UNDERSTANDING THE ROLE OF STANDING GENETIC VARIATION IN FUNCTIONAL GENETICS AND COMPENSATORY EVOLUTION

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Zoology—Doctor of Philosophy
Ecology Evolutionary Biology and Behavior—Dual Major

2014

ABSTRACT

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Conventionally the phenotypic outcome of a mutation is considered to be due to a specific DNA lesion. But it has long been known that mutational effects can be conditional on environment (GxE) and genetic background (GxG). Thus it is standard practice to perform experiments by controlling for rearing environment and using co-isogenic strains. Though such a controlled approach has been very successful in enabling many discoveries, by not considering conditional effects our understanding of biological systems is incomplete. My research utilized conditionality in terms of genetic background and standing genetic variation therein to understand whether mutational interactions can themselves be background dependent. I demonstrated that a majority of mutational interactions identified via a dominant modifier screen are background dependent. Extending this idea of contingency in terms of standing genetic variation to the phenomenon of compensatory evolution in the presence of deleterious mutations, I demonstrated that natural populations of *Drosophila melanogaster* possess standing genetic variation for compensatory alleles to ameliorate even severe phenotypic defects. I further demonstrated that, despite considerable standing variation to ameliorate the focal phenotype perturbed by the mutation, natural selection exploits alternative evolutionary trajectories to recover fitness. Additionally this model system also allowed me to understand that loss of sexual signaling can be compensated by modulating behavioural and life history traits.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Ian Dworkin for giving me the opportunity to work on these wonderful projects. Thank you for all the ideas and discussions that helped shape my research. I am grateful to my current and former committee members, Dr. Barry Williams, Dr. Titus Brown, Dr. Richard Lenski and Dr. Alexander Shingleton for the helpful discussions, ideas and support they provided during my Ph.D. I would also like to thank Dr. Lenski for accepting to be on my committee on a very short notice. I would like to thank the Department of Zoology, the Program in Ecology, Evolutionary Biology and Behavior (EEBB) and the BEACON center for providing financial support. I am also extremely thankful to the office staff from Zoology (Lisa, Mike, Sue, Debbie and Julia), EEBB (Pat) and BEACON (Connie) for their help with all the terrifying paperwork throughout my Ph.D. I would like to especially thank all current and former members of the Dworkin lab- David, Mike, Will, Chris, Alycia, Cody, Lin, Amanda, Anne, Abhijna, Sarah, Mauricio, Megan (Fritz and Cermak), Christian, Alex, Emma and everyone else for providing a great environment for research and supporting me in various capacities ranging from actual help in the lab to lending patient ears when I needed to talk to someone. I would like to thank everyone at BioSci- Sonya, John, Tom, Sachi and Sibyl for giving me an opportunity to teach and supporting me. Thanks to my teachers, family and friends especially my mother, father, grandmother and Dharni for your unconditional love, support and blessings. Finally thank you Ferzin, for everything - most of all for being so patient and tolerant.

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KEY TO SYMBOLS OR ABBREVIATIONS

sd	*scalloped*
vg	*vestigial*
rho	*rhomboid*
FVWA	Ancestral wild-type
FVW	Evolved wild-type
BASE	Unevolved mutant ancestors
CNS	Natural selection populations
CAS	Artificial selection populations
NASC	Control for artificial selection

CHAPTER 1: INTRODUCTION: IMPLICATIONS OF GENETIC BACKGROUND AND OTHER CONDITIONAL EFFECTS INFLUENCING MUTATIONAL PHENOTYPES IN GENETICS AND EVOLUTION

1.1 Premise

The central theme of genetics is to understand genotype-phenotype (G-P) relationships. From a functional genetic perspective understanding G-P relationship involves identifying the number, structure and function of genes that influence particular phenotypes. A functional genetic approach typically involves identifying a phenotype or biological process of interest and screening or selecting for mutant organisms that have particular lesions in the DNA due to which they are defective in that trait. Subsequently, mutants can be classified and further analyzed to understand the nature of the DNA lesion and its exact role in influencing the phenotype of interest [1]. Much of our inferences regarding biological processes and pathways have been derived from such screens based on how genetic variants causally affect the phenotypic outcome. Most of the biological discoveries due to such systematic analysis in the laboratory also owe their success to a scientific approach that controls for multiple aspects in the experiment. For example, in most functional genetic studies, experimental organisms are grown under controlled external environment and are often isogenic for everything but the mutation that is the focus of the study.

Another approach to studying genotype-phenotype relationship involves utilizing natural genetic variation in organisms. This approach to understanding complex traits usually involves identifying the genotypic states of multiple loci via markers and then associating them with the trait status [2]. This forms much of the basis of genome wide association (GWA) and QTL mapping studies. Such an approach may even be extended to include transcriptional or gene expression states where the genomic variation is correlated to differences in gene expression that is further associated with trait variation. Loci influencing several common diseases have been identified via such approaches. In this approach, often the effects of an allele are averaged across multiple backgrounds.

But it has long been recognized that the phenotypic effect of an allele is conditionally influenced by genetic background, external environment, epigenetics, ploidy and numerous other factors [3]. Thus the linear genetic logic of 'phenotypic outcome is a function of only the causal DNA variant(s)' may not always be true. The interaction between a focal allele and other loci in the background is known to influence the expressivity of a variety of traits in many organisms. For example, the long established Samarkand and Oregon-R lab strains of *Drosophila melanogaster* have qualitatively similar, wild type wing morphology. But when the *scalloped*^{E3} mutation is introduced it affects the wing phenotype with the Oregon-R background more strongly than the Samarkand background [4]. The rearing environment can also affect how a phenotype is expressed. For example, the mutant *obake* allele causes antennal duplications in *Drosophila melanogaster.* But when the mutant

larvae are raised under high density, the resulting adults look completely wild type [5]. These examples represent how the phenotypic outcome of a large effect mutation can be conditionally altered. But this can also happen when considering QTLs that influence trait variation. For example, variation in fitness associated traits like sporulation efficiency in between two strains of yeast- oak tree and vineyard strains- was accounted by epistasis between four QTNs (Quantitative Trait Nucleotide). These interactions explained the reduced sporulation efficiency in the vineyard strain compared to the oak tree strain [6]. Similarly, QTL mapping in different populations of *Arabidopsis thaliana* raised under different ecologically relevant photoperiods demonstrated that 27-50% of QTLs showed GxE effects across 13 inflorescence traits [7].

From a myopic perspective, all these factors can be identified as individually influencing the phenotypic outcome in any particular investigation. But the actual genotype-phenotype relationship can be far more complex and such conditional effects may not be mutually exclusive. For instance in the above example of the vineyard and oak tree yeast strains, interaction between four segregating QTNs was responsible for the variation in the sporulation efficiency. But upon further dissection it was found that these interactions were themselves dependent on the genetic background and the rearing environment thereby exhibiting a complex QTN: QTN: genetic background: environment interaction [8]. If conditional effects are ubiquitous, then designing an experiment to account for all these effects can be

extremely complicated, if not impossible. It can thus have the following implications on genetic and evolutionary analysis.

1.2 Implications on Genetic analysis

It could be argued, how important are these effects if investigators recognize and tightly control for them? If the results of an experiment are always interpreted within the scope of a given context, then it should not matter. But in many studies there is a tendency to generalize the results or not account for such effects often leading to contradictory results. For example, the onset as well as progression of some forms of lung and breast cancers are dependent on interaction between certain risk alleles such as *KRAS2* and *BRCA-1/2* respectively with the genetic background and environment [9,10]. Additionally cancers are extremely heterogeneous involving many pathways that can be influenced by variable mutations, environment and epigenetic effects [11–14]. Failing to account for these in addition to high propensity for cancers to develop resistance can lead to failure of treatments [15]. Similarly studies investigating lifespan and longevity are extremely susceptible to conditional effects. For example, failure to account for such effects in *Drosophila melanogaster* and *Caenorhabditis elegans* have often led to contradictory and irreproducible results when genetic background and environmental effects were taken into consideration [16,17]. Thus, by not accounting for such effects or even by controlling for them, biological conclusions may lack generality.

Accounting for conditional effects is extremely important when designing modifier or sensitization screen where perturbation in a genetic network is utilized to uncover genetic interactions with other loci in the genome. Such mutational interactions form much of the basis for constructing interaction networks underlying genotype-phenotype relationships. But if such interactions themselves are context dependent then by not considering these effects the inferences regarding network topologies are incomplete. For example, epistatic interactions that influence lifespan in *Drosophila melanogaster* are themselves modified by diet [18]. In almost all model organisms different strains are well established for specific studies. For example, in mice, the C57BL/6 strain is used for studying various phenotypes and the '129' strain used in creating targeted gene knockouts. Often hybrids, or F2 populations between them are used to study various learning behaviours [19]. But in these strains there is background dependent variation in many of the behaviours even in the "wild-type" stocks that could cause even more variable results when mutations are introduced [19,20]. Furthermore, the current '129' strain is actually a complex hybrid created by combining different strains at various time points since its origin in 1928 to the present [21]. Often sub-strains created from '129' at different historical time points have very high among line variation in many traits, for instance, in an extreme case skin grafts are rejected among these sub-strains in immunological studies [21]. But, in many cases, even if the phenotypes are qualitatively similar, such lines are generated from distinct collections/populations and could have different fixed alleles that could underlie complex genetic interactions. For instance, a genome-wide survey for conditionally

essential genes for viability in two yeast strains demonstrated that interactions between four or more loci are required to explain the variation in penetrance [22].

Additionally questions like whether certain genes or backgrounds are more sensitive to mutational or environmental perturbations consequently exhibiting conditional effects, can only be answered by further empirical investigations. Such considerations are essential to understanding the nature of genetic networks. For example, mutations in genes with a large number of connections in a network (for example Hsp90) have the potential to exhibit enhancement of variation in other traits [23]. Similarly, these considerations are also important with respect to choosing an appropriate background for a given screen since number and type modifiers recovered will heavily depend on the background and conditions used. Thus considering conditional effects can also be potentially utilized to identify novel loci, gene-networks or regulatory mechanisms underlying a given phenotype.

1.3 Implications on Evolutionary analysis

Understanding conditionality of genetic effects is essential for our understanding of evolutionary processes and trajectories. Historical contingency in adaptation is a type of conditional effect on evolutionary timescale, where the phenotypic effects of new mutations are dependent upon either potentiating or permissive mutations that have arisen earlier during the evolutionary process. For instance, *Escherichia coli* is naturally unable to utilize citrate. But in an experimental evolution study in the presence of both glucose and citrate, citrate utilizing variants (Cit^+) evolved after

~31,000 generations. Further analysis revealed that potentiating mutations in the Cit^+ background had allowed the evolution of the Cit^+ variants and epistatic interactions between two or more mutations had permitted the expression of citrate utilizing phenotype [24,25]. This implies that the fate of an allele entering the population depends on the genetic background in which it arises because whether it will provide a beneficial or a deleterious fitness effect which can be further acted upon by selection is contingent upon the genetic background. For instance, the neuraminidase H274Y mutation confers oseltamivir resistance but is severely deleterious and reduces the fitness of the N1 influenza virus. But permissive mutational changes to the genetic background allowed H1N1 to tolerate deleterious effects subsequent H274Y mutation making this mutation beneficial [26]. This also implies that such conditional effects can create distinct genotypic fitness peaks separated by lower fitness valleys. Thus, such peaks can only be accessed by certain mutational trajectories where occurrence of later mutations are entirely contingent upon prior fixation of other alleles.

The main implication from an evolutionary perspective is that conditional effects can influence the selection coefficient of an allele in the population. Direct evidence for this comes from at least two studies, one involving *Arabidopsis thaliana* and the other in *Drosophila melanogaster*. In the former case plants derived from two distinct ecotypes were subjected to viability and fertility selection and genome-wide patterns of selection were estimated. It was found that selection coefficients for several weak to moderately selected loci were background dependent [27].

Similarly in flies with more polymorphic genetic background, selection against several known mutant alleles was stronger [28].

Another interesting implication concerns the presence of the seemingly silent changes in the background that can be expressed upon occurrence of new mutations. Such cryptic variation can also be expressed upon environmental changes. For instance, classic studies in *Drosophila melanogaster* using heat shock to induce the crossveinless phenotype in wings and ether vapours to induce homeotic transformations of the haltere to a wing [29–32]. Upon selection for such phenocopies, it was demonstrated that artificial selection could proceed rapidly, increasing the frequency of the phenotypes. Furthermore, these "mutant" phenotypes could subsequently become expressed without heat shock or ether exposure. These studies demonstrate that, when genetic systems are environmentally perturbed beyond the 'normal' conditions, hidden variation can be released which can be potentially selected upon. In addition to cryptic genetic variation many natural populations harbor allelic variants and segregating modifiers. Combined, these constitute standing genetic variation (i.e. Standing Genetic Variation = cryptic genetic variation + background dependent modifiers). Thus in such cases, the populations can potentially adapt rapidly to a novel selection pressure since beneficial alleles might be already present in the population [33]. Additionally, the effects of drift could be minimized as these alleles might be at higher initial frequencies as compared the 1/2N frequency for a new mutation.

What causes the spread of such conditional alleles/ variants in populations? These alleles could be pleiotropic on fitness-associated traits even under invariant conditions. For example, chaperones such as *Hsp90* are required protein folding under normal conditions [34]. Alternatively selection could cause the spread of such alleles as seen in the case of a λ phage-*E. coli* coevolution study where natural selection caused the spread of three permissive mutations in the population [35]. The occurrence of a fourth mutation on this background further allowed the virus to exploit a novel receptor for infection. Alternatively, it could be a more stochastic process such as developmental systems drift, where the phenotypes are invariant but the underlying molecular pathways have diverged as seen in the vulval development of *Caenorhabditis* species [36,37]. They could also be a general property of complex biological systems that have undergone past exposures to fluctuating environments. For example, yeast and bacteria evolve cross-stress protection to different extents when grown under single stressors. While none of these scenarios are mutually exclusive, more empirical evidence is required to address this issue [38,39].

1.4 Current Thesis: Integrating and utilizing conditional effects in genetic and evolutionary analyses

I have sought to address the idea of context-dependence via genetic background using *Drosophila melanogaster* wing development and mating behaviour in naturally caught as well as inbred lab populations (where relevant).

In chapter 2 of this thesis, I investigated what proportion of interactions between mutations are background dependent. Though studies have shown the importance of genetic background effects in such interactions, whether they are common is not known. Using mutation in the *scalloped* gene that perturbs wing development, I performed a genome wide dominant modifier screen in two distinct and commonly used isogenic strains. I demonstrate that ~74% of all modifiers of the sd^{E3} phenotype are background-dependent due in part to differential sensitivity to genetic perturbation as well as strain specific modifiers.

In chapter 3 of this thesis, I investigated the role of standing genetic variation on the process of compensatory evolution after the introduction of specific wing development mutants in a large natural population. Additionally I have also investigated whether fitness recovery occurs in mutated populations via recovery in the focal phenotype (wing defect) or by evolution in other traits. I demonstrate that there exists standing genetic variation for compensatory alleles that influence the recovery of the focal wing defect via artificial selection. But despite this, natural selection mediates compensation via evolution in pleiotropic traits associated with the mutation. Furthermore, I also demonstrate that organisms can compensate for the lack of sexual signaling due to a morphological defect via behavioural and life history compensation.

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CHAPTER 2: THE CONDITIONAL NATURE OF GENETIC INTERACTIONS: THE CONSEQUENCES OF WILD-TYPE BACKGROUNDS ON MUTATIONAL INTERACTIONS IN A GENOME-WIDE MODIFIER SCREEN.

Adapted from publication with the same title DOI: 10.1371/journal.pgen.1003661

2.1 Abstract

The phenotypic outcome of a mutation cannot be simply mapped onto the underlying DNA variant. Instead, the phenotype is a function of the allele, the genetic background in which it occurs and the environment where the mutational effects are expressed. While the influence of genetic background on the expressivity of individual mutations is recognized, its consequences on the interactions between genes, or the genetic network they form is largely unknown. The description of genetic networks is essential for much of biology; yet if, and how the topologies of such networks are influenced by background is unknown. Furthermore, a comprehensive examination of the background dependent nature of genetic interactions may lead to identification of novel modifiers of biological processes. Previous work in *Drosophila melanogaster* demonstrated that wild-type genetic background influences the effects of an allele of *scalloped (sd)*, with respect to both its principal effect on wing development and its interactions with a mutation in *optomotor blind*. In this study I address whether the background dependence of mutational interactions is a general property of genetic systems by performing a genome wide dominant modifier screen of the sd^{E3} allele in two wild-type genetic backgrounds using molecularly defined deletions. I demonstrate that ~74% of all

modifiers of the sd^{E3} phenotype are background-dependent due in part to differential sensitivity to genetic perturbation. These background dependent interactions include some with qualitative differences in the phenotypic outcome, as well as instances of sign epistasis. This suggests that genetic interactions are often contingent on genetic background, with flexibility in genetic networks due to segregating variation in populations. Such background dependent effects can substantially alter conclusions about how genes influence biological processes, the potential for genetic screens in alternative wild-type backgrounds identifying new loci that contribute to trait expression, and the inferences of the topology of genetic networks.

2.2 Introduction

Fundamental to the logic of genetic analysis is that the observed variation in a phenotype for a genetically mediated trait is causally linked to one or more DNA lesions/variants. However, it is well known that the phenotypic effects of many individual mutant alleles are context dependent, with respect to environmental influences, as well as the "wild-type" genetic background in which the mutation is observed. Indeed, genetic background has long been known to influence observed phenotypic expression across traits, organisms, and a range of allelic effects, including hypomorphs, amorphs/nulls and neomorphs [1,2,3,4,5,6,7,8,9]. These results make it clear that the phenotypic effects of a mutation (i.e. penetrance and expressivity) are themselves “complex traits”, subject to environmental and polygenic influences [1]. Far beyond being a minor curiosity in genetics, the background dependent effects of a number of mutations have been at the heart of debates over the conclusions and the ability to replicate key findings from several studies, including the genetics of life span [10,11,12,13,14], stress tolerance [15,16,17] and pigmentation [18,19,20].

Although the basic influence of genetic background on the expressivity of mutations is well documented, the wider consequences of such influences are poorly understood [21]. In particular, the extent to which wild-type background influences the magnitude and sign of genetic interactions remains unclear. Research to date addressing this question [4,22,23], has largely focused on a small set of mutations, and defined genetic backgrounds. Recent work has demonstrated that the

magnitude of genetic interactions can be influenced by environmental factors [24], and even ploidy level [25]. Yet the generality of such findings remains unclear. Thus this remains an essential, but poorly explored area of fundamental genetics, as our understanding of gene interactions, and our inferences of the topology of genetic networks are often derived from genetic interactions [26,27,28,29,30,31,32,33]. In addition, modifier screens have been extremely important, and have identified large numbers of genes that interact to influence the visible expression of the phenotype of the focal mutation, even when the modifier may not have a visible phenotype by itself [34,35]. It was previously shown that the phenotypic effects of an allele of the *scalloped* gene (sd^{E3}) in *Drosophila melanogaster* is profoundly influenced by wild-type genetic background (Figure 1B), with effects extending to wing disc transcriptional profiles [36]. One gene that was transcriptionally regulated in a background-dependent matter, *optomotor blind*/*bifid* (*omb/bi*), was then examined in a double mutant combination with sd^{E3}. It was demonstrated that the phenotypic consequence of the interaction between these mutations was markedly influenced by wild-type genetic background. In one wild-type background the double mutant combination resembled the individual sd^{E3} phenotype, while in the other wild-type background, the *omb* mutation behaved as a strong synthetic enhancer of *sd* [36].

These findings clearly demonstrate the influence of wild-type genetic background on this genetic interaction, but an important challenge is to determine whether such context dependent effects are widespread. To address this question I performed a genome wide-screen for dominant modifiers of sd^{E3} using two wild-type genetic

backgrounds. My results suggest that the majority (~74%) of all modifiers are background-dependent. The background-dependence of the modifier alleles are in part due to the wild-type strains differing in overall sensitivity to mutational perturbations. Using a subset of the deletions spanning the range of modifier effects, I observed that these effects were consistent using an additional allele, sd^{ETX4}. Furthermore, I show that the deletion effects are a result of the interaction with mutations at the *sd* locus, and not a simple consequence of haplo-insufficiency in the genomic region of the deletion. I also demonstrate that the background-dependence of modifiers for sd^{E3} is linked to the same genomic regions that contribute directly to the background-dependent effects of the allele itself. I argue that the phenotypic expressivity of mutations can be considered a quantitative trait, and a more comprehensive, context-dependent view of the effects of mutations needs to emerge.

2.3 Materials and methods

2.3.1 Fly stocks

The Oregon-R strain was originally obtained from the Bloomington stock center, while Samarkand was obtained from the lab of Dr. Trudy Mackay. For both strains, we further inbred them to near isogenicity, and tested via a panel of 30 polymorphic markers to confirm there was no contamination or residual heterozygosity. A combination of sequencing and PCR-based genotyping suggests that these two strains have an approximately 2% divergence from one another, and that all

sequenced regions examined to date are a subset of variation from natural populations. The X-linked sd^{E3} mutant allele (obtained from the Drosophila stock center, Bloomington IN), used in this study is caused by a P{*w*[E] *ry*[1t7.2]=wE} transposon located in the third intron of the *sd* gene [55]. This mutant allele was introgressed into two lab wild-type strains, Oregon-R and Samarkand, both marked with *white (w)*, by repeated backcrosses involving homozygous mutant female and the wild type male for over 20 generations [36]. These lines have been subjected to extensive genotyping to verify the extent of the introgression, and to avoid contamination. The sd^{ETX4} and vg^{F02736} alleles were also obtained from the Bloomington stock center, and were introgressed for 20 generations into each wild-type strain.

Deletion lines (obtained from Bloomington stock center): I utilized the DrosDel [37] and Exelixis/BSC [38] collections of lines that have defined segmental deletions collectively spanning ~90% of the autosomes, with an average deletion size of 400kb and 140kb respectively. Deletion panels were generated in isogenic backgrounds and include overlapping as well as nested deletions within and between each panel. The progenitor wild-type strains (one for DrosDel & one for Exelixis/BSC) were used in crosses to generate background-specific control flies. While spontaneous loss of the tip of chromosome 2L, containing *l(2)gl* could potentially confound the results of our screen [56], my tests of a subset of these deletions did not demonstrate non-complementation with *l(2)gl*. Thus it is unlikely that this is a confounding factor in my analysis.

2.3.2 Dominant modifier screen

Crosses: To assess the influence of wild-type background on genetic interactions I used a dominant modifier screen, and examined *sd* mutant hemizygotes who were heterozygous for the deletions. Deletion lines (see above), and their isogenic wild-type progenitor strains were crossed to homozygous sd^{E3} mutant females (Figure 1). Flies were allowed to mate and lay eggs for 3-4 days and then transferred into fresh vials for a backup. All crosses were performed at 24˚C. For each deletion, sd^{E3}/**Y**; Deletion/+ male progeny were scored in each genetic background (Oregon-R and Samarkand) for enhancement or suppression of the sd^{E3} phenotype (Figure 1A). Thus I scored flies hemizygous for *sd*, and heterozygous for the deletions. Deletion crosses were performed in large blocks, involving 25 to 100 deletions per block (paired across backgrounds), and for each block a simultaneous set of control crosses with the progenitor wild-type strains for DrosDel and Exelixis flies was also performed. Nevertheless, there was negligible variation in the wing phenotypes of the flies resulting from the control crosses across all the blocks (not shown). However, for appropriate inferences, phenotypic analysis for all crosses within a block were made with respect to specific sets of control crosses from within that same block. I screened between (5-20) flies for each cross (crosses with fewer than 5 progeny were re-tested), with a mean/median of 8.2/7 flies per cross. Any deletion that showed evidence for modification (see below) of sd^{E3} was re-tested (new crosses) to verify the phenotypic effects. Crosses performed with DrosDel deletions on chromosome arm 3L showed a marked increase in the number of modifiers relative to other arms (22/59 compared to 37/228 for the rest of the

chromosome arms for the DrosDel collection). Thus putative modifiers on 3L were re-tested 3 times each, with consistent results, suggesting that these modifiers are unlikely due to a sampling artefact. In total 780 deletions were tested, with 18,167 flies scored.

