CGV and genetic assimilation in *Drosophila melanogaster*

Cryptic genetic variation and its contribution to genetic assimilation in natural populations of *Drosophila melanogaster*

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Abstract

Over 65 years ago, Waddington demonstrated phenotypically plastic traits can evolve to become constitutive, a process he termed genetic assimilation. Experiments demonstrated genetic assimilation evolves rapidly, with the response in large part due to segregating genetic variation only expressed in rare/novel environments, but otherwise phenotypically cryptic. Despite previous work suggesting a substantial role of cryptic genetic variation contributing to the evolution of genetic assimilation, some have argued for a prominent role for new mutations of large effect concurrent with selection. Less concerned by the relative contribution of CGV or new variants, Waddington aimed to test the role of canalization, an evolved form of robustness. While canalization has been extensively studied, its role in the evolution of genetic assimilation is disputed, in part because explicit tests of evolved robustness are lacking. To address these questions, we recreated Waddington's selection experiments on an environmentally sensitive change in *Drosophila* wing morphology (crossvein development), using many independently evolved replicate lineages. Using these we show that 1) CGV has potentially pleiotropic and fitness consequences in natural populations and may not be always be "cryptic". 2) CGV, but not new variants of large effect are largely responsible for the evolved response demonstrated using both genomic and genetic approaches. 3) Using both environmental manipulations and mutagenesis of the evolved lineages that there is no evidence for evolved changes in canalization contributing to genetic assimilation.

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Declaration of Authorship

The chapters of this thesis are part of a broader paper encompassing all of the thesis work, which will be submitted by a group. All experiments and fly husbandry were done by Sarah Marzec. Analyses were done by Sarah Marzec and Ian Dworkin. Exceptions to this included work for the first mutagenesis experiment (chapter 4) which was completed by Amy Chang and analyses of wing shape and size data for pleiotropy (chapter 2) and environmental canalization (chapter 4) done by Katie Pelletier and Ian Dworkin. Katie Pelletier also assisted by performing wing dissections and taking images for the wing shape and size analyses.

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List of Abbreviations

bp – Base pair
CGV – Cryptic genetic variation
CVL – Crossveinless
GA – Genetic assimilation
HSP90 – Heat shock protein 90
LD – Lab domestication
SGV – Standing genetic variation

Chapter 1: The role of cryptic genetic variation, plasticity, and genetic assimilation in rapid evolution

Rapid Evolution

Many avenues are possible for rapid evolution to occur, but always central is the available genetic variation. Genetic variation is the substrate upon which natural selection acts resulting in adaptive evolution. However, the provenance of genetic variation contributing to adaptive evolution remains a subject of some controversy, with examples of both *de novo* mutations concurrent with selection (Cai et al. 2008; Heinen et al. 2009) and standing genetic variation (Colosimo et al. 2005; Jones et al. 2012). Standing genetic variation is genetic variation present in a population providing immediate "fuel" when selection changes (i.e. changes in the ecological conditions of the population). This can be contrasted to *de novo* mutations that occur during the selective process, resulting in adaptive evolution being mutationally limited. Whether available standing genetic variation is sufficient to enable a population to reach a new adaptive peak, or whether such variation is depleted (Blows and Hoffmann 2005) during the early adaptive phases resulting in adaptive changes being mutationally limited is often unclear.

However, most standard quantitative population genetic models assume a polygenic response, where there are many segregating alleles of small to moderate

effect. If these are the source for rapid evolution under strong selection, we'd have certain implications. A case of many alleles of small effect already segregating in the population would result in a very strong phenotypic response to selection that would have little effect on allele frequencies and would not deplete genetic variation. Alternatively, the genetic basis of adaptive change is a result of only a few alleles of large effect segregating in the natural population. The difference here is that there will an initial substantial and rapid effect on both mean trait values and allele frequencies. In such a case, reaching the new adaptive peak may ultimately be mutationally limited. Examples of adaptation exist for both many alleles of small effect (Sawamura et al. 2000; Fishman et al. 2002) and fewer alleles of large effect (Albertson et al. 2003; Rogers and Bernatchez 2007; Steiner et al. 2007). In either case, rapid evolution is initially facilitated by existing variation in the population.

Understanding the source of genetic variation for rapid evolution, and the mechanisms by which it occurs, is not only of academic interest. In light of climate change and rapidly changing environments, the opportunities organismal populations can take to adapt and overcome becomes important in terms of conservation and evolutionary rescue[1].

There are limited examples of evolutionary rescue occurring in nature. Threespine sticklebacks in Lake Washington, USA displayed demographic shifts to

[1] Evolutionary rescue is the process by which a population that would otherwise become extinct due to demographic changes can genetically adapt, through either existing genetic variation in the populations, new mutations or gene flow (Carlson et al. 2014).

heavily armored morphologies after bioremediation allowed for increased water clarity suggesting stronger predator efficiency (Kitano et al. 2008). Australian black snakes have adapted a resistance to the venom of invasive cane toads, demonstrating rapid evolution in potentially no more than 23 generations (Phillips and Shine 2006). More recently, genome scans of little brown bats exposed to the fungal pathogen causing white-nose syndrome have shown some adaptive potential to overcome population decline through natural selection acting using standing genetic variation (Auteri and Knowles 2020). Although there are fewer studied examples in nature, overall there is support for standing genetic variation to increase probability of evolutionary rescue through theory and models (Gomulkiewicz and Holt 1995; Barrett and Schluter 2008; vander Wal et al. 2013) and laboratory studies (Agashe et al. 2011; Lachapelle and Bell 2012; Ramsayer et al. 2013).

This introduction is to address the joint contribution of cryptic genetic variation, plasticity, and genetic assimilation as potential sources of variation and mechanisms for rapid evolution. Although there is overlap between these concepts, we will present them individually and then integrate them.

Cryptic genetic variation as a source of genetic variation

Questions remain about how standing genetic variation is maintained in populations, and whether this variation contributes substantially to adaptive evolution.

Traditional models examining maintenance of genetic variation in populations suggest that much of this standing genetic variation is due mutation-selection balance, which predicts that most alleles will be neutral to deleterious (and recessive) (Zhang and Hill 2005). When populations are already close to a fitness optimum, dominant and large effect mutation are unlikely to put the population any closer to the optimum, either through direction (away from the optimum) or magnitude (perhaps overshooting the optimum). Under "Haldane's sieve", there is a decreased probability of establishing recessive beneficial mutations in populations (Turner 1981). In contrast, additive or dominant beneficial mutations are expected to increase in frequency relatively quickly. Under both these models, few loci will be segregating beneficial alleles (beneficial under the current environment) in populations at any given time (Barrett and Schluter 2008). If these models are correct, most genetic variation in populations is due to alleles segregating at low frequencies, and may be recessive for effects on fitness (García-Dorado and Caballero 2000; Manna et al. 2011) and generally somewhat deleterious or neutral. Alternatively, populations may be segregating alleles that are conditionally neutral. Conditionally neutral alleles can occur in a variety of situations. Alleles that influence phenotypes may not influence fitness in all environments, making them neutral in certain environments. However, alleles can also be phenotypically silent under a common range of environments that organisms experience, and only under rare environmental (or genetic) conditions can these alleles influence phenotypic variation

for traits (and potentially fitness). Either situation can include potentially beneficial alleles that are conditionally neutral.

Cryptic genetic variation (CGV) is standing genetic variation that has little to no effect on the phenotypes of a population under common conditions (environmental or genetic), yet can lead to heritable phenotypic variation under novel or rare conditions. CGV can contribute to traits that already show phenotypic and genetic variation. There are cases where CGV results in phenotypic variation for traits that were previously invariable. For instance, the introduction of the *scute* mutation in *Drosophila* can change scutellar bristle number from an invariant number (four) to a variable number of bristles. The variation generated by the introduction of the *scute* mutation can be selected upon (Rendel 1959), demonstrating genetic variation existed in the population for what was seemingly an invariant trait. CGV can also be seen in situations where there is existing phenotypic variation but result in an increased amount of variation. For instance, populations of spadefoot tadpoles show some variation in body size, gut length, developmental stage under normal conditions (a detritus diet) but show increased phenotypic (and genetic) variation when switched to a rarer environment (a carnivorous diet) (Ledón-Rettig et al. 2008, 2010). In some cases, traits may already be variable, but a release of CGV can result in an increase of the heritable variation for a trait, leading to an increase in the phenotypic variation influenced by genetic effects. Oceanic stickleback fish raised in both high (normal condition) and low (novel condition) salinities show differences in the additive genetic variation impacting body size

(McGuigan et al. 2011). However, the sticklebacks raised in both salinities actually had comparable amounts of overall phenotypic variation, but the high salinity (normal environment) reared sticklebacks had almost no genetic variance allowing for the phenotypic variance, showing body size was based mostly on environment. The low salinity (novel environment) reared sticklebacks showed substantial additive genetic variation contributing to body size representing a release of CGV in the novel environment.

Rare conditions include stresses on the population like environmental stresses, where existing alleles are conditional on the environment. Rearing dung flies under thermal environments that are rare in nature releases CGV for spermathecae number (Berger et al. 2011). Here, the environmental change is the rearing temperature of the dung flies, outside the "typical" range experienced by the population, resulting in changes to the additive genetic variation for spermathecae number. This may seem equivalent to phenotypic plasticity, and plasticity may be a way for CGV to accumulate in populations (Palmer 2012). Phenotypic plasticity (discussed below in more detail) refers to the mean change in the phenotypic value across different environments. Gene-by-environment interactions demonstrate that there is genetic variation for how organisms respond to the environment. The distinguishing factor of CGV in gene-by-environment interaction, as opposed to standing genetic variation (SGV), is that the "inducing" environment is relatively rare in nature, and so these variants are rarely expressed for a trait and thus exposed to selection, unlike SGV which is frequently

phenotypically expressed. These variants that ultimately contribute to CGV accumulate when populations are in a normal environment for a long time and then CGV is exposed in rare or novel environments.

Abnormal conditions can also include genetic stressors, such as novel alleles interacting with the genetic background. For instance, the introduction of the *tabby* mutant caused increased variability in the number of vibrissae appearing in mice (usually fixed at nineteen) and this could be selected upon to increase and decrease vibrissae number (Dun and Fraser 1958). Similarly, mutations to the anchor cell for the vulva in nematodes (*Caenorhabditis elegans*) caused a release of CGV resulting in both timing differences in induction and the number of cells that reached specific vulval fates (Milloz et al. 2008).

Despite a recent resurgence in the study of CGV, concepts of heritable changes in response to induction, such as the “Baldwin effect“, have been around since the late 19th century (Baldwin 1896). The “Baldwin effect” encompasses two ideas; “organic selection” where a plastic response helps an individual survive an environment within its lifetime and “orthoplasy” where heritable variation directs evolution. Baldwin did not believe the initial phenotypes resulting from environmental cues were heritable, but instead genetic variation in the population could be selected on in the same direction as plasticity (Crispo 2007). Half a century after Baldwin, a more Darwinian approach to plasticity in environments would be introduced by Dobzhansky with his idea of “concealed genetic variability” which may include beneficial alleles in new conditions

(Dobzhansky 1941). One of the first empirical examples of this hidden genetic variation (CGV) came in 1953. Waddington demonstrated that persistent selection (over many generations) on an environmentally induced (plastic) phenotype eventually resulted in individuals expressing the environmentally contingent phenotype, but without the requirement of the environmental induction (Waddington 1953b). Waddington used as a model system, the environmentally sensitive nature of the penetrance of crossveins on the wings of *Drosophila melanogaster*. Under a wide range of rearing temperatures, the wing will develop normally with both a complete anterior and posterior crossvein (**Fig. 1**). However, under high temperature stress during a critical period during pupation, the resulting adult wing can develop with an incomplete or missing posterior crossvein (i.e. crossveinless (CVL) phenocopy[2] **Fig. 1**).

While Waddington was initially unaware of this, this phenotype is observed in field caught populations of *Drosophila melanogaster* at very low frequencies (less than 1%). However, under high temperature stress, the frequency of CVL flies in the population increased substantially (up to 5-10% in the population). Artificial selection on flies displaying CVL (while maintaining the temperature stress each generation) increased the frequency of CVL in later generations, demonstrating that the presence of genetic variation for the CVL phenocopy in natural populations despite its near absence in field collected flies. This demonstrated that there was cryptic variation for the CVL

[2] "Phenocopies" are environmentally induced phenotypes that mimic mutations (coined by (Goldschmidt 1940)). In this thesis, phenocopy and phenotype will both be used when referring to the environmentally induced trait of *Drosophila melanogaster* crossveinless wing.

phenocopy segregating in natural populations. Remarkably, after ~14 generations[3] of artificial selection on the temperature induced CVL phenotype, Waddington observed flies with the CVL phenotype, despite these individuals having not experienced the temperature stress. Via genetic crosses, he generated a lineage which increased in CVL frequency with selection every generation all without the requirement of the (initially necessary) environmental stimulus. That is, the initially environmentally induced phenotype became largely genetically fixed (and independent of the environment). He coined the term genetic assimilation to describe this process, which I will discuss in detail below.

The contribution of CGV to evolutionary change is discussed mostly because there are gaps in the understanding of its role (McGuigan and Sgrò 2009) and importance. There are questions of both the contribution of CGV, as compared to SGV, to adaptation and whether these variants are likely to be beneficial when revealed. Some suggest that CGV is random and thus may mostly be neutral and deleterious, whereas others consider the "pre-adaptive" (or "exaptive" (Gould and Vrba 1982)) potential to CGV that would allow it to be, on average enriched for beneficial alleles. Some models show under biologically plausible parameters, the pool of CGV can be enriched for potential beneficial variants and have some advantageous effect in the new environment (Eshel and Matessi 1998; Masel 2006). This is predicted because it is likely

[3] The generational timepoints for when genetically assimilated flies were first observed are different in Waddington's publications. He lists it as taking either 12 generations (Waddington 1952) or 14 generations (Waddington 1953b).

that the environments that reveal CGV in populations are not completely novel to these populations. Instead they are environments that the populations have experienced occasionally in the past and very deleterious alleles may have been previously been purged from the population, and beneficial alleles may have increased somewhat in frequency. For instance, Wright (Wright 1931, 1956) maintained that new environmental conditions could sometimes resemble rarer niches from the old environment (e.g. population experiences in a heterogenous environment), which means it's possible there was already some selection for beneficial alleles. However, when considering the release of CGV due to genetic changes, this historical filtering of highly deleterious variants is less straight-forward.

Although there is a need for more empirical work to address the contribution of CGV to adaptive response, some examples exist that show CGV has some evolutionary benefit. For instance, populations of RNA enzymes that harbor CGV have been shown to more quickly adapt to new substrates than populations that don't harbor cryptic variants (Hayden et al. 2011). There is also implication for CGV in phenotypic evolution of domesticated maize as the ancestor, teosinte, has been shown to have CGV for several invariant traits (specifically those differentiating maize and teosinte) which may have allowed for rapid domestication (Lauter and Doebley 2002).

Also under debate in the sometimes vague cutoff between what is considered a response to standing genetic variation versus what is actually cryptic. CGV is standing genetic variation but termed "cryptic" when it is only (phenotypically) expressed in rare

environments. However, as discussed above, populations can encounter these environments and potentially become pre-adapted to them. This could then prompt questions of when an environment is considered rare. In the experiment by Waddington (1952, 1953b, above), the alleles for the crossveinless phenocopy were originally thought to be entirely cryptic. Yet, the crossveinless phenotype does appear at very low frequency in natural populations meaning that it could be considered SGV. Furthermore, different natural populations have varying rates of crossveinless frequency (Lack et al. 2016) which suggests that the alleles underlying this trait would not be considered "cryptic" at all for some populations. It may not be important to create a divide between what is actually hidden variation. The usefulness of calling it CGV is in describing how often we see it. In future studies for CGV, we should consider it as an extension of SGV with understanding that it is SGV we would not typically see under normal circumstances. There are several models and empirical examples demonstrating standing genetic variation facilitating in adaptation. However, it is important to consider the genetic variation that is "hidden" under certain circumstances, and labeling it as CGV allows us to distinguish the population's response in rare and/or novel circumstances.

Plasticity's role in evolution

Genetic variation may be the substrate underlying phenotypes that natural selection acts on, but genetic influences on phenotype must be viewed through the

environment in which organisms develop. It is the combination of genotype, environment, and genotype-environment interactions that work through developmental space and shape how organisms will look phenotypically. All aspects of organismal fitness can be affected by genotype-by-environment interactions including morphology (Pfennig and Murphy 2002; Binning et al. 2010), behavior (Cotman and Berchtold 2002; Torres-Dowdall et al. 2012), and life history (Visser et al. 2009; Foster et al. 2015). Phenotypic plasticity is the ability of a single genotype to produce various phenotypes dependent on environment. This can take the form on continuous phenotypic trait values along an environmental gradient (such as organism color developments due to condition-dependence (Ruell et al. 2013) or temperature (Assis et al. 2020)) or discrete morphs triggered through some environmental shifts (i.e. threshold traits, sometimes known as polyphenisms) (threshold traits reviewed in (Roff 1996)). Plasticity can be useful for an organism in that it can offers ways to respond to heterogeneous and/or changing environments.

Plasticity can be envisioned as a reaction norm for an individual genotype. Each reaction norm is a single genotype expressing different phenotypic values along an environmental continuum. Reaction norms are typically depicted as continuous lines or curves, although there are some types of plasticity with discrete traits such as threshold traits. If a reaction norm is flat, meaning there is no environmental effect on phenotype, then that trait is canalized (often called macro-environmental canalization), or robust to environmental changes. Populations can be represented by collections of these reaction

norms accounting for the different individuals. There can be several variations of plasticity in populations. In a simple example, there may be individuals that all have a common slope, but with different intercepts (**Fig. 1.2a**). In this case there is genetic variation present for the mean phenotype but not the plasticity, or slope. In response to selection for greater trait value, we would expect only certain reactions norms to prevail and overall, mean trait value would be greater (**Fig. 1.2b**). However, there would be no effect on slope of these reaction norms as there is no genetic variation. We can also look at this trait with reaction norms where the degree of plasticity varies as well (**Fig 1.2c**). In this case, under selection for greater trait value, we could see multiple scenarios. Potentially we could see an increase in plastic response so that the mean reaction norm has a steeper slope than before evolution (**Fig. 1.2d**). This could show an increase in variance. It should be noted that plasticity can evolve without shifts in mean phenotypic value, such that the variance of phenotypic values increase around the mean. Alternatively, there may be a reduction in the mean plasticity of the population (**Fig. 1.2e**). Here, there would be a shift in the mean trait value.

Adaptive plasticity is plasticity in the same direction as an optimal trait for the specific environment. Genetic variation for plasticity (including adaptive plasticity) could allow for organisms to weather abrupt changes into novel environments, where they may otherwise go extinct. For instance, a relatively new colony of coastal dark-eyed juncos has a longer breeding seasons (of which the timing and length is plastic) than the ancestral population, allowing for increased fledgling production and maintenance of

population size during colonization (Yeh and Price 2004). Red squirrels have adapted to a changing climate of warmer spring by breeding earlier, a plastic response due to increasing food abundance (Réale et al. 2003). There are several empirical studies and models that show that plasticity is advantageous and can enhance overall species fitness and longevity (Price et al. 2003; Chevin et al. 2010; Scheiner and Holt 2012).

Price et al. (2003) make the argument that certain amounts of plasticity can aid in evolution. If there is little to no plasticity (genotype-by-environment interaction), then populations could potentially go extinct in encountering new environments. Additionally, small amounts of plasticity may mean there are no shifts of population fitness off of smaller adaptive peaks. However, if plasticity is too high, then the plastic response may encompass the new fitness peak. Stabilizing selection would act on the population, resulting in less genetic differentiation for this trait with less opportunity for evolutionary change. Moderate levels of plasticity could not only allow for populations to persist into new environments, but leaves room for evolution to occur. Here, plasticity should not encompass the new adaptive peak and directional selection can act of the population so that it evolves towards the new peak. The Price et al. (2003) model showed that an intermediate level of plasticity allows for an adaptive peak shift.

Although there is no debate over that environment can influence organismal development and phenotype, there has been controversy over plasticity's role in facilitating or constraining evolution (Pigliucci and Murren 2003; Diamond and Martin 2016). However, "plasticity-led" (also referred to as "plasticity-first") evolution is a

theory that is increasingly discussed (Schlichting and Wund 2014; Levis and Pfennig 2016, 2020; Schneider and Meyer 2017; Jones and Robinson 2018). Under this theory, plasticity precedes and facilitates evolutionary adaptation, resulting in some cases as genetic assimilation. This is unlike a mutation-driven model where selection acts on new variants and that result in changes in plasticity and phenotype. Basically, the difference between these two modes in whether there is environmental influence over the development of organisms that can allow for adaptation versus some mutational effect on development. Advocates of plasticity-led evolution suggest that environmentally-induced traits could be more evolutionary relevant than mutational ones for several reasons: 1) *de novo* variants occur in single individuals unlike new traits (or changes to trait means) that are environmentally-induced and can occur in many individuals at once because of genotype-by-environment interactions, 2) the segregating variation for environmentally-influenced traits can be enriched for beneficial variants which could improve adaptive response, and 3) plasticity can allow for CGV to accumulate and be released under certain conditions (Levis and Pfennig 2016). As discussed previously, there is quite a bit of overlap between causes and consequences of CGV and plasticity. CGV is often thought of in contexts of release due to environmental influence (substantial changes to the environment). This segregating variation can allow populations exposed to new or rare environments to have plastic responses that help increase phenotypic variation for which some of may be adaptive. There is even some

evidence for beneficial plastic responses under “stressful” conditions (Badyaev 2005; Palmer 2012).

