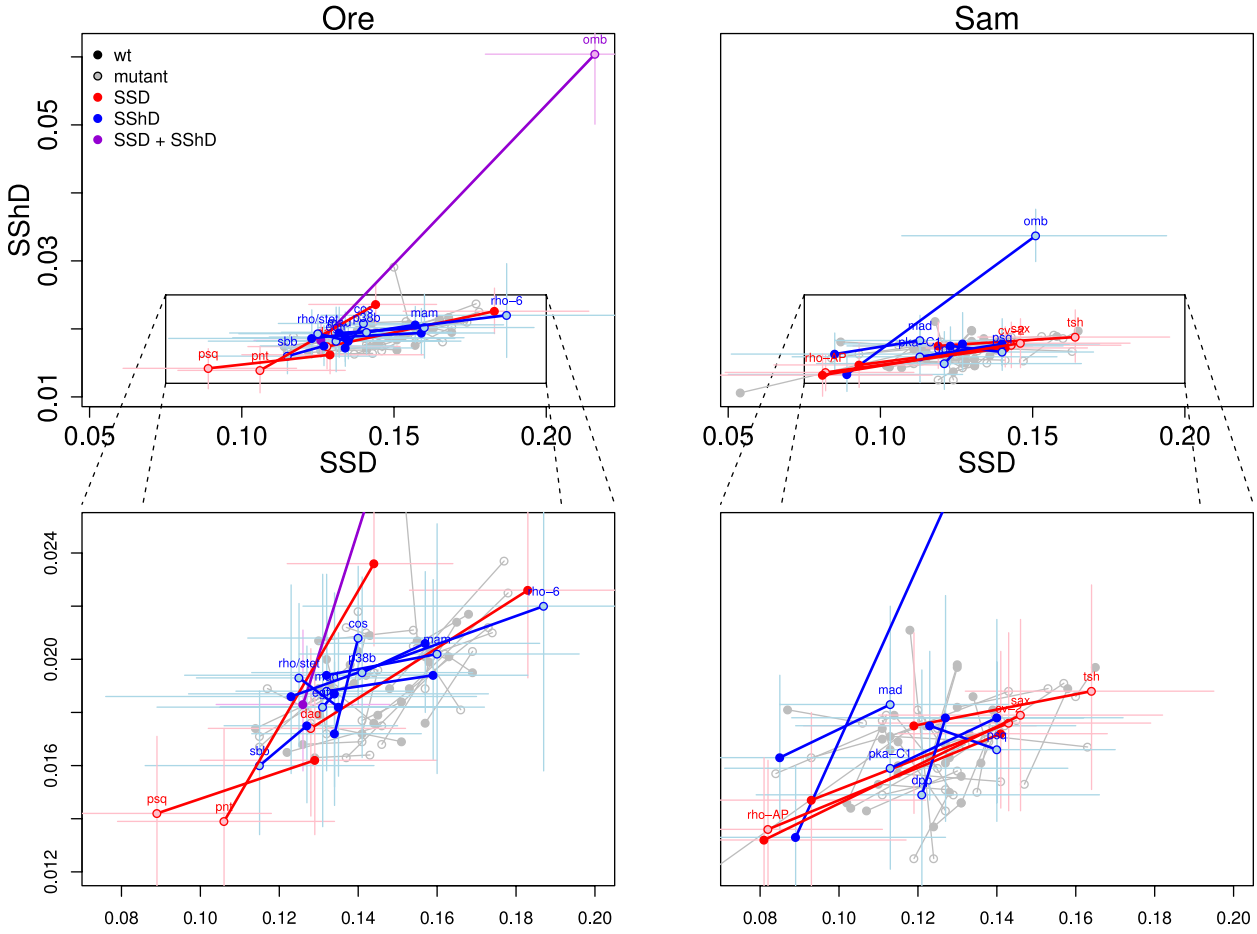

Figure A1. Magnitude of SSD and SShD for 42 mutants in Oregon-R (left) and Samarkand (right) wild type backgrounds, after correcting for the influence of allometry (shape on size). A common allometry relationship was assumed across genotype and sex within each background and line combination. Residuals from the allometric model were then used for the analysis. This figure is otherwise identical to Figure 2 (which does not correct for allometry). The effect of each mutant is mapped out in a size-and-shape dimorphism space. Genotypic means for each mutant are indicated by point style and connected by a solid line. SSD is plotted on each x-axis for all plots and SShD is displayed on the y-axis. The plots above display the entire range of variation observed, while those below display only the area with the highest density of points. Lines with significant sex-by-genotype effects are highlighted as follows: effect on both size and shape, shape only and size only. Only significant genes (after sequential Bonferroni correction) from the linear models are colored. Error bars are 95% confidence intervals (unadjusted alpha). All gene names are displayed lower-case, regardless of dominance.

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