Scoring Technique: For initial assessment of phenotypic modification I developed a semi-quantitative analysis similar to that used by other investigators [57], grouping the progressive loss of wing tissue based on shape and size (proxy for severity of mutation) into 10 categories from A through J (nominal scores of 1-10) such that, category "A" represented a wild type wing phenotype and "J" represented a severely reduced wing phenotype (Figure 5). Pure Samarkand sd^{E3} individuals were generally category D while Oregon-R sd^{E3} individuals were category H, with relatively minor variation in these scores. The rationale for such a semi-quantitative approach was two-fold. First, I wished to mirror the genetic screen approaches used in many functional genetic studies (using qualitative or semi-quantitative measures), and second this allowed us to screen a much larger panel of lines. As discussed below, these semi-quantitative measures correlated well with quantitative measures of wing size.

To mimic a traditional genetic screen I assessed interactions based largely on non-overlap distributions of phenotypes, comparing genotypes bearing deletions to their co-isogenic wild-types. While this likely underestimates the number of true interactions of the deletions with sd^{E3}, it was done so that the observed effects were

of an almost qualitative nature (as is often done for visual screens). As discussed above, all putative modifiers were verified at least once with an independent replication cross.

In addition, I also utilized a more quantitative approach, fitting the data to the following linear model:

$$Y_{ijk} = \mu + B_i + D_j + B \times D_{ij} + \varepsilon_{ijk}$$

where Y is the semi-quantitative measure of size (1-10), B is the wild-type genetic background (Oregon-R and Samarkand) and D is the deletion (deletion bearing chromosome, or co-isogenic wild-type). I evaluated the results from the linear model. While each cross was performed independently, given that so many crosses were performed, the results (with respect to significant "hits") were examined with unadjusted p-values, as well as using several methods to control for multiple comparisons (FDR and Holm/Sequential Bonferroni). The analysis was performed using the lm() function and p.adjust() in R (V 2.12).

2.3.3 Quantification of size and shape

To validate the primary findings of this study, I repeated crosses, and quantified wing size for a subset of 44 deletions, spanning the direction and magnitude of effects (background dependent-independent, suppressor-enhancer, as well as negative controls) observed in the genome-wide screen. A single wing from each of 5 male flies (*w* sd^{E3}/**Y**; Deletion/+) was dissected and mounted in glycerol, for both

backgrounds. For the isogenic wild-type control strain, 30 individuals were used from each background-specific set of crosses to better ascertain the degree of variability. Images of the wings were captured using an Olympus DP30BW camera mounted on an Olympus BW51 microscope. Six landmarks (Figure 6) were digitized using tpsDIG software [58] and centroid size was used as a measure of wing size. The landmarks were specifically chosen as they could be discerned on all wings (Figure 6). To quantitatively verify the background-dependent effects of a given deletion on wing size (Figure 4) the following model was used:

$$Y_{ijk} = \mu + B_i + D_j + B \times D_{ij} + \varepsilon_{ijk}$$

where Y is the Centroid Size, B is the background and D is the deletion. The analysis was performed using the lm function in R (V 2.12) and 95% confidence intervals were constructed using confint(). Significance was determined by non-overlapping confidence intervals with controls.

The quantitative measure of wing size used for this analysis, correlates well with the semi-quantitative method and results used for the initial screening (r=0.82, CI:0.69-0.9 in Oregon-R, r=0.78, CI:0.63-0.87 in Samarkand). This suggests high repeatability of the initial screen, as well as the semi-quantitative measure of wing size.

To ascertain whether there was a commensurate effect of the genomic deletions in "wild-type" wings (as opposed to the mutant phenotype caused by *sd* mutants), I quantified wing size in females heterozygous for the focal sd^{E3} mutation with each

deletion (*w sd^{E3}*/*w sd^{+};* Deletion/+) digitizing the same 6 landmarks on the wing. For size estimation, I utilized the same set of 6 landmarks as described above.

2.3.4 Generation and crossing of "large-wing" and "small-wing" backcross lines of *sd^{E3}*

Potentially the genomic regions (from the wild-type strains) that influence the genetic interaction between the deletions and *sd*^{E3} could be independent of those regions that influence the variation for phenotypic expressivity of the *sd*^{E3} mutation itself. To test this lines that had "high expressivity" *sd^{E3}* phenotypes in an otherwise "low expressivity" background (Figure 7) were generated. A backcross-selection procedure was used to introgress the modifiers that contribute to the "large wing" phenotype from the Samarkand background into the "small wing" background of Oregon-R and vice-versa (Figure 7). Upon generation of these lines, I repeated the dominant modifier screen as described above using a subset of the 44 confirmed modifiers and negative controls. These lines were used in identical crosses to those outlined above, with *sd^{E3}*/**Y**; Deletion/+ individuals examined.

2.3.5 Fine Scale mapping

To narrow down several genomic regions to a set of a few candidate genes I utilized an additional set of overlapping deletions in DrosDel, Exelixis and BSC strains followed by use of P-element insertional mutations co-isogenic with the Exelixis

panel of lines. I utilized this approach for four genomic regions (49E1, 57B3-B5, 63F2-F7, and 86E13-E16) detailed in Supplemental Table 2.

2.4 Results

2.4.1 The majority of dominant modifiers of sd^{E3} are dependent upon wild-type genetic background in which they are observed

Genetic modifier screens are powerful tools to both identify interacting factors that contribute to signaling networks, as well as to infer their topology. This approach has considerably shaped our understanding of the genetic basis of many traits, across numerous organisms. However little is known about how wild-type genetic background influences such genetic interactions. It was previously demonstrated that the genetic interaction between mutations in two genes, *sd* and *omb*, is dependent on genetic background [36]. To determine if such an effect is a general phenomenon I performed an analysis of genome-wide genetic interactions between the sd^{E3} mutation and deletions generated in otherwise isogenic backgrounds spanning the autosomes of *Drosophila*.

Deletions spanning a number of putative candidate genes (*Dll*, *wg*, *vg*) previously demonstrated to interact with *sd* modify the sd^{E3} phenotype were initially verified. In each of these instances the deletions confirmed previous expectations for the interaction (supplemental figure 1b). I then screened the autosomes, with two independent sets of genomic deletions, DrosDel [37] and Exelixis/BSC [38,39], each

generated in an independent isogenic progenitor background (Fig. 1b). In total 723 deletion-bearing strains (spanning ~ 90% of the autosomal genome) were crossed to sd^{E3} in each wild-type background. F1 males hemizygous for the sd^{E3} mutation and heterozygous for the deficiencies were scored.

For the 198 deletion strains that consistently modified the sd^{E3} wing phenotype, ~ 74% of the observed effects were dependent on wild-type (Oregon-R vs. Samarkand) genetic background. Frequently, the background contingency was a result of severe effects in one wild-type genetic background, with modest or no effects in the other (Figure 1A and 2, Figure 3A). A complete list of modifier regions, and putative candidate genes can be found in supplementary table S1 (doi:10.1371/journal.pgen.1003661.s008). An example of the physical location and contribution of these effects is illustrated using the left arm of chromosome 3 (Figure 3, Figure 8), where background-independent and -dependent effects are illustrated, including some deletions with opposing effects in terms of modifying the sd^{E3} phenotype.

These results were confirmed using a linear model (ANOVA), by asking what proportion of all "significant" modifiers also had a "significant" interaction effect between genetic background and the deletion. Based upon these criteria ~79% of modifiers demonstrated background dependence. While each cross was performed independently, there were a very large number of crosses performed, and each deletion bearing genotype was compared to a common reference from with the

block of crosses (see methods). Therefore, several methods that adjust for multiple comparisons were also utilized. While these methods will decrease the number of deletions deemed modifiers using standard comparisons (i.e. α=0.05), this study is primarily interested in the proportion of such modifiers that are due to background dependent effects. Using False Discovery Rate (FDR) the same frequency (~78%) as with unadjusted p-values was observed, while with the sequential Bonferroni (Holm) it was ~68%. Regardless of the exact approach used, it is clear that the vast majority of modifiers recovered are background dependent.

I performed this screen using two different sets of deletions, each of which varied in the size of the deletion. I observed little association between deletion size and severity of phenotypic modification (Samarkand: correlation-0.09 & -0.08 using Exelixis & DrosDel; Oregon: -0.061 & -0.067 using Exelixis & DrosDel deletions respectively, Figure 9). The lack of association between size of deletion and magnitude of effect suggests that it is unlikely that the observed effects are due to the number of genes perturbed in each deletion.

These key results suggest that at least in sensitization screens, and possibly for many studies of genetic interaction, wild-type genetic background will have profound influences on the range of phenotypes observed and the modifiers that are identified, with only a subset of modifiers being background-independent. Using Flymine and Droid [40,41] as well as literature mining we examined all of the previously identified genes that act as genetic modifiers, protein-protein interacting

partners, or are targets of transcriptional regulation by SD. From these sources I collated evidence for 19 genes that were covered by deletions in this screen (i.e. excluding genes on the X), and all but one (*sens*) were recovered as genetically interacting with sd^{E3} (Figure 3B). However, more than 50% of these specific loci demonstrated background-specific interactions with sd^{E3}, including *vg*, which is known to physically interact with SD to form a heterodimer, and is transcriptionally regulated by this complex. Several well-known genetically or physically interacting genes (such as *salm* and *yki*) showed surprisingly mild enhancement of the phenotype, which may also be a result of the particular wild-type backgrounds used in this study. These findings suggest that even for well-characterized interacting genes, the influence of genetic background can be substantial, consistent with the flexible nature of genetic interactions. An important caveat to this interpretation is that many of these deletions may contain more than one gene. This could potentially mean that the interaction is due to both to the deletion of the focal gene as well as other loci nearby. Yet, as described above, we observed no evidence for a relationship between deletion size and magnitude of effect, suggesting that this may be a minor contributing factor.

2.4.2 Variation in the extent of epistatic effects is in part due to differences among the wild-types in sensitivity to mutational perturbation

To further validate, refine, and extend our analysis I quantified a subset of 44 of the Exelixis deletion lines that spanned the range of modifier phenotypes across both

severity and background-dependence. Interestingly (Figure 4), the background-dependent interactions are clearly a result of both specific differences with respect to the nature of sensitizing mutational effects in each background, as well as to the degree of sensitivity to mutational perturbation. Indeed, the sd^{E3}/**Y**; Deletion/+ combinations in the Oregon-R wild-type background demonstrated considerably more variation between deletion strains, compared to the same genotypes in Samarkand (Figure 4). Despite the fact that the sd^{E3} mutation in the Oregon-R background had more severe loss of wing tissue (Figure 1, Figure 5), the range of both enhancement and suppression exceed that of the same mutation in the Samarkand background (Figure 4). The between deletion co-efficient of variation (CV) for wing size in the Oregon-R background is approximately double that (0.34) of the Samarkand background (0.15). These results were confirmed using a Levene's test with a non-parametric bootstrap. Despite the differences in both degree and spectrum of sensitivity, there was still a moderate correlation of effects of the sd^{E3}/**Y**; Deletion/+ combinations (0.66, CI(0.46,0.8)) across the two wild-type backgrounds. These data indicate many of the modifiers are acting in the same direction, although vary for magnitude of effect. Interestingly, even the non-genetic component of phenotypic variation observed for Oregon-R sd^{E3}/**Y**; +/+ in crosses to the wild-type deletion progenitor shows considerably greater phenotypic variation for wing size compared to Samarkand (Figure 4), although it is unclear if this is related to the changes in between line variation (robustness).

While the semi-quantitative measure of wing size used for the initial screen, and quantitative measure described above are highly correlated (see methods), a few putative modifier regions failed to replicate so in the tertiary validation cross with quantitative measures. Similarly a few deletion lines that were expected to not have an effect (based on the initial screen), did have one with the quantitative measure. However these potential false positives and negatives are few, of similar numbers, and thus are not expected to influence the overall conclusions.

2.4.3 The influence of the deletions for modifying sd^{E3} is not correlated with their effect on wild-type wing size.

One possible explanation for these results would be that the deletions influenced wing size, *per se*, and the results were not a specific consequence of the interaction between *sd* and the deletion. To investigate this I quantitatively examined females who were heterozygous for the sd^{E3} mutation and for the deletions (i.e. sd^{E3}/+ ; Deletion/+) across each genetic background. These females have qualitatively "wild-type" wings, and previous work did not observe an effect of sd^{E3} on wing size in females as heterozygotes [42] (although it did influence wing shape). Therefore I quantified these females across the same set of deletions as described above. If the deletions were not generally acting as modifiers of the "sensitized" *sd* mutant phenotype in hemizygous males, but as general modulators of size, then a strong positive correlation between the effects on size in males and females (sd^{E3}/+ ; Deletion/+ vs. sd^{E3}/**Y** ; Deletion/+) should be expected. The correlation between Samarkand and Oregon-R sd^{E3}/+ ; Deletion/+ females was ~ 0.8, suggesting that the

effects of the deletions on overall wing size is similar across backgrounds. However the correlations within each background (i.e. sd^{E3}/+; Deletion/+ vs. sd^{E3}/**Y**; Deletion/+) were 0.22, (CI -0.08, 0.49), and 0.21, (CI -0.08, 0.48) respectively, and neither case was significantly different from 0. The lack of a correlation indicates that the influence of the deletions in sd^{E3} hemizygous males is largely independent of any effects on overall wing size. More importantly the CV for wing size in females (across deletions) for both backgrounds was ~ 0.03, which is 5X and 10X less than that observed for sd^{E3} hemizygotes in Samarkand and Oregon-R respectively (Figure 10). This suggests that most of the phenotypic variation for wing size due to the deletion is observed when the backgrounds are "sensitized" with the *sd* mutation, while having relatively little influence on wild-type wing size.

2.4.4 Loci influencing background dependent interactions are linked to those influencing phenotypic expressivity of *sd*

A fundamental question to address is whether the loci influencing the background-specific genetic interactions are the same as those that modulate phenotypic expressivity for wing size of the focal sd^{E3} mutation. To address this question a set of backcross lines between Oregon-R and Samarkand (both fixed for sd^{E3}) were generated, where "long" wings were selected in the backcross to the Oregon-R background, and "short" wings in backcrosses to the Samarkand background (Figure 7). Using ~30 SNPs polymorphic across backgrounds, it was verified that these backcross lineages showed expected genotypes for more than 90% of markers

(i.e. phenotypically short wings but with Samarkand genotypes). Among the molecular markers that did introgress, include those tightly linked to the unknown causal loci on 2R near cytological band 48 and at the centromere of 3L [36]. If the loci modulating the magnitude of the genetic interactions were caused by genes other than those influencing the background-specific disruption of wing development, no correlation should be predicted between sd^{E3}/**Y**; Deletion/+ in Oregon-R and the equivalent genotype from the "short" backcross (with an otherwise Samarkand background). Similar logic prevails for the Samarkand and the "long" phenotype. However, even using semi-quantitative measures, it is clear that these are highly correlated; 0.82 (CI 0.66-0.91) and 0.86 (CI 0.73-0.93) respectively. These results are consistent with the loci influencing the background-dependent genetic interactions being the same as those influencing the background-dependent effects on the phenotypic expressivity of the focal sd^{E3} mutation.

2.4.5 Background dependent interactions are consistent across additional alleles of *sd*

The results described above demonstrate that the loci that influence the background dependent nature are linked to those influencing phenotypic expressivity of the mutation itself. However, it was unclear if the observations were due to some particular properties of the sd^{E3} allele, or a more general function of perturbation at the *sd* locus. To address this, I retested a subset (29) of the deletions spanning the range of phenotypic effects with sd^{E3}, using an additional allele sd^{ETX4}, across each

genetic background. The phenotypic consequences of sd^{ETX4}, while background-dependent, are somewhat weaker than sd^{E3} (Figure 11). Despite these phenotypic differences, there was a moderate to high correlation across the modifiers' effects on these two alleles. In the Oregon-R and Samarkand wild-type genetic backgrounds respectively, the correlation between the effects of the deletions on the phenotypes of the sd^{E3} and sd^{ETX4} allele was 0.66 (CI 0.38-0.82), and 0.76 (CI 0.55-0.88). In addition the general pattern of greater sensitivity to mutational perturbation by modifiers of the *sd* phenotype appears to be generally maintained (Figure 11). These results demonstrate that even across multiple alleles, the background dependence of the modifiers is maintained.

2.4.6 *vestigial* (*vg*) interacts with *sd* in a background dependent manner

Although the primary goal of this study was to explore the flexibility in genetic interactions, not to identify candidate genes, for confirmatory purposes, I examined several genomic regions that demonstrated background-dependent or -independent modifiers (Supplementary table S2-doi:10.1371/journal.pgen.1003661.s009). Interestingly, one region, 49E1, contained *vg*, which encodes a SD-regulated transcriptional factor that forms a heterodimer with SD. Fine mapping, followed by the use of candidate insertional mutants (co-isogenic to the Exelixis deletions) confirmed that the vg^{F02736} allele behaved as a background-dependent enhancer with strong enhancement in Samarkand, but very weak enhancement in Oregon-R. I followed this up by introgressing this allele into both the Samarkand and Oregon-R

background. Again I observed background-specific enhancement of the *sd* phenotype. Other fine mapping regions suggest several candidate genes, although for at least one region, no obvious candidate gene could be determined (Supplementary Table 2- doi:10.1371/journal.pgen.1003661.s009).

2.5 Discussion

Genetic modifier screens have provided an indispensible tool for identifying interacting sets of genes, providing an early glimpse into the underlying genetic network and a point of entry for further molecular characterization. Much of our knowledge of network topology has depended on the use and interpretation of such genetic interactions [43], and such information is included in many common databases and graphical representations of networks such as in FlyMine and DroID [40,41] as well as flybase[44]. The importance of modifier screens cannot be over-stated for the identification of interacting genes. Yet the generality of networks defined by these interactions is unclear, given that such screens (and thus the nature of the interactions) are generally performed in isogenic wild-type backgrounds to prevent numerous artefactual findings. In this study, I demonstrated that the majority of such genetic interactions are dependent on wild-type genetic background. These results suggest that different wild-type strains vary in their general sensitivity to mutational perturbation, as well as having strain specific responses to such modifiers (Figure 3, Figure 4, Figure 8). Both of these factors contribute to both quantitative and qualitative changes in the observed phenotypic effects across the focal *sd* mutations and the deletions. While the

majority of the observed background dependent effects changed the magnitude of the interaction, I did observe several instances of sign epistasis, where the deletion would modify the phenotypic expressivity of the *sd* allele in opposite ways in the different backgrounds. This genotypic conditionality suggests that genetic networks may be quite flexible, with segregating variation in natural populations influencing magnitude and possibly sign of interactions. Indeed, such context dependence in genetic interactions, whether due to genetic background, or other factors needs to be recognized as a likely general phenomenon.

It is probable that the results presented here under-estimate the degree of background dependent genetic interactions. In this study I screened for dominant modifiers of the *sd* mutations, and only two wild-type strains were used heterozygous against common isogenic tester strains. It is to be expected that double mutant combinations in each homozygous genetic background would demonstrate even more background dependence from the phenotypic expression of recessive alleles, as has been examined for particular pairs of interacting loci in a few model systems [4,23]. Yet in this relatively simple design, ~74% of modifiers were background-dependent (Figure 2A, Figure 3A). Even for functionally characterized genes that interact with *sd*, over 50% demonstrated interactions that were background-dependent (Figure 3B). The results were consistent both across multiple alleles of *sd* (figure 11), and across backcross-introgression lines (figure 7). In addition the results were consistent when I moved from particular deletions to individual mutations. The well-known interacting factor *vg* demonstrated

background-specific interactions from the segmental deletion containing it, to an individual mutation in the gene, with strong enhancement in Samarkand but mild effects in Oregon-R, similar to previous observations between *sd* and *omb* [36].

Overall, the observed background-dependence was due to a combination of both sensitivity of the wild-type background to mutational perturbation, as well as specific patterns of interactions between deletions and the sd^{E3} mutation. Despite the principal effect of sd^{E3} being more severe in Oregon-R than in Samarkand, both the suppressors and enhancers recovered were also of greater magnitude in the Oregon-R background (Figure 4). The choice of a particular wild-type background for sensitization screens could lead to profoundly different interpretations with respect to the number and nature of modifiers recovered. This is of some concern when it is acknowledged that wild-type strains with the same names may not be genetically identical across different labs due to new mutations, bottlenecks, recombination and contamination. Thus the inferences made from studies of pairwise mutational interactions may be difficult to generalize, and may in part explain why the same allelic combinations can result in different phenotypic outcomes. In this study, it was not just change in magnitude of the genetic interactions, but in some instances the sign (i.e. enhancer vs. suppressor) of the interaction that was contingent on the genetic background. Such findings may explain why attempts to replicate findings of genetic effects can be difficult. Despite the obvious complications, the background-dependent nature of these effects has a beneficial aspect; new loci can be identified by performing modifier screens in

additional wild-type backgrounds. Indeed with many wild-type strains being sequenced to perform genome wide associations, this may provide an additional tool for rapid identification of new interacting loci. Additionally, the use of RNAi across multiple genetic backgrounds may be able to facilitate such studies [45]. However interpretation of such complex results may require a new population level context in which to interpret such data.

There are outstanding questions that this study is unable to address. The background dependent nature of the genetic interactions could be the result of a "third-order" effect between the *sd* mutation, the hemizygous allele uncovered over the deletion and other loci across each wild-type genetic background. An alternative, and perhaps simpler explanation would be of differential quantitative complementation uncovered by the deletion [46]. In such cases, the variation in the degree of the modification of the focal mutation (*sd*) is a direct result of the alleles that differ across backgrounds uncovered by the deletion. While these results could be a combination of both explanations, it is likely that without very high resolution mapping of the genomic regions, or test of specific polymorphisms will it be possible to determine the relative contribution of each type of interaction. However the previous work that motivated this current study, namely the background dependent interaction between *sd* and *Omb* was clearly due to a third order effect [36]. Understanding the degree to which increasingly higher order epistasis contributes to phenotypic variation is under-explored but of great importance [47].

One curious finding of this study was that the background (Oregon-R) that demonstrated the higher degree of phenotypic expressivity of the focal *sd* mutations, showed increased sensitivity to mutational perturbation (both enhancers and suppressors) as well as greater phenotypic variation within line. Recent work has demonstrated that loci can influence trait variability ("noise") directly [48,49,50], including naturally occurring variants in the *Hsp90* gene of Drosophila [51]. Indeed even cell-to-cell variation, and variation in penetrance appears to have a complex genetic architecture [48] influenced by variability in gene expression [52]. It is unclear whether the loci that contribute to increased phenotypic "noise" also contribute to the amplified sensitivity to mutational perturbation as seen in the Oregon-R vs. Samarkand wild-type backgrounds. In previous work Oregon-R does have higher levels of phenotypic variation in quantitative measures of wing shape, but no increased sensitivity to weak (heterozygous) mutational perturbation [42]. However the focal mutations used in the current study (sd^{E3} and sd^{ETX4}) represented more severe perturbations to wing development, so this may not provide an adequate comparison. Regardless, this remains an unanswered question, and a potential link between so-called variance controlling genes and sensitivity to perturbation would have important implications for the genetic architecture of canalization and robustness [5,53].

One constraint of the current study is that a hypomorph of moderate phenotypic effect was utilized, as opposed to a null allele. While a formal definition of functional epistasis (sensu [54]) requires the use of null alleles, most interaction screens utilize

alleles of comparable (hypomorphic) effect to allow the recovery of both enhancers and suppressors. Nevertheless, previous work has demonstrated that null alleles can also show background-dependence effects in the primary effect of the mutation, including on development, growth and viability [1,2], and the current results demonstrate that these conditional effects are likely to be reflected in the genetic interactions between mutations as well. In addition I demonstrated that the quantitative effects observed with the interaction between sd^{E3} and segmental deletions in each wild-type genetic background were correlated when observed across another (weaker) allele, sd^{ETX4}, suggesting that such effects are not due to a particular allele. I also demonstrated that the effects of these interactions are tightly linked to the same genomic regions that contribute to the primary background-dependent phenotypic effects of the mutations. Thus at least for this system, the genetic variants fixed between the wild-type backgrounds that influence the phenotypic expressivity of the mutation itself appear to be the same as those that modulate both the magnitude, and potentially the sign of genetic interactions between mutations.