The relative importance and contribution of plasticity-led evolution remains actively debated (Via et al. 1995; de Jong 2005; Laland et al. 2014). Much of this stemming from the limited direct evidence for it in nature (and difficulty in being able to demonstrate it). Some examples of plasticity assisting in evolution have been demonstrated in the lab (Waddington 1953b; Bateman 1959a; Sollars et al. 2003; Suzuki and Nijhout 2006), but could be argued to be less ecologically relevant (given the simplified nature of most lab environments) especially in cases where artificial selection has been employed. Although there is a relatively modest amount of direct empirical evidence in natural populations, some field work is suggestive of plasticity-led (Losos et al. 2000; Wund et al. 2008). For instance, Daphnia that have underwent rapid adaptation when fish predators were introduced show a reduced plastic response and less melanin expression, compared with Daphnia without selective pressures from predators (Scoville and Pfrender 2010).

Levis & Pfennig (2016) present several criteria for how to assess if a trait evolved through plasticity-led evolution: 1) the trait is environmentally induced in the ancestor (or proxy for ancestor), 2) CGV for the trait in the ancestor is revealed under novel environmental conditions, 3) the trait will have some new form or function in the evolved lineages, and 4) the trait will have undergone adaptive refinement in the evolved lineages. The evolution of carnivore traits in ancestrally omnivore spadefoot

toad tadpoles matches each of these criteria. An ancestral proxy for the *Spea* species, *Scaphiopus couchii*[4], when fed a carnivorous diet shows more plasticity for gut length than the *Spea* tadpoles (Ledón-Rettig et al. 2008) and displayed CGV with greater heritability for size and gut length (Ledón-Rettig et al. 2009, 2010). More recently, adaptive refinement of the carnivore traits in *Spea* tadpoles has been shown to be driven by frequency-dependent selection (Levis and Pfennig 2019).

As plasticity-led evolution is highly associated with CGV, studying CGV in nature and understanding how it can contribute to adaptive response may be one of the best ways to address plasticity's potential role in evolution. Specifically understanding the contribution of CGV as compared to new mutations could help identify cases of plasticity-led evolution. However, at the end of the evolutionary process, the signatures behind these two modes may be indifferentiable, and in that case genetic sampling during several timepoints throughout the evolutionary process are advisable. Since plasticity-led evolution will source from CGV, its selection signature should be similar to that of selection of standing genetic variation. Overall there is a need to for more theoretical and empirical studies to help determine the signatures and patterns behind plasticity-led and "genes-first" evolution (Kovaka 2019).

[4] Ancestral character state reconstruction suggests non-plastic *Spea* ancestor (generating solely omnivore morphs), however Levis & Pfennig (2016) acknowledge that *Sc. couchii* may not be truly representative of the ancestral state.

Genetic assimilation as a process in rapid evolution

Mean plasticity in populations can be selected on in a few simple directions: to increase, maintain, or decrease the slope of the reaction norm. Representing the individuals of a population as multiple reaction norms (i.e. each reaction norm is a genotype), we can envision the mean reaction norm for this population shifting as some individuals are selected as more favorable. If plasticity is to be maintained in populations, then either it must be neutral or there must be selection for varying traits with reliable response to the environment. However, either situation may result in a reduction of plasticity or loss of the genetic variation for plasticity. If neutral, genetic drift can reduce variation in population. When under selection, there must be selection for differing phenotypes along the environmental gradient. If these phenotypes are not expressed often enough (due to not receiving environmental cues), plasticity can be diminished or lost if genetic drift and mutational degradation occur under relaxed selection (Masel et al. 2007). Additionally, when plasticity is in adaptive directions for certain environments, there can be selection for a singular trait value under those circumstances (rather than to maintain plasticity). There is not selection for plasticity *per se*, but for specific trait values under those environmental conditions. This selection could act to reduce plasticity, by selecting on specific trait values instead of maintaining plasticity. In terms of a reaction norm, this would result in a flattening of the slope so there is less variation in phenotype across environments. With enough selection under certain circumstances, this can lead to traits becoming constitutively expressed

regardless of future changing environments. Genetic assimilation is the process where traits that are revealed by environmental stimuli are selected on so that they become genetically fixed and are expressed even with a return to the normal environment. In this context, genetic assimilation is the loss of ancestral plasticity.

Genetic assimilation has been demonstrated multiple times in laboratory settings beginning with Waddington's classic experiment (Waddington 1952, 1953b, 1956; Bateman 1959b,a; Rendel 1959; Ho et al. 1983; te Velde et al. 1988; Rohner et al. 2013; Fanti et al. 2017). Although genetic assimilation certainly can evolve in the lab, there is some controversy over what role it would have during adaptive evolution in nature. To directly study if a population went through genetic assimilation, you would have to know that 1) there was ancestral plasticity under some environmental cue and 2) that because of directional selection for a trait, there was a reduction of that plasticity, until alleles fixed and/or the selected phenotype became constitutive (see below). This would involve a multigenerational study in natural populations to identify if genetic assimilation truly occurred. Instead, some have tried to make inferences of the evolution of genetic assimilation through viewing cases where there is a loss of ancestral plasticity.

Some researchers have taken a comparative/historical approach to understanding if there are occurrences of genetic assimilation through loss of plasticity. Palmer has suggested that studying bilateral asymmetry may provide evidence for evolution by genetic assimilation (Palmer 1996, 2004). He proposes two evolutionary

pathways to directional asymmetry. In the first, mutation and selection allow symmetric populations to evolve directly towards directional asymmetry. In the second pathway, mutation causes symmetry without a bias (i.e. populations with antisymmetry, in which dextral and sinistral forms are equally frequent) and then a secondary stage of mutations allows for the this non-heritable antisymmetry to become genetically heritable directional asymmetry, and therefore genetically assimilated. Several clades have followed this alternative pathway and seemed to have evolved via genetic assimilation (Palmer 1996, 2004).

Related, in a phylogenetic comparative approach, some *Acacia* species have potentially genetically assimilated extrafloral nectar secretion (Heil et al. 2004). The ancestral state is induced extrafloral nectar secretion as suggested by the phylogeny of the genus *Acacia*. This nectar secretion is used to attract ants as a form of indirect resistance against herbivores. However, some species have evolved constitutive expression extrafloral nectar secretion (Heil et al. 2004).

Others have examined among population levels of plasticity as a lens into evolution via genetic assimilation. Ancestrally, Australian tiger snakes have occupied mainland Australia with populations branching off and colonizing islands during different points in history. Older populations (those that branched off the ancestral population earlier) of these island tiger snakes that have evolved less plasticity in the rate of their jaw growth than evolutionarily “younger” populations, likely in response to more specific prey available (Aubret and Shine 2009). Sword (Sword 2002) suggests that

the varying levels of population-level reaction norms in either palatable or unpalatable grasshoppers is consistent with genetic assimilation of aposematism in the naturally occurring populations.

Mechanisms that lead to the evolution of genetic assimilation:

The proximate mechanisms that underlie the evolution of genetic assimilation are not fully understood. Some of this is because there are not many studies that have investigated the evolution of genetic assimilation as it occurs. Waddington demonstrated genetic assimilation of the bithorax phenotype in fruit flies (Waddington 1956), and Gibson & Hogness would later identify a naturally occurring polymorphism that could allow for the increase in the bithorax phenocopy, perhaps with potential to contribute to genetic assimilation (Gibson and Hogness 1996). Other laboratory studies have also implicated standing genetic variation as contributing to the evolution of genetic assimilation (Jarosz and Lindquist 2010; Hayden et al. 2011). Alternatively, new mutations concurrent with selection (Specchia et al. 2010; Fanti et al. 2017) or epigenetic factors (Sollars et al. 2003; True et al. 2004) have also been shown to contribute. It may be harder to identify mechanisms for the evolution of genetic assimilation in nature. However, a natural study identified potential genetic assimilation occurring during an adaptive radiation in cichlid fishes finding two candidate genes, *gif* and *alas1*, that may have contributed to reduced plasticity in the adaptive radiations (Gunter et al. 2017). However, the researchers did not investigate what caused the differences in expression of these genes, meaning that even if this is an example of

genetic assimilation like they suggest, the mechanism for how it occurred isn't determined.

There are multiple mechanisms that in principle could allow for the evolution of genetic assimilation (which are discussed below) including changes in canalization, the standard liability threshold model, new mutations during selection and epigenetics allowing for genetic assimilation. Although genetic assimilation is often redefined to be thought of simply as a loss of ancestral plasticity, the mechanisms that underlie it may not be so simple to define. Some of this confusion is due to limited experimental work or conflicting examples. However, these models are also not mutually exclusive, which may result in further complexities in understanding the evolution of genetic assimilation.

Canalization as a mechanism for genetic assimilation:

First, we will give particular consideration to a canalization as a mechanism leading to the evolution of genetic assimilation, as the original selection experiment by Waddington (1952, 1953b, described above) was designed to test this theory. In brief, Waddington took a population of flies that was invariant for a trait (all flies had wings with complete crossveins), exposed this population to an extreme environment stimulus (heat stress) and selected on flies that now had variable phenotype (some expressed broken or completely missing crossveins). After 12-14 generations of selection, this trait evolved to become constitutive. Waddington believed the initial population represented a canalized one because it was invariable for the trait (**Fig. 1.3a**) and that the extreme

environmental stress of the heat shock de-canalized the population meaning that there was more phenotypic variability (**Fig. 1.3b**). Most importantly, Waddington interpreted the results, that the crossveinless wings became constitutive (genetically assimilated) through rapid re-canalization around this new trait optimum he had artificially selected for (**Fig. 1.3c,d**), making the phenotype invariable again. It should be noted that decanalization and re-canalization could be occurring on different time scales, with some suggesting that decanalization happens on a much shorter time scale (Gibson and Lacek 2020), and this is discussed more in Chapter 5. Waddington thought that canalized traits could, by the process in which they are buffered from genetic changes, accumulate genetic variation that would not be expressed at the phenotypic level and hidden from selective pressures. This CGV was a source of genetic variation that could be revealed under more extreme environmental or genetic conditions as heritable phenotypes and could be selected upon. The genetic variants were always there but not phenotypically expressed until some threshold of “change” is met, either through genetic mutation or environmental “stress”.

Waddington believed populations could evolve to harbor CGV to help with changing conditions and environments (Waddington 1957, 1961). This genetic variation could be expressed as heritable phenotypic variation in circumstances when organisms were exposed to environmental influences that exceeded the capacity of its evolved environmental or genetic buffering canalization. In this way, canalization, or more specifically its breakdown could provide the genetic substrate for adaptation.

Waddington also hypothesized that after this de-canalization, organisms could adapt through reduction in variability around the new trait optimum (re-canalize). Waddington's classic selection experiment was meant to test the prediction of genetic assimilation via underlying changes in a population's capacity for canalization, or simply through changes in canalization. Genetic assimilation would result from these changes in canalization. Although canalization definitely occurs, it is untested whether the genetic assimilation from Waddington's experiment is a result of changes in canalization in the evolving populations.

Considerable research has focused on the evolutionary and genetic mechanisms that may enable or facilitate canalization. However, in its modern incarnation, canalization is studied through changes in sensitivity to both environment and genetic effects (Stearns et al. 1995; Debat et al. 2009; Szöllosi and Derényi 2009; Lehner 2010; Lack et al. 2016; Groth et al. 2018). Researchers tend to view canalization in terms of relative trait variability, the tendency to vary (not to be confused with observed variance, the amount of change), of a genotypes' sensitivity to mutations and environmental changes (Gibson and Wagner 2000). Highly canalized genotypes would be less sensitive to changes and show more variability than those genotypes that are less canalized. Lande suggests that after some extraordinary environmental shift prompts phenotypic changes, canalization can be measured by accounting for variance in the intermediate environments (Lande 2009). It should be noted that the resiliency of phenotypes to change is studied under different names, with several researchers using

"buffering" (Rutherford 2000; Sangster et al. 2008) or "robustness" (de Visser et al. 2003; Wagner 2008) to explain the phenomenon of the invariability of traits under a range of conditions. Buffering and robustness are not perfectly synonymous with canalization. Buffering tends to refer to mechanisms that can allow for trait stability. Canalization is perhaps a more specific case of developmental robustness, where robustness is a broad term to encompass the persistence of traits undergoing changes in conditions. Regardless, in some cases these are used interchangeably or with less specificity and so should be considered more carefully.

Many researchers study canalization as the ability of populations (or genotypes) to "buffer" against environmental or genetic changes of varying magnitudes, assessing the variability in response to these changes. For instance, many studies have been devoted to the concept of potential universal buffering mechanism of genetic variation, a heat-shock protein, HSP90. HSP90 has been implicated in maintaining robustness for many systems, and its impairment has shown examples of decanalization in *Drosophila* (Rutherford and Lindquist 1998) , yeast (Jarosz and Lindquist 2010), Arabidopsis (Queitsch et al. 2002), and cavefish (Rohner et al. 2013). However, although these cases show disruption leads to more variability in phenotype, it is possible they may not be representative of adaptive canalization *per se*. Most importantly for the evolution of genetic assimilation, Waddington believed that canalization evolved from natural selection towards a particular fitness optimum (Waddington 1942, 1961). It is perhaps better to think of Waddington's canalization is terms of adaptation. For instance, a

recently adapted high-altitude population of *Drosophila melanogaster* in sub-Saharan Africa show less genetic robustness than their low-altitude putatively ancestral like counterparts (Lack et al. 2016). The high-altitude populations display more mutational defects in wing morphology, and additional mutagenesis experiments showing this is not simply the result of a larger genetic load, but they are more sensitive to new mutations. This suggests the low-altitude population (ancestral) is relatively canalized and that concurrent with adaptation to high altitude environments, the population became decanalized (as a result of pleiotropic effects in this instance).

Waddington treated susceptibility to both environmental and genetic stressors and perturbations as basically equivalent for canalization (1961). However, we now know there are evolutionary reasons to consider them separately. There is theory (Wagner et al. 1997; de Visser et al. 2003) and empirical evidence (Lehner 2010) that suggest genetic and environmental canalization may be quite distinct from an evolutionary perspective (with environmental canalization being relatively difficult to evolve). Under some models genetic and environmental canalization will be correlated, the so called the “congruence hypothesis”. However, there is alternative theory saying they are separable (Masel and Siegal 2009) and several empirical studies that demonstrate that genetic and environmental canalization do not have to be correlated including work in *E. coli* (Cooper et al. 2006) and *Drosophila* (Milton et al. 2003).

There are also multiple ways that environmental canalization can be measured including within-individual variation (fluctuating asymmetry), variation among

individuals of the same genotype (micro-environmental variation), and a reaction norms approach, looking across common and different environments (macro-environmental variation) (Dworkin 2005). However, each of these are not necessarily that same and can result in different variability of phenotypes (Pesevski and Dworkin 2020), and represent distinct developmental and evolutionary mechanisms.

The liability threshold model and the evolution of genetic assimilation:

One of the most parsimonious models for the evolution of genetic assimilation is a relatively well-accepted one, the standard threshold liability model (**Fig. 4**). In liability models, discrete phenotypic polymorphism can actually have a polygenic basis as a result of an underlying continuous "liability" (typically depicted as a normal distribution) (Wright 1934b,a; Dempster and Lerner 1950; Landauer 1958; Falconer 1960; Falconer and Mackay 1996; Lynch and Walsh 1997). Liability collectively describes all the genetic and environmental factors that contribute to the development of a trait. Typically, these models are used to describe the underlying genetics of threshold traits. Threshold traits are discrete phenotypes that follow some continuous distribution (liability). When the combination of genetic and environmental effects for this latent "liability" trait exceed the threshold, the alternative state for the trait is expressed. Threshold traits are evidenced in many organisms (reviewed by (Roff 1996)). In a genetic context, the liability could be made up of many small effect alleles that act additively to contribute to a trait value (Dempster and Lerner 1950; Falconer 1965, 1967; Falconer and Mackay 1996; Roff et al. 1997; Ostrowski et al. 2000).

In consideration of Waddington's work, the evolution of genetic assimilation could be due to the selection of individuals at the extreme of the liability distribution resulting in a mean population shift, or in evolution of the threshold itself[5]. Waddington's own graduate student, who completed further work on the genetic assimilation of venation phenocopies, argued that the process of genetic assimilation was possible through a standard threshold liability model (Bateman 1959a). Under normal conditions the distribution for the population's underlying liability (for example amount of gene product, or total amount of protein activity) is lower than the normal threshold that would allow for the trait to be phenotypically expressed. Environmental influences can shift this threshold; in this case heat stress lowers the threshold so that individuals of the population with lower liability will develop the trait. Selection for the trait could allow for the accumulation of alleles in the population and this would shift the underlying liability so that it may pass even the normal threshold and thus become constitutive in those individuals regardless of environmental influences. There are others that argued that liability could be an effective explanation for genetic assimilation without necessitating canalization (Landauer 1958; Stern 1958; Falconer 1960). Schmalhausen (Schmalhausen 1949) and others (Rendel 1959; Milkman 1961) said that the persistent selection on the phenocopy would enable the accumulation of

[5] Shifts in mean underlying liability have been demonstrated when selecting on hormones inducing wing polyphenisms in crickets (Fairbairn and Yadlowski 1997). However threshold shifts have also been demonstrated in these crickets where lower hormone titers are necessary for morph shifts in different populations (Roff and Fairbairn 1999).

many alleles (in aggregate within individuals) that could ultimately contribute to a sufficient increase (or decrease) in the activity of a developmental genetic network, exceeding the "normal" threshold for environmentally dependent induction. Waddington discussed how selection could accumulate alleles for the trait present in the initial population and did so in the context of threshold traits (Waddington 1961), which seems to match the liability model.

However, Waddington disagreed with proponents of the liability model believing that they were using different language to describe the same ideas. Waddington's biggest criticism was that the liability threshold model could not account for situations where threshold don't seem to be involved, giving an example of a trait that is influenced by genes only under certain environmental cues (Waddington 1961). However, the biggest discrepancy between Waddington's changes in canalization model (Waddington 1961) and Bateman's liability model (Bateman 1959a) seems to be within the last step of recanalization for genetic assimilation. Under the threshold liability model, genetic assimilation could still be the result of an initial decanalization step but does not necessarily require recanalization for genetic assimilation. Here, decanalization could increase the variance of the liability for the population, potentially allowing for an increased number of individuals to fall past the threshold and display the trait (even without shifts in trait threshold) (as suggested by (Gibson and Lacek 2020)). While liability is accepted in many contexts (such as for many threshold traits), neither it nor canalization has been studied explicitly for Waddington's experiment.

The role of *de novo* mutations in the evolution of genetic assimilation:

An alternative possibility for the evolution of genetic assimilation is that, concurrent with selection for a specific trait value while it remains plastic, new mutations can occur which essentially having that same phenotypic effect as the plastic response and can allow for this trait to be constitutively expressed. Although Bateman proposed the threshold model as a mechanism for genetic assimilation of some traits (Bateman 1959a), some of her other work on the genetic assimilation of *dumpy* in fruit flies suggested that this was due to a *de novo* variant potentially induced by the heat-stress treatment (Bateman 1959b). Unlike in her previous work on venation phenotypes where selection produced a reliable increase in phenotype frequency and genetic assimilated individuals were observed relatively early (Bateman 1959a), the frequency of dumpy phenotype was erratic during the starting generations and genetically assimilated individuals were only observed starting in generation 25. Bateman identified a single large effect mutation in the fully assimilated population that failed to complement with a known *dumpy* mutant allele, and suggested that the heat-stress treatment was likely the reason for this spontaneous mutation that appeared in the populations at generation 25 (Bateman 1959b; Waddington 1961). Bateman (Bateman 1959a) also examined this question for *Drosophila* wing venation phenotypes using a starting population of flies who all were genetically identical (so any response would be due to new mutations alone). She neither observed an increase in posterior crossveinless frequency nor any evidence of genetic assimilation of the crossveinless

phenotype, suggesting little evidence for the contribution of new mutations during these short time scale artificial selection experiments.

Interestingly, heat stress can increase mutation rates as demonstrated in several experiments (Lindgren 1972; Ito et al. 2011; Cappucci et al. 2019) which supports the argument for a role of new mutations as a mechanism for genetic assimilation. Similarly, heat stress (Ratner et al. 1992; Vasilyeva et al. 1999; Lerman et al. 2003; Barah et al. 2013; Ito et al. 2013; Quadrana et al. 2019) and stress response in general (Negi et al. 2016; Horváth et al. 2017) are linked to increased transposable element mobilization which also supports the possibility for selection of new mutations. Additionally, there is support for HSP90 suppressing transposable element activity (Ryan et al. 2016) which lends more connection between heat stress and increased mutation rate. That said, there are several examples where neither extreme temperatures (Alonso-González et al. 2006) nor heat stress (Arnault and Biemont 1989; Arnault et al. 1997) seem to increase transposable element activity in *Drosophila*.