While the positive and negative implications for modifier (and other genomic) screens is clear, the potential flexibility of genetic networks given segregating variation in a population needs to also be considered. In particular an allele entering a population (either as a new mutation, or as a result of introgression from another population or species) may not have a “fixed” effect on fitness; instead the

genetically contingent effects of the allele result in a distribution of phenotypic effects, including a possible change in sign (i.e. from deleterious to beneficial).

APPENDIX

Chromosome arm	2L	2R	3L	3R
Modifier proportion in DrosDel (Background dependent/ Total # of modifiers)	8/9 = 89%	10/15 = 67%	22/30= 73%	19/25= 76%
Lines Screened	94	39	59	95
Modifier proportion in Exelixis (Background dependent/ Total # of modifiers)	20/33= 60%	19/25= 76%	17/23= 74%	32/38= 84%
Lines Screened	125	82	84	145

Table 1: Summary of modifiers recovered

Number of background dependent and independent modifiers recovered by chromosome arm and deletion collection. Similar results were obtained from the linear model, adjusting for multiple contrasts (see results).

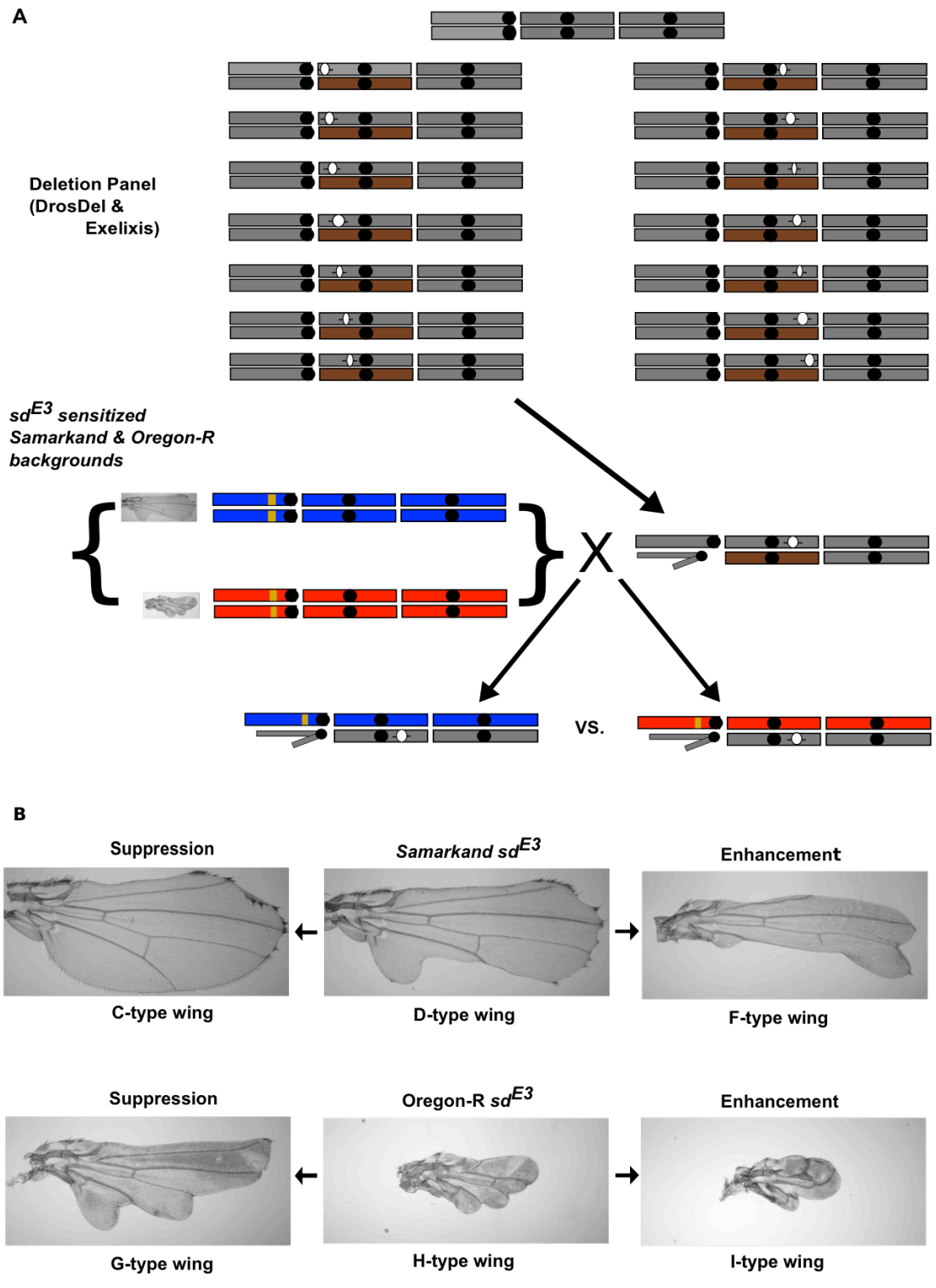


Figure 1: Genetic background effects influence *sd*^{E3}, and are used for a dominant modifier screen.

Figure 1 (cont'd)

A) Outline of the modifier screen employed in this study (illustrated for 2nd chromosome deletions). Using the DrosDel and Exelixis Deletion collections, male deletion–bearing (denoted with -()-) flies were crossed to females homozygous for the sd^{E3} mutation from each wild-type genetic background, Samarkand (blue) and Oregon-R (red). Male offspring that were hemizygous for the sd^{E3} allele and heterozygous at all other loci, including the deletion, were compared between the two genetic backgrounds. Thus we were scoring male flies hemizygous for sd^{E3}, and heterozygous for the mutation. The co-isogenic progenitor wild-type strains was used for control crosses. Each grey rectangle represents a chromosome (X, 2 & 3 from left to right), with centromeres (black dots), and balancer chromosomes (brown rectangles). Yellow represents the sd^{E3} mutation and closely linked genomic region on the X chromosome. B) The effect of genetic background on the phenotypic expression of the sd^{E3} allele, and examples of suppression and enhancement of this allele in each background. Letters beside each image represent the semi-quantitative scores assigned to wings (all figures taken at 40X magnification).

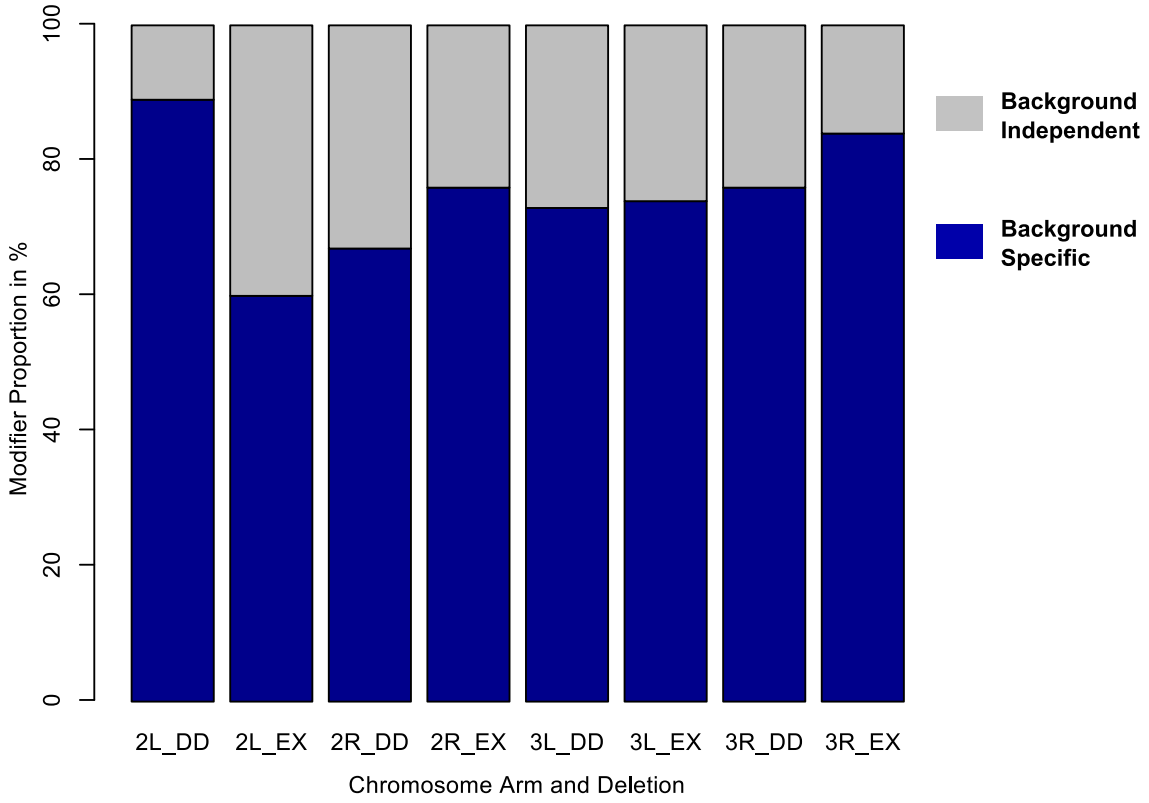


Figure 2: The majority of autosomal modifiers of *sd*^{E3} are background-dependent.

A) Proportion of deletions that modify the *sd*^{E3} phenotype in a background-dependent or -independent manner, by chromosome arm and deletion collection. DD= DrosDel collection. EX = Exelixis collection. Numbers at the bottom of each bar indicate whether the effects are in autosomal chromosome two or three, while the letters L and R represent whether the effects are found on the left or right chromosome arms, respectively. B) *This will be table 1 inset into the figure*.

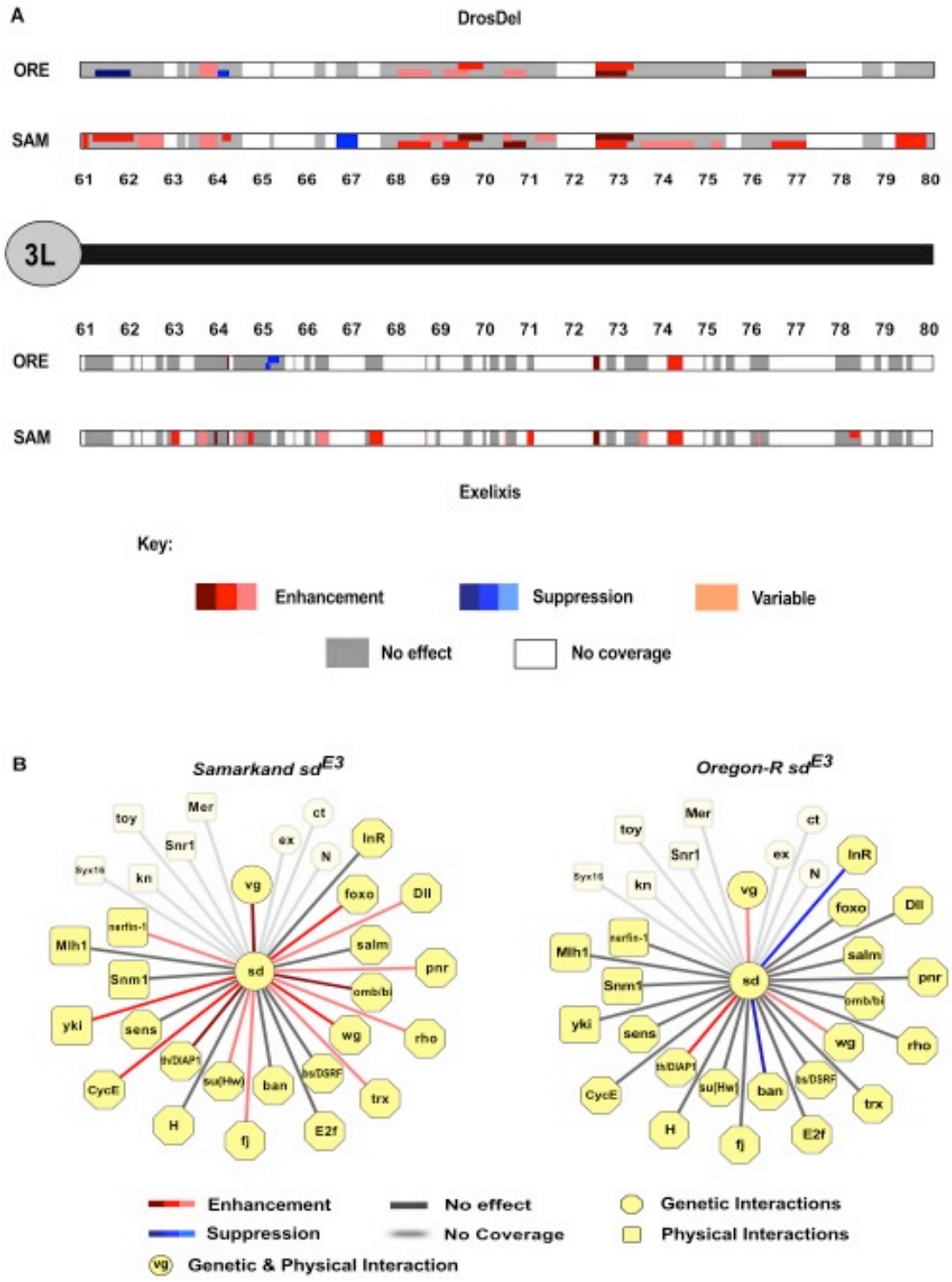


Figure 3: Genomic distribution of background-dependent and independent modifiers of *sd*^{E3}.

Figure 3 (cont'd)

A) Example of the distribution of background-dependent and -independent modifiers of sd^{E3} on the left arm of chromosome 3 for each deletion collection. The cytological location (61-80) of all deletions on the left arm of chromosome 3 are shown. Regions with no coverage are left blank (white). While there are several locations that show co-enhancement or suppression for Samarkand (SAM) or Oregon-R (ORE), most show an effect in only one background, and occasionally opposite effects (i.e. between 61-62 in DrosDel), consistent with sign epistasis. In a given collection where there were two deletions with overlapping genomic locations (or were nested), the regions in the figure are divided vertically to show the effect of each deletion. The remaining chromosome arms are shown in figure 8) Evidence for background dependent interactions for *apriori* known interacting loci. For deletions that covered the known interacting factors of *sd*, we show the background dependent effects [59]. Unlike the finding for the genome as a whole, there appears to be more synthetic enhancers in Samarkand than Oregon-R.

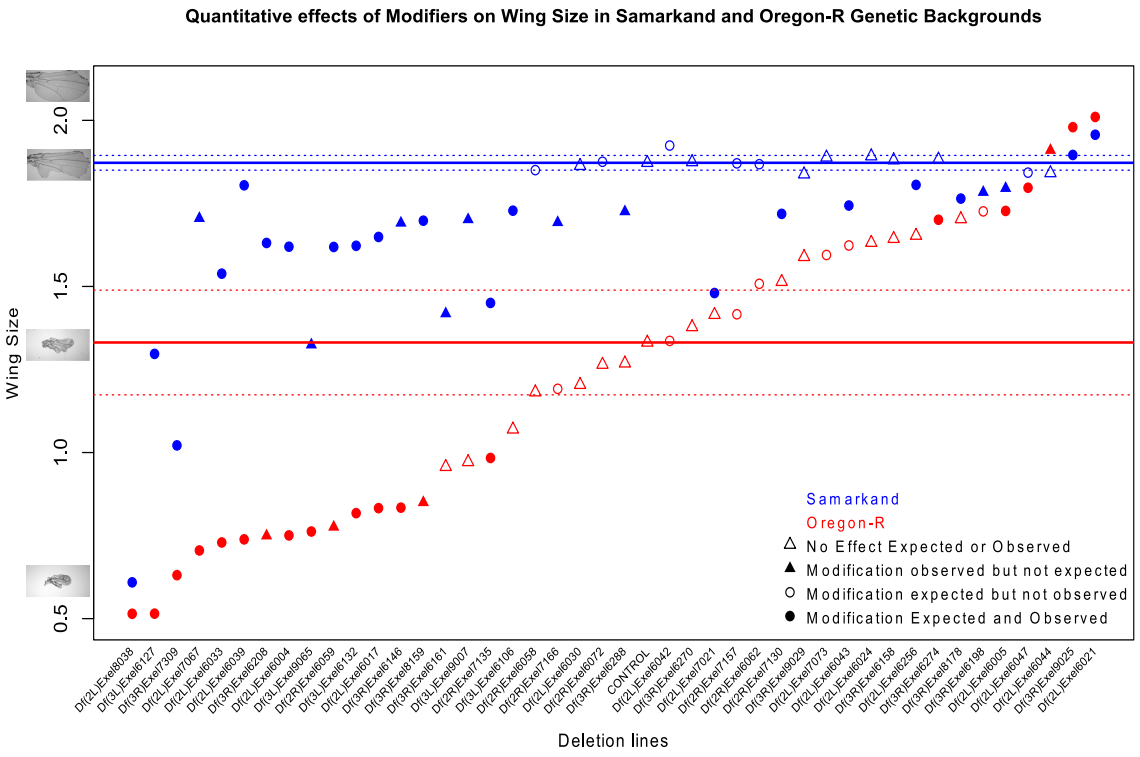


Figure 4: Background dependence is partially a consequence of strain specific sensitivity to genetic perturbation.

Quantitative effects of a subset of 44 deletions on the modification of the sd^{E3} phenotype are shown. The deletions are rank-ordered based on wing size in the Oregon-R background. Enhancement and suppression of the sd^{E3} phenotype is much greater in the Oregon-R background, relative to Samarkand, in both absolute (shown) and relative terms (not shown). Solid and stippled lines (blue and red) represent the mean and 95% confidence interval, respectively, for wing size in the control *sd* hemizygous males (sd^{E3}/**Y**). Circles represent deletions with an *a priori* expectation of modification based on the initial semi-quantitative screen, while triangles represent deletions with no observed effect in that screen. Filled symbols

Figure 4 (cont'd)

represent a significant observed effect in the quantitative screen. The Y axis shows a measure of wing size using centroid size (see methods).

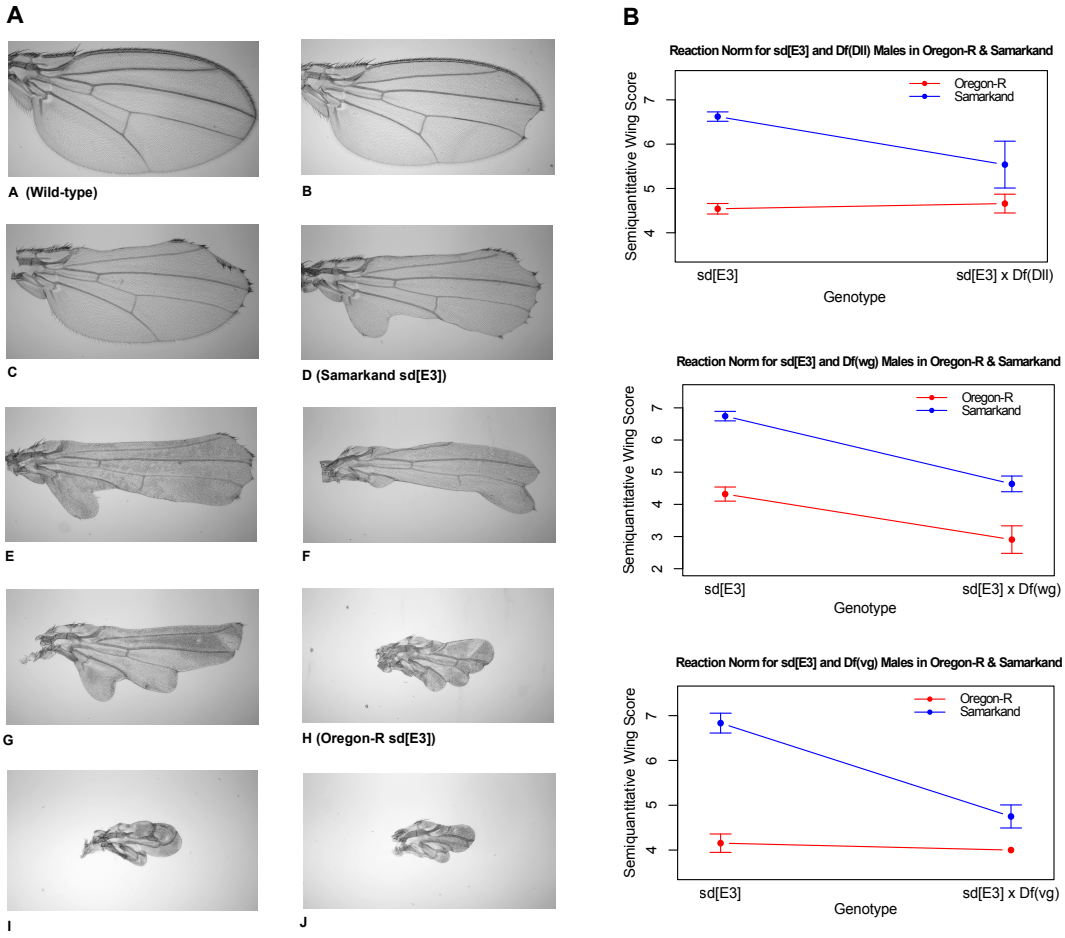
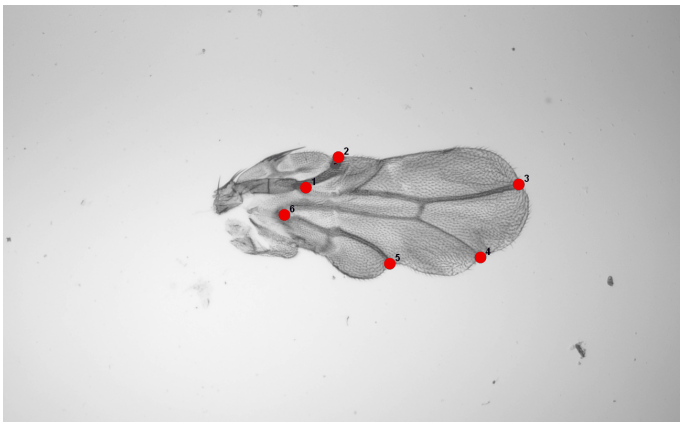
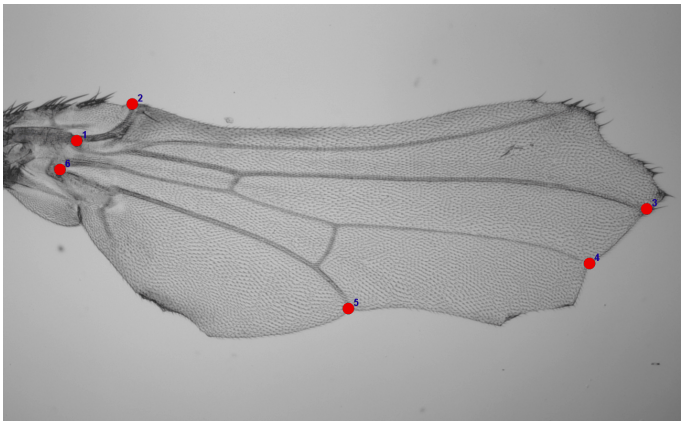


Figure 5: A) The Semi-Quantitative Scoring Scheme used for the primary screen for the modifiers of sd^{E3}. The semi-quantitative scoring scheme used for this study was similar to other ones previously used (see methods), allowing for rapid phenotyping of the wings. A comparison of quantitative and semi-quantitative methods with a test data set were highly correlated (not shown). B) Reaction norms from deletions uncovering known interacting genes with *sd.*



Oregon-R sd^{E3}



Samarkand sd^{E3}

Figure 6: Landmarks used to quantify wing size.

To quantify wing size in this study we utilized the centroid size calculated from 6 landmarks. These landmarks could be unambiguously found in all specimens that we examined in this study. It is worth noting that for mutations (not used in this study) that influence wing development more severely, these 6 landmarks could not be scored (not shown).

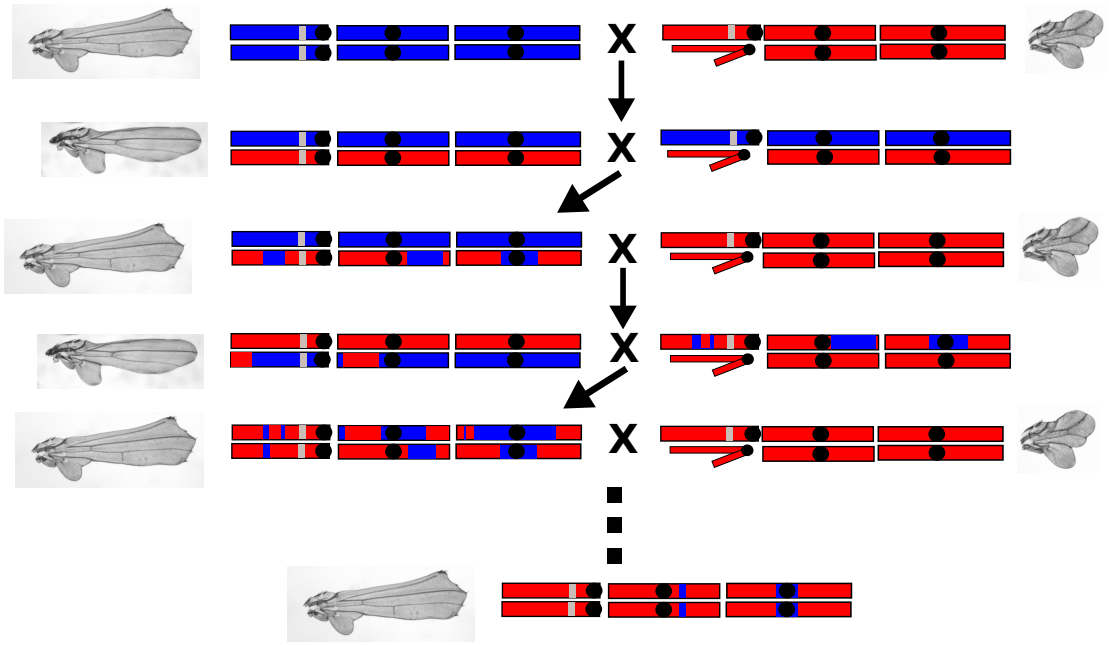


Figure 7: Backcross-selection procedure across wild-type backgrounds with *sd*^{E3} to introgress "long" and "short" alleles.

The alleles that contribute to the background dependence of the genetic interactions between *sd*^{E3} and the autosomal deletions could potentially be the same as those that contribute to the variation in expressivity in the *sd* phenotype. If this hypothesis is false, then we would predict no association between the genomic regions that contribute to variation for *sd* expressivity and the nature of genetic interactions across backgrounds. To test this, we utilized a backcross-selection procedure to move the genomic regions conferring "long" wings into an otherwise "short" Oregon-R background. Individuals from the Samarkand and Oregon-R background bearing the *sd*^{E3} allele were crossed together, and F1 flies were mated *interse* to produce an F2 population segregating alleles influencing the expressivity of the *sd* wing phenotypes. Flies with the largest wings (most Samarkand *sd*^{E3} like)

Figure 7 (cont'd)

were then crossed to Oregon-R sd^{E3} individuals, as well as the reciprocal for the shortest wings (crossed to Oregon-R). This two generation procedure was repeated for 12 cycles for the flies being selected for "short" wings, and 19 cycles for those for the "long" wings. This approach allows for the introgression of the alleles influencing *sd* expressivity from one background to the other. A panel of 30 SNP markers known to be polymorphic between Oregon-R and Samarkand were then used to verify the extent of the introgressions.

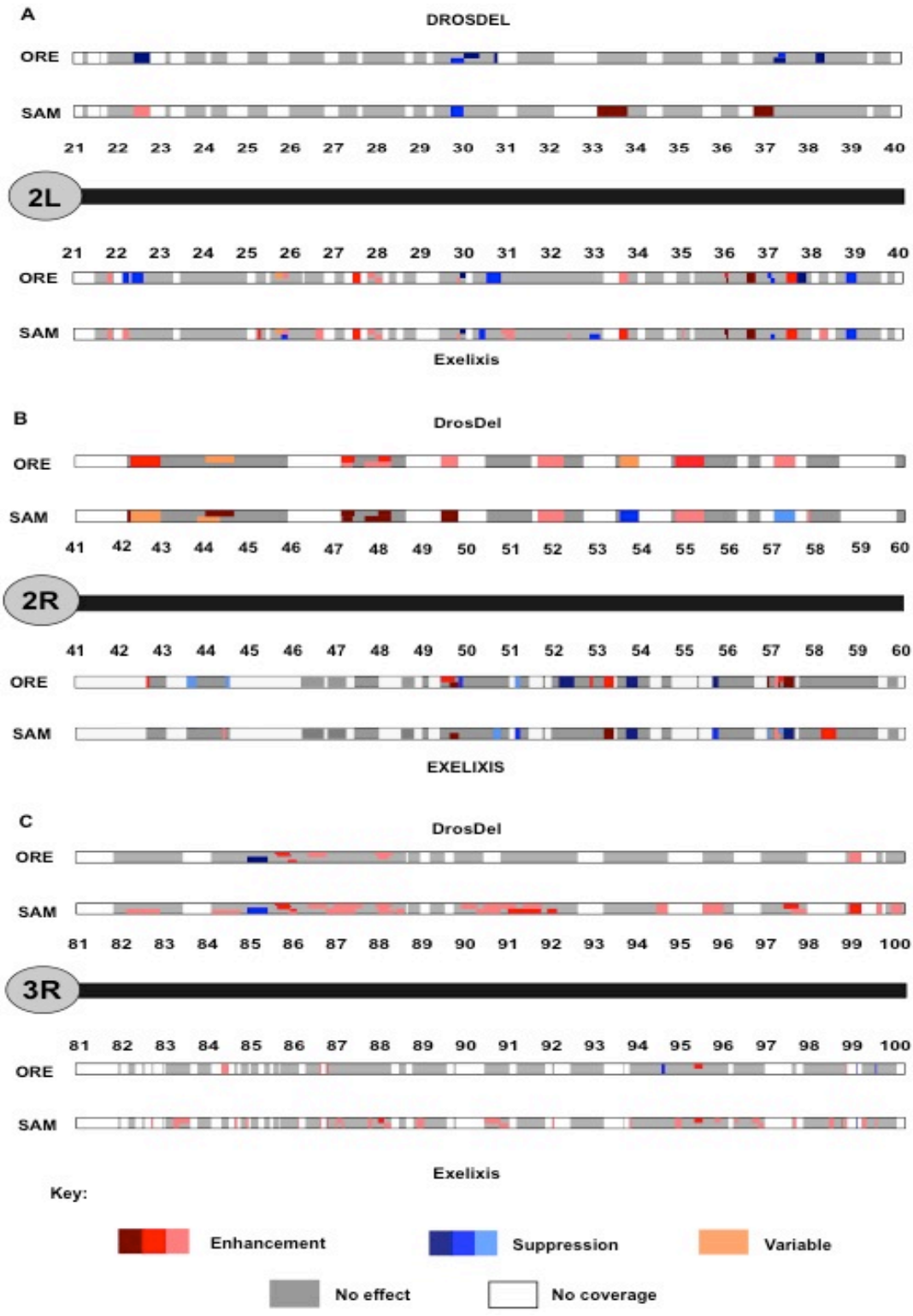


Figure 8: Distribution of modifiers on other chromosome arms.

Figure 8 (cont'd)

Figure legend and description as for figure 3A. A) Chromosome arm 2L. B) Chromosome arm 2R. C) Chromosome arm 3R.

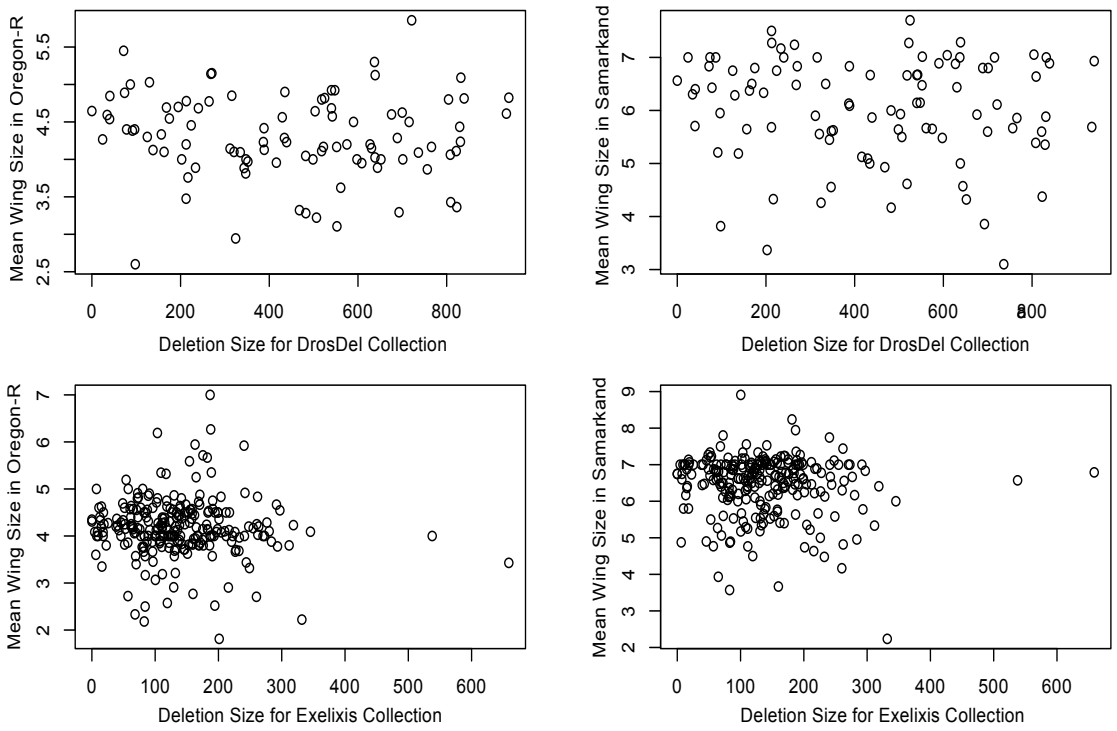


Figure 9: No association between size of the genomic deletion and magnitude of effect as a modifier of *sd*^{E3} was observed.

To determine whether the deletions generally uncovered a single or multiple modifier alleles of *sd*^{E3}, we examined the relationship between the magnitude of the effect of the deletion on the wing phenotype, and the size of the deletions (in kbp). As seen in these figures, there is no association between them, suggesting that across the set of screened lines, each deletion is likely only uncovering a single modifier allele. However particular individual deletions may have more than one modifier, and modifiers that act in opposite directions.

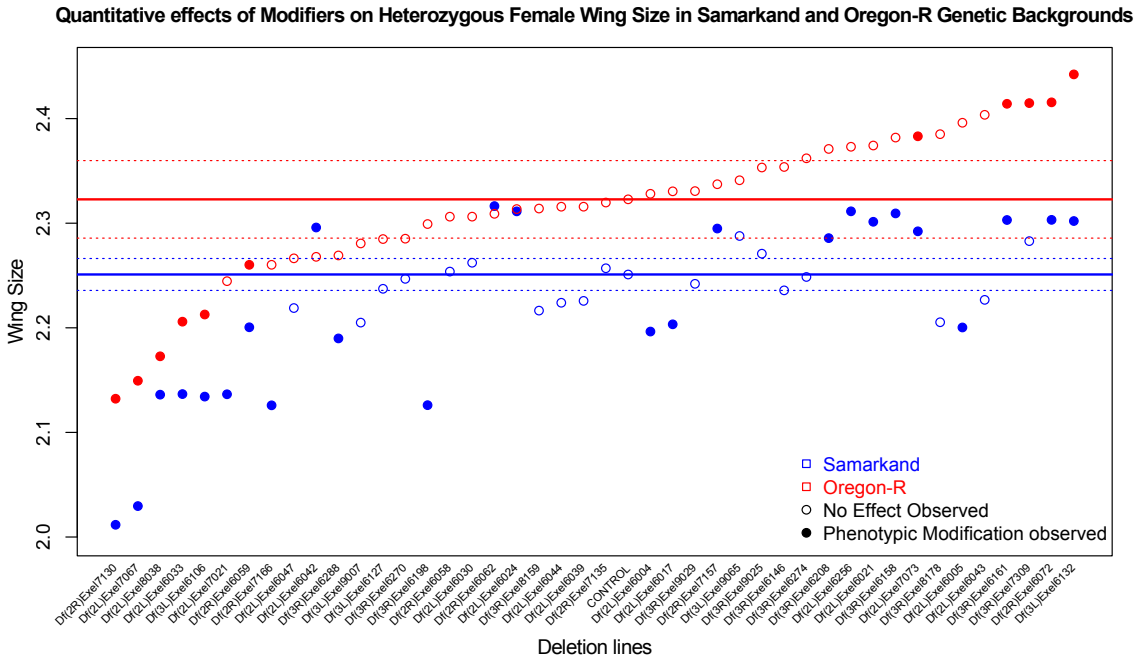


Figure 10: The effects on wing size of 44 deletions in females heterozygous for *sd*^{E3}.

To determine the extent of the phenotypic effects of the genomic deletions on wild-type wing sizes, we examined the effects of 44 of the deletions (the same ones used for Figure 4) in *sd*^{E3}/+; Deletion/+ females in each background. While the mean wing size differed across wild-type backgrounds, the range of phenotypic effects around each mean was similar (see text). Importantly, the coefficient of variation across strains was ~10X smaller for wing size for wild-type wings, than for the wings of *sd*^{E3} hemizygous males.

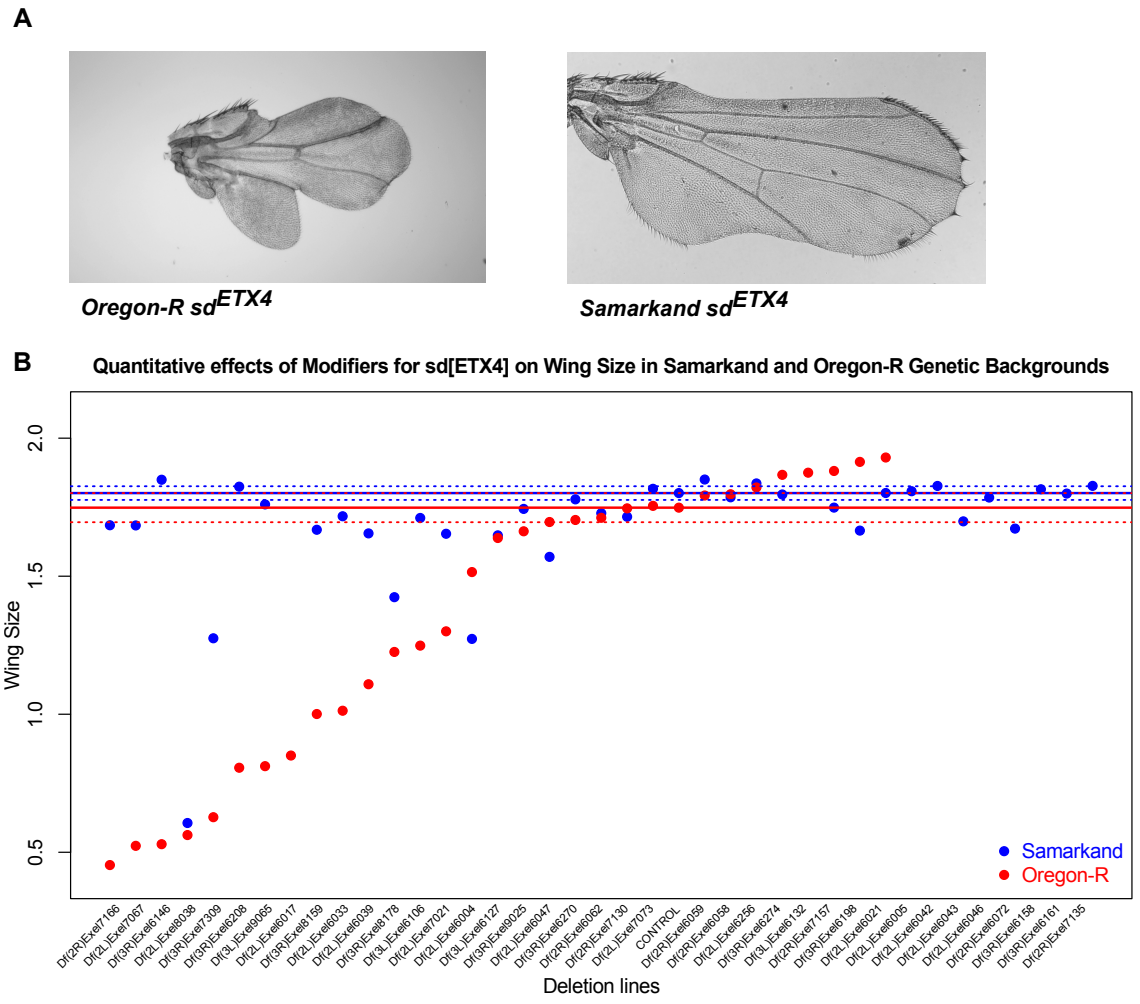


Figure 11: The background dependent effects on the *sd*^{ETX4} allele.

To determine whether the findings observed for the background dependence of the genetic interactions of the *sd*^{E3} allele with the deletions would hold across other alleles, we introgress an additional allele, *sd*^{ETX4}, into both Samarkand and Oregon-R, and re-examined a subset of the deletions. A) *sd*^{ETX4} also shows profound background dependence with respect to the expressivity of the *sd* phenotype. As described in the text, the results were significantly correlated across alleles. Interestingly the background dependent expressivity of *sd*^{ETX4} is substantially

Figure 11 (cont'd)

weakened in crosses with the Exelixis Deletion progenitor strain. However, the background dependence of the genetic interactions appears to be at least as extreme as that observed for sd^{E3} (B).

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CHAPTER 3: COMPENSATORY EVOLUTION VIA STANDING GENETIC VARIATION: DISTINCT TRAJECTORIES TO PHENOTYPIC AND FITNESS RECOVERY

3.1 Abstract

Fitness decline due to deleterious mutations can be ameliorated via conditional epistatic interactions with second-site compensatory mutations. But it is unclear whether fitness compensation occurs by directly ameliorating the phenotypic effects of the mutations. Furthermore, while compensatory evolution driven by new mutations is relatively well studied, less is known about compensatory adaptation from standing (cryptic) genetic variation. To address these issues, we individually fixed mutations that perturb wing development to different extents, in a large natural population of *Drosophila melanogaster*. Using these populations, we independently performed both artificial selection directly for phenotypic compensation of the perturbation of wing form, and experimental evolution under the influence of natural selection. We observed a rapid phenotypic compensation of the wing due to artificial selection and no phenotypic compensation of wing morphology in the natural selection lineages. However, natural selection resulted in modifications in courtship behavior and several life history traits associated with fitness that were not observed in control or artificial selection lineages. Our results demonstrate that there is considerable segregating compensatory genetic variation in natural populations that can influence phenotypic and fitness compensation via distinct mechanisms.

3.2 Introduction

Populations are constantly exposed to deleterious mutations that can potentially cause phenotypic defects and a reduction in fitness [1–3]. For instance, estimates of deleterious mutation rates per diploid genome in many organisms including fruit flies, nematodes and humans have been reported to be more than 1 per individual per generation [1,3–11] . In both sexual and asexual populations, the distributions of deleterious fitness effects are complex and not necessarily unimodal [9]. Selection purges deleterious mutations, and the strength of selection determines their frequency in populations and rate of loss. Furthermore, the rate of loss of deleterious mutations can also depend on whether they are influenced by the opportunity for mate choice and sexual selection [12–16]. There have been conflicting empirical evidence from *Drosophila melanogaster* regarding the role of sexual selection in reducing deleterious mutations with some cases showing that sexual selection is effective in accelerating the rate of loss of deleterious mutations [14,16] while others reports show no effects [17,18]. Recent evidence suggests that the evolutionary history of the base population used in a study can influence the relative contribution of natural and sexual selection towards rate of loss deleterious alleles [19]. In *Drosophila melanogaster* populations that were adapted to a particular condition, sexual selection was in conflict with natural selection while in populations that had an influx of deleterious alleles (thus not at an adaptive peak) sexual selection reinforced natural selection in providing a fitness benefit.

But deleterious mutations can often rise to high frequencies or even get fixed in populations, owing to genetic drift and bottlenecks [20–23]. Deleterious mutations can also increase in frequency as a result of hitchhiking with beneficial alleles, antagonistic pleiotropy or a change in environment causing a previously beneficial or neutral allele to become deleterious [24–27]. In such cases, fitness can be recovered via unconditionally beneficial mutations or by epistatic interactions with second site compensatory mutations that conditionally reduce the fitness cost associated with the original deleterious mutation. The distinction between unconditionally beneficial and compensatory mutations depends on the evolutionary history of the population and genetic/ genomic context- a compensatory mutation being neutral or deleterious by itself and only beneficial when co-occurring in the deleterious background [20,22,23,28]. Although, compensatory evolution occurs due to antagonistic interaction between two or more deleterious mutations, it is not necessary that all interactions between deleterious mutations be compensatory [29]. From an evolutionary perspective the following important questions regarding the process of compensatory evolution warrant empirical investigations:

1) Does fitness compensation occur via aggregation of multiple small effects mutations or via few large effect mutations?
2) Does the magnitude of compensation depend on the magnitude and number of initial deleterious mutations?
3) How does standing genetic variation contribute to compensatory adaptation?

4) Does fitness compensation occur by ameliorating the same phenotype perturbed by a given mutation or via evolution in other phenotypes?
5) How repeatable is compensatory evolution with respect to its occurrence and (genetic) mechanism under a given selection regime?

The evolutionary consequences of compensatory mutations can be understood using experimental evolution in model systems. Deleterious alleles are fixed by either repeated bottlenecks [20,22,23] or by introducing specific mutations in model organisms [28,30,31]. In the former case, multiple deleterious mutations can potentially be fixed while in the latter the number and target of mutations can be controlled. This distinction becomes important if understanding the mechanism of compensatory evolution is an objective of the study. But in both cases, low fitness populations are created that can then be experimentally evolved. This provides the opportunity for compensatory mutations to potentially arise and recover fitness. Another approach can be to expose populations to a novel/ stressful environment (for example pathogens exposed to antibiotics) [32–34]. In such cases the initial population will be of lower fitness and will start adapting via beneficial mutations in the new environment. These mutations can have negative pleiotropic consequences if the environment changes back to the original/ benign state. But prolonged exposure to the novel/stressful environment can provide an opportunity for compensatory mutations to arise and reduce the associated pleiotropic fitness cost.

Compensatory evolution in microbial systems: Experimental evolution in microorganisms provides a powerful system to address many of the above questions. In microbial systems, compensatory evolution is studied by starting with a single mutant genotype and allowing for fitness recovery to occur via new compensatory mutations. For example, the bacteriophage Φ*6* was propagated using single plaques for several generations [20]. After 40 generations of such a severe bottlenecking, a drastic decline in fitness occurred. This fitness decline was most likely due to the fixation of a single large effect mutation. This mutant clone was then allowed to recover fitness for about 100 generations via population expansion using multiple plaques, that is, at multiple population sizes. All populations recovered fitness via compensatory evolution. Interestingly, the recovered populations exhibited a positive relationship between the step size and population size. At lower population sizes (<1000), the evolution occurred via multiple steps indicating the presence of multiple small effects compensatory mutations while at larger population sizes (>=1000) compensatory evolution occurred via larger steps. While this experiment demonstrated that for a large effect deleterious mutation, there could potentially be several compensatory mutations, it did not directly manipulate the effect size or the number of the initial mutation. The generality of compensatory adaptation with respect to the effect size and number of initial deleterious mutations was investigated using *Escherichia coli* (*E. coli*) [28]. In this study, multiple mutant lines with small vs. large effect size mutations and single vs. double mutants were created by mutagenesis. When the mutant populations were allowed to evolve for 200 generations, compensatory evolution occurred in all

lineages indicating that it is a pervasive phenomenon. The relative gains in fitness were proportional to the effect size of the original mutation or pairs of mutations, that is, more deleterious the mutations higher the fitness gain. Additionally, replicate populations of a given genotype were consistent in their evolutionary response. In the above study, although the deleterious mutations were deliberately introduced into *E. coli*, they were primarily via random insertions and not targeted. A recent study in *E. coli* specifically targeted the key metabolic enzyme phosphoglucose isomerase (*pgi*) [30]. Loss of this enzyme causes growth defects by negatively influencing glycolysis and creating a redox imbalance in the cell. When multiple replicates of *pgi* knockouts were evolved for 50 days (>300 generations) on M9 minimal media with glucose these mutant strains developed compensatory mutations that influenced the stress response as well as the transhydorgenase genes that catalyse an important redox reaction in the cell. This suggested that from a mechanistic perspective, compensatory mutations arise to compensate for the fitness defects due the focal mutations in a pleiotropic manner. Yet another study where the stress response sigma factor *rpoS* was deleted in *E. coli* demonstrated that compensatory mutations arose during experimental evolution in a stressful environment that changed the expression pattern of the downstream genes from an *rpoS* dependent to an independent mode of expression [31]. Thus, in this case, compensatory evolution involved changes in the same pathway as the focal defect.