The reduction of HSP90 activity leading to an increase in transposon mobilization has been suggested as a mechanism for genetic assimilation (Specchia et al. 2010). Impairment of HSP90 has been linked with increased phenotypic variance of several traits in fruit flies (Rutherford and Lindquist 1998), although there is not a distinction between variation due to SGV or new mutations. Specchia et al. show that functional alterations of HSP90 lead to increased transposon activity (2010), claiming these new

mutations as an alternative mechanism to genetic assimilation as opposed to selection on SGV.

More recently, this same group claimed to directly address the mechanisms behind genetic assimilation in Waddington's experiments and proposed that heat stress induced selectable phenotypic variants and caused new mutations to occur which were co-selected, eventually allowing for genetic assimilation (Fanti et al. 2017). However, unlike the Waddington and Bateman venation experiments (Waddington 1952, 1953b; Bateman 1959a) which saw a phenocopy response in the first generations, Fanti et al. heat-stressed populations for several generation until they found phenocopies that resembled mutations (2017). The discrepancy in generational appearance of these traits during heat-stress is important in considering the contribution of transposable elements to genetic assimilation. Transposable elements do play a role in adaptive response in nature (Lanciano and Mirouze 2018; Schrader and Schmitz 2019), but may need to more carefully considered as a mechanism in genetic assimilation, which is discussed further in chapter 3. Additionally, it is important to consider that new mutations and rare segregating variants of large effect may have similar signatures of selection and so identifying if selected alleles were present in ancestral populations is important to understanding the source of genetic variation for the evolution of genetic assimilation.

Epigenetic mechanisms as a step towards genetic assimilation:

Some researchers have suggested that epigenetic mechanisms may provide a way for populations to respond to the new stimulus before new mutations occur in the population. Nishikawa & Kinjo argue that modeling shows a combination of epigenetic and genetic factors through a "cooperative model" allow for genetic assimilation (Nishikawa and Kinjo 2018). Here, phenotypic changes induced by epigenetics are replaced by small-effect genetic mutations during natural selection. Similarly, other models suggest that epigenetic mechanisms for changes in plasticity are eventually replaced by genetic changes as a mechanism for genetic assimilation (Kronholm and Collins 2016; Danchin et al. 2019). One empirical example of this is the epigenetic silencing of URA3 expression in Saccharomyces cerevisiae under selection eventually resulting in genetic assimilation by mutation for reduced expression (Stajic et al. 2019). Additionally, yeast have epigenetically triggered impairment of translation termination with introduction of a prion [*PSI*$^{+}$], and have several occurrences of genetically assimilated conversion of 3' UTR into coding regions with loss of [*PSI*$^{+}$] after some generations (Giacomelli et al. 2007). Here, the researchers suggest a possible mechanism may be that mutations in the stop codon follow the epigenetic impairment of translation termination, which would mean including regions past the stop coon in further translation even when [*PSI*$^{+}$] is lost. Some also suggest that epigenetic buffering, epigenetic modifications allowing for phenotypes to be resistant to environmental

changes, could allow for rapid adaptation and biased mutations leading to genetic assimilation (O'Dea et al. 2016).

Can genetic assimilation facilitate evolution?

Waddington maintained that genetic assimilation played an important evolutionary role (Waddington 1957, 1961). He was less specific in separating genetic assimilation from the initial plastic response to new environments, and thus discussed its role in both allowing for phenotypic innovations and for rapid adaption to the new environments. Some examples of genetic assimilation having evolutionary benefit exist. For instance, Badyaev et al. describe a potential case of GA for the coevolution carotenoids and feather structure in finches (Badyaev et al. 2017). They describe that carotenoid-based ornamentation could be going through phases where external carotenoids (those gained through diet) induce phenotypes and can lead to genetic assimilation where redundancy allows for the same derived carotenoid to be produced which integrates with feather structure and growth. This process could restart with introduction to a new environment and new dietary carotenoid.

Moving forward, we must consider what steps we need to take to study genetic assimilation in an evolutionary context. As discussed above, it could be hard to observe genetic assimilation as it occurs in nature. Previous work that suggest cases of genetic assimilation in nature has done so by looking for loss of ancestral plasticity. Perhaps by studying these examples and finding the mechanisms behind the genetic assimilation in

those populations, we can have a better understanding of more universal molecular mechanisms behind genetic assimilation. However, it will be hard to disentangle the contribution of standing genetic variation (by way of the liability model) or new mutations if we study populations only after genetic assimilation has occurred. For this reason, it may be beneficial to study examples in the lab and relate these to nature.

When considering the evolutionary implications of genetic assimilation, most researchers combine both the initial plastic response and then the reduction of this plasticity (the latter of which is genetic assimilation). However, it may be beneficial to study these two processes separately in nature since they'd occur on arguably smaller timescales. Understanding the source of variation for plasticity that could be adaptive and understanding the mechanisms behind reducing plasticity as part of adaption can be separable undertakings. Yet if generalizable mechanisms can be identified for these two facets, then combining these ideas will be very relevant when considering ways that populations adapt to new environments. Regardless of the exact process studied, genetic assimilation can be considered like other quantitative traits and thus the molecular mechanisms underlying it can be identified with genome scans or other genetic tools.

Specific thesis goals

This thesis will answer remaining questions from Waddington's classic selection experiment on crossveinless *Drosophila melanogaster* wings (1952, 1953). In his work,

Waddington showed an example of cryptic genetic variation that was revealed through heat-stress and displayed increase of phenotypic variability (i.e. presence of CVL wings where typically all wings have crossveins). Chapter 2 will address if the cryptic genetic variation that influences the CVL phenocopy response is neutral (and thus truly "cryptic") with respects to phenotype and fitness, or if this variation maintained in the population through some pleiotropic effects.

Waddington also demonstrated that with selection for the CVL phenocopy over several generations, heat-stress was no longer necessary to elicit the CVL trait. Instead CVL became constitutive, a process he termed genetic assimilation (Waddington 1953). Some work was done to show CGV played a role in CVL phenocopy response (Bateman 1959), that the trait was polygenic (Milkman 1965b), and that some known alleles influencing crossvein formation interact with generated CVL selection and genetic assimilation lineages (Milkman 1960). However, it is unknown how polygenic the CVL phenocopy is and whether these alleles contribute to genetic assimilation of the trait. Chapter 3 will address what the genetic architecture behind both the CVL phenocopy response is and if it the same for genetic assimilation of the trait (i.e. are the alleles the same, or do new mutation allow for genetic assimilation).

Waddington originally completed this selection experiment as a test for his model of evolution through changes in canalization (Waddington 1942, 1961). Although canalization is known to occur (Lack et al. 2016; Groth et al. 2018), it is unknow if genetic assimilation evolves through changes in canalization or if other models such as

the liability threshold model or new mutations concurrent with selection are better suited to explain genetic assimilation in Waddington's selection experiment. Chapter 4 will address Waddington's proposed model and if changes in canalization are necessary for genetic assimilation.

Figures

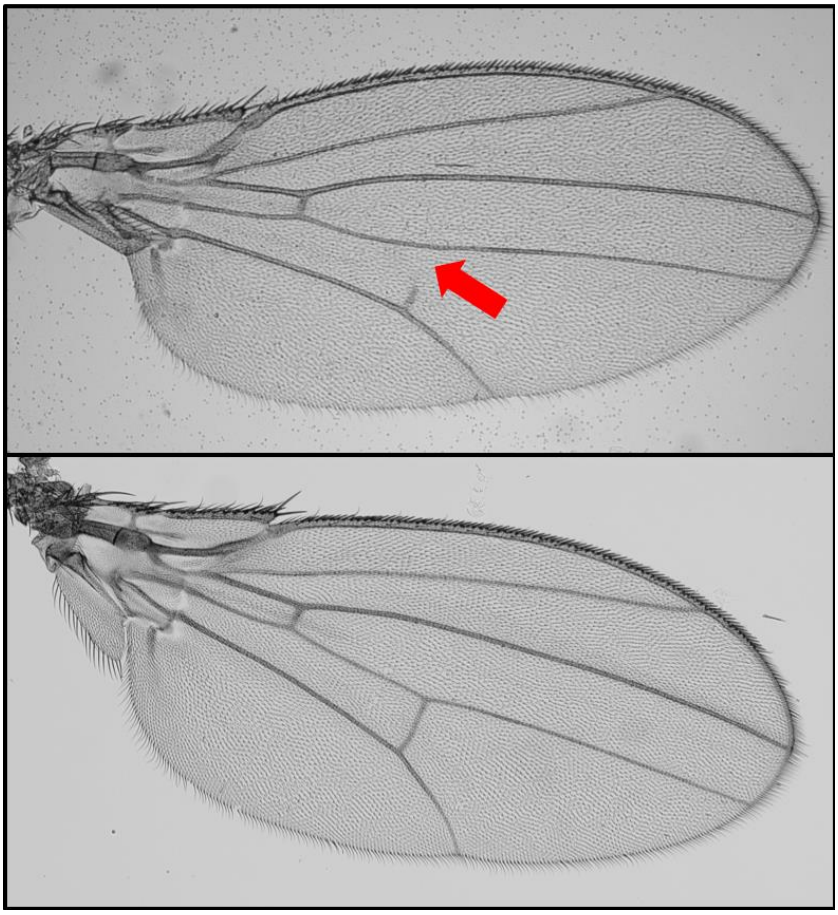

Figure 1.1: Depiction of wings. Crossveinless (top) and normal (bottom) wings. Red arrow points to partially missing posterior crossvein.

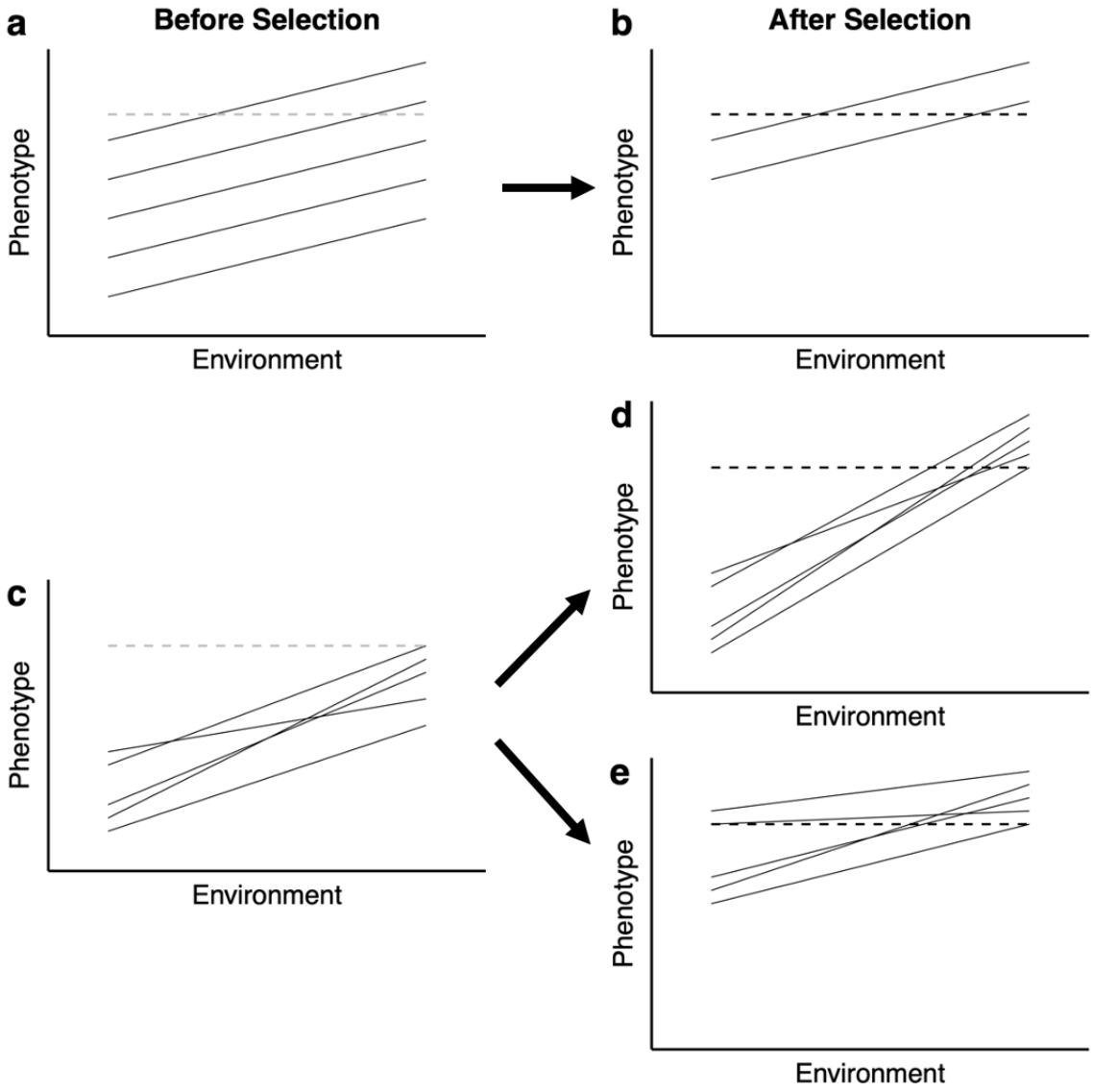

Figure 1.2: Plasticity under selection. Reaction norms representing individual genotypes in a population are solid lines. Dashed lines (gray for before selection and black for after selection) represent a hypothetical threshold for fitness optimum along the environmental gradient. a) In a simplistic scenario all individuals have the same plasticity (parallel slopes with different intercepts) and b) after selection only those above the optimum survive. c) In a complex scenario with varying plasticity that can be selected on to change, d) plasticity can either increase resulting in steeper slopes after selection or e) plasticity can decrease resulting in a mean trait change for the population.

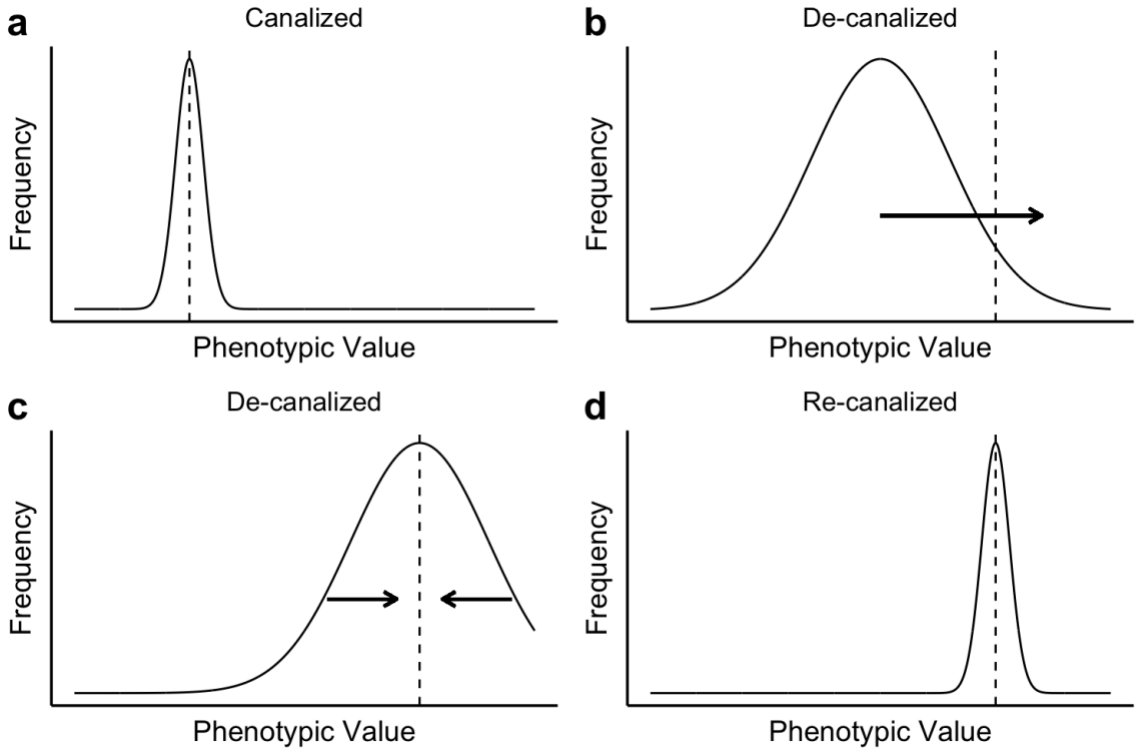

Figure 1.3: Waddington's canalization model explaining genetic assimilation. a) Canalized system with phenotypic values distributed around the optimum (represented by a dashed line). b) De-canalized system, where a new optimum is introduced and results in the disruption of canalization. Arrow represents selection shifting phenotypic mean. c) The distribution of phenotype shifts towards the new optimum and stabilizing selection occurs. d) Re-canalized system with phenotypic values distributed around the new optimum.

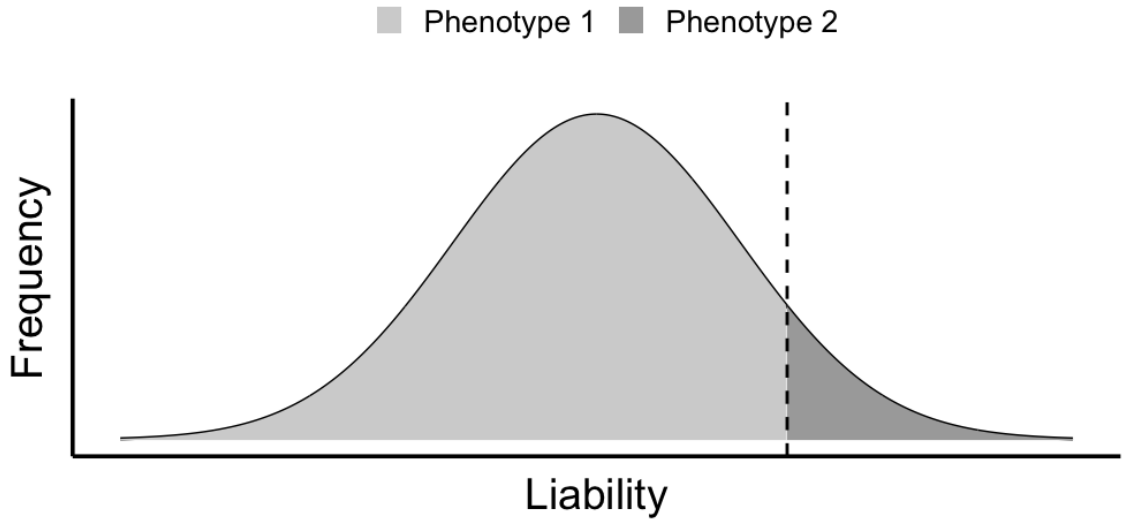

Figure 1.4: Depiction of the threshold liability model. Phenotype is influenced by underlying liability (e.g. gene product) for the trait. After a certain threshold, depicted by a dashed line, there is a shift in phenotype expressed.

Chapter 2: Cryptic Genetic Variation – Allelic variation contributing to the selective response may not always be "cryptic"

Introduction

Waddington provided one of the most established examples of rapid evolution during the 1950s through his work on *Drosophila melanogaster* wing morphology (Waddington 1952, 1953b). He demonstrated that persistent selection (over many generations) on an environmentally induced (plastic) phenotype eventually resulted in individuals expressing the environmentally contingent phenotype, but without the requirement of the environmental induction (Waddington 1953b). Waddington used as a model system, the environmentally sensitive nature of the penetrance of crossveins on the wings of *Drosophila* melanogaster. Under a wide range of rearing temperatures, the wing will develop normally with both a complete anterior and posterior crossvein. However, under high temperature stress during a critical period during pupation, the resulting adult wing can develop with an incomplete or missing posterior crossvein. While Waddington was initially unaware of this, this phenotype is observed in field caught populations of *Drosophila melanogaster* at very low frequencies. Under artificial selection during high temperature stress for the crossveinless (CVL) phenotype, the penetrance of CVL will increase in the population. This demonstrates that the CVL

phenotype is heritable and that there is genetic variation for this trait. As this genetic variation does not seem to affect phenotype under normal rearing temperatures, but does so for under a high temperature stress environment, it is considered an example of cryptic genetic variation.

Cryptic genetic variation is standing genetic variation that has little to no effect on phenotype under normal conditions, but that can lead to an increase in heritable phenotypic variation under rare and/or novel conditions. Although cryptic genetic variation is thought to be conditionally neutral as it is "hidden" under typical circumstances, there are additional possibilities. Some segregating variants are known to have pleiotropic effects (while varying in magnitude across traits), and this is likely to be quite common. Cryptic genetic variation may thus represent additional pleiotropic effects of these variants that are context dependent (Paaby and Gibson 2016). While there is not much experimental work addressing this question, a recent study demonstrated that in some instances, "apparent" CGV may actually not be "cryptic" at all, but has previously unmeasured pleiotropic effects (Duveau and Félix 2012).