A more medically relevant form of compensatory evolution is present in the phenomenon of antibiotic resistance in microorganisms. In these cases, based on

studies in several viruses, bacteria and fungi, the general consensus is that initially when resistance emerges in a population, it is at a selective advantage when the antibiotic is present [32–36]. But in the absence of the antibiotic though, the resistance locus has a negative pleiotropic effect on growth and survival. But over several generations of continued antibiotic application, this cost of resistance is ameliorated by compensatory mutations that are conditionally beneficial in the resistant background. In many of these cases as well, the mechanism by which compensation occurs at the molecular level varies, ranging in spectrum from compensation in the same target molecule or by compensation in the interacting partners or other aspects of the resistance pathway.

Compensatory evolution in metazoan systems: Many mutations are characterized by their deleterious effects on a focal organismal phenotype, for example, survival or growth characteristics in microbial systems. Consequently, from a phenotypic perspective, this could lead one to consider that any recovery in fitness is mediated via recovery in the same trait influenced by the deleterious mutations. But animal systems offer more diverse categories of phenotypes or fitness components that can be pleitropically affected by mutations [37]. Thus a mutation that deleteriously influences such a phenotype can potentially be compensated for by evolution in other traits. An interesting example demonstrating such a pleiotropic recovery of fitness was in natural populations of Hawaiian field crickets, *Teleogryllus oceanicus* [38]. Male crickets produce a sexual signal (mating call or song) to attract mates. But a parasitoid fly, *Ormia ochracea,* also uses the mating calls to find and lay eggs with

lethal consequences to the host crickets. In less than 20 generations after parasitization, over 90% of the males had evolved a flatwing morphology, which renders them mute with respect to the mating call. This is clearly deleterious with respect to sexual signalling although it serves to protect males from the parasitoid. Upon further investigation, it was demonstrated that the flatwing morphology could rise and be maintained in the population due to evolution in both male and female behaviours that predated the loss of sexual signalling. The females in these populations had reduced mate choice and mated more readily with the flatwing males [39]. Additionally, the males exhibited a satellite behaviour where they would approach the few remaining normal wing males producing the call and would intercept the females that were attracted by the calls [40].

Another case where compensatory evolution occurred in natural populations was during the emergence of diazinon resistance in blowflies, *Lucilia cuprina.* When resistance initially arose in blowflies there was also an associated fitness cost for it in the absence of the pesticide. The resistant flies had lower survivorship and higher developmental asymmetry [25,41]. But in the continued presence of diazinon, compensatory mutations arose that reduced the cost of resistance even in the absence of diazinon. In this case the fitness and the asymmetry modifier were mapped to the same gene/ gene complex that was not linked to the original resistance mutation but probably functioned jointly during development [42].

The potential for compensatory evolution is also demonstrated in the nematode model organism of *Caenorhabditis elegans.* In these studies, distinct mutation

accumulation (MA) lines were created by propagating individuals for many generations thereby fixing multiple deleterious mutations of varying effect sizes [22,23]. The accumulated deleterious mutations affected egg to adult survivorship and fecundity, both important components of fitness. When the mutation accumulation phase was followed by population expansion, fitness compensation was observed to occur rapidly by compensatory mutations in several lines. But in this case neither the compensatory response with respect to fitness nor the mechanism by which it arose were similar between different lines [22,43]. Furthermore, between two studies that used the same lines, there were often different responses ranging from complete extinction for a given lineage in one study to acquiring different magnitudes of fitness gains in another. This was attributed the fact that different mutations could be randomly fixed during population expansion that could influence fitness recovery.

In sexual systems that harbour standing genetic variation, recombination among individuals can allow many more allelic combinations to be tested with a given deleterious mutation, potentially contributing to compensatory evolution. Thus the waiting time for a compensatory interaction to occur with a deleterious mutation may potentially be reduced. Natural populations of *D. melanogaster* harbour abundant standing genetic variation that can modify the phenotypic consequences of a mutant allele. In this model system, there are few studies that utilize artificial selection and experimental evolution to understand the effects of selection on populations with fixed, defined mutations. Mutant flies with wing vein defects

where the mutations caused gaps in the longitudinal veins or where the mutations caused extra vein materials to appear, were artificially selected to either enhance or suppress the phenotype [44–47]. These populations rapidly responded to selection in as early as generation 5 indicating the presence of compensatory segregating modifiers that can influence the phenotype. Another striking effect was seen with the *vg*^{1} mutation that causes severe reduction of the wing tissue, where artificial selection completely recovered the phenotype to wild-type [48]. These evolved lineages showed delayed development indicating the pleiotropic consequences of the modifiers that influenced the recovery of the wing defect. Thus while the above studies demonstrate compensation of the focal phenotypic effects of the mutation, they do not provide detailed analyses of other fitness consequences. It needs to be further clarified that many mutations are characterized by their effects on a given phenotype i.e. the focal phenotype. This can consequently lead one to consider that any recovery in fitness also is mediated via recovery in the same trait. But as discussed above in the case of the *T.oceanicus*, such mutations often have pleiotropic effects on other traits where compensation can occur [38–40]. Thus, in the *D. melanogaster* example, do the individuals with compensated wings also have similar reproductive success as wild-type? In contrast another study using a different mutation *nub*^{1} in *D. melanogaster*, which also causes severe reduction of the wing tissue, analysed the influence of natural selection on multiple fitness components like juvenile viability and reproductive success among others. But this study lacked replication and did not mention the effects of evolution on the wing phenotype [58].

I wanted to understand the role of standing genetic variation in compensatory evolution and importantly whether compensation in fitness occurs via recovery in the focal phenotype affected by a mutation or by evolution in other traits pleiotropically influenced by the mutation. To address these issues I individually fixed mutation in the *vestigial* gene, (vg^{1}) that results in a truncated wing and mutations in the *rhomboid* ($rho^{ve\text{-}1}$) and *net* (net^{1}) genes that result in minor wing venation defects, in large natural population of *Drosophila melanogaster.* Using the mutant populations, I created replicated treatments of artificial selection, selecting only for phenotypic compensation of the wing (i.e. recovery of the wild-type phenotype). I also generated replicated experimental evolution treatments, with natural selection altering the population (and no artificial selection on the wings). Wings are a target of both natural and sexual selection and perturbing wing development can cause flight defects, influence courtship signalling [37,49,50], aggression [51–53] and anti-predation. Thus wings are a target of both natural and sexual selection. Furthermore natural populations of D. melanogaster are known to harbour segregating variation influencing wing morphology. Thus, standing genetic variation for compensatory alleles of the wing phenotype may potentially cause a rapid recovery of the focal phenotype and fitness in both the selection regimes. But if the wing defect does not recover, fitness recovery could be potentially via evolution in these behavioural traits i.e. behavioural compensation to overcome the lack of wing-mediated signalling.

In the artificial selection lineages I observed a very rapid and almost complete compensation of the wing phenotype consistent with segregating variation for compensatory alleles that can ameliorate even severe morphological defects. Interestingly, there was no wing recovery in the experimental evolution lineages. In these lineages, I observed rapid compensatory evolution for behavioural traits that influence mating behaviour as well as other aspects of life history and fitness components. Thus I demonstrate that there is considerable standing genetic variation for phenotypic and fitness compensation. Furthermore, in populations perturbed by deleterious mutations, fitness recovery can occur independent of phenotypic compensation in the focal trait affected by the mutation. Interestingly, if such a trait mediates sexual signalling, behavioural compensation can evolve to compensate for the loss of sexual signalling.

3.3 Materials and methods

3.3.1 Drosophila strains

Mutations: The autosomal mutations *vestigial*^{1} (vg^{1}), rhomboid$^{ve\text{-}1}$ (*rho*$^{ve\text{-}1}$) and *net*^{1} (Figure 5) were originally obtained from the Bloomington stock center. *vg*^{1} was first introgressed into a synthetic outbred population prior to being introgressed into the natural population described below.

Origin and maintenance of the Drosophila Population: The large natural population of phenotypically wild-type *Drosophila melanogaster* was caught from Fennville

Winery in West Michigan (GPS co-ordinates: 42.578919, -86.144936) and a lab population was initiated using ~500 single pair matings (with non-virgin females). *D. simulans* which was present at low frequency of about ~5% was screened out and discarded. After screening, the progeny from the single pairs were mixed together and introduced ~1500 individuals in a large cage (32.5cm^3) obtained from BugDorm (BD43030F) to establish the FVW Ancestral (FVWA) population. This population was maintained at an adult density of ~1500-3000 at 23^{0}C (+/- 1^{0}C), and 30-50% Relative Humidity (RH). The adults were allowed to lay eggs in 10 bottles with 50-60ml food for 2-3 days and the bottles were incubated in a Percival (Model: I41VLC8) incubator at 24^{0}C and 65% RH throughout the larval stages. Upon onset of adult eclosion, the bottles were transferred into a fresh cage and the eclosion process was allowed to occur for 10-12 additional days. After eclosion, the old bottles discarded and the population density reduced to ~1500-3000 individuals, that formed the breeders for the next generation. All flies and larvae were maintained at 12hr Light/Dark cycle.

Introgression of deleterious alleles into the FVWA population: After allowing the *Drosophila melanogaster* FVW population to lab adapt for 2 generations the mutations were introgressed into FVW by repeated backcrossing to form 1 replicate of 'Base' mutant population per mutation. Each population level backcross cycle consisted of mating ~150-200 mutant males to 300-400 virgin FVW females to create F1 hybrids. 600-800 randomly chosen F1 flies were mated to recover recombinant F2 mutant homozygotes. I performed 10 cycles of the backcross for

vestigial^{1} (*vg*^{1}), and 8 cycles each for *rhomboid*$^{ve\text{-}1}$ (*rho*$^{ve\text{-}1}$) and *net*^{1}. This generated populations of flies that should be segregating much of the natural variation in the FVW population except for near the focal locus itself. These Base populations were maintained in small 17cm^{3} cages (BugDorm -BD41415) at an adult population density of 300-400 flies. From the Base mutant population, 4 replicates of Natural Selection (CNS) lineage, 3 replicates of Artificial Selection (CAS) lineage and 3 replicates of population-size matched Control for Artificial Selection (NASC) lineage were generated. In addition, 3 replicates of FVW controls were also created to control for natural selection to the overall lab rearing conditions. All adult flies were maintained in small cages in similar conditions as FVWA.

3.3.2 Selection Procedure

Natural Selection (CNS) & Controls: These lineages were initiated by allowing 500 adults to lay eggs for 5-7 days in 4 bottles with 50-60ml food and allowed to develop in the incubator. 2-4 after eclosion of the first few adult flies, the bottles were transferred to the cage for 5-7 days of further eclosion. After this, the old bottles were replaced with fresh, yeasted bottles for egg laying. There was no direct control on the population density and the populations evolved high adult and larval densities as the experiment progressed. The average population size for *vg*^{1}= ~2000, *rho*$^{ve\text{-}1}$ and *net*^{1} = ~3000 based on census every 8-10 generations. For the FVW control replicates the population size was estimated to be ~4000. After females laid eggs, and larvae emerged (to form the next generation), adults were stored in 70% ethanol for future morphological quantification. I also re-introgressed the base

mutant stock to the original lab adapted, natural, wild-type population (FVWA) every 8-10 generations for 3 backcross cycles as described above and especially before any comparative testing assays to generate the equivalent of an 'unevolved ancestral control' Base mutant population.

Artificial selection (CAS) & Non-selection Controls (NASC): Adult flies were allowed to mate and lay eggs for 2 days in 5-6 bottles with 50-60ml food following which, development occurred in the incubator. After adults emerged, ~1000-1200 individuals were screened, and individuals were selected phenotypically that possessed the least severe phenotypic effects of the mutation, with respect to wing morphology. Effectively, the *vg*^{1} CAS flies were selected for longest and widest wing phenotypes, while rho and net were selected for the most (relatively) "wild type" venation patterns. From this, ~55 pairs of the selected individuals were used for breeding for the next generation. Selection was performed visually under light CO_2 anaesthesia on Leica M125 microscope. The NASC controls in this case, were formed by allowing ~55 pairs of adults that were randomly chosen out of ~1000-1200 to breed for the next generation. In both cases the collected flies were maintained at 18^0C with 65% RH and 12hr Light/Dark cycle for 5-7 days before being introduced into cages. After every round of egg laying the adults were stored in 70%.

3.3.3 Measuring wing and body (thorax) size

A single wing was dissected from each of 15-individuals/sex/population/selection regime/replicate every four generations during the course of evolution and

mounted in 70% glycerol/30% PBS (with phenol as a preservative) for a total of 30 observations. Images of the wings were captured using an Olympus DP30BW camera mounted on an Olympus BW51 microscope using DP controller image capture software (v3.1.1). The wing area was then obtained using a custom macro in ImageJ software (v1.43u). Thorax of every fly was laterally imaged prior to wing dissection using Leica M125 microscope under a magnification of 63x. Body size was measured as the length of the thorax i.e. from the tip of the thorax to the first humeral bristle using ImageJ software (v1.43u).

3.3.4 Selection estimates

Estimation of strength of selection against vestigial^1: An assay to calculate the selection coefficient for the *vg^1* mutation in the BASE population under conditions with and without mate choice was perfromed. Each treatment was initiated with three replicates of 100 males and 100 females with 98 being *vg^1*/ *vg^1*, 18 wt/wt and 84 *vg^1*/ wt. So the initial frequency of *vg^1* mutation was 0.7. For each replicate in the mate choice treatment, 20 vials, each vial containing 5 males and 5 virgin females were set up. For each replicate in the treatment without mate choice 100 vials with single mating pairs were set up. In both cases, flies were lightly anesthetized on a CO_2 plate and randomly assigned to a vial. The flies were allowed to mate for 3 days in a Percival (Model: I41VLC8) incubator at 18^0C and 65% RH with 12hr Light/Dark cycle, following which the males were discarded and the females were transferred to the small cages. They were allowed to oviposit for 4 days in 4 lightly yeasted food bottles (with ~50-60ml fly food each). The females were then discarded and the

bottles placed at 18^0C and 65% RH with 12hr Light/Dark cycle. Upon eclosion, males and virgin females were collected for a period of 4 days and phenotyped. After collection the flies were randomly placed into vials with or without mate choice as described above. In addition, to determine the exact *vg*^{1} allele frequency, a test cross was performed every 3 generations by independently mating 50 phenotypically wild-type females from each replicate to homozygous *vg*^{1} male. I assumed Hardy-Weinberg equilibrium and based on the allele frequency estimated from the homozygotes I calculated the selection coefficient per generation [s = 1-(q'/q)] and the average throughout the experiment. I fit the following model for analysis,

$$Y = \mu + \beta_{Treatment} + \beta_{Generation} + \beta_{Population\ X\ Generation} + \varepsilon$$

Where Y was the allele frequency and $\beta_{Population}$ and $\beta_{Generation}$ were the coefficients for the mate choice treatment (present or absent) and generation.

Estimation of selection on the Wing: Previous evidence has demonstrated that wing size is a target of selection with respect to female mate choice [50,54–57]. Yet it was unclear if selection still occurred on the wings of individuals bearing the *vg*^{1} mutation. To determine whether phenotypic variation in wing size for *vg*^{1} individuals were a target of selection, I experimentally generated an F2 panel by mating CAS flies to Base population. The resulting population encompassed the entire variation spectrum from being phenotypically *vg*^{1} like (5-10% wing) to having almost wild-type like wings (100%). A choice assay was performed by providing a female from the base population with 2 males- one with a 5-10% (of

wild type size) wing and another with a 10-15% wing. All assays were performed in vials with 10ml food. Virgin males and females were collected using light CO_2 anaesthesia. All flies were given at least 24 hours to recover from exposure to CO_2. 3-6 days post eclosion the appropriate age matched flies were randomly aspirated into vials for the assay. Successful mating pairs were separated and the wing area of the left and right wing as well as body size of both the successful and unsuccessful males were measured. I fit following model for analysis,

$$Y = \mu + \beta_{Status} + +\beta_{Body\ Size} + \beta_{Block} + \beta_{Status\ X\ Block}\ \beta_{Body\ Size\ X\ Block} + \varepsilon$$

Where Y is the average wing area and β_{Status} represents the coefficient representing the mating success status and β_{Block} represents the coefficient for blocking effects.

3.3.5 Behavioural and Life- history assays

Prior to all assays described below, the base populations were reintrogressed for at least one generation (irrespective of previous introgressions) into the FVWA population. Also, 1000 flies from each population were collected separately under light CO_2 anaesthesia and maintained under common conditions for one generation to eliminate parental effects. Eggs for all of the life- history assays were collected on 2% grape juice agar plates (with 50-60% grape juice).

Mating Assays: I performed courtship assays for the artificial selection, natural selection, Base vg^1 and FVW control populations, for all of the replicates of the vg^1 mutation. Each of the 4 populations was reared at moderate-high larval rearing

density. Once adults emerged, they were communally maintained as 15 virgin males or virgin females per vial). They were age matched (3-6 days after ecclosion) and received exactly the same conditions of light, food and humidity. For the assay I introduced 15 pairs into a cage and scored the number of flies courting or copulating every 5 minutes for a total duration of 70 minutes. I also placed a small amount of food in the cage. In order to differentiate between male persistence and female choice, I performed similar cage courtship assays between a specific sex from the selection lineages and the corresponding opposite sex from control lineages.

Larval Competitive ability: Eggs from every replicate of FVW (both the original as well as ones undergoing selection), CAS, CNS, NASC and Base populations were placed in a food vial (15ml food) with equal number of eggs from a common competitor population (Inbred, lab strain: Samarkand wild-type marked with white-allele) at low (25+25) and high (150+150) total density. Upon emergence, we scored and categorized the number of flies that phenotypically resembled the population under consideration (red eyes) or the common competitor (white eyes). We also performed the same experiment by competing CNS and BASE populations against FVW populations as the common competitor. I fit the following model for analysis,

$$pr\left(Y_{Treatment,Common\ competitor}\right) = logit^{-1}(\beta_0 + \beta_{Treatment\ /Replicate} + \beta_{Density} + \beta_{Treatment\ X\ Density} + \beta_{Block})$$

Where the left hand term is the proportion of the treatment flies surviving over the common competitor. $\beta_{Treatment\ /Replicate}$ represents the coefficient for the treatment

as defined by the selection regime accounting for replicate effects. $\beta_{Density}$ represents the coefficient for density (low vs. high) and β_{Block} represents the coefficient for the blocking effect.

Egg to adult viability and development time: Eggs from every replicate of FVW, CAS, CNS, NASC and Base populations were placed in a food vial (15ml food) at low (50) and high (300) densities. To determine viability, we scored and calculated the proportion of flies that eclosed to the number of eggs placed in the vial. We also recorded the time to first and last eclosion and duration between them, which gave us the egg to adult development time for a particular population. I used the following model for analysis,

$$pr(Y_{Survived}) =$$

$$logit^{-1}(\beta_0 + \beta_{Treatment\ /Replicate} + \beta_{Density} + \beta_{Treatment\ X\ Density} + \beta_{Block})$$

Where the left hand term is the proportion of the treatment flies alive. $\beta_{Treatment\ /Replicate}$ represents the coefficient for the treatment as defined by the selection regime accounting for replicate effects. $\beta_{Density}$ represents the coefficient for density (low vs. high) and β_{Block} represents the coefficient for the blocking effect.

To model the development time data, I used the same model separately for both densities,

$$Y = \mu + \beta_{Day} + \beta_{Treatment\ /Replicate} + \beta_{Treatment\ X\ Day} + \beta_{Block} + \varepsilon$$

Where Y is the proportion of the total flies ecclosed. β_{Day} is the coefficient for the day of ecclosion. $\beta_{Treatment\ /Replicate}$ represents the coefficient for the treatment as defined by the selection regime accounting for replicate effects. β_{Block} represents the coefficient for the blocking effect.

Testing Female Fecundity: 20 virgin females from every replicate of FVW, CAS, CNS, NASC and Base populations were introduced with conspecific virgin males 2-3 days after eclosion, as single pairs in a minimally yeasted food vial (<10ml food) for 3 days. This three-day mating period was to partially mimic the waiting time in selection treatments before new bottles are added. After this 3-day mating period, each single pair was transferred to a fresh vial (~10ml food) to lay eggs- termed as day 1- for 24 hours. On day 2, the single pair was transferred to another minimally yeasted fresh food vial. This single pair was transferred into fresh food vial without yeast for another 2 days. To determine female fecundity we counted the total number of eggs laid over 4 days. I used the following model for analysis,

$$Y = \mu + \beta_{Treatment\ /Rplicate} + \beta_{Density} + \beta_{Treatment\ X\ Density} + \beta_{Block} + \varepsilon$$

Where Y is the total number of eggs laid. $\beta_{Density}$ is the coefficient representing the density at which the flies were raised. $\beta_{Treatment\ /Replicate}$ represents the coefficient for the treatment as defined by the selection regime accounting for replicate effects. β_{Block} represents the coefficient for the blocking effect.

Statistical analyses: All statistical analysis were performed in R v3.1.1 using the lm() and glm() functions. This was followed by extracting and plotting the relevant coefficients using the effects() package v3.0-0. Other plots were generated using the sciplot() package v1.1-0

3.4 Results

3.4.1 *vg¹* has stronger deleterious effects in the presence of mate choice

vg¹ allele has a strong wing defect phenotype, but we wanted to confirm whether it also had a strong deleterious effect on fitness. Additionally, since wings are involved in sexual signalling, we also wanted to determine the extent to which the potential for mate choice would influence the deleterious nature of the allele. The frequency of the *vg¹* allele is rapidly reduced in all treatments consistent with severe deleterious consequences on fitness (Figure 6). On average, the selection against *vg¹* was stronger (s= 0.25) in the presence of mate choice. In this treatment its mean allelic frequency reduced from 0.7 to 0.21, consistent with the known role of wings in sexual selection. In the absence of mate choice, on average, selection against *vg¹* was reduced by half (s= 0.12) and its mean frequency reduced from 0.7 to 0.41. But reduction in fitness, even in the absence of mate choice also indicated that *vg¹* had deleterious effects independent of sexual selection.

3.4.2 Rapid recovery of near wild type wing morphology for mutants under artificial selection demonstrates that segregating variation for compensatory effects is present in the population

To understand whether standing genetic variation influences the recovery of the wing morphology, I performed artificial selection and also allowed populations to evolve under the influence of natural selection. There was rapid and sexually dimorphic recovery of the wing morphology in all of the lineages artificially selected for the most "wild type" wing morphologies. For the *vg*^{1} mutation, the males compensated more rapidly than females (Figure 7). In these populations the wings compensated to almost wild-type phenotype as early as the 14th generation and by generation 32 the mean wing area increased to ~1.5 mm^2 from the initial ~0.15 mm^2 at generation 1. Such a rapid recovery is consistent with the presence of standing genetic variation for compensatory alleles for the developmental perturbations of these mutations on wing development. Furthermore, the increase in wing size is not correlated with increases in body size, which has remained relatively consistent throughout the evolutionary process (Figure 8). I observed similar rapid wing-phenotype recovery in *rho*$^{ve\text{-}1}$ and *net*^{1} CAS lineages. But there was no striking sexual dimorphism in compensatory response with either of these mutations (Figure 9).