It remains to be determined whether the CVL contributing alleles are in fact truly "cryptic", i.e. whether are generally conditionally neutral (in common environments) or have pleiotropic (but unmeasured) effects, and thus are not phenotypically neutral at all. CGV must somehow be maintained in populations once it has accumulated. If neutral with respect to variation and fitness, then the fate of alleles contributing to CGV is primarily the result of genetic drift. However, the alleles we identify as CGV in novel

environments (meaning they have more noticeable effects in novel environments) might also have some pleiotropic effects (including effects on fitness) under normal circumstances. If this is the case, we need to understand how natural selection is maintaining their frequencies in populations. Although most of the experiments have been conducted in the lab, previous research has demonstrated that *Drosophila* larvae and pupae are exposed to equivalent temperature stress in nature (Feder et al. 1997), resulting in similar phenotypes, suggesting that the model of CVL flies is potentially ecologically relevant.

Here, we have repeated Waddington's classic experiment generating populations with increased penetrance of CVL and populations selected for normal wings. We used these to conduct several fitness assays and measures of traits potentially associated with the alleles contributing to CVL in order to assess the aggregate effect of these alleles on various aspects of fitness and phenotype.

Methods

Establishment and Maintenance of *Drosophila* Populations and Selection Lineages

Wild *Drosophila melanogaster* were collected at Country Mill Orchard (Michigan, USA 42°38'8.5"N 84°47'32.3"W) in Fall of 2015. Field collected females were individually placed into vials so that male offspring could be used to distinguish *D. melanogaster* from *D. simulans*. Two male and two female progeny from each field collected *D. melanogaster* female were used to generate the lab population. Progeny from over

1000 field collected D. melanogaster were used to establish this population. After establishing the base population, field collected individuals were stored in 70% ethanol (-20°C) for future genotyping. All flies were maintained using standard cornmeal media (recipe https://github.com/DworkinLab/Protocols/blob/master/Recipes.md).

The population was maintained for 3 generations until being split into replicates for each selection regime: a control group subject only to lab conditions, representing a control for lab adaptation and genetic drift (***lab domestication***), as well as selection regimes exposed to high temperature stress (***up-selection*** and ***down-selection***). The lab domestication lineages were important because lab populations are known to begin rapidly adapting within a few generations (Reed et al. 2014; Hoffmann and Ross 2018). There was a total of 12 replicate selection lineages: 6 up-selection replicates, 3 down-selection replicates, and 3 lab domestication replicates. Each replicate lineage was initiated with 50-pairs of flies and allowed to mate for one day in cages (bugdorm 17.5cm^3) maintained at 21°C. Flies laid eggs over the next five days, with one bottle switched out once a day to maintain low to moderate larval density, maintaining genetic diversity. Cultures in bottles were maintained at 24.5°C. Pupae for the up-selection and down-selection lineages were heat-stressed (described below) and then returned to 24.5°C. Eclosed flies were sorted daily for sex and kept at 18.5°C to allow for minimal development gaps between the flies eclosing early/later in the week. The selection regime described was completed on a three-week cycle for each generation, where week 1 was egg-laying, week 2 was for pupae collection and heat-stress exposures for

the up-selection and down-selection lineages, and week 3 was for collecting and sorting adults. This was repeated for each generation of selection.

Establishment of artificially selected lineages.

Staging of pupae and high temperature exposure.

The critical window for the temperature mediated crossveinless phenocopy occurs during early-mid pupal development (Milkman 1962). We used standard procedures to procure staged cohorts of *Drosophila*. Pupae develop an air bubble causing them to float to the water surface at 8 hours past pupation if developing at 24.5°C. Pupae from media bottles were collected and age of pupae were estimated using a series of two floatings in water. Pupae aged at 8±2 hours past pupation were retained to be used for temperature experiments.

Based on previously published work (Milkman 1962) and pilot experiments (**Table A1**) to examine both the penetrance of the crossveinless phenocopy as well as viability, pupae were exposed to 37.5°C for four hours at 24±2 hours past pupation. This approach differs from Waddington's in that we used a lower temperature stress. We made these changes as Waddington's original procedure had a substantial impact on viability which would cause a strong selective response in its own right as well as contribute to increased genetic drift, both of which could confound analysis and interpretation of experiments. Our collection of pupae from several replicate lineages necessitated a ±2 hour window on pupae age, and a longer duration of heat stress

increased the overlap with the critical development stage for the majority of the pupae. The length and magnitude of the temperature shock we used are similar to numerous other studies which examined aspects of the crossveinless phenocopy (Milkman 1962). Heat-stress exposure was performed by placing pupae on moistened paper-towel in plastic vials with plugs. The vials were submerged to just below the top of the vial in waterbaths within a plastic rack.

Virgin adults were sorted for crossveinless phenotype defined as having at least one break in the posterior crossvein of either wing. It should be noted that although we treated this as a discrete trait (present or missing crossvein), there are differences in severity of missing crossvein and this could be considered as a continuous trait. Up-selected flies were selected for having crossveinless wings while down-selected lineages were selected for having wild type crossveins. In the first few generations, some replicate lineages did not have 50 pairs of individuals with loss of crossvein (up-selection only) and were supplemented with individuals from the same replicate. This was done to make sure that population size was not reduced. Individuals from the lab domesticated lineages were randomly selected at the same time. 50-pairs were chosen from the selected flies for each replicate lineage to initiate the next generation. Parental flies from each generation were stored in 70% ethanol at -20°C.

Generation of Genetically Assimilated Lineages

Genetically assimilated flies (crossveinless flies that developed ***without*** high temperature stress) were first examined and observed in generation 15. Starting in generation 17, a subset of pupae from each up-selected replicate lineage were allowed to develop without high-temperature stress. Crossveinless flies were selected to start matching assimilated lineages. These assimilated lineages were maintained separately from their corresponding up-selected lineages (and from other assimilated lineages) but were supplemented with additional assimilated flies from their corresponding up selection lineages for the beginning generations since we could not find 50 male-female pairs per genetically assimilated lineage at the start to maintain equivalent census population sizes. By generation 3 of assimilation (generation 20 of selection lineages), lineages no longer needed to be supplemented. Assimilated lineages were maintained with selection, but without heat stress.

Estimating fitness effects

Relaxed Selection on phenocopy penetrance

At generation 18, each of the six replicates of up-selected lineages were split into three sets (treatments). One set acted as a control and continued the normal high-temperature stress and selection protocol. The second set was exposed to high-temperature stress, but no selection was performed. The third set had neither exposure to high-temperature stress nor selection. Each set of lineages were otherwise maintained normally for five generations. Progeny of the fifth generation of this

experiment (parallel to generation 23 of main experiment) were exposed to high-temperature stress and CVL frequencies determined by counting 100 flies /replicate/sex and analyzed using a logistic mixed effect model with treatment as fixed effect, and random effects of lineage nested within treatment using glmmTMB (v0.2.3). All analyses were performed in R (v3.5.0).

Fitness component assays

Fitness assays were done with subsets of individuals from lineages unexposed to temperature stress for two generations prior to experiments to avoid confounding maternal effects of heat stress. Assays were done with all replicate selection lineages except assimilated.

Viability

Individuals were split from selection lineages at generation 28. Eggs from each replicate selection lineage were placed in vials at low or high (50 vs. 300 eggs) density. We used both densities to mimic natural as well as lab evolved conditions. 10 replicate vials per density/replicate lineage within each evolutionary treatment were used. Viability was measured as proportion of surviving adults. A logistic mixed model was fit with treatment, density and their interaction as fixed effects. Independent random effects were fit for collection date, individual (egg picker), and replicate lineage nested within treatment, including “random slopes” for density. This was done in lme4 (v1.1-19) using glmer(), and Anova() in car (v3.0-2).

Competitive Ability

Eggs from each replicate selection lineage were placed in vials at either low or high density (50/300 eggs) with half the eggs from a marked competitor; the recessive *scute*^{1} allele introgressed into the ancestral background population. There were 10 replicate vials/density/replicate treatment, split from selection lineages at generation 38. This was repeated with a second block in generation 45. Competitive viability was analyzed in a similar manner to viability, with the addition of uncorrelated random effects for experimental blocks and effects of "person" transferring common competitors to vials.

Fecundity

Females (n=21-24) from each replicate selection lineage (lab-adapted, up-selected, and down-selected) and larval density were collected from the viability assay. Each female was placed in a vial and mated for 24 hours with a male from the same treatment/density. The pair of flies was then flipped into fresh vials each day for 5 days. At the end, all five vials were collected, and total eggs counted for each female. Switching the female into a new vial each day facilitated egg counting and mirrored artificial selection protocols, where bottles were transferred daily. Female thorax length was imaged (Leica MZ12.5 microscope, 6.3x magnification) and measured (ImageJ v1.50f) as size influences female fecundity. A linear mixed model was fit with treatment,

density, their interaction size as fixed effects, thorax length as a covariate and replicate lineage nested within treatment as a random effect using lmer().

Competitive Mating Ability

Individuals were split from selection lineages at generation 40. The marked competitor population with *scute*^{1} mutation was used because it was easily distinguishable and had no other previously known fitness effects. A male from each treatment lineage and a *scute* male were placed in a vial with a single *scute*^{1} female (n=37-50). Replicate vials were set up in a balanced block design with equal numbers of lineage replicates. Females were allowed to lay eggs for 3 days then all three flies were switched to a new vial to keep density low. Progeny were counted and sorted for *scute*^{1}, totaled over 6 days, to estimate proportion sired by each male. A generalized linear mixed model was fit with treatment as a fixed effect. Independent random effects were fit for replicate lineage nested within treatment, vial replicate nested within replicate lineage and treatment, and block using glmer().

Pleiotropic (correlated) effects of CVL alleles on body size, wing size and shape.

Body Size

Using the flies from the fecundity assay described above, thorax length was used to examine correlated effects of CVL selection on size. A linear mixed model was fit with

treatment, density and their interaction as fixed effects, with random effects for replicate lineages within treatment for both intercept and density.

Wing Size and Shape

Animals were stored in 70% EtOH until right wings from females (low density only) were dissected, mounted in 70% glycerol in PBS and imaged (Olympus DP80 camera mounted on an Olympus BX43 microscope, using a 4X objective total 40X magnification). Images were captured with cellSens Standard (V1.14) at 4080 x 3072 pixels (0.0005375 mm/px). Landmarks were obtained using a modified version of Houle *et al* 2003 and Pitchers et al (2019) using Wings (v. 3.72), and CPR to extract Procrustes superimposed configurations with 12 landmarks (excluding posterior crossveins) and 33 semi-landmarks plus centroid size. 480 individuals (average 20/vial/replicate), were included. To examine correlated effects on wing size we used the same model described above for thorax length, without effects of density. The median form of Levene's statistic was used for variability in wing size (examined on both linear and log scale). A similar model to that described above was used but fit using an inverse link function and assuming Gamma distributed error with glmmTMB().

Analysis of wing shape was done in geomorph (v. 3.1.3 (Adams et al. 2019)), with fixed effects of centroid size, treatment, the interaction between treatment and replicate lineage and the interaction between treatment, replicate lineage and vial replicate on shape residuals. For hypothesis testing, a model without the treatment

effect term was used as the null model. The effect of treatment for both mean shape (using distance between vectors) and variance (using disparity) was tested with these models.

Part 2:

We address whether there were differences between the UP-selection and their corresponding assimilated lineages for patterns of variability. For this experiment, flies saved from the "Relaxing Selection on Flies" experiment described above were used for non-heat-stressed up-selected lineages along with lab-adapted lineages from the matching generation (F23) and genetically assimilated flies of the corresponding generation (F6). Wings from female flies were dissected and shape data was collected as described above. A total of 291 individuals were included, averaging 19.5 individuals for each replicate lineage. The effect of treatment on wing size was tested using a mixed model with a fixed effect of treatment and a random effect of replicate lineage. To test the hypothesis that treatment has an effect on wing size variance, a generalized linear model was used with the same predictors as the size model above. For shape, a model with terms for centroid size, treatment and replicate lineages was fit using the geomorph package. For hypothesis testing of the effect of treatment on mean shape change and variance, the null model removed the treatment term.

Results and Discussion

We replicated Waddington's experiment with modifications to facilitate the experimental design; we adjusted the temperature of the heat stress exposure to allow for maximal viability of individuals and for greater sample size to fall in the correct developmental stage during heat stress. After exposure to developmental heat stress each generation, individuals derived from a natural population were selected for loss of the posterior crossvein (hereafter "up-selection"), or its maintenance ("down-selection"). Unlike most previous studies (which had very limited replication of selection lineages) we generated six independent replicates of up-selection and three down-selection lineages, to facilitate biological and statistical inferences. Consistent with previous findings, we observed a rapid response to selection for increased penetrance of the CVL phenotype under developmental heat stress (**Fig. 2.1**). All up-selection lineages experienced a temporary decrease in crossveinless frequency for several generations starting at generation 10, due to a move in laboratory space and slightly re-stabilization in lab environmental conditions. Notably once the lab condition was stabilized, the populations all returned to the expected trajectory. We observed genetically assimilation in each up-selection lineage and from these propagated matching independent genetically assimilated lineages (**Fig. 2.1**). Additionally, we generated three lab domestication lineages (with population sizes matching up-selection and down-selection) to account for laboratory adaptation and genetic drift during selection.

If the response to selection is a result of standing genetic variation in natural populations, it is necessary to ask whether the allelic effects are truly "cryptic" in environments without temperature stress. If so, the expectation would be that their frequencies in natural populations are maintained under mutation-drift balance. Alternatively, the allelic variants have unmeasured pleiotropic effects (Duveau and Félix 2012), and are maintained in the population in part due to selection. In this scenario we would predict a correlation between response to selection for loss of crossveins and other traits or fitness. Utilizing these lineages, we sought to determine the aggregate fitness consequences of alleles contributing to the response. A previous study suggested that the alleles contributing to the CVL phenocopy response were potentially deleterious (Bateman 1959a) by examining how phenocopy penetrance varied under relaxed selection. We used a similar form of relaxed selection, but also included a treatment where there was continued heat-stress and relaxed selection. In either case with relaxed selection, we observed a decrease in CVL frequency relative to the starting generation (**Fig. 2.2**), consistent with a deleterious effect of these alleles in aggregate in the up-selection lineages.

If alleles contributing to the crossveinless phenocopy response are maintained by selection in natural populations, we may see contrasting effects on fitness components. We examined three fitness components of *Drosophila*: viability, fecundity, and male mating success. The up-selection lineages showed reduced viability compared with the down-selection lineages at low density, with no significant differences among

selection lineages at high densities (**Fig. 2.3**). To confirm those effects, we did a subsequent and similar experiment examining competitive viability against a marked competitor and observed consistent effects (**Fig. 2.4**). We also examined both fecundity and male mating success in parallel experiments. While there was considerable variation, we did not see substantial treatment effects for either (**Fig. 2.5,2.6**). However, for the competitive male mating assay, we found that our marked common competitor was not as relatively fit as the selection flies, meaning that it had poor success with all treatments; potentially using a better competitor could have resulted in more separation of selection lineages mating success. These results suggest that -- in aggregate -- alleles that contribute to this response may in fact be deleterious independent of heat stress, at least in these genetic backgrounds and conditions.

We also examined whether these alleles have correlated or pleiotropic effects on specific traits. The number of measurable traits is impossibly large, but using the 83 genes in Flybase with identified roles in crossvein development, gene ontology enrichment suggests that many have additional roles during organismal development including cell proliferation, body size and other aspects of wing development. Indeed, wing size, shape, and development are known to be influenced by variants in genes that contribute to crossvein development (Debat et al. 2006; Dworkin and Gibson 2006; Matsuda and Shimmi 2012; Shimmi et al. 2014). While there was considerable variation in body size, wing size and wing shape we observed no evidence for consistent

treatment level effects on mean trait values or changes in variation for either body size (**Fig. 2.7**) or wing size or shape (**Fig. 2.8, Table 2.1, 2.2**).

While we were unable to demonstrate what specific traits the fitness effects are linked to, our results suggest that the allelic variants influencing the crossveinless phenocopy response influence fitness in aggregate, and are not necessarily phenotypically "cryptic". These alleles in aggregate have some deleterious effect as we see in reduction in crossveinless frequency during relaxed selection. Specifically, we see deleterious effect for viability. The deleterious nature of these alleles in aggregate is not unexpected as these alleles in aggregate are not maintained at high frequency in populations; the crossveinless phenotype occurs relatively rarely in natural populations (less than 1% in wild-caught *Drosophila* populations). Future work plans to address how recent temperature history affects the maintenance of these alleles in populations, which may give some insight into whether CGV can be enriched for beneficial alleles in response to certain environmental stimuli.

As the crossveinless trait is highly polygenic (see Chapter 3), it could be that we are underestimating phenotypic effects as the pleiotropic contributions of alleles influencing CVL could vary in direction. Further, it may be that the fitness effects of the alleles influencing viability are maintained due to other forms of selection (i.e. density or frequency dependence). It should be noted that the allelic combinations brought about by selection may be very rare in nature, and thus the phenotypic effects could be largely cryptic in such circumstances.

Figures

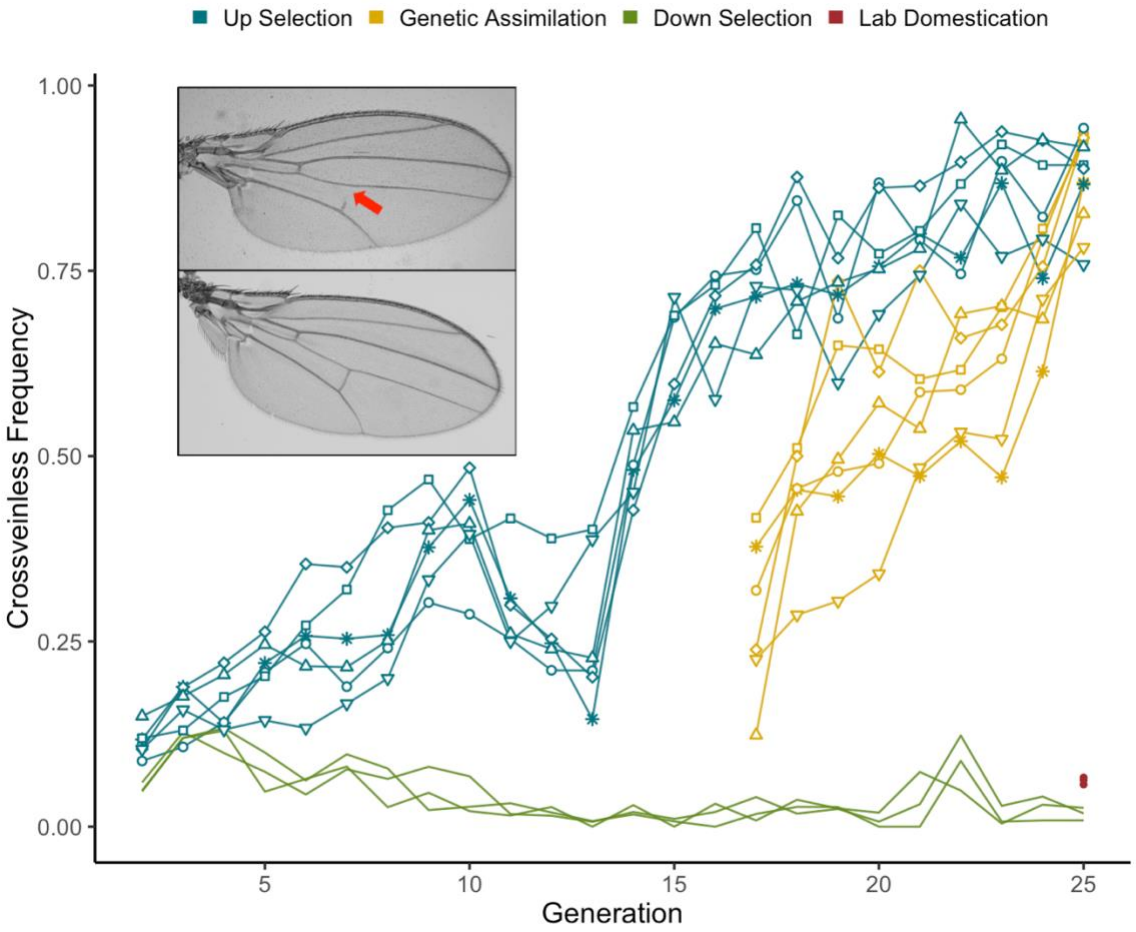

Figure 2.1: Alleles associated with CVL show strong response to selection.Crossveinless frequency is the proportion of flies for each lineage that showed the CVL phenocopy (defined as a fly with one or more breaks in one or both of the posterior crossveins, see picture) for that generation. Shapes on each up selection replicate lineage correspond to the matching genetically assimilated replicate lineage. Lab domestication lineages are shown only for generation 25 where they were heat-stressed to confirm maintenance of CVL alleles in these populations.

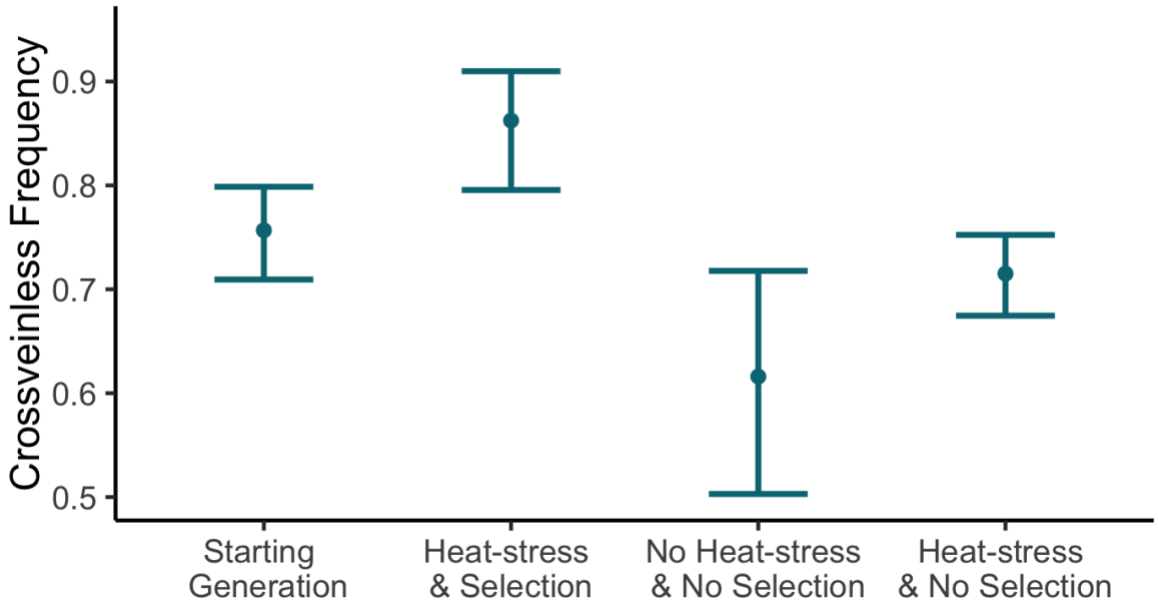

Figure 2.2: Relaxation of selection for five generations reduces the frequency of the CVL phenocopy response. Frequency of CVL after 5 generations of relaxed selection. The starting generation is the average CVL frequency of generations 17, 18, and 19. Heat-stress and selection are the lineages continued for the normal procedure of artificial selection. 200 individuals were counted for all six replicate lineages for each treatment. Error bars are 95% confidence intervals. ANOVA from the generalized linear model shows that all treatments differed significantly from the starting generation, heat-stress & selection ($p<0.0001$), no heat-stress & no selection ($p<0.0001$) and heat-stress & no selection ($p<0.01$).

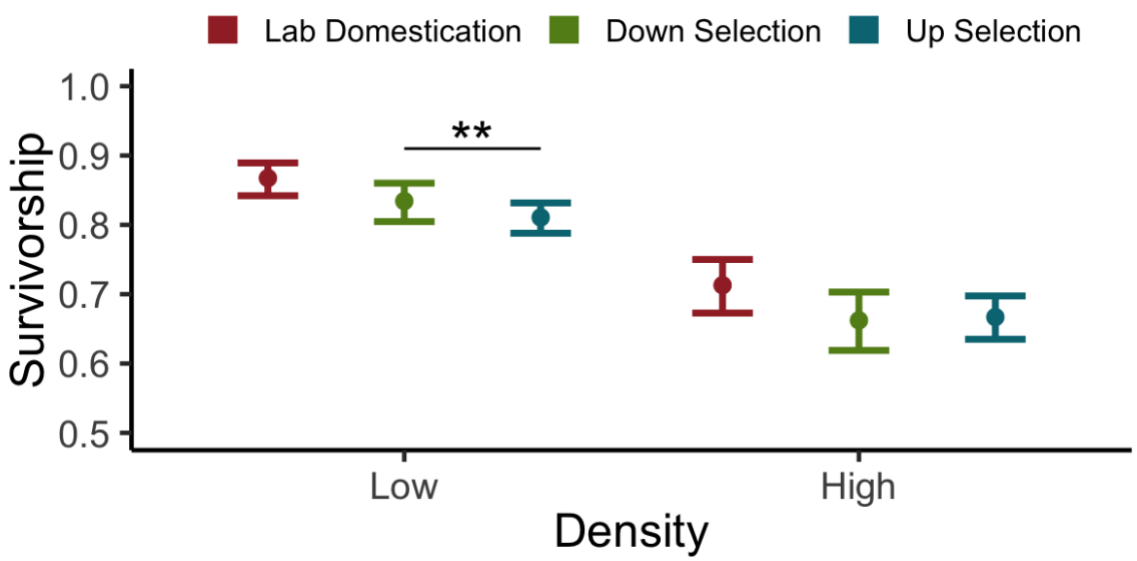

Figure 2.3: Increased frequency of CVL alleles in a population is associated with reduced viability. Percent survivorship of the three selection regimes. High density had 300 eggs and low density had 50 eggs per vial. For each density: n_{LD}=30, n_{DOWN}=30, n_{UP}=60 vials (10 vials per replicate lineage). Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model (**P < 0.01).

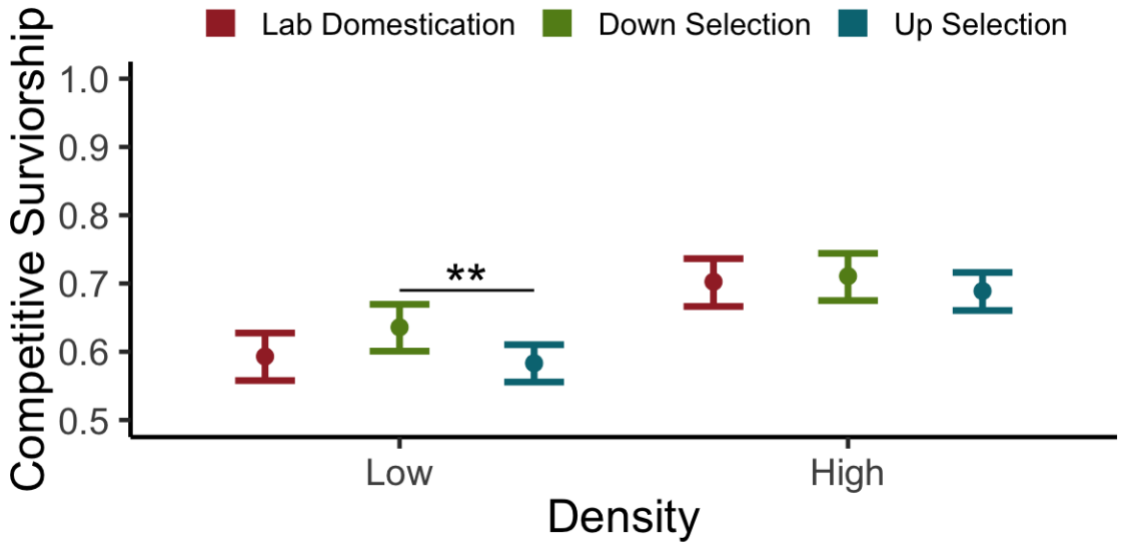

Figure 2.4: Increased frequency of CVL alleles in a population is associated with reduced competitive viability. Relative percent survivorship of the three selection regimes. High density had 300 eggs and low density had 50 eggs per vial, half treatment eggs and half common competitor. For each density: n_{LD}=30, n_{DOWN}=30, n_{UP}=60 vials (10 vials per replicate lineage). Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model (**P < 0.01).

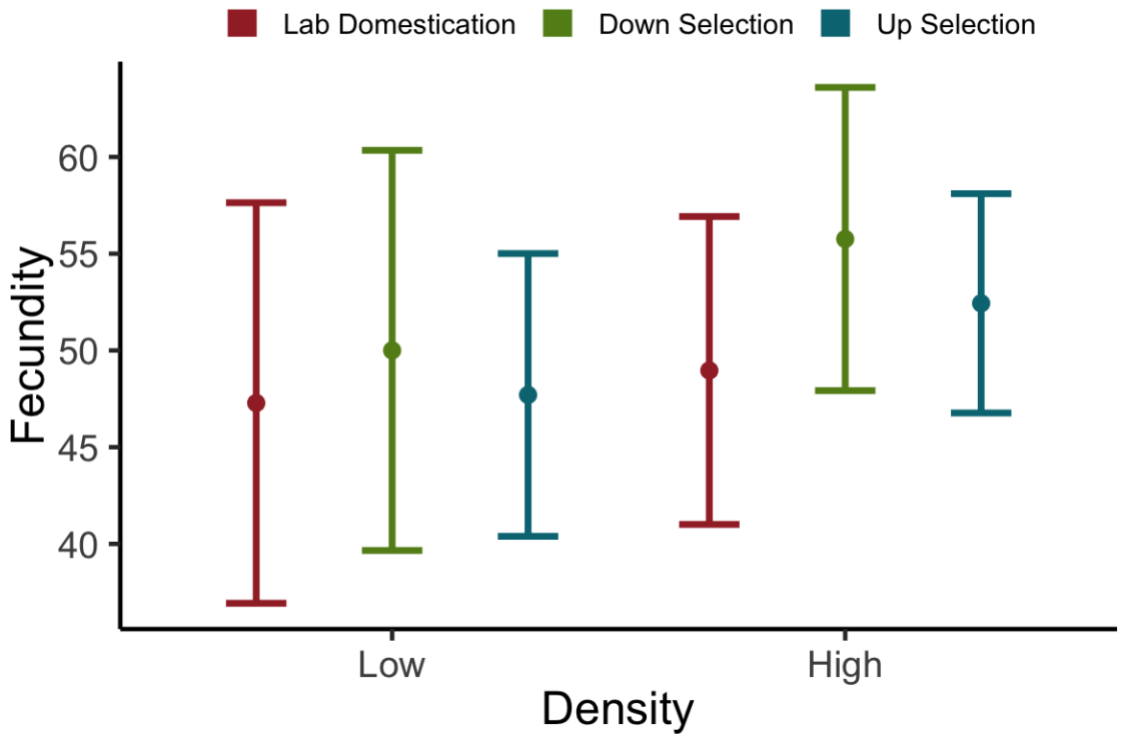

Figure 2.5: Variation in fecundity does not appear to be associated with frequency of CVL alleles. Fecundity of the three selection regimes. Females were raised at either high or low densities before egg laying. For low density: n_{LD}=67, n_{DOWN}=68, n_{UP}=138 females. For low density: n_{LD}=62, n_{DOWN}=64, n_{UP}=126 females. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model; none of the treatments show significant differences from each other at either density ($p>0.1$).

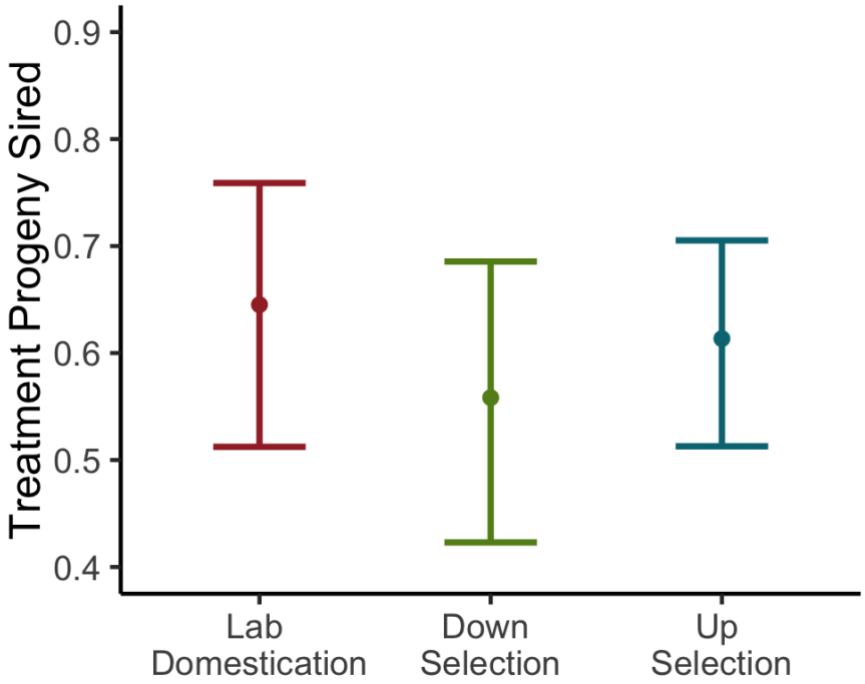

Figure 2.6: Variation in male competitive ability does not appear to be associated with frequency of CVL alleles. The observed proportion of treatment for each selection lineage. n=37-50 per replicate lineage in a treatment. Competitor females were kept with a treatment and competitor male for 6 days and all progeny from that time was counted. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model; none of the treatments show significant differences from each other at either density ($p>0.1$).

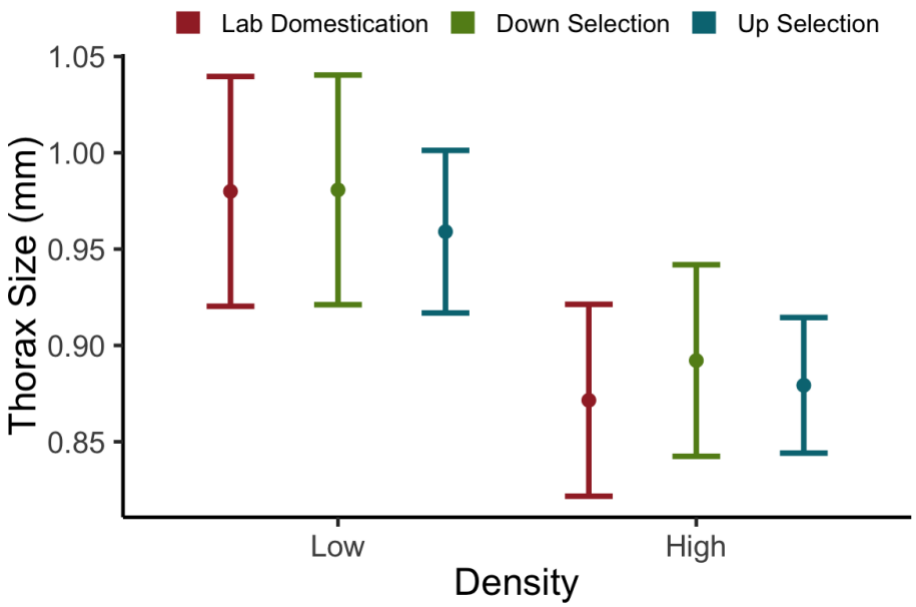

Figure 2.7: Variation in body size does not appear to be correlated with frequency of CVL alleles. Thorax length (as a proxy for body size) was used to examine correlated effects. Flies were taken from the fecundity assay (High density: n_{LD}=67, n_{DOWN}=68, n_{UP}=138 females. Low density: n_{LD}=62, n_{DOWN}=64, n_{UP}=126 females). Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model; none of the treatments show significant differences from each other at either density ($p>0.1$).

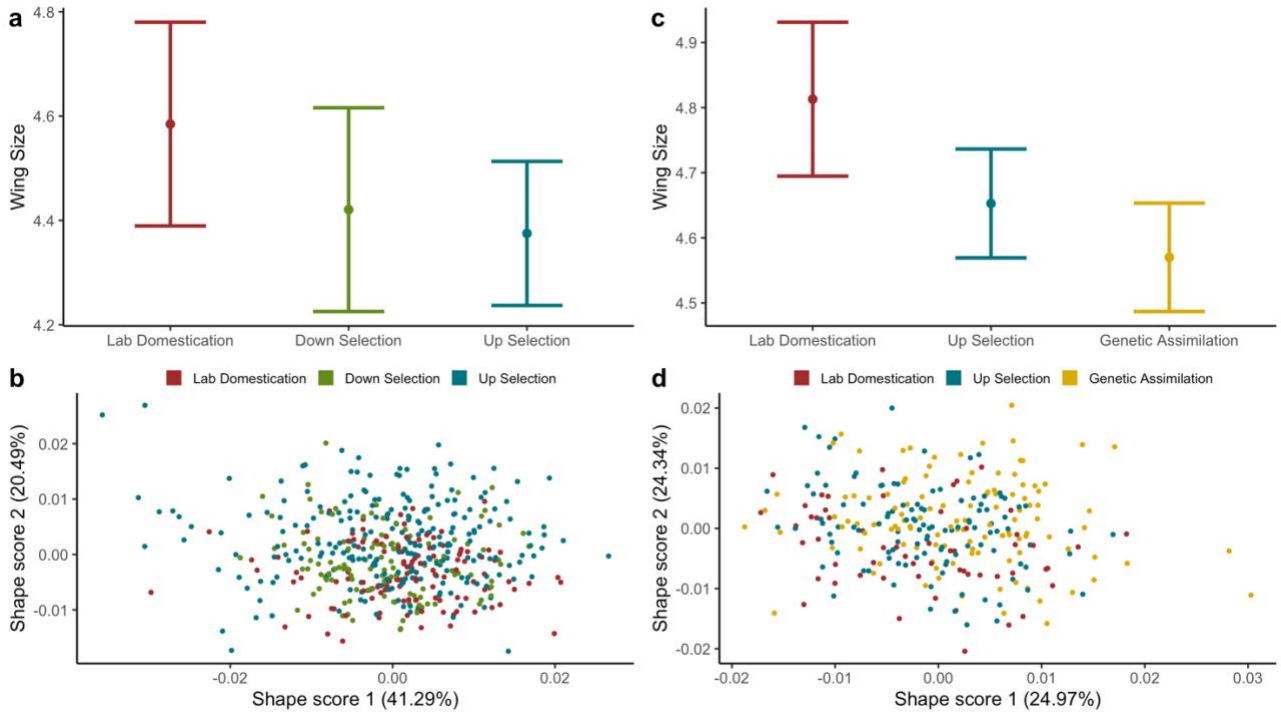

Figure 2.8: Variation in wing size and shape does not appear to be correlated with frequency of CVL alleles. Wing size (centroid size) and shape scores for two sets of comparisons among the selection lineages. The first group (a,b) was used for comparison between up-selection and down-selection (n~160/group), and the second group was (c,d) for up-selection and genetic assimilation (n~97/group). Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model. Levene's statistic was used to estimate variability and showed no difference between treatment for either set of selection lineages. Shape scores are projections of observed data onto vectors defined by PCs of fitted values.

Table 2.1: Results from ANOVA on deviations from the mean landmark configuration of the entire wing for comparison of lab domestication, down-selection, and up-selection lineages. Treatment refers to selection lineage, replicate refers to replicate of selection lineage, and vial refers to vial replicate.

	df	SS	MS	R^2	F	Z	P
Centroid Size	1	0.009	0.009	0.052	32.26	7.34	0.001
Treatment	2	0.006	0.003	0.033	1.41	0.98	0.153
Treatment x Replicate	9	0.018	0.002	0.104	2.45	3.66	0.001
Treatment x Replicate x Vial	12	0.010	0.001	0.057	2.96	7.23	0.001
Residuals	455	0.123	0.000	0.727			

Table 2.2: Results from ANOVA on deviations from the mean landmark configuration of the entire wing for comparison of lab domestication, up-selection, and genetic assimilation lineages. Treatment refers to selection lineage, replicate refers to replicate of selection lineage nested within treatment (not an interaction), and vial refers to vial replicate.

	df	SS	MS	R^2	F	Z	P
Centroid Size	1	0.003	0.003	0.029	10.03	5.38	0.001
Treatment	2	0.004	0.002	0.038	1.92	1.75	0.045
Treatment x replicate	12	0.012	0.001	0.119	3.38	8.32	0.001
Residuals	275	0.078	0.000	0.807			

Chapter 3: Genetic assimilation of loss of crossveins is due to a polygenic response, not *de novo* mutations of large effect

Introduction

Waddington's work on the genetic assimilation of CVL in *Drosophila* wings raised several questions about how the assimilation was possible. Was the mechanism for genetic assimilation due to segregating variation or new mutations? If it was segregating variation, why was this variation maintained in natural populations (i.e. was it neutral or pleiotropic)? Further research into this model has shown that the CVL phenocopy could not be environmentally induced when starting with an isogenic strain, meaning the genetic variation allowing for the induction CVL phenocopy in natural populations is already present (Bateman 1959a). However, Bateman did not examine whether there were differences in the genetic basis between the increase in frequency of the phenocopy response compared with the genetically assimilated lineages derived from those lineages (whether new unconditional mutations occurred during the selection process, and these were ultimately responsible for the genetic basis of the genetically assimilated CVL phenotype). Later studies show that the genetically assimilated lines had the same alleles affecting the phenocopy in both the selected lineages and the genetically assimilated lineages (Milkman 1961), and that the genetically assimilated lineages could be selected on to return to the wild-type wing (as long as they were not fixed for the alleles causing CVL) (Milkman 1965a). The CVL phenocopy is polygenic and

due to a combination of commonly occurring alleles rather than individual rare alleles (Milkman 1965b). There are several alleles that are known to affect crossvein formation, including *crossveinless* (*cv*) and these can be targeted directly as candidate genes affecting the CVL phenocopy. However, past studies have shown that few of the alleles mediating the CVL phenocopy in the genetically assimilated lineages map to known candidate genes for CVL (Mohler 1965b, 1967). Thus, there is a critical need to determine the genetic architecture of the phenocopy and assimilation response to help to understand why these alleles are segregating in natural population. There is also evidence of only partial genetic parallelism in response to selection, even for lineages originating from the same natural population (Mohler 1965b,a). The allelic variation that contributes to the selection response for the increase in the CVL phenocopy, and then to the genetic assimilation of the CVL phenocopy, seems to occur in many different natural populations in both Europe (populations used by Waddington and Bateman) and the North America (populations used by Milkman and Mohler). However, there are still questions on the degree of polygenicity of the CVL phenotype is, whether it is a few major genes influenced by penetrance genes or if it is just very polygenic.