3.4.3 Despite standing genetic variation for compensatory alleles on wing morphology, natural selection proceeds by compensation independent of wing development

Interestingly, I did not observe any recovery of the wings in the CNS lineages (Figure 7). In contrast, the wing phenotype appeared to have reduced further, suggesting an alternative evolutionary trajectory in which the wings may not be as important. Indeed, there is some evidence of a small decrease in wing size for the *vg*^{1} lineages. Similarly the populations allowed to evolve by natural selection that possessed the *rho* and *net* alleles also did not demonstrate any phenotypic recovery. Thus despite demonstrating the availability of segregating genetic variation to compensate for the perturbations to wing morphology, this was not the route natural selection took and I sought to examine what alternative phenotypic routes natural selection utilized.

3.4.4 Wings may be a potential target of sexual selection even in *vg*^{1} populations

As there was no phenotypic compensation of the wing defect in CNS populations, we wanted to confirm whether wings were indeed a target of selection in *vg*^{1} populations Since our rearing procedure did not require improved flight performance of *vg*^{1} individuals, I focused on whether wing size was important for successful mating in these populations. Previous work has demonstrated that the magnitude of wing clipping is linearly related to copulation latency [50]. However, as the *vg*^{1} mutations' phenotypic consequence is the result of a developmental perturbation, it remained a formal possibility that the variation for wing size in the mutant population was insufficient to observe selection for mate choice. Mate choice

experiments were performed utilizing males with *vg*^{1} mutation, but that varied by 10-15% for wing size (Figure 10). Mate choice experiments demonstrated that on average successful males had slightly larger wings (0.13mm^2) as compared to the unsuccessful males (0.128mm^2). While the experimental evidence was very weak, it still demonstrates that wing size could potentially be under selection with respect to mate choice.

3.4.5 Behavioural compensation in the courtship behaviour in natural selection populations potentially mediates fitness recovery

I investigated how populations with defective wings, lacking wing-mediated sexual signalling could recover for the loss of their courtship signal. Since I did not observe any changes in the wing phenotype in the natural selection lineages, any potential recovery in fitness was independent of recovery in the wing morphology. I investigated whether CNS populations had evolved changes in their mating behaviour to compensate for the lack of wing-mediated sexual signalling. I performed behavioural assays with *vg*^{1} selection and associated control populations. Our population level mating assays demonstrated that a significantly higher proportion of males from the CNS populations (~50%) were engaged in courtship at any given time as compared to the "unevolved" Base population as well as the FVW control populations at any given time (~30% for each) (Figure 11). This increase in courtship also resulted in increased copulation in the natural selection lineages as compared to the "unevolved" Base populations although it did not achieve wild type levels (Figure 12).

I also wanted to understand whether this change in the mating behaviour was due to increase in male persistence or decrease in female choice. Utilizing a similar population level scheme, when I assayed flies from the CNS lineages with the control lineages, we observed that the CNS males courted consistently more irrespective of the females provided as compared to the "unevolved" Base population (Figure 13). This increase in courtship also resulted in increased copulation in the natural selection lineages as compared to the "unevolved" Base populations although it did not achieve wild type levels (Figure 14). This indicated that behavioural changes had occurred mainly due to increased male persistence in the CNS populations. This demonstrated that the CNS populations had compensated for the lack of wing mediated sexual signalling via an increase to courtship persistence for the males of these populations.

I used a similar set of population assays to determine whether artificially selected flies had also recovered the wing-mediated behaviour. In addition to the wings, vg^{1} mutation also affects the wing-associated musculature that mediates the wing-vibrations during courtship. Thus, even if the wings recover it is not necessary that the associated musculature and hence the ability to produce the courtship song recovered upon artificial selection. I observed that the proportion of CAS flies courting and copulating were almost comparable to the wild-type flies (Figure 11-14). This demonstrated that the compensation for defect in morphology could also recover associated performance (and associated behaviours). I have not explicitly

tested whether males from the artificial selection lineages produce a courtship song equivalent to that of the wild-type males.

3.4.6 Life history compensation in natural but not artificial selection populations

Since *vg*^{1} had deleterious fitness consequences independent of sexual selection, I wanted to investigate whether any pre-adult components of fitness had evolved during the selection process. I examined, egg to adult survivorship and development time, larval competitive ability with a common competitor and female fecundity at low as well as high densities using *vg*^{1} mutant and associated control populations.

My results demonstrated egg to adult survivorship had increased in CNS populations as compared to the BASE mutant populations, and was almost comparable to that of wild-type at both densities. Interestingly, the egg to adult survivorship was severely reduced in the artificial selection (CAS) lineages, compared to almost all of the populations at both densities. This suggested potential antagonistic pleiotropy due the alleles that recovered the wing defect. While the controls for artificial selection (NASC) were also somewhat reduced, they remained significantly higher than the CAS artificial selection lineages at high densities. I observed minimal differences between the wild-type controls and the ancestral wild-type flies at high density while it was more variable at low density (Figure 15).

For the larval competition assay we used the inbred white-eyed Samarkand strain as

the common competitor. This strain was substantially weaker and survived much worse than all the other treatment populations (Figure 16). But the natural selection lineages showed higher larval competitive ability at high density as compared to the "unevolved" Base as well as the artificial selection lineages. I performed an additional assay, where the CNS and BASE populations were competed against FVW as the common competitor. The CNS populations tested, survived significantly better at both densities as compared to BASE when competed against FVW. This provided a clearer demonstration that the CNS populations had evolved higher larval competitive ability (Figure 17).

I observed negligible difference in total female fecundity among lineages. Furthermore, there was no plasticity associated with total female fecundity whether the parents of the females were raised under low or high density (Figure 18). But accounting for body size differences between treatments for just one of the replicates per treatment, there is weak evidence for higher fecundity in the natural selection lineages as compared to other lineages at high density (Figure 19). Egg to adult developmental time (from egg to eclosion as adults) did not appear to evolve among treatments substantially (Figure 20-21).

3.5 Discussion

Compensatory evolution in microbial and nematode systems demonstrates the ubiquity of *de novo* compensatory mutations contributing to adaptation. But we do not yet have a clear understanding of how selection utilizes standing genetic

variation to compensate for both the phenotypic and fitness defects due to a mutation. I thus sought to investigate the role of standing genetic variation in compensatory evolution and whether fitness compensation occurred via recovery of the focal phenotype perturbed by the mutation or by evolution in other traits. To address these, I fixed several mutations, in a large natural population of *Drosophila melanogaster* and subjected them to two distinct selection regimes of artificial selection and natural selection. Focusing mainly on one of the mutations, vg^1, during the course of selection I assayed and found evidence for rapid compensatory evolution in morphological, behavioural and life-history traits in these populations contingent upon the selection regime.

To confirm whether vg^1 indeed had fitness defects that could be potentially compensated for, the strength of selection against this allele was tested in a polymorphic background with the wild-type allele. While the frequency of the vg^1 allele reduced in treatments with and without mate choice, when mate choice was present, sexual selection and natural selection acted potentially synergistically to accelerate the loss of the mutant allele as compared to the no mate choice treatment. My results also showed that vg^1 allele was potentially influencing different stages of life cycle that could be influenced by sexual or natural selection, i.e. the adult and the juvenile stages respectively. Thus compensatory evolution could also potentially influence these stages distinctly- an idea that has not been explored before.

Wing morphology in Drosophila is a potential target of selection and it has also been demonstrated that there is substantial segregating variation influencing wing size and shape in Drosophila [59–62]. The rapid recovery of almost completely wild type wing morphology in all replicates of CAS populations upon artificial selection in as early as 14 generations is consistent with compensatory alleles segregating in the natural population. My results are consistent with a previous study showing the rapid evolution of larger wings upon artificial selection using the same vestigial mutation introgressed into a different natural population [48]. The pattern of compensation was sexually dimorphic for vg^1, with males recovering faster than females. To my knowledge, this is the first evidence of sexual dimorphism in the rate of compensatory evolution, and it could probably arise due to overexpression of a sex-linked modifier. But whether such segregating variation is available to natural selection for mitigating a severely deleterious mutation that is fixed in the population is not known. The lack of wing recovery in all the natural selection lineages might suggest that these modifiers may be rare or require being in rare combinations to compensate for the wing defect. Alternatively, the wings in the BASE vg^1 might have substantially weakened selection. It is also possible that there is more standing variation as well as stronger selection for other fitness-associated traits that have evolved and imposed a constraint thereby inhibiting recovery of the wings. While neither of these explanations are mutually exclusive, I tried to address the more parsimonious hypothesis of whether selection operates on vg^1 wings. This was especially relevant in my study since there was no direct selection on the flight-capability and any selection in these populations would be most likely due to sexual

selection on the use during courtship as a sexual signal. A critical aspect of courtship and mating success in Drosophila involves acoustic wing vibrations, which also depends on the wing size as well as shape [37,50]. While it is difficult (due to very small wing size) to investigate whether *vg*^{1} flies vibrate their wings during courtship, I tested whether males in the *vg*^{1} population with slightly larger wings had higher mating success. This was indeed the case; where on average males with larger wings were more successful in mating. But this effect was extremely weak and may not be biologically significant. Thus, based on both my results and past experiments [37,50], while sexual selection could potentially act to increase wing size, it is weak. Additionally, instead of ameliorating the effects of the *vg*^{1} mutation, the natural selection lineages seem to be further undergoing a gradual reduction in wing size. There is evidence in other species for selection for wing loss and flightlessness from naturally occurring as well as lab generated mutations [63,64]. While I did not explicitly select for flightlessness, my results suggest that the natural selection populations have evolved along another trajectory (discussed below) and reduced the importance of wing-mediated fitness gain, at least with respect to mate choice.

I investigated whether flies from the natural selection lineages had modified their mating behaviour to compensate for the lack of sexual signalling. Upon testing the mating behaviour I observed that the rate of courtship was higher in the naturally selected populations relative to the ”ancestral” population and other control lineages. This led to an increase in successful copulations, for the naturally selected

lineages, relative to the "ancestral" mutant population, although not to the wild type levels. Furthermore this modification of courtship was largely due to increased male persistence. No changes in the female behaviour in the CNS populations were detected and could have a number of potential explanations. I presumably tested female attraction based on how many males courted or copulated with females without mate choice rather than explicitly providing mate choice and hence lacked the sensitivity to detect subtle changes in female behaviour. Alternatively, these populations were maintained at high population densities that can variably influence mating behaviour in populations ranging convenience polyandry (reduction in female choice) to increased female reluctance [65,66]. Indeed in other species, there is evidence of increased male persistence potentially reducing female co-operation [67]. Although it would be interesting to understand the manner in which mate choice and female receptivity could have evolved in these lineages, it would not substantially change the general conclusion regarding behavioural evolution to compensate for loss in sexual signalling in the CNS populations.

In contrast, recovery of wings may not necessarily guarantee the recovery of flight or wing-mediated courtship and consequently mating success, since there was no direct selection on either of these abilities. But the CAS lineages could utilize their indirect flight muscles and as evidenced from the courtship assay were almost as successful as the wild-type flies in acquiring mates. This result has two potential implications; from an evolutionary perspective, if an allele perturbs a phenotype causing an associated fitness decline, then recovering such a phenotype can also

recover the fitness cost associated with its perturbation. From a developmental perspective, if an allele influencing a phenotype is perturbed without perturbation in other aspects of the underlying developmental pathways, then compensating for the effects of the allele should also consistently recover the phenotype. For instance, when considering evolutionary transitions between wingless and winged morphs, if the underlying developmental pathways are conserved and present in a wingless morph then persistent and strong selection might be able to recover winged phenotypes [68–71].

In holometabolous insects like *Drosophila melanogaster*, the sexual phase is distinct and temporally and spatially separated from the juvenile phase. The larval phase is important in resource acquisition and thus there is a potential for natural selection to act independently on this stage of the life cycle; on aspects like survivorship and larval competition. The amount of resources acquired in the larval stages also influences adult condition and many aspects of adult derived fitness components. I found that the egg to adult survivorship in naturally selected populations had substantially increased their survivorship compared to the BASE population although it was significantly less than either of the wild-type populations. The artificially selected populations have the lowest egg to adult survivorship suggesting that the alleles that provide phenotypic compensation of the wing phenotype might have negative pleiotropic consequences on survivorship. While the larval competition assay suffered from reduced sensitivity due to a poor choice of common competitor, it was still clear that the naturally selected populations were more

competitive than either the artificially selected lineages or the "ancestral" base population. Additionally, experiments with the FVW wild type population as the common competitor demonstrated that naturally selected populations were more competitive than the BASE populations in larval competition. This is consistent with other experiments testing adaptation to high larval density where larval competitive ability evolved [72,73]. Interestingly, the choice of competitors used also influences the total survivorship with less total survivorship when an inbred strain was used as common competitors. This potentially suggests an interaction between selection due competition and genotypic diversity in a given environment influencing total productivity at a population level [74] although it remains to be tested further.

I did not find any changes in the relative developmental rates between different populations at either low or high density, though previous evidence suggests developmental rates could potentially evolve in a density dependent manner [73,75]. This can be explained by the fact that the high-density populations were allowed to eclose for a period of 10-12 days after the first eclosion thereby reducing the strength of selection on both early development and early fecundity or any correlation between them. Similarly, in the artificial selection lineages (low-moderate density), artificial selection was performed over 7-9 days as the adults eclosed. This allowed for the possibility that more phenotypically compensated flies (in terms of wing morphology) might develop at a slower rate. Thus the artificial selection procedure could have reduced selection, on both early development and early fecundity.

The lack of difference in absolute female fecundity, which is also shown to evolve at different densities in previous studies [73], could also have a number of potential explanations. The CNS populations evolved under high density as both larvae and adults. Also I did not test long-term fecundity or female fecundity directly at two densities, but rather tested the effects of larval density on individual adult female fecundity. Besides experimental design reducing the sensitivity of detecting differences there is a possibility that the absolute female fecundity is indeed not different among the populations, which has broader implications. From a developmental point of view, the compensation for the wing phenotype could potentially impose a developmental cost on other traits such as body size that consequently determines female fecundity [76]. But my results show that there is no correlation between body size and wing size in the CAS populations, which suggests there is no competition for resources between development of the recovered wings and general body size. Consequently, larger wings should not impose a cost on fecundity in these populations, which is observed in my study. It thus supports evidence from *Onthophagus sagittarius* in which production of larger horns does not engender a cost on female fecundity [77]. In the CNS populations however, while the absolute fecundity is similar to other treatments, I have limited evidence that after accounting for body size, fecundity could be higher at high density as compared to all other populations. Thus, if body size were directly correlated with fecundity, then reduction in body size without any change in fecundity would suggest evolution in this trait. This is plausible if we consider the evidence for reduction in the wing

morphology in these populations. There exists indirect evidence that relaxation of selection on flight can increase fecundity selection by reducing trade-offs in resource allocation [68,69,78]. This is hypothesized for the repeated evolution of flightlessness. While I do not directly test resource allocation trade-offs in our populations, it is possible that further reducing flight-related structures can provide extra resources towards fecundity. Thus, not only the continued presence of vestigial wings but further reduction in flight-associated structures may even be adaptive in these populations. But body size was accounted for only one of the replicates in all treatments and to demonstrate whether evolution in fecundity is general in the natural selection populations, further tests on all replicates are necessary.

While there was some variation among replicates, for all phenotypes the direction of response was identical. Thus consistent repeated parallel evolution was achieved in all populations under the same selection pressure. I also observed two novel phenotypes during the evolutionary process and the subsequent assays, although I have not quantified these yet. In only one of the replicates in the CAS lineages, there was a consistent development of extra-vein material between L3 and L4 longitudinal veins as the wings recovered. This also shows that while the general trend of the evolutionary response is same, there can be novel phenotypes generated potentially due to some historical contingency. Additionally, during the fecundity assays, in all of the reduced wing lineages we observed a change in the oviposition behaviour. After two days of oviposition on the food surface, the females switched to laying

eggs on the walls of the vials. While this was present in only vg^{1} flies with reduced wings and not in flies with wild-type or compensated wings, it is not clear whether this is a general pleiotropic response for being flightless (to avoid getting stuck in the food) or it suggests a previously unknown pleiotropic role for the vg^{1} allele in the oviposition behaviour. It also remains to be studied whether there was a difference between the natural selection and BASE populations in this behaviour.

I also performed similar selection with mutations in *rhomboid* ($rho^{ve\text{-}1}$) and *net* (net^{1}) genes. These mutations are present in low frequencies in natural populations and previous selection experiments with these have produced rapid phenotypic response by artificial selection [44–46,79]. My results are consistent with previous experiments and show that segregating compensatory modifiers can influence recovery of the wing phenotype in these populations in as early as 3-5 generations. Additionally, I also demonstrate that CNS lineages of these mutations do not recover the wing morphology. I have not quantitated whether the mutational effects have enhanced in these populations. While vg^{1} is a strong perturbation with severe fitness consequences, the other alleles are relatively weak and likely have relatively smaller effects on fitness. From an evolutionary perspective it is understandable that selection for a complete recovery could be weak since there is negligible fitness benefit. But I have not performed exhaustive tests of these lineages and am not aware of fitness consequences of the mutant alleles or evolution in other traits.

In conclusion, my study demonstrates that under identical selection, rapid and consistently repeatable compensatory evolution can occur from standing genetic variation across multiple mutations (with severe or weak effects). Phenotypic as well as fitness compensation in mutationally perturbed populations can occur via distinct mechanisms influenced by both natural and sexual selection on distinct stages of the life-cycle. In this case, the artificial selection lineages rapidly recovered the focal defect in the wing phenotype consequently recovering flight and courtship defect but have lower survivorship. The natural selection lineages seem to have taken an alternative evolutionary trajectory by increasing courtship behaviour and survivorship without recovery of the wing phenotype. Thus as shown in my study, organisms can compensate for the loss of sexual signalling via compensation in behavioural and life-history traits. A potential avenue of further research could be to understand whether these distinct trajectories were due to an evolutionary constraint imposed by the severe perturbation (a deep valley) in combination with relatively weak selection on the recovery of the wing phenotype in the CNS lineages? It would be interesting to perform experimental evolution where in addition to sexual selection at high density, if there was also a direct stronger selection on the wing phenotype in the natural selection lineages. Finally, it would be interesting to understand the underlying genetic architecture and mechanistic details that have produced such striking phenotypic evolution in terms of morphological development, behaviour and life-history.

APPENDIX

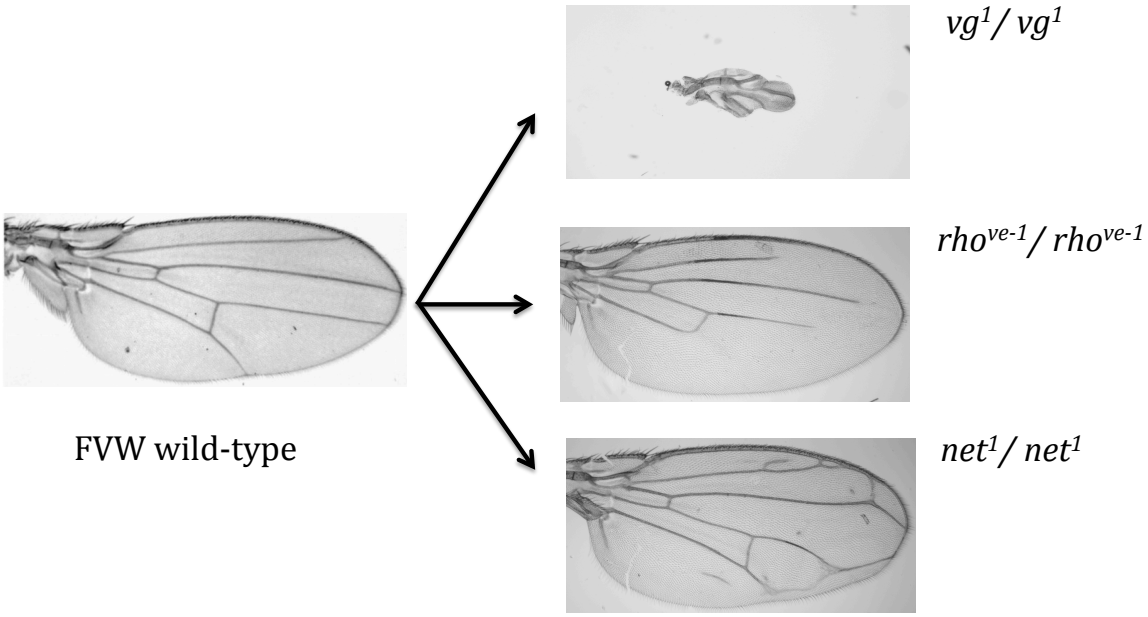

Figure 12. Effects of the mutant alleles on the wing phenotypes.

The homozygous phenotypes of three autosomal mutations introgressed into the FVW natural populations. These mutant populations were used to initiate the selection treatments.

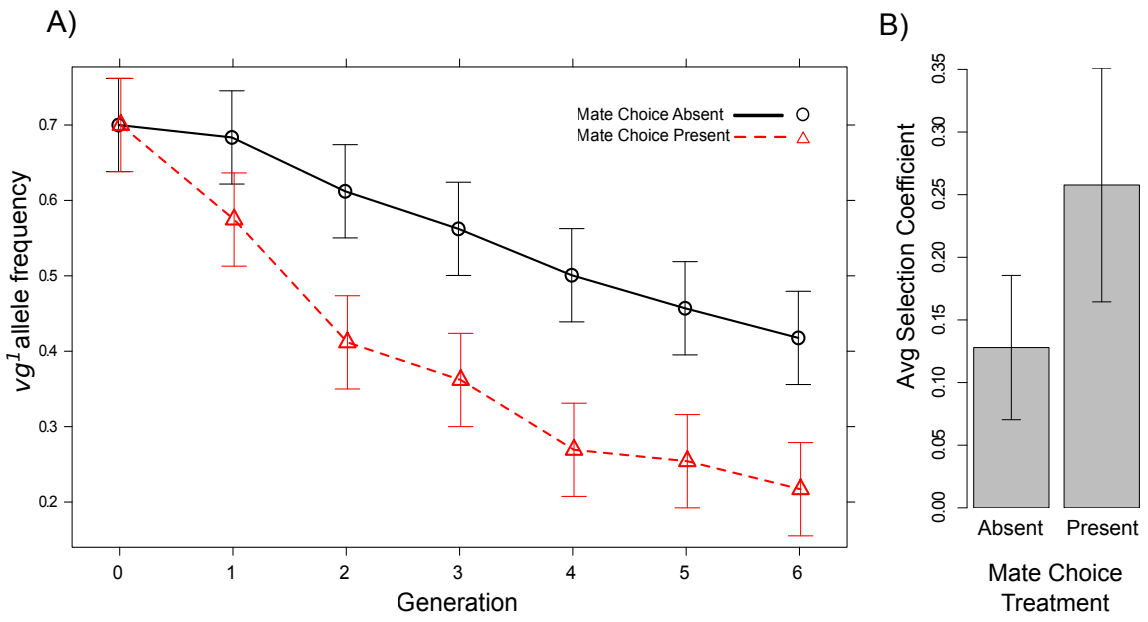

Figure 13. Rate of loss of *vg*^{1} is accelerated in the presence of mate choice

A) The mutant allele frequency decreases over multiple generations both in the presence and absence of mate choice. The rate of loss of the mutant allele is more accelerated in the presence of mate choice . The open circles and triangles represent the means and the error bars represent 95% CI from three replicates each of the mate choice absent and present treatments respectively. B) The average selection calculated over the course of the experiment demonstrates that the selection coeffecient for the mutant allele in presence of mate choice is almost twice in magnitude as compared to the absence of mate choice. The grey bars represent the mean and the error bars represent the 95% CI.