Since the advent of molecular genetic and genomic approaches, little work has been done to dissect the genetic architecture of a genetically assimilated trait, although genetic markers have been shown to be correlated with increased frequency of a phenocopy (Gibson and Hogness 1996). Additionally, a proposed “universal” mechanism by the inhibition of the heat shock chaperone protein HSP90, which normally acts as a

buffering mechanism and can contribute to the accumulation of CGV (Rutherford and Lindquist 1998). How general and relevant this mechanism is for the CVL phenocopy and genetic assimilation of this trait is unclear. Although past work has shown segregating allelic variants are necessary for increasing penetrance of the CVL phenotype (Bateman 1959a) and were suspected to contribute to genetic assimilation (such as like liability model (Stern 1958; Bateman 1959a)), recent work has encouraged debate into the contribution of new mutations during the selection process allowing for genetic assimilation of the trait. More specifically, it has been argued that high-temperature stress could increase transposable element activity resulting in *de novo* variants of large effect (Specchia et al. 2010; Fanti et al. 2017). While the conditions for this are plausible, it has not been conclusively demonstrated or refuted in any case of a genetically assimilated trait, including for the CVL phenotype.

Here, we aimed to understand the genetic architecture behind both increased phenotypic response and genetic assimilation. We investigated the influence of known alleles affect crossvein development and used deep resequencing of the ancestral population and all lineages at multiple time points during the selection process to assess relative contribution of CGV and *de novo* mutations to the genetic architecture for the assimilated phenotype.

Methods

Isolation of DNA

Isolation of DNA was done with a modified Qiagen kit protocol for "Purification of total DNA from insects using the DNeasy Blood & Tissue Kit - using a mortar and pestle". 100 individuals from each replicate lineage were used in the DNA extraction (50 males and 50 females) for pooled sequencing. Flies were prepared in groups of 25 individuals (by sex). After DNA quantification, the four groups making up a selection lineage replicate were combined to have equal DNA contributions. The Ancestral population was sampled and sequenced with a total of 400 individual flies to capture genetic diversity and rare variants in the founding population, but with the same group (25) and pool (100) sizes for sequencing.

Genomic Analysis

To examine the genomic consequences of selection and assimilation, we sequenced all up-selection, down-selection, and lab domestication replicate lineages at generation 10 and generation 23. Genetic assimilation lineages were sampled at generation 8, corresponding to generation 23 of other selection lineages. This was done at the Michigan State University RTSF Genomics Core using the Illumina Truseq Nano DNA library preparation kit and samples were run over two days on 4 lanes (4x2 lanes total) Illumina HiSeq flow cell. We generated 125bp paired reads with an average insert size of 700bp. We obtained ~140X genome coverage for each selection lineage replicate (at each time point) and 600X coverage for the ancestor population. Reads were

mapped to the *D. melanogaster* reference genome (r5.57) using bwa (v0.7.8) and novoalign (v3.07.00). We used two mappers which had both been evaluated for pool-seq data and used the intersection to reduce false-positive polymorphisms (recommended in (Kofler et al. 2016b)). PCR duplicates were removed with Picard (v1.131) and GATK (v3.4-46) was used for indel realignment. Nucleotide diversity (Tajima's pi) and F_{ST} were calculated with PoPoolation (v1.2.2, (Kofler et al. 2011a)) and PoPoolation2 (v1.201, (Kofler et al. 2011b)), respectively (--pool-size set to individuals per replicate, --max-coverage set to approximately double the average genome coverage per replicate). To address the contribution of segregating variation versus new mutations selected in the genetic assimilation populations, a custom R script was used to check if selected alleles were present in the ancestor.

We also checked for selection by finding regions with reduced nucleotide diversity. To account for the genetic drift experienced by each population, we counted reductions in Tajima's pi for each genetic assimilation lineage when it was larger than that of the lab domestication lineages. All reductions in Tajima's pi are in reference to the ancestral nucleotide diversity and calculated over 500bp windows.

Crosses among lineages

Reciprocal crosses were performed among selection lineages, and CVL phenotype frequency determined in F1 progeny and compared to frequencies of corresponding "pure" replicate lineages. Heat stress was applied as described above.

Up-selected lineages were crossed in a balanced incomplete round-robin design due to constraints of having to heat-stress pupae (**Table A2**). We performed each cross at two generational time-points (corresponding to generations 22 and 23) and at least 100 flies were scored for each sex/cross.

For genetically assimilated lineages all possible crossing of lineages were done, but otherwise the details of the crosses (but without heat shock) as described above. We performed each cross at two generation time-points (corresponding generations for assimilated lineages are 12 and 13) and at least 100 flies were scored for each sex per cross. To quantify differences between pure lineages and "hybrids" between them for both the up-selection and assimilation crosses, a logistic mixed model was fit using CVL counts, with pure/hybrid and block as fixed effects, and independent random effects for lineage from both the male and female parents.

Quantitative Complementation Tests - with co-isogenic deletion lines.

Using FlyBase (~December 2017) , we identified the ~81 known genes influencing crossvein development via loss-of-function (mutant or RNAi) perturbation. Using this set, we identified deletion lines within each of the Exelixis and DrosDel Deficiency collections. While these collections had independent progenitor strains, all deletions within collection are otherwise co-isogenic to their respective progenitor. We identified 76 deletion lines, spanning 78 genes. Every genetically assimilated replicate lineage was crossed with each deletion line (n = 2 independent crosses) and its respective progenitor

control lineages (n = 2 crosses per block). Given the large number of experimental crosses required (>900), multiple experimental blocks were required, with independent control crosses within each block. Males of autosomal deletion lines were crossed with females of genetically assimilated lineages and all progeny containing the deleted chromosome region were scored for CVL frequency. Females of X-chromosome deletion lines were crossed with males of genetically assimilated lineages and female progeny were scored for CVL frequency. Twelve deletion lines were further tested with a higher number of replicate crosses per lineage (n=4). Measures of CVL frequency were done as stated above.

To confirm that the effects observed were not due to haploinsufficiency of the deletion, Males from 10 autosomal deletion lines (and females from 2, X-chromosome deletion lines) were crossed to 3 lab-adapted lineages (LD1: n = 2 vials, LD2: n=1, LD3: n=1). Among F1 progeny, no individuals with the CVL were observed.

To serve as a background matched control to examine dominance of the CVL phenotype we crossed each genetically assimilated lineage with the 3 lab-adapted lineages reciprocally (n = 4 independent crosses each) and CVL frequency recorded among F1. For each deletion we fit a logistic regression (counts of CVL and crossvein flies) with genotype (deletion/wild type), assimilated lineage and their interaction. For several sets of crosses we observed complete separation during modeling. As such we adjusted all counts by adding one to both counts of total CVL and crossvein flies to enable model convergence.

Results and Discussion

The increase in penetrance of the temperature induced loss of the crossvein under artificial selection is known to be due to a polygenic response, largely alleles of individually small effect (Milkman 1960, 1962). However, several models have been proposed for the evolution of genetic assimilation. Waddington and others (Bateman 1959a; Rendel 1959; Milkman 1961; Waddington 1961) discussed the relative contribution of standing genetic variation, both in terms of a polygenic response (many alleles of small or moderate effect interacting) and the contribution of rare alleles of large effect (i.e. "Mendelian" mutations). They also recognized the potential contribution of new mutations of large effect occurring concurrent with the selective response. In the polygenic response model, genetic assimilation occurs when the frequency of alleles across genes increase sufficiently that individuals (on average) have enough copies of alleles to result in a threshold effect (i.e. a liability model (Landauer 1958; Stern 1958; Bateman 1959a)). Alternatively, artificial selection can also select on individually rare segregating alleles of large phenotypic effects that cause crossveinlessness, and are responsible for genetic assimilation. Similarly, new mutations of large effect that occur concurrent with selection produce similar results.

Early tests of these models favored a polygenic response based on standing genetic variation (Bateman 1959a; Milkman 1960; Mohler 1965a). However, crossveinless individuals are observed in natural populations at low (>1%) frequency,

and spontaneous mutations in genes involved with crossvein development occur (Milkman 1964; Boyer et al. 1973). Additionally, recent work has emphasized that heat stress (Ito et al. 2011; Cappucci et al. 2019), transposable elements (Quadrana et al. 2019) and their interaction result in high mobilization rates, and suggest new mutations are responsible for genetic assimilation (Specchia et al. 2010; Fanti et al. 2017). Interestingly Bateman examined the contribution of new mutations in 1959 (Bateman 1959a). Repeating the selection experiment using an isogenic strain (with no genetic variation), she observed no increase in frequency of the CVL phenotype nor genetic assimilation. Yet given current knowledge of TE mobilization rates under heat stress (Ryan et al. 2017), this hypothesis has resurfaced.

To address the relative contribution of SGV vs new (or rare) mutations of large effect, we took multiple independent genetic and genomic approaches. From crosses between all genetically assimilated lineages we observed that F1 individuals from crosses between lineages showed only slightly reduced frequencies of loss of crossveins (**Fig. 3.1**). This is similar to the results of crosses among the up-selection lineages. If the results were due to different new mutations in each independent lineage, then crosses would show highly reduced crossveinless penetrance, unless they had considerable dominance. However, if crossveinless is due to a polygenic response with partial parallel response of alleles, crosses would be expected to show intermediate penetrance similar to those of the “pure” lineages. Importantly, crosses of these lineages back to “control” (lab domestication) lineages showed low levels of crossveinless (**Fig. 3.2**), inconsistent

with substantial dominance. Overall there is evidence that is consistent with partial but incomplete genetic parallelism of the selection response among replicate lineages.

We next examined the role of potential candidate genes known to influence crossvein development. Previous work suggests that distinct genomic regions contribute in different genetic assimilation experiments, often associated with genes influencing crossvein development (Milkman 1960; Mohler 1965b). However, when most of these studies of the genetic assimilation of crossveinlessness were performed, the number of known genes influencing this developmental process was severely limited. We screened Flybase and identified 81 genes with known loss-of-function (or RNAi) phenotypes influencing crossvein development (as described above). We obtained co-isogenic control and deletion lines for 78 of these (spanning the focal and nearby genes) and performed quantitative complementation analysis for all deletions by all 6 independent genetically assimilated lineages. This approach examines the relative dosage of allelic effects. If new variants of large effect contributed, we would expect few of these genes to interact within each lineage (i.e. just the gene with the new mutation would fail to complement), with perhaps different genes interacting with each lineage. Under a polygenic response with partial parallelism in response we would expect many genes to interact with the replicate lineages and seeing partially shared response among the replicate lineages. We observed evidence that many genomic deletion regions influence the penetrance of the CVL phenotype (**Fig. 3.3-3.4**), varying among replicate lineages. Consistent with the results from the crosses amongst lineages, we observed evidence

for polygenic response and incomplete parallelism amongst the assimilated lineages (i.e. **Fig. 3.5**). Some of the genomic regions contributing to assimilation in our lineages were also observed in previous studies, yet not all; we tested *crossveinless*, *crossveinless-c*, *crossveinless-d*, *dachs*, and *dachsous* as Milkman did (Milkman 1960). Importantly, the deletions we checked had no effects on CVL frequency themselves under heterozygous conditions crossed to lab domesticated lineages, suggesting effects were not due to dosage of these genes *per se.* Our observation of incomplete parallelism (i.e. different genetically assimilated lineages in our experiment utilizing alleles in different combinations that ultimately result in assimilation of crossveinless phenotype) is consistent with SGV in that there is sharing of some groups of alleles. Our results are also inconsistent with a single allele of large dominant effect as in the heterozygous state they have low penetrance (**Fig. 3.2**).

We used genome wide scans to examine the response in an unbiased manner (i.e. not dependent on candidate genes). If the response is polygenic due to a combination of segregating variants spread across many genes from the ancestral population, we predict many small changes in patterns of genetic differentiation across the genome. Importantly this provides an explicit test of the model of new variants of large effect (and for very rare segregating variants), even if their identity and position remain unknown. If genetic assimilation was due to new mutations of large effect followed by strong and rapid truncation or threshold selection (with the selected variant rising to near fixation or fixed), it would be associated with the signature of a hard

sweep, substantially reducing nucleotide diversity in linked regions. We sequenced the ancestral, genetic assimilation lineages, and all selection lineages at two timepoints during artificial selection. Although we recognize that the replicate lineages may have different regions contributing to varying degrees, from a statistical standpoint we were interested in regions that occurred in at least a few of the lineages and so viewed the lineages together with average F_{ST} (**Figure 3.6**). We observed evidence of many regions in the genome contributing (**Fig. 3.6-3.9**), further support for a large polygenic response and not just due to a small number of mutations of individually large effects. Genomic scans also provided a strong test of the potential role of either rare variants or *new* variants of large effect and we found that much of the selected variants were segregating in the ancestor (**Table 3.1**). Although we tried to take a conservative approach in removing spurious sites (using two mappers, indel realignment, etc.), Table 3.1 shows that we have a very high number of selected variants. Likely, many of these are artefacts introduced by mapping, and the the proportion of segregating variation we report represents a lower bound of the contribution of standing genetic variation. While we observed a small reduction in overall nucleotide diversity (relative to the ancestor) as expected due to drift, we do not observe any large genomic regions of reduced variation indicative of a hard sweep (**Fig. 3.10-3.12**). We do however observe smaller regions of reduced variation (**Table 3.2, 3.3**). These are consistent with soft sweeps, polygenic responses and potentially some new variants of small phenotypic effects contributing and in the process of rising in frequency (but not yet approaching fixation).

Recent work that explored the basis of Waddington's genetic assimilation, claimed that heat-induced transposable elements led to new mutations and allowed for constitutive expression of phenotypes (Fanti et al. 2017). However, based on work by Bateman (1959a), we know response to CVL phenocopy is due to segregating variation. We also know that this trait and genetic assimilation both seem to be polygenic (Milkman 1965b; Mohler 1965b, 1967). Our own work in this system is consistent with the CVL phenocopy response and genetic assimilation being a largely polygenic response from standing genetic variation. Genomic scans for regions of selection, through looking at both reduced nucleotide diversity and F_{ST}, show many regions across the genome contributing. Additionally, the quantitative complementation tests show that the many genes spanning the genome influence crossveinless frequency and that much of these responses are shared among genetic assimilation lineages, suggesting that the genetic assimilation lineages have some overlap in the alleles influencing CVL. In support of that, hybrid cross among both up selection and genetic assimilation lineages show little reduction in crossveinless frequency (relative to pure crosses) which is consistent with many of the same alleles being present in the separate lineages. Overall our results are not consistent with new mutations (or very rare segregating alleles) of large phenotypic effect accounting for genetic assimilation for the crossveinless phenotype. Instead the results suggest a largely polygenic response contributing to the increase in CVL phenocopy penetrance and genetic assimilation. Future work plans to identify some of the specific causal variants contributing to genetic assimilation.

Figures

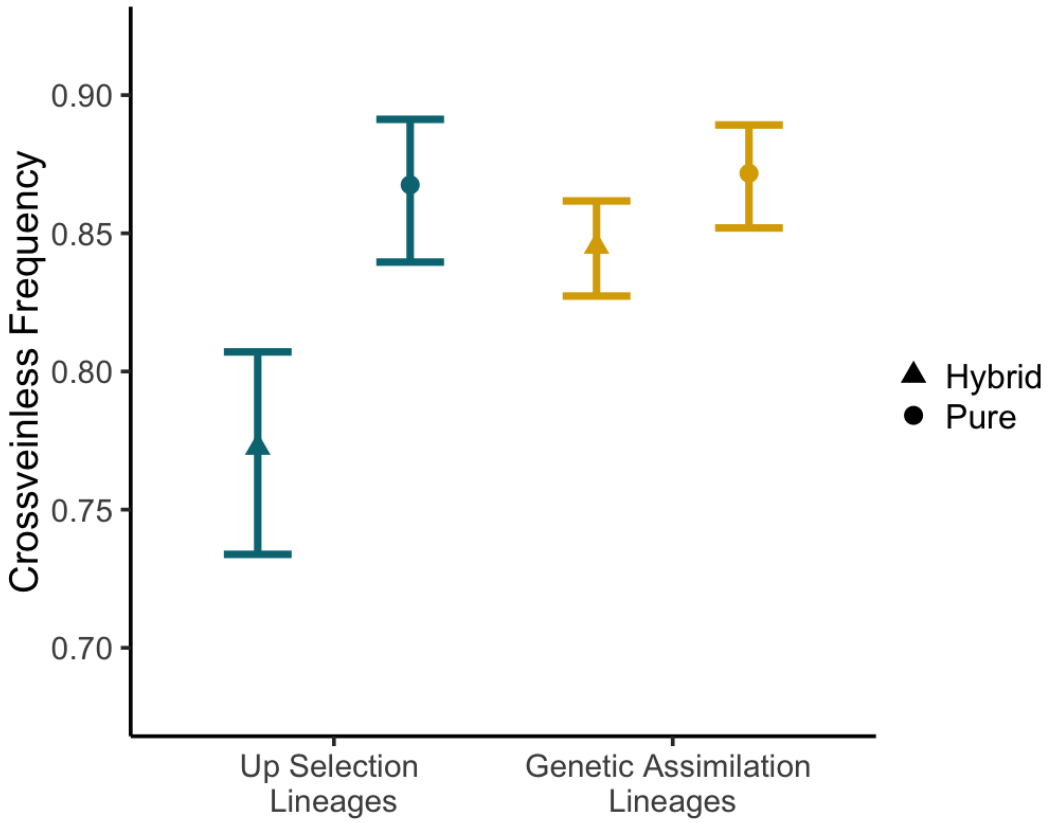

Figure 3.1: Incomplete parallelism among lineages in response to artificial selection or genetic assimilation. Partial di-allele crosses between the replicate genetically assimilated lineages were performed. Pure populations are those with parents from the same replicate lineage. Hybrid populations are those with parents from different replicate lineages. Crosses were done over 2 generational timepoints. Up selection lineages: n_{hybrid}=24, n_{pure}=12. Genetic assimilation lineages: n_{hybrid}=60, n_{pure}=12 Error bars are 95% significantly by the purity of the cross ($p<0.05$).

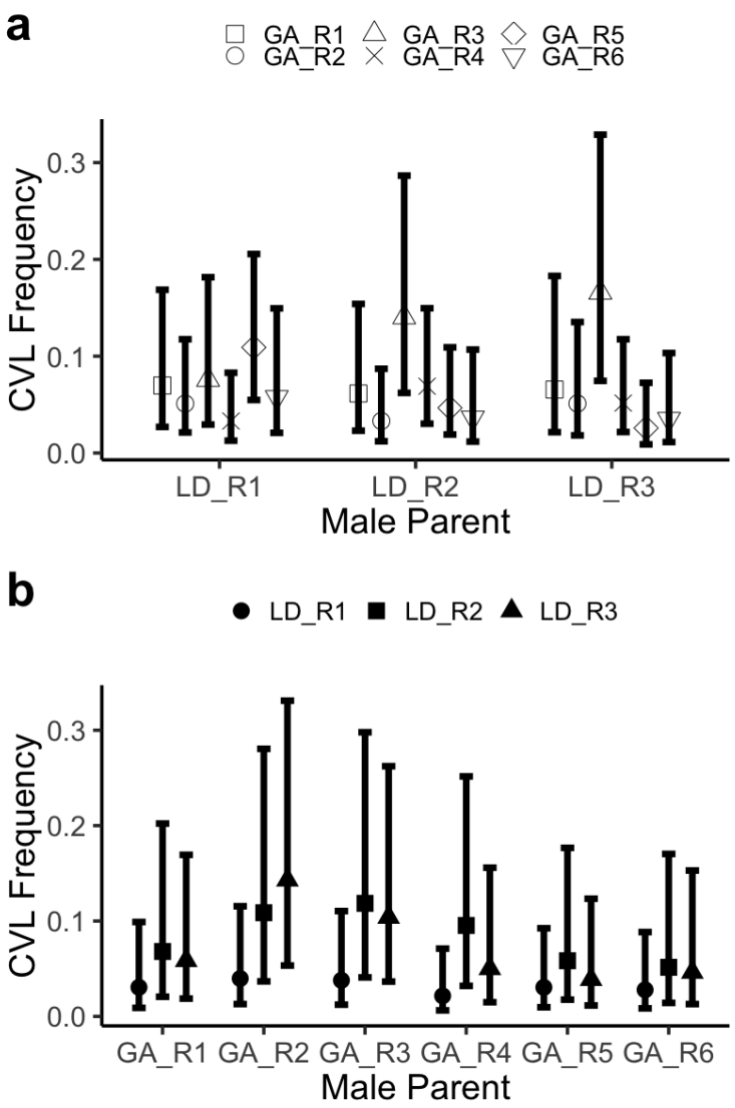

Figure 3.2: Low crossveinless frequency among hybrid progeny of genetic assimilation and lab domestication lineages. a) Male lab domestication and female genetic assimilation parents and b) the reciprocal male genetic assimilation and female lab domestication parent lineages (n=4). Error bars are 95% CIs on estimated effects from a generalized linear mixed model.

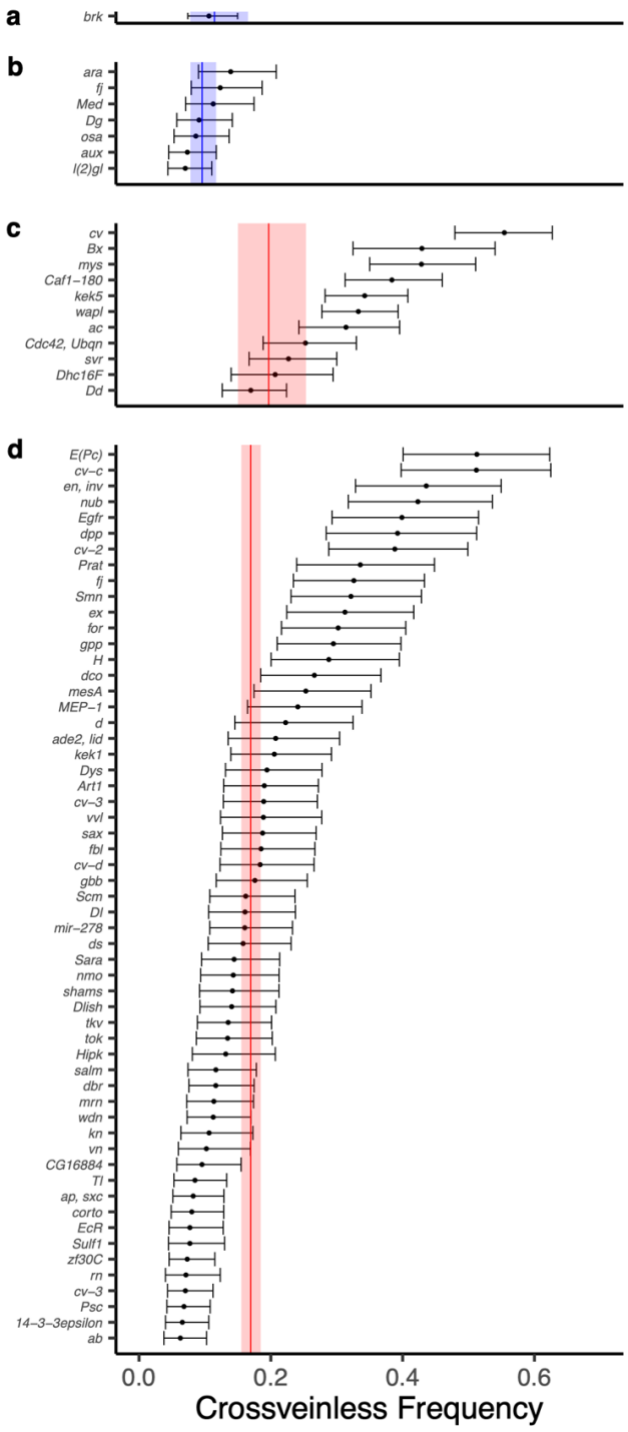

Figure 3.3: Deletion line crosses average for all genetic assimilation lineages. Genes of interest in each deletion region for a) DrosDel X chromosomes, b) DrosDel autosomes, c) Exelixis X chromosomes, and d) Exelixis autosomes (n=12 crosses for each deletion). Blue/red solid lines represent DrosDel/Exelixis progenitor means with shaded rectangles as 95% CIs. Error bars are 95% CIs on estimated effects from a generalized linear mixed model.

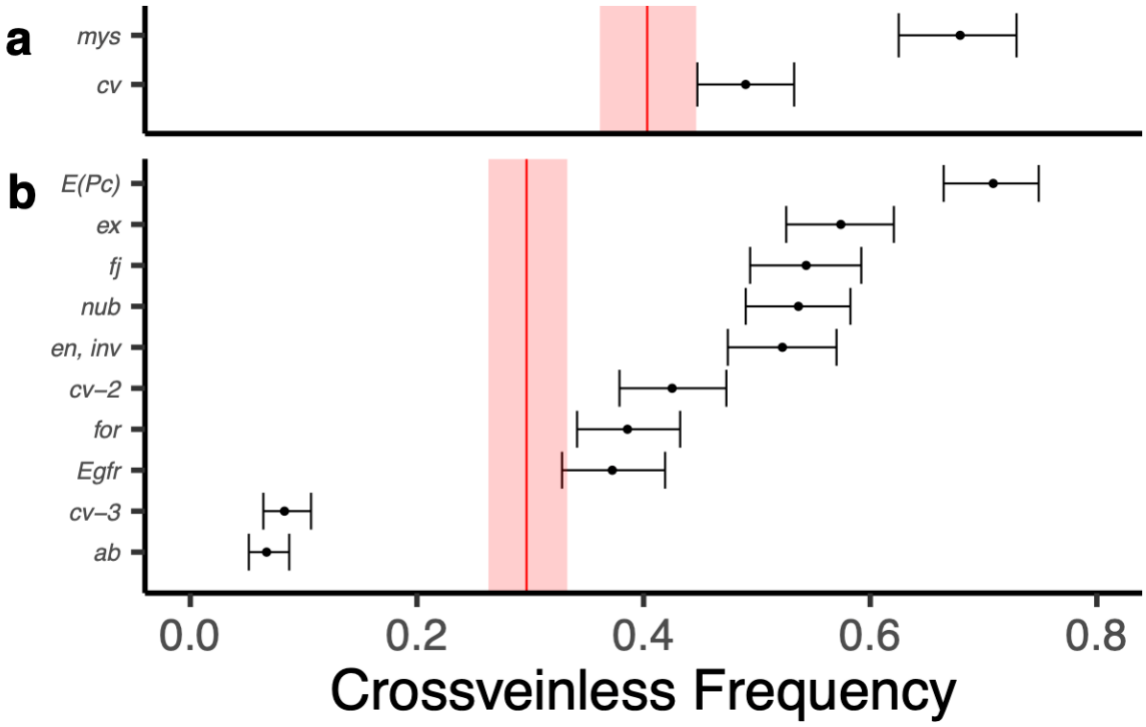

Figure 3.4: Deletion line crosses average for all genetic assimilation lineages in second experiment for further testing. Genes of interest in each deletion region for a) Exelixis X chromosomes and b) Exelixis autosomes (n=24 crosses for each deletion). Red solid lines represent Exelixis progenitor means with shaded rectangles as 95% CIs. Error bars are 95% CIs on estimated effects from a generalized linear mixed model.

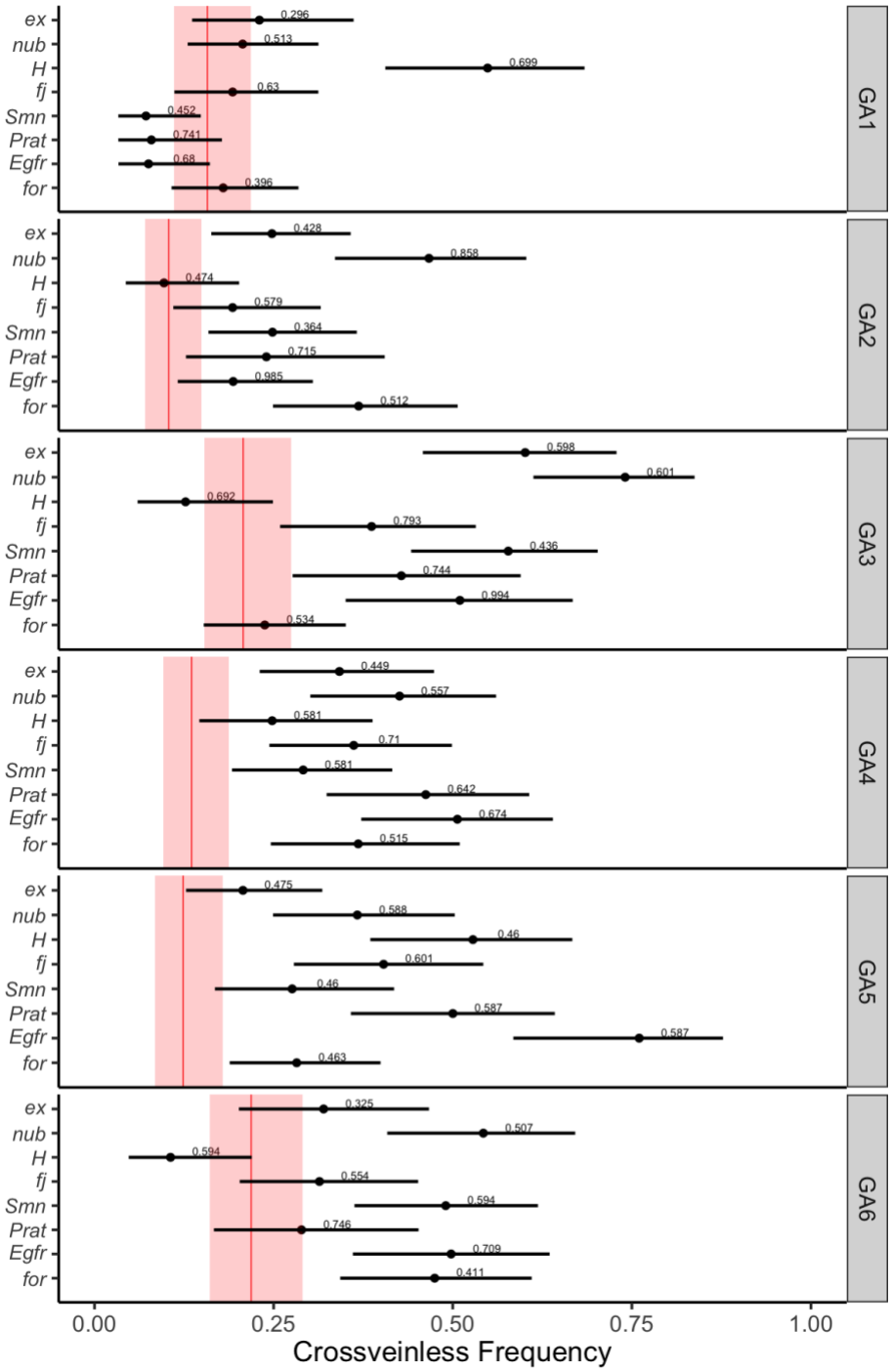

Figure 3.5: Demonstrating a subset of deletion line crosses for each genetic assimilation lineage. Genes of interest in each deletion region for each genetic assimilation lineage (n=4 for each deletion). Red solid lines represent Exelixis progenitor means with shaded rectangles as 95% CIs. Error bars are 95% CIs on estimated effects from a generalized linear mixed model. Numbers with each deletion cross are the highest F_{ST} within the deletion region for the genetic assimilation lineage compared with the ancestor population.

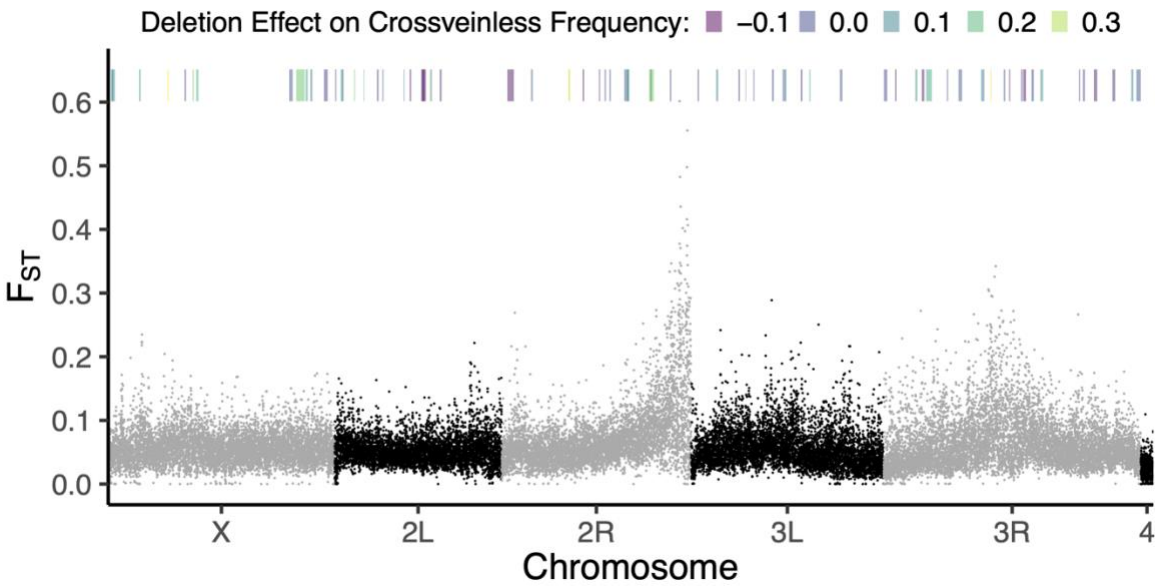

Figure 3.6: CVL phenotype is influenced by many alleles spanning the genome. F_{ST} was calculated using PoPoolation2 (Kofler et al. 2011b) on 500 base-pair windows for comparing the genetic assimilation lineages with the ancestor population for two separate mapping software and taking the minimum F_{ST} value. The average effect of each deletion line influencing the CVL frequency in the genetic assimilation lineages (compared to control progenitor crosses) is depicted for the range of the genome that deletion spans.

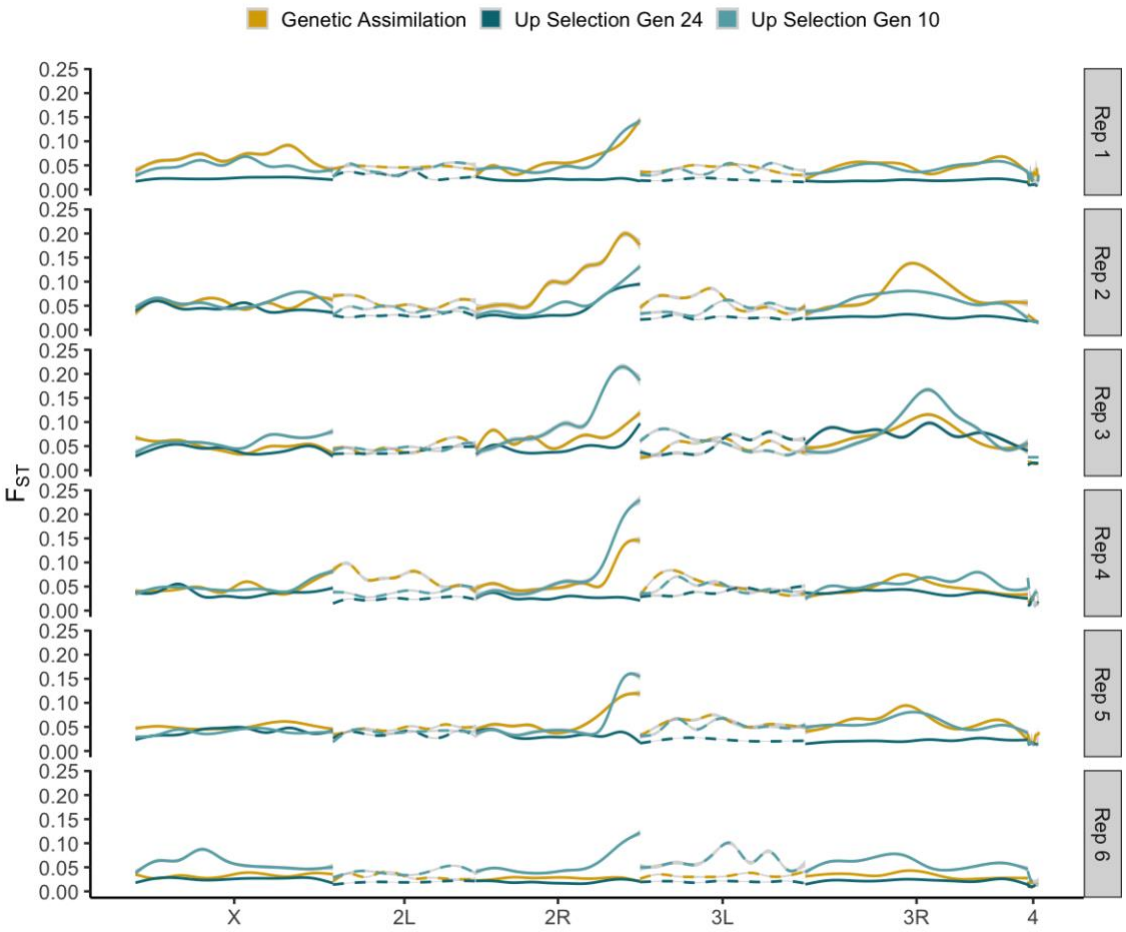

Figure 3.7: F_{ST} for up-selection lineages at two time points during artificial selection and genetic assimilation lineages. F_{ST} was calculated using PoPoolation2 (Kofler et al. 2011b) on 500 base-pair windows in comparison with the ancestral population for two separate mapping software and taking the minimum F_{ST} value. Plots were generated with geom_smooth() using method 'gam' and formula 'y~s(x,bs="cs")' in R.

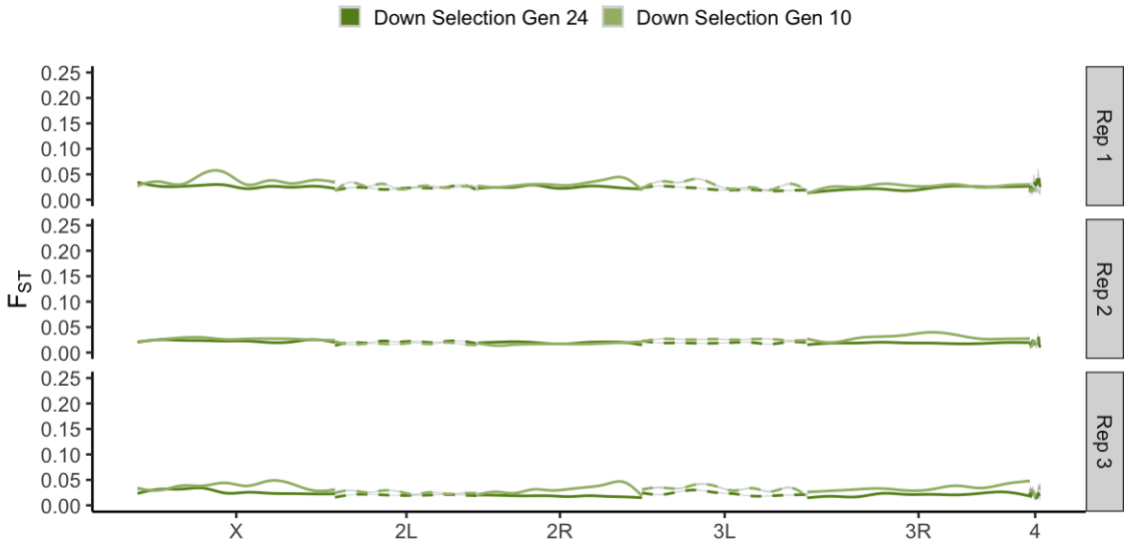

Figure 3.8: F_{ST} for down-selection lineages at two time points during artificial selection. F_{ST} was calculated using PoPoolation2 (Kofler et al. 2011b) on 500 base-pair windows in comparison with the ancestral population for two separate mapping software and taking the minimum F_{ST} value. Plots were generated with geom_smooth() using method 'gam' and formula 'y~s(x,bs="cs")' in R.

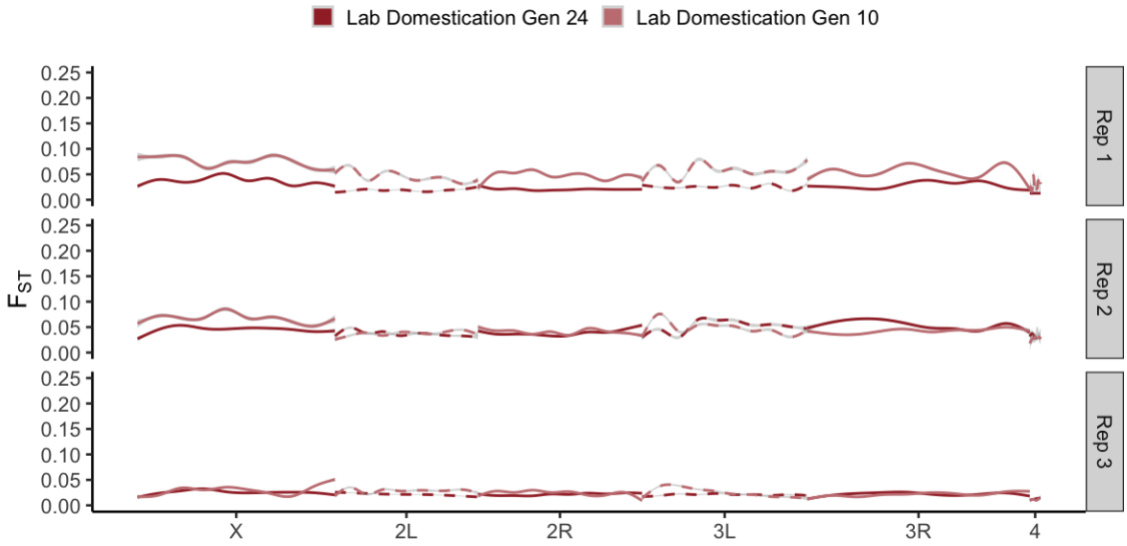

Figure 3.9: F_{ST} for lab domestication lineages at two time points during artificial selection. F_{ST} was calculated using PoPoolation2 (Kofler et al. 2011b) on 500 base-pair windows in comparison with the ancestral population for two separate mapping software and taking the minimum F_{ST} value. Plots were generated with geom_smooth() using method 'gam' and formula 'y~s(x,bs="cs")' in R.