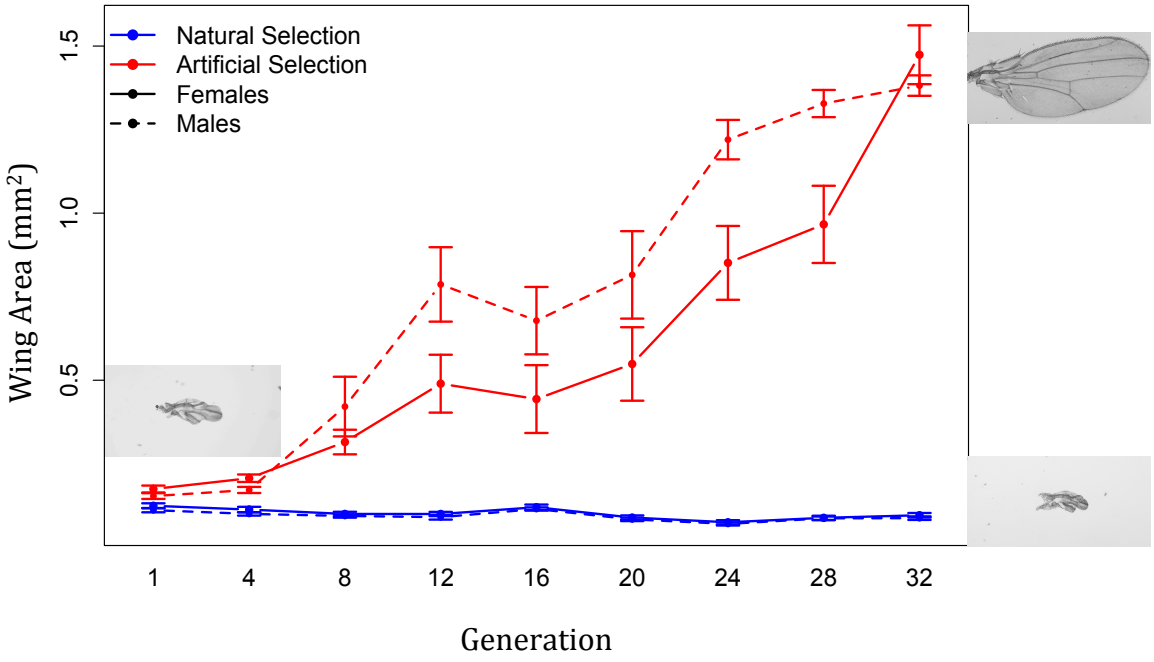

Figure 14. Rapid response of the wing phenotype to artificial selection demonstrates the presence of standing genetic variation for compensatory alleles

Measure of wing area every four generation during evolution demonstrating differences in selection response to artificial and natural selection. The solid and stippled lines represent the evolutionary response exhibited by females and males respectively. Closed circles represent the mean wing area of 15 individuals per sex per selection regime and the error bars represent the 95% CI.

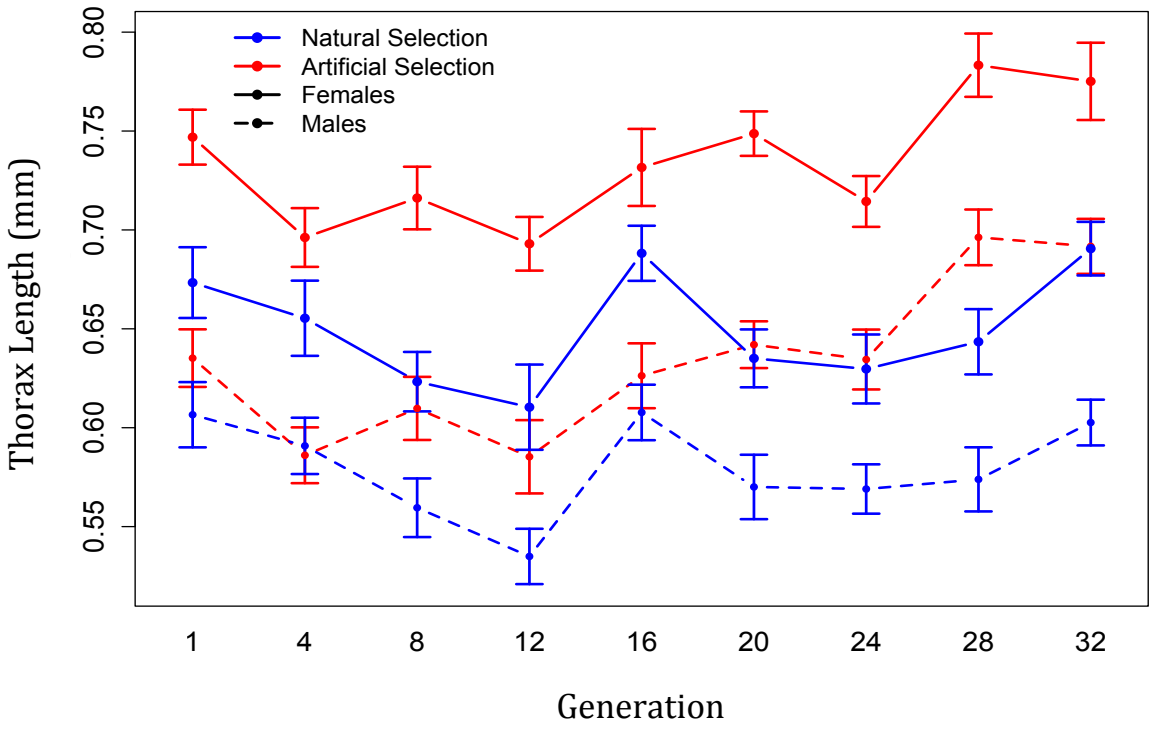

Figure 15. No correlated response in body size to explain the evolution in wing size

Measure of thorax length every four generation during evolution demonstrating relatively little change in body size in both selection regimes. The solid and stippled lines represent the evolutionary response exhibited by females and males respectively. Closed circles represent the mean thorax length of 15 individuals per sex per selection regime and the error bars represent the 95% CI.

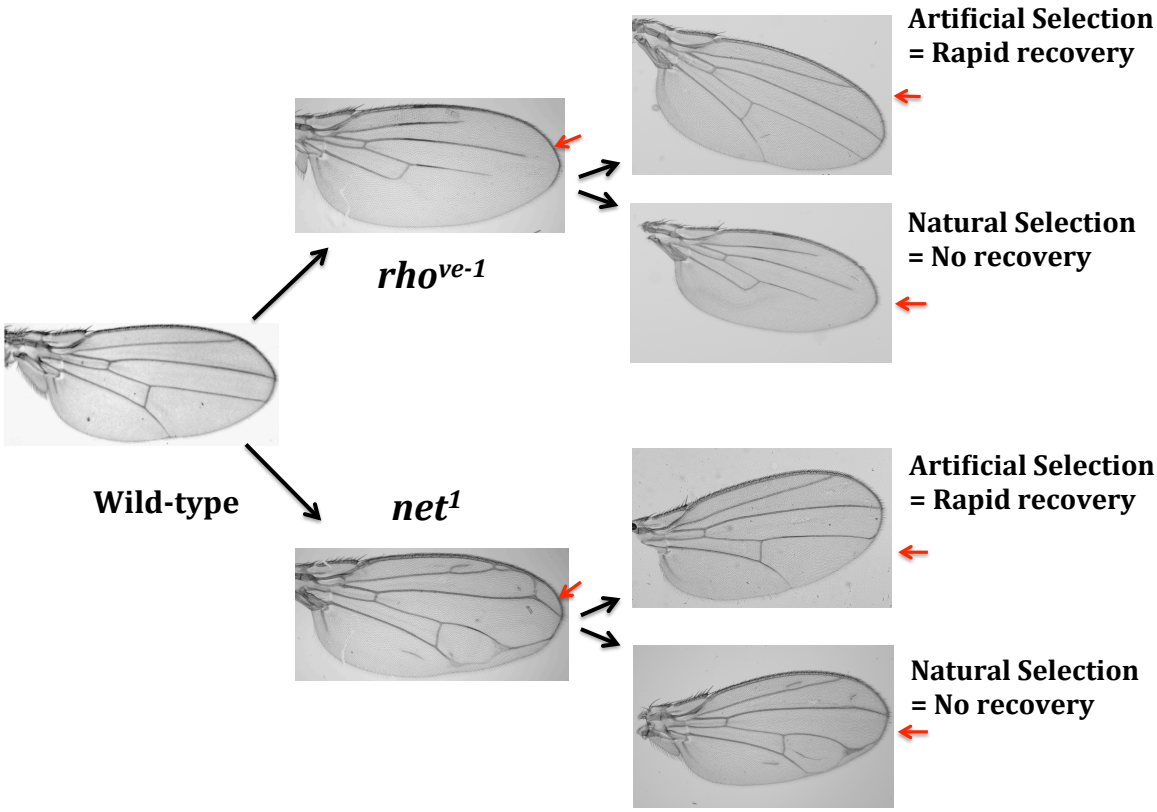

Figure 16. Phenotypes of the wings for *rho*$^{ve-1}$ and *net*^{1} mutations after 24 generations of evolution

Qualitative analysis of the wings reveal similar pattern of recovery of the wing defect by artificial selection and not by natural selection

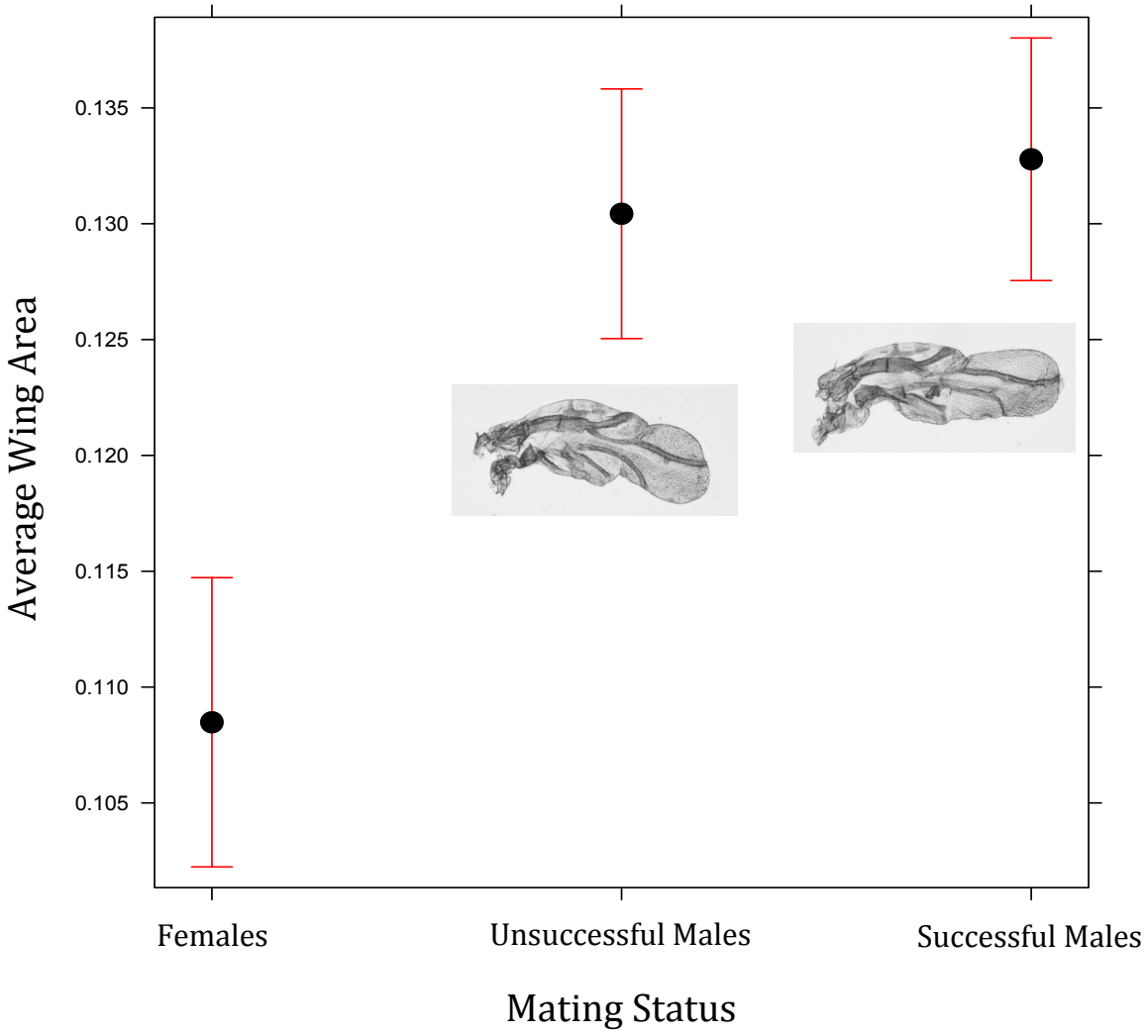

Figure 17. Female Mate Choice: Successful males have relatively larger wings

Wing area measured from 200 trials with females provided a choice between males with small vs. large wings demonstrates that the successful males have larger wings. Circles represent the average wing area for each category and the error bars represent the 95% CI. The wing images shown are representative of the mean wing sizes for both successful (winners) and unsuccessful (losers) males.

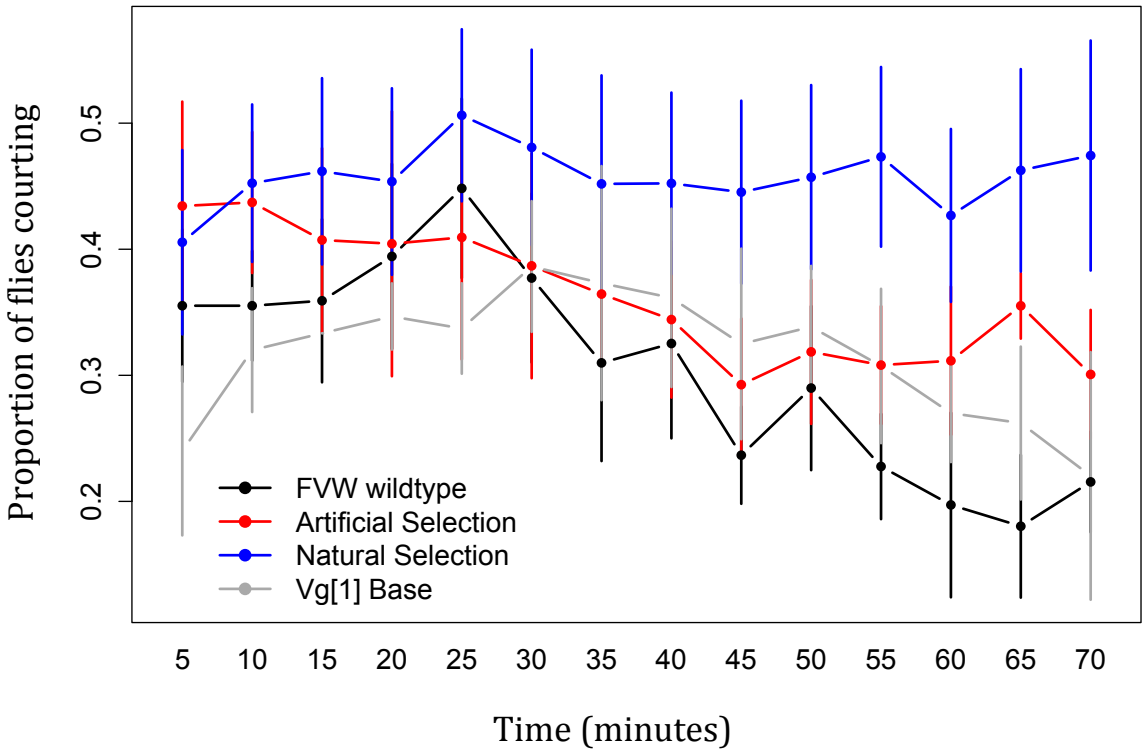

Figure 18. Proportion of flies courting from the natural selection lineages is consistently higher than all the other populations

Time series depicting the variation in courtship behaviour in all the populations demonstrates that the natural selection lineages perform the courtship higher than all other populations during the assay period. The circles represent the average proportion over multiple blocks and replicates for each population and the error bars represent 95% CI.

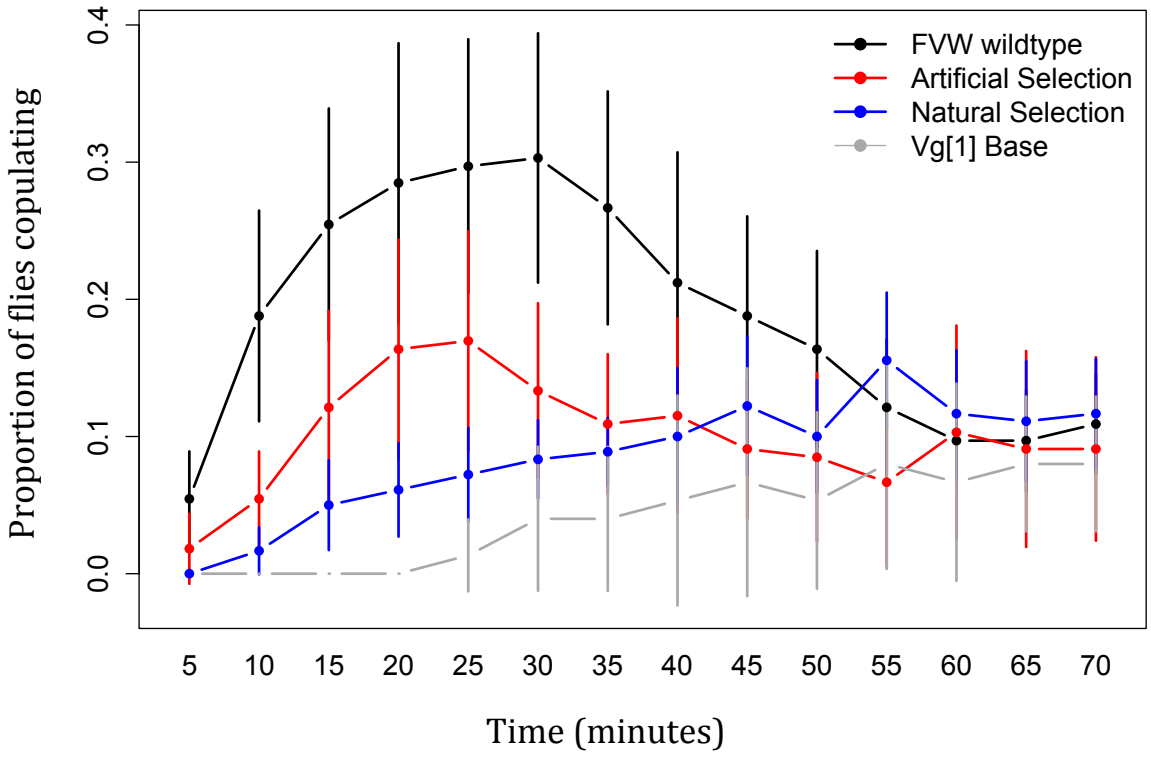

Figure 19. Proportion of flies copulating from the natural selection lineages is consistently higher than the unevolved base mutant.

Time series depicting the variation in copulation in all the populations demonstrates that the natural selection lineages perform copulate more than the base mutant population during the assay period. The circles represent the average proportion over multiple blocks and replicates for each population and the error bars represent 95% CI.

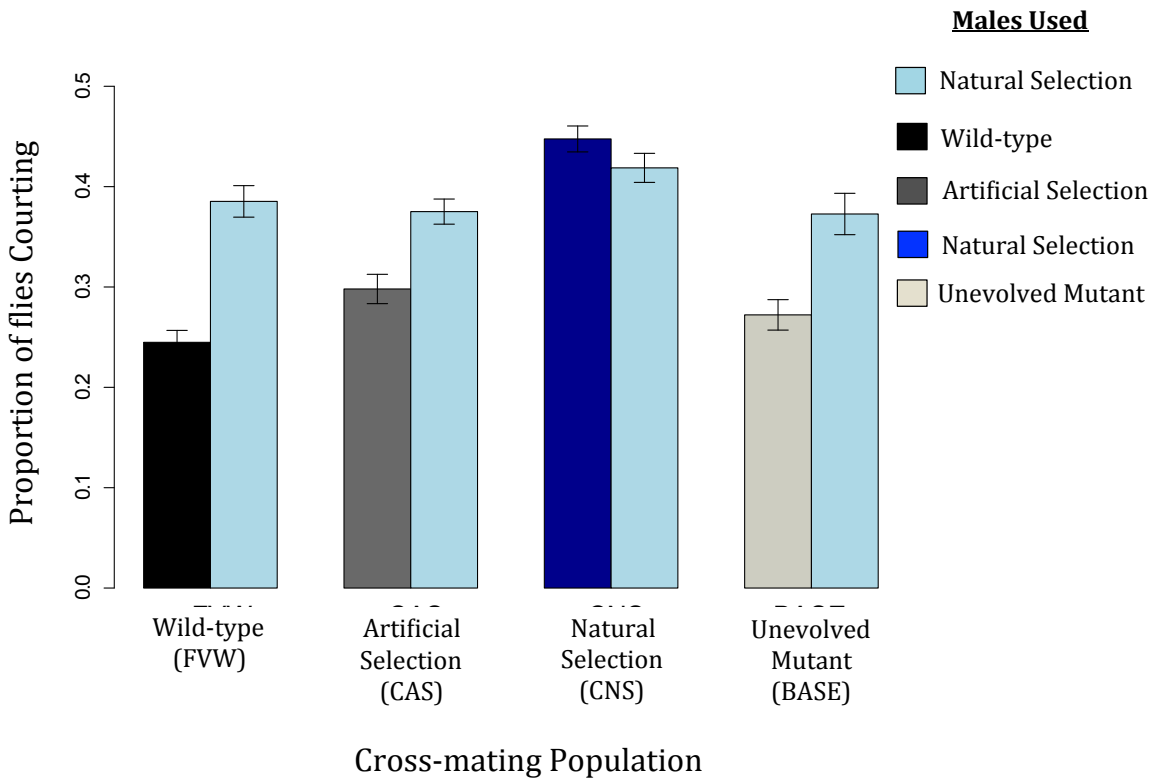

Figure 20. Proportion of males courting from the natural selection lineages is consistently high irrespective of the female provided

Graph representing the results from the "Cross mating" assay where a given set natural selection flies were reciprocally assayed for mating behaviour with flies from all other treatments. The X-axis represents the other treatment groups that were used for the assay with the natural selection lineages. The bars represent the average proportion of flies courting over multiple blocks and replicates for each population and the error bars represent 95% CI.

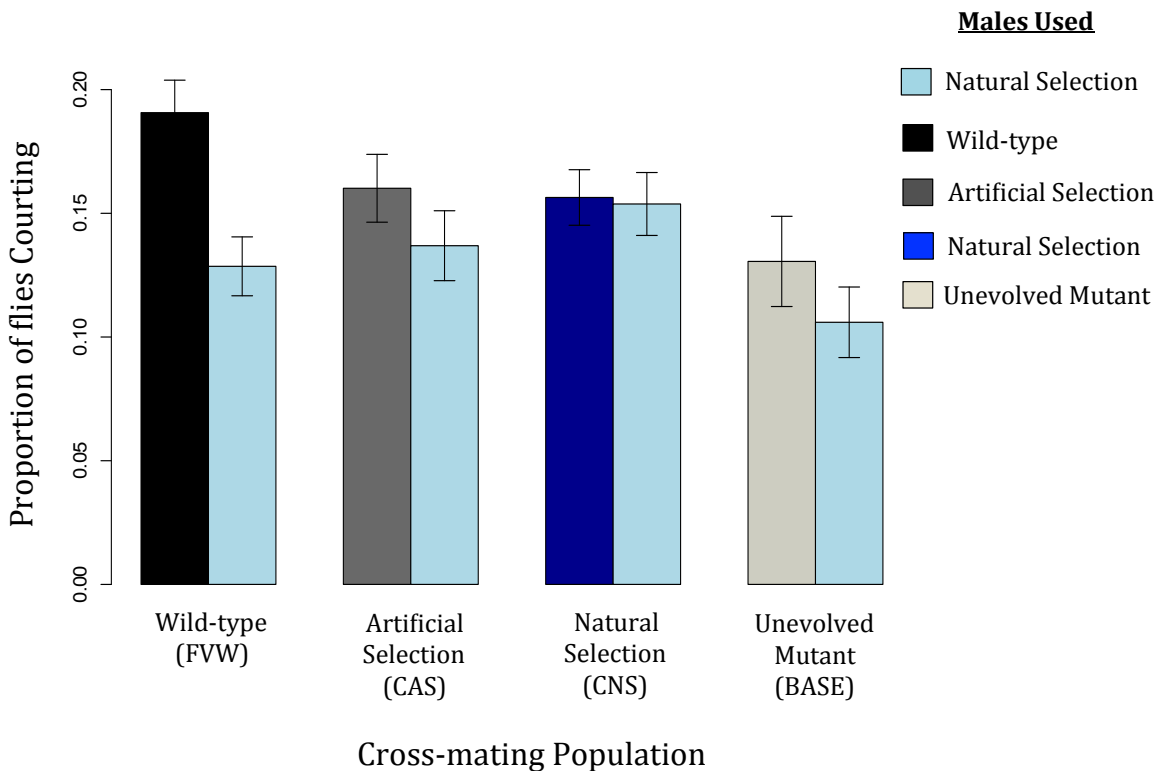

Figure 21. Proportion of males copulating from the natural selection lineages is higher than the unevolved base population

Graph representing the results from the "Cross mating" assay where a given set natural selection flies were reciprocally assayed for mating behaviour with flies from all other treatments. The X-axis represents the other treatment groups that were used for the assay with the natural selection lineages. The bars represent the average proportion of flies copulating over multiple blocks and replicates for each population and the error bars represent 95% CI.

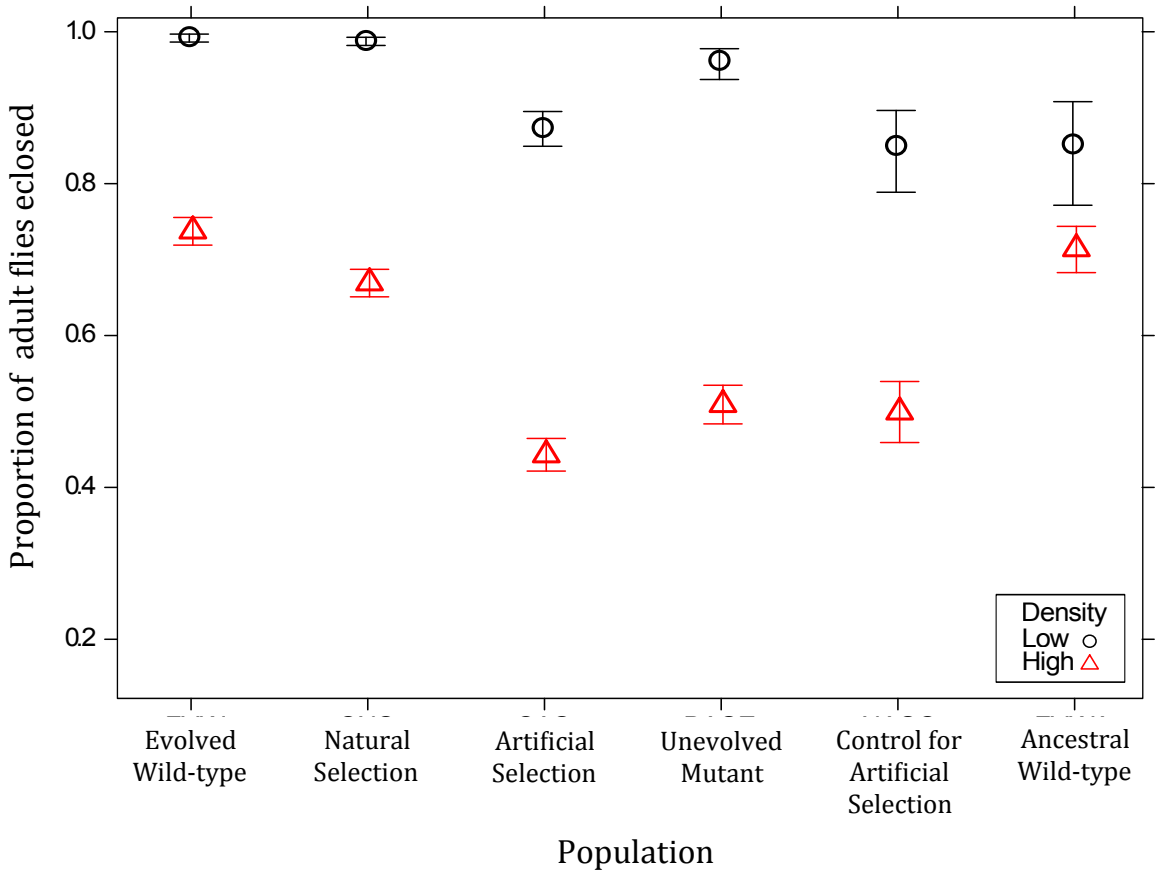

Figure 22. Egg to adult survivorship has evolved in the natural selection lineages

The proportion of natural selection flies that survived to adulthood from eggs at low (50 eggs/vial) and high (300 eggs/ vial) density is significantly higher as compared to the unevolved base mutant lineage. The open circles and triangles represent the average proportion of survival across multiple blocks and replicates at low and high density respectively. The error bars represent 95% CI.

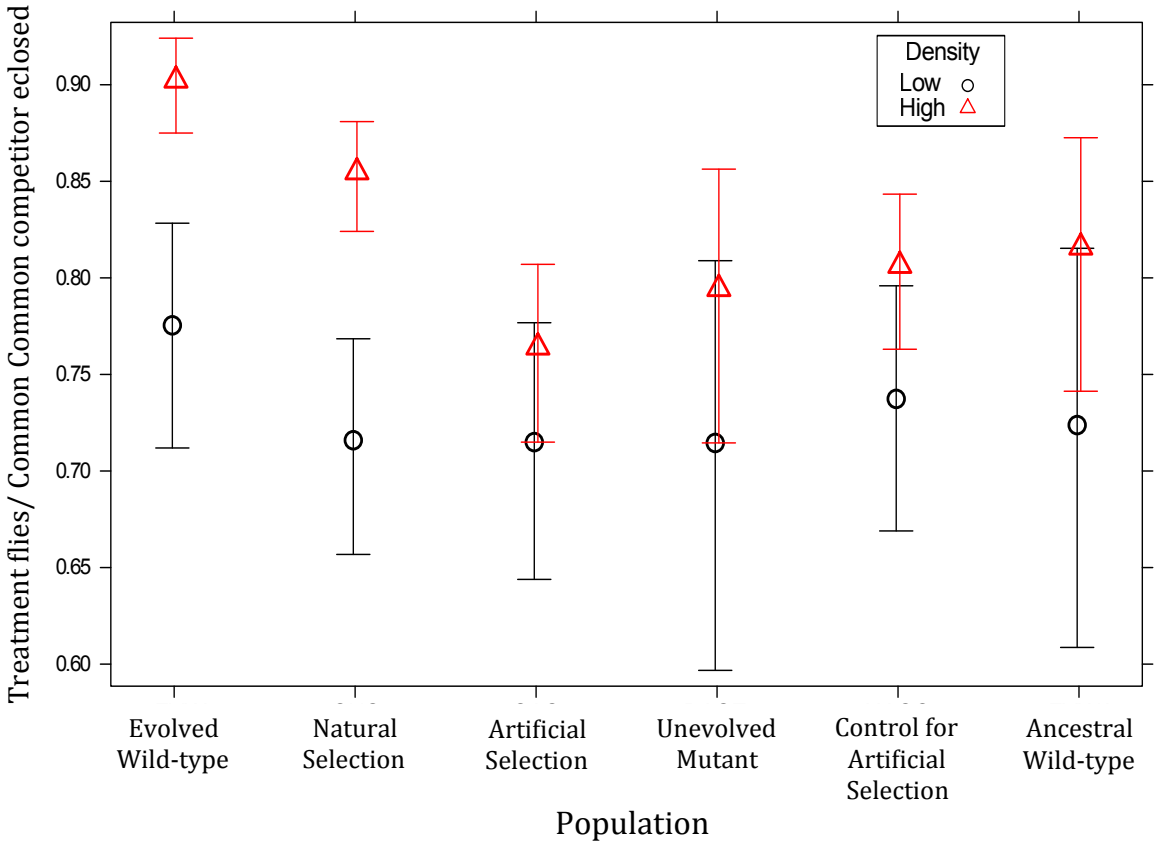

Figure 23. Potential evolution in larval competitive ability at high density in the natural selection lineages against a common inbred competitor

The proportion of natural selection flies that survived to adulthood as compared to the common inbred competitor from eggs at high (150+150 eggs/ vial) density is higher as compared to the unevolved base mutant lineage. The open circles and triangles represent the average proportion of survival across multiple blocks and replicates at low and high density respectively. The error bars represent 95% CI.

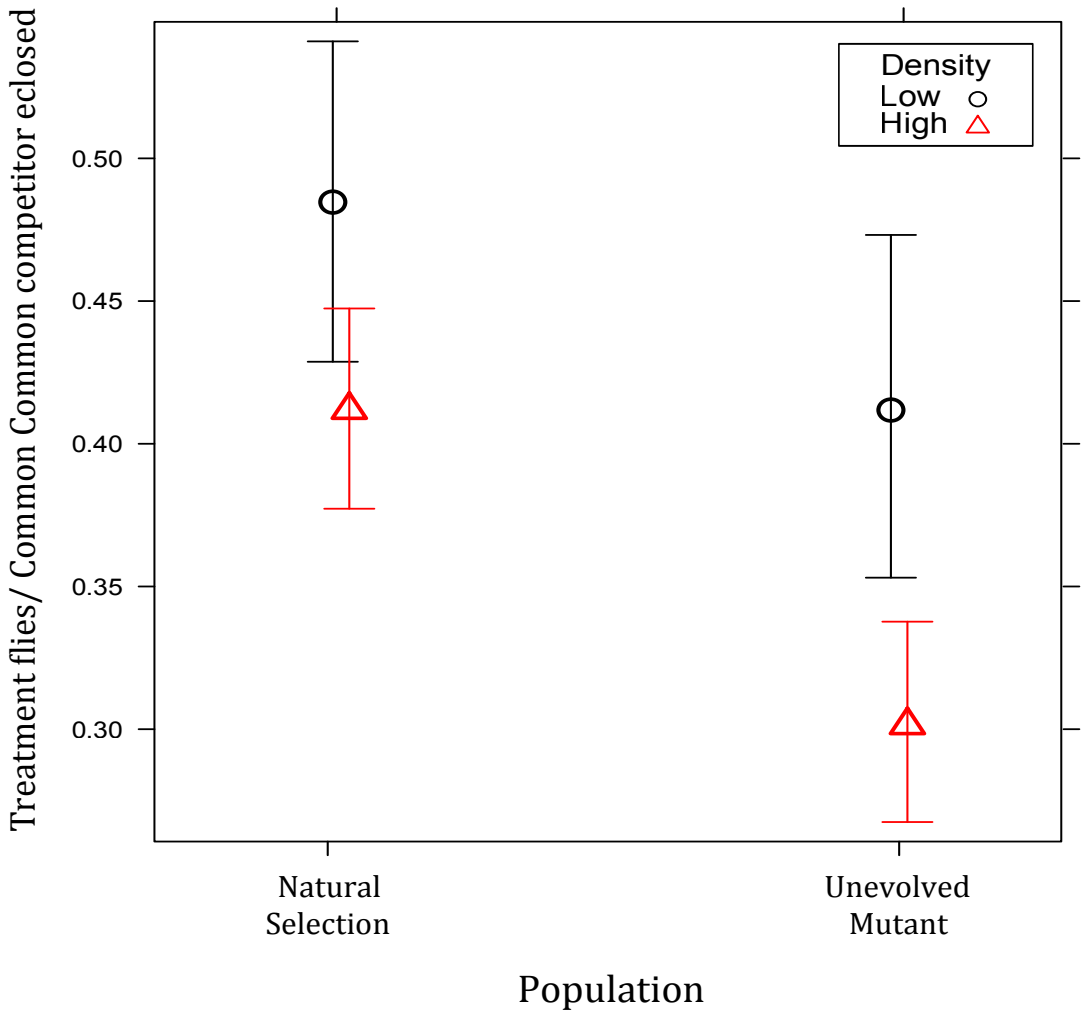

Figure 24. Clear demonstration of evolved larval competitive ability in natural selection lineages when competed against the FVW wild type

The proportion of natural selection flies that survived to adulthood as compared to the common FVW wild-type competitor from eggs at high (150+150 eggs/ vial) density is higher as compared to the unevolved base mutant lineage. The open circles and triangles represent the average proportion of survival across multiple blocks and replicates at low and high density respectively. The error bars represent 95% CI.

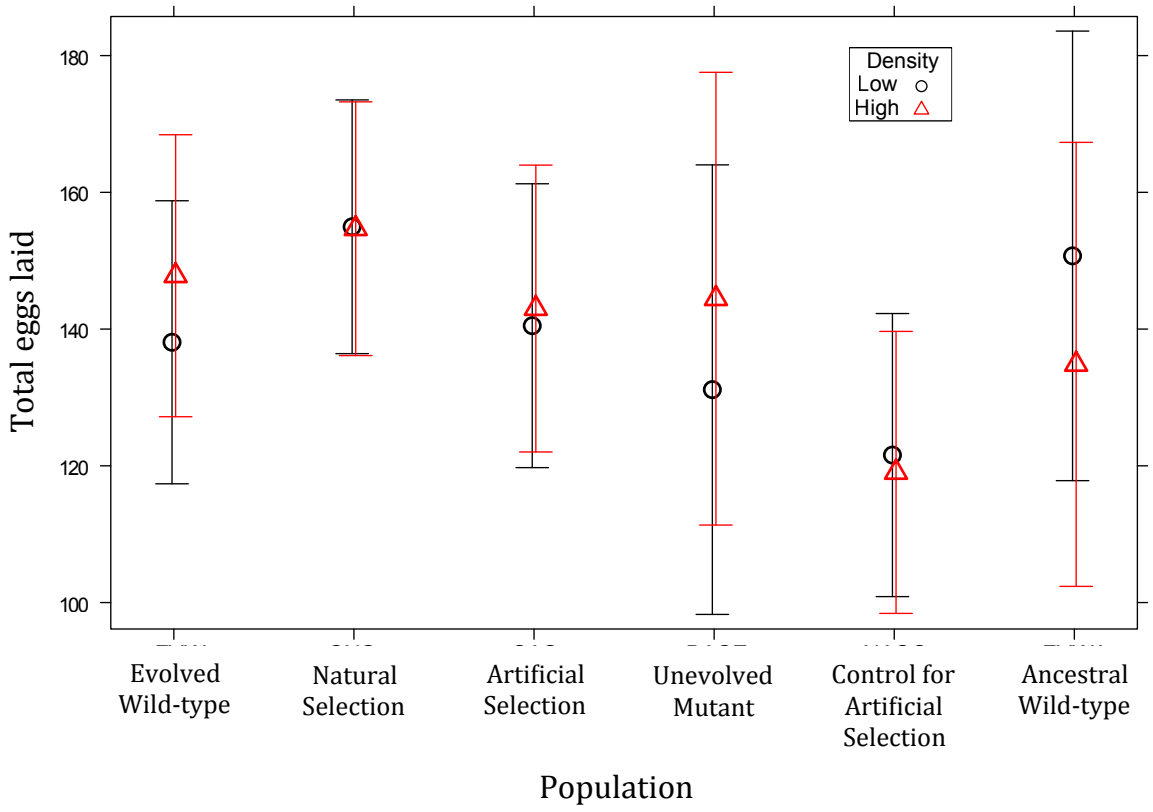

Figure 25. No change in fecundity in artificial selection lineages demonstrates negligible fitness cost of wing size evolution on fecundity.

The open circles and triangles represent the average proportion of survival across multiple blocks and replicates at low and high density respectively. The error bars represent 95% CI.

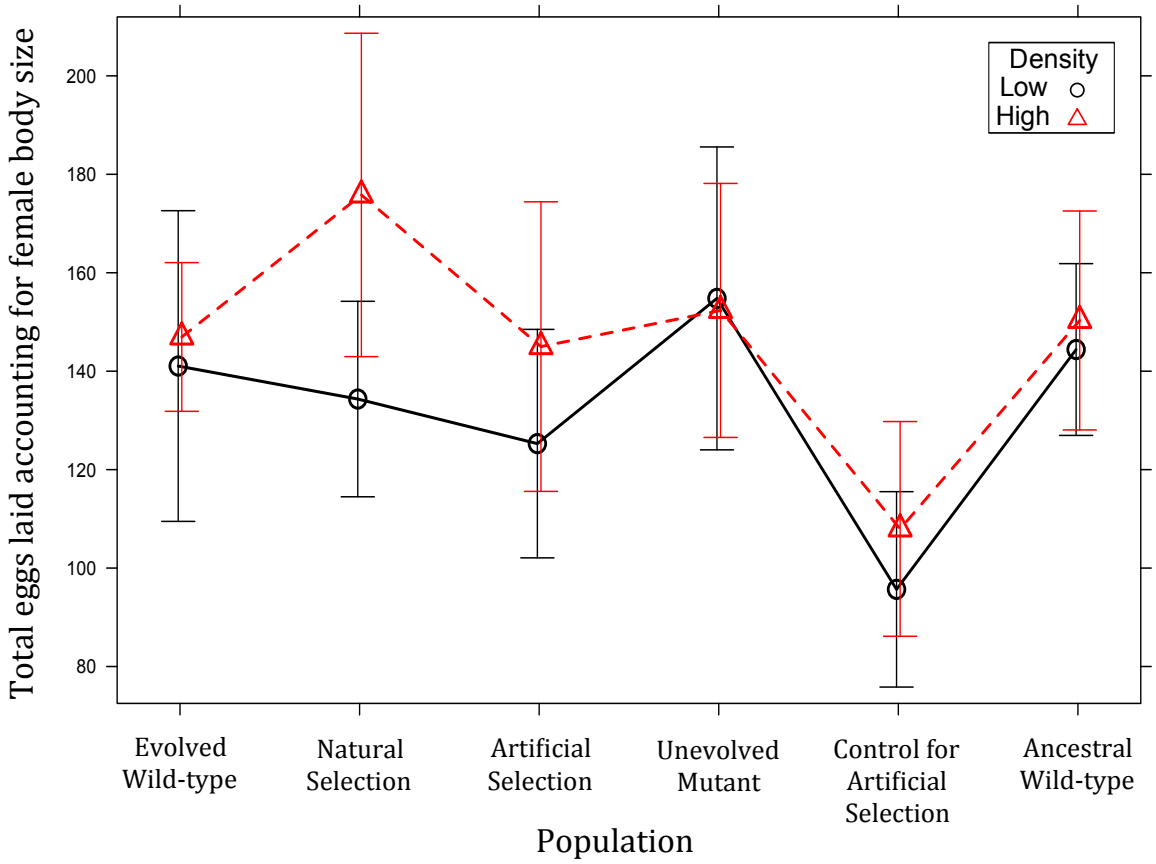

Figure 26. Higher Fecundity after accounting for female body size suggests evolution in this component of fitness via natural selection.

The open circles and triangles represent the average proportion of survival across multiple blocks and replicates at low and high density respectively. The error bars represent 95% CI.

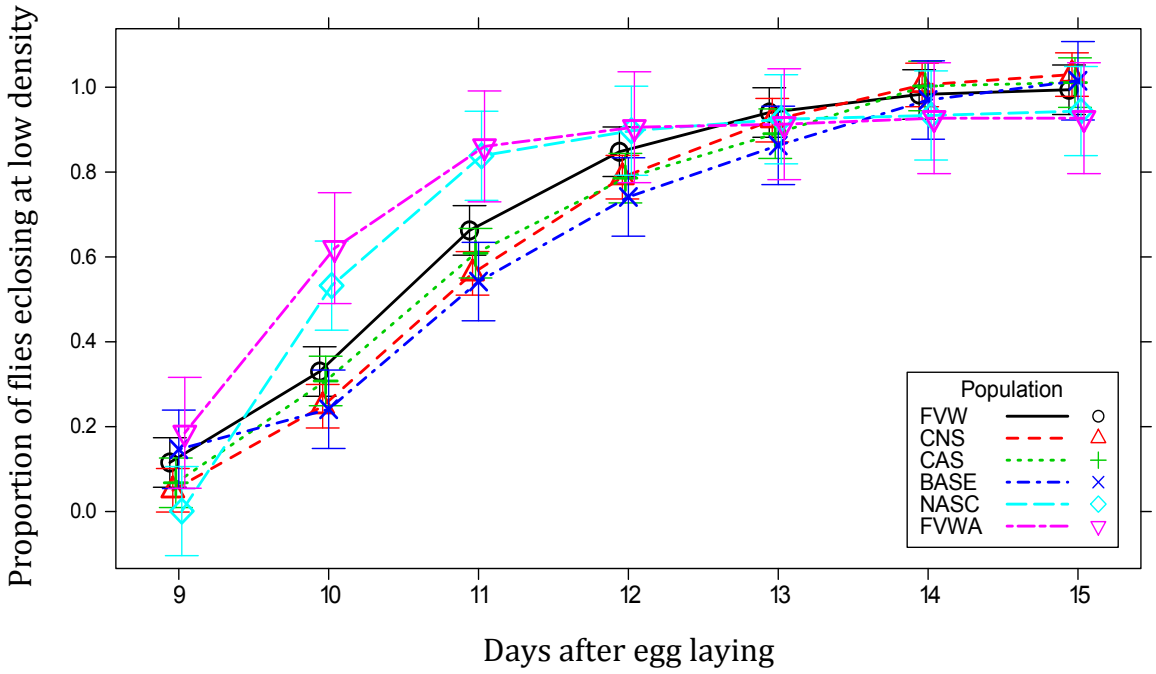

Figure 27. No evolution in egg to adult development time at low density

The symbols represent the average proportion of flies ecclosed per day for each treatment across multiple replicates at low density. The error bars represent 95% CI.

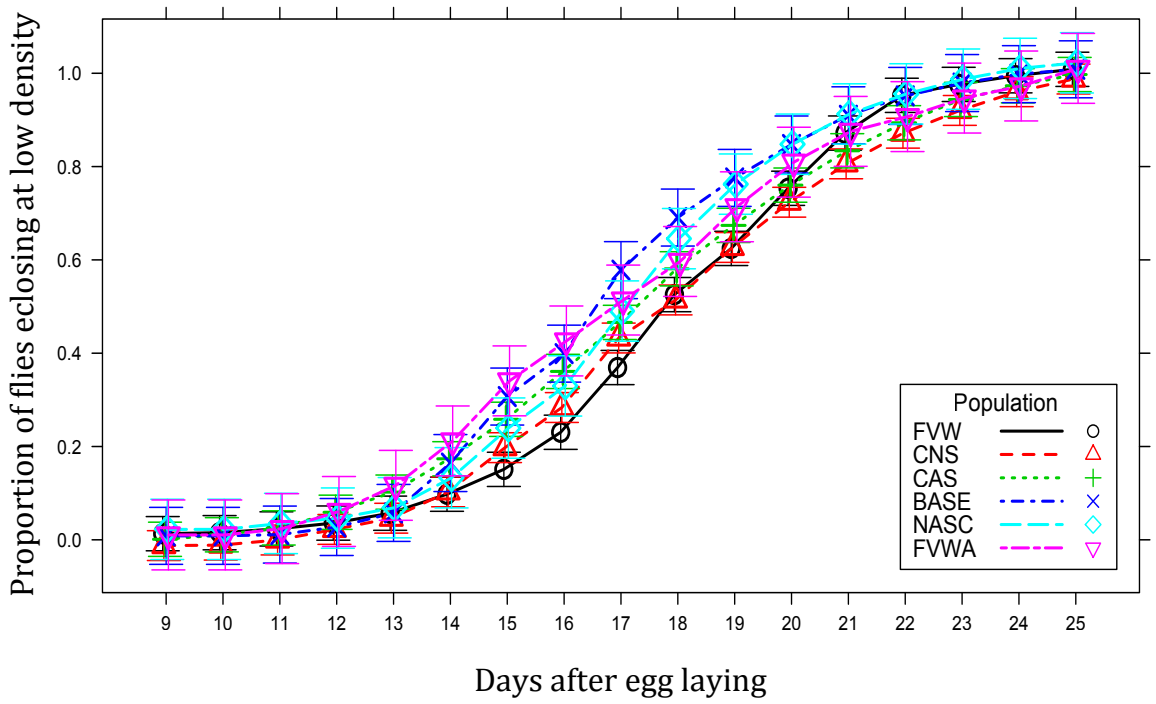

Figure 28. No evolution in egg to adult development time at high density

The symbols represent the average proportion of flies ecclosed per day for each treatment across multiple replicates at high density. The error bars represent 95% CI.

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