Table 3.1: Proportion of selected variants that are segregating in the ancestor. All variants that had an F_{ST} higher than 0.3 (F_{ST} used was minimum F_{ST} between two mappers, novoalign and BWA, calculated using PoPoolation2) were checked to see if present in the ancestral population. Major allele is allele with higher frequency than minor allele.

Genetic Assimilation Replicate Lineage	Major allele is segregating	Major allele is new mutation	Minor allele is segregating	Minor allele is new mutation	Proportion of major allele segregating	Proportion of minor allele segregating
1	22590	495	19365	55	97.86%	99.72%
2	15669	146	11576	51	99.08%	99.56%
3	53986	1006	45231	80	98.17%	99.82%
4	26701	535	24757	163	98.04%	99.35%
5	21753	266	18191	69	98.79%	99.62%
6	17659	213	13011	93	98.81%	99.29%

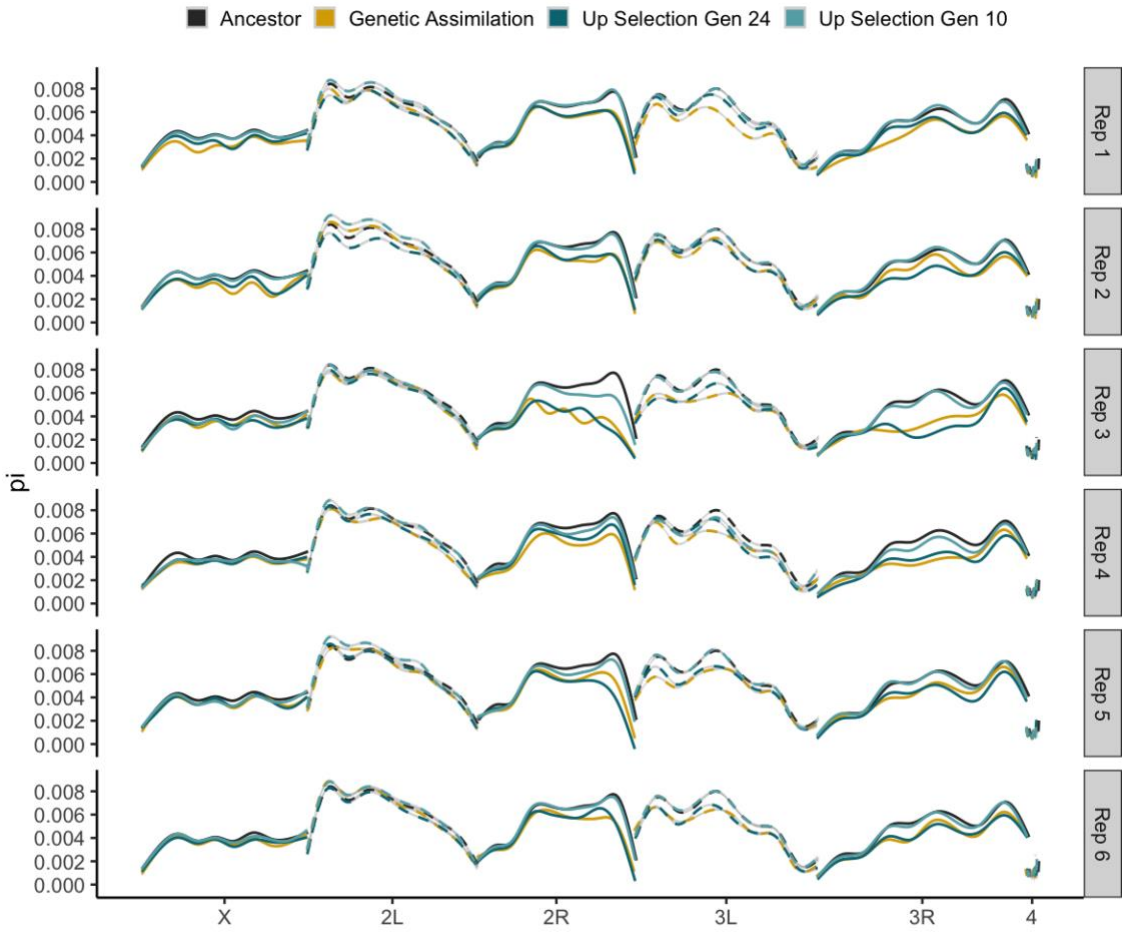

Figure 3.10: Nucleotide diversity (Tajima's pi) for up-selection lineages at two time points during artificial selection and genetic assimilation lineages. Tajima's pi was calculated using PoPoolation (Kofler et al. 2011a) on 500 base-pair windows for two separate mapping software and taking the minimum pi value. Plots were generated with geom_smooth() using method 'gam' and formula 'y~s(x,bs="cs")' in R.

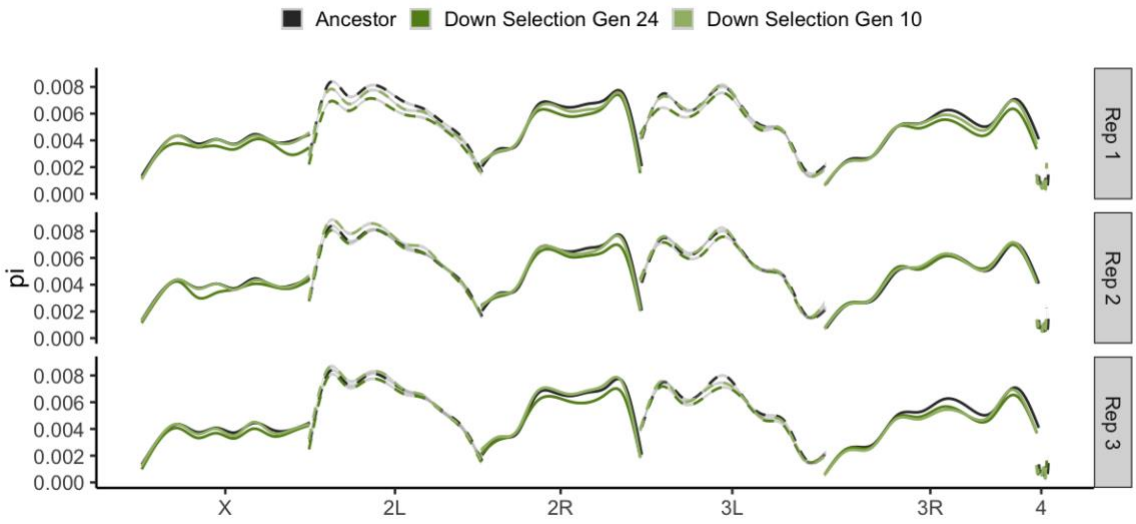

Figure 3.11: Nucleotide diversity (Tajima's pi) for down-selection lineages at two time points during artificial selection. Tajima's pi was calculated using PoPoolation (Kofler et al. 2011a) on 500 base-pair windows for two separate mapping software and taking the minimum pi value. Plots were generated with geom_smooth() using method 'gam' and formula 'y~s(x,bs="cs")' in R.

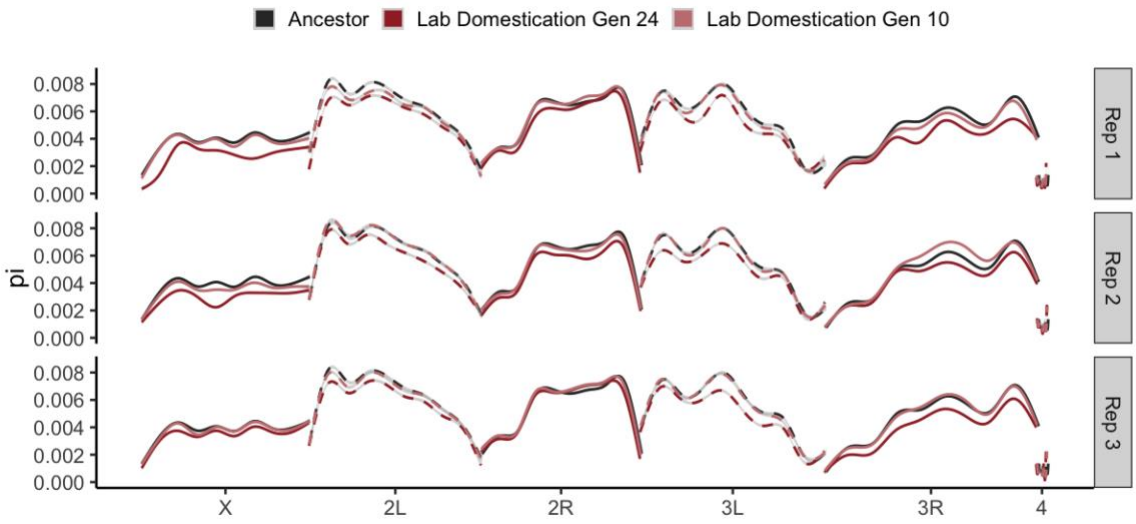

Figure 3.12: Nucleotide diversity (Tajima's pi) for lab domestication lineages at two time points during artificial selection. Tajima's pi was calculated using PoPoolation (Kofler et al. 2011a) on 500 base-pair windows for two separate mapping software and taking the minimum pi value. Plots were generated with geom_smooth() using method 'gam' and formula 'y~s(x,bs="cs")' in R.

Table 3.2: Top 20+ largest regions for reduction in nucleotide diversity (Tajima's pi). Reductions in Tajima's pi were defined for each genetic assimilation lineages when showing a larger reduction from the ancestral pi as compared to the lab domestication lineages.

Genetic Assimilation Replicate 1		Genetic Assimilation Replicate 2		Genetic Assimilation Replicate 3	
Chromosome:Position	Size (Mb)	Chromosome:Position	Size (Mb)	Chromosome:Position	Size (Mb)
2R:20171250-20188750	0.0175	2R:20370250-20386250	0.016	2R:18702750-18783250	0.0805
2R:20370250-20386250	0.016	X:21377750-21393250	0.0155	2R:18658750-18691250	0.0325
2R:20027750-20043250	0.0155	2R:20027750-20043250	0.0155	3R:10808250-10840250	0.032
3L:22444750-22459750	0.015	2R:20124250-20137750	0.0135	3L:3163750-3189750	0.026
2R:20124250-20137750	0.0135	2R:20415750-20429250	0.0135	2R:20323750-20349750	0.026
2R:20415750-20429250	0.0135	2R:15545750-15558250	0.0125	2R:19470750-19494250	0.0235
2R:19565750-19578250	0.0125	2L:22339250-22351250	0.012	2R:20192250-20215750	0.0235
2R:20204250-20215750	0.0115	2R:20338250-20350250	0.012	3L:9608250-9630750	0.0225
3R:9241750-9252250	0.0105	3R:5540250-5551750	0.0115	2R:17312750-17334250	0.0215
3R:9714250-9724750	0.0105	3R:9241250-9252750	0.0115	3R:16445750-16467250	0.0215
X:11705750-11714750	0.009	2R:20171250-20182250	0.011	2R:19372750-19393750	0.021
X:14743250-14752250	0.009	2R:20387250-20397750	0.0105	2R:13762250-13782750	0.0205
X:20912750-20921750	0.009	2L:19727250-19737250	0.01	2R:18273750-18294250	0.0205
3L:22708250-22717250	0.009	3L:4466250-4476250	0.01	2R:20083750-20104250	0.0205
3L:24044750-24053750	0.009	2R:18005250-18015250	0.01	2R:17948250-17968250	0.02
2R:20104750-20113750	0.009	X:13004750-13013750	0.009	3R:10634250-10654250	0.02
2R:20151250-20160250	0.009	X:14965250-14974250	0.009	2R:19660250-19679750	0.0195
2R:20387250-20396250	0.009	2R:1517750-1526750	0.009	2R:20494750-20513750	0.019
3R:9645250-9654250	0.009	2R:17347250-17356250	0.009	2R:18421750-18440250	0.0185
3R:9756750-9765750	0.009	X:7620250-7628750	0.0085	2R:19506250-19524750	0.0185
		X:17453250-17461750	0.0085		
		2R:16191750-16200250	0.0085		
		2R:16712750-16721250	0.0085		
		2R:20147250-20155750	0.0085		
		2R:20482250-20490750	0.0085		
		3R:20845250-20853750	0.0085		

Table 3.2 (cont.): Top 20+ largest regions for reduction in nucleotide diversity (Tajima's pi).

Genetic Assimilation Replicate 4		Genetic Assimilation Replicate 5		Genetic Assimilation Replicate 6	
Chromosome:Position	Size (Mb)	Chromosome:Position	Size (Mb)	Chromosome:Position	Size (Mb)
2R:14515250-14529750	0.0145	2R:20120250-20217750	0.0975	2R:20171250-20198250	0.027
3R:11438250-11452250	0.014	2R:20370250-20413250	0.043	3L:22497250-22518750	0.0215
2R:17343250-17356250	0.013	2R:20288250-20322250	0.034	2R:20338250-20354750	0.0165
2R:12887250-12898250	0.011	2R:20331750-20354750	0.023	2R:20370250-20386250	0.016
3R:14682750-14693750	0.011	2R:20098250-20119250	0.021	2R:20123250-20137750	0.0145
3R:10674750-10685250	0.0105	2R:20417750-20431750	0.014	2R:19285750-19299750	0.014
3R:15424250-15434750	0.0105	2R:20355750-20369250	0.0135	2R:20146250-20160250	0.014
3L:10295750-10305750	0.01	2R:20218750-20231750	0.013	3L:22446250-22458750	0.0125
2R:17167250-17177250	0.01	2R:20274250-20287250	0.013	2R:19223750-19235750	0.012
3R:12176750-12186750	0.01	2R:20251250-20263250	0.012	2R:20398750-20410750	0.012
3R:17418250-17428250	0.01	X:22036250-22047250	0.011	2R:20204250-20215750	0.0115
2R:18005750-18015250	0.0095	2R:19405750-19416750	0.011	3R:9241750-9252750	0.011
2L:18019750-18028750	0.009	3L:3169750-3180250	0.0105	2R:18004250-18014750	0.0105
2L:20200750-20209750	0.009	X:20912250-20922250	0.01	2R:20387250-20397750	0.0105
3R:15482750-15491750	0.009	2R:19223750-19233750	0.01	2L:19750750-19760750	0.01
3R:2940250-2948750	0.0085	2R:20470250-20480250	0.01	3L:24044750-24054750	0.01
3R:7705250-7713750	0.0085	3R:13062750-13072250	0.0095	2L:22342750-22352250	0.0095
3R:10550250-10558750	0.0085	X:21388250-21397250	0.009	3L:24062250-24071750	0.0095
3R:10570750-10579250	0.0085	2R:17051750-17060750	0.009	2R:17345250-17354750	0.0095
2L:18609750-18617750	0.008	2R:18006750-18015750	0.009	2R:20422250-20431750	0.0095
2R:16705250-16713250	0.008	3R:10634750-10643750	0.009		
3R:7646250-7654250	0.008				
3R:10734750-10742750	0.008				
3R:12051750-12059750	0.008				
3R:15334250-15342250	0.008				

Chapter 4: Evolved changes in canalization is not necessary for the evolution of a genetically assimilated trait

Introduction

Canalization, a form of evolved organismal robustness, can facilitate the accumulation of cryptic genetic variation (CGV) (Waddington 1942). Waddington hypothesized that canalizing mechanisms and their evolution could facilitate rapid phenotypic evolution. He suggested that under "normal" conditions, the developmental basis for many phenotypes are relatively canalized, meaning they express little phenotypic variance (or are invariant) to modest changes in the environment, yet under more extreme environmental or genetic changes, some developmental genetic thresholds can be altered causing the release of CGV for these phenotypes. Persistent selection on the phenotype can lead to a change in the threshold and re-canalization for the new phenotypic value (**Fig. 1.3**). Waddington conducted his classic selection experiment as a test for this model of evolution through changes in canalization. He experimentally showed that artificial selection for increased frequency of an ancestrally environmentally induced trait (loss of wing crossveins in *Drosophila*), resulted in the evolution of constitutive trait expression, a process he termed genetic assimilation (Waddington 1953b).

Although Waddington proposed genetic assimilation occurs through changes in canalization, alternative models have been suggested such as the liability threshold

model (Landauer 1958; Stern 1958; Bateman 1959a) and new mutations being concurrently selected (Specchia et al. 2010; Fanti et al. 2017). However, no explicit tests have occurred for evolution through changes in canalization in Waddington's classic selection experiment.

Here, we looked at the evolved differences in the degree on both genetic and environmental canalization for the artificially selected crossveinless lineages and genetically assimilated lineages, as a test of Waddington's proposed model for evolution in changes in canalization. Past work in canalization has often used observations of "buffering" and the release of CGV as suggestive of canalization. To address genetic canalization, we used a mutagenesis assay because recent work has shown that examining the effects of new mutation is an effective way to measure canalization (Hermisson and Wagner 2004; Geiler-Samerotte et al. 2019). Additionally, we looked at non-mutagenized controls from the mutagenesis assay to investigate if there was an increase in mutations for the heat-stressed populations; this would occur if heat-stress induced transposable element activity caused new mutations, specifically associated with genetic assimilation (such as with (Fanti et al. 2017)). To address environmental canalization, we used sensitivity to rearing temperatures, as the crossveinless system is affected by developmental temperature-stress.

Methods

Mutagenesis (Genetic Canalization)

Males (G0) from three replicate lineages of the up-selection, lab domesticated, and genetic assimilation selection treatments were starved for 12 hours (with wet cotton ball for hydration), exposed to 25mM EMS (in 1% sucrose solution) for 8 hours and allowed to recover for 24 hours. This exposure level is expected to modestly increase mutation rate relative to typical mutagenesis experiments. After, they were mated to virgin females of the same lineage. Along with the mutagenized males, control males were fed with a 1% sucrose solution and then mated with virgin females of the same lineage (Mutagenized n=23-46; Control n=8-10 for each lineage). F1 progeny from each F0 cross were split into 3 single pair crosses. F2 Males (for X-linked mutations), and F3 males and females were scored for eye {color, shape, size, roughness} and wing {shape, size, scalloping, curling, crumpling, pigmentation and venation including anterior crossveins} phenotypes. See **Fig. A1** for flowchart of experimental procedure.

A second mutagenesis experiment was performed to examine both the effects on down-selection treatment (compared with UP and assimilated) and to examine the effects of a longer EMS exposure (16hrs) using males from three replicate lineages of each selection treatment (up-selection, down-selection, lab domesticated, and genetic assimilation). G0 males were mated to virgins as above (Mutagenized n=9-10; Control n=5 for each lineage). F1 progeny for each were split into 2 single pair crosses. Phenotypic scoring as described above.

We fit two models to account for sensitivity to mutagenesis, using different methods to account for phenotypes. Variation in penetrance of mutations can be evidence of canalization. If there is variation in the amount of canalization, there may be variable penetrance for mutant alleles. Thus we wanted to consider the total number of mutant phenotypes found in a replicate. However, it is hard to disentangle variable penetrance with the number of the individuals that are segregating with genotypes that would express mutant alleles. Therefore, we also considered whether replicates contained any mutations at all. Both models were fit with mutagenized treatment, selection treatment, and generation as fixed effects. Random effects were fit with block, replicate lineage nested with selection treatment, and starting males nested within block, replicate lineage, and selection treatment. Mutant rate (estimate of identifying phenotypes per individuals) was calculated with phenotypes counted per vial using a negative binomial model (log link) in glmmTMB (v0.2.3). Probability of mutants (estimate of frequency of phenotypes per vial) was calculated as a Bernoulli trial (if any phenotypes were observed among progeny in the vials) using glmer() and Anova() in car (v3.0-2).

In the second mutagenesis experiment, there were little to no eye phenotypes observed in the control vials for the selection lineages. Therefore, the mutant rate model for eye phenotypes was adjusted with a score of 1 to account for the complete separation. Additionally, the probability of mutants modeled for eye phenotypes gave

very large confidence intervals (spanning 0 to 1) for selection lineages with no phenotypes scored.

Environmental Canalization

All artificially selected and genetically assimilated lineages were raised in low-density vials (n=3 vials each) at 6 different temperatures: 31C, 29C, 24.5C, 21C, 18C, and 16C. Adult flies were collected when they eclosed. Animals were stored in 70% EtOH and right wings were dissected from both males and females and imaged as described above (chapter 2). For wing size, we examined variation in temperature induced plasticity among the evolved lineages (macro-environmental canalization) fitting size as a function of evolutionary treatment, temperature, and their interaction with replicate lineage nested within treatment allowing temperature plasticity to vary by lineage using lme4() and glmmTMB(). To address micro-environmental canalization, Levene's statistic was calculated as above (chapter 2) additionally accounting for rearing temperature using the same model as for plasticity in wing size but with an inverse link, assuming gamma distributed variation and with uncorrelated random effects. For shape data, landmark and semi-landmark data was recorded in the same way, with the exception that Wings (v. 4.11.22) was used to fit splines. A total of 2870 individuals (females) were included, an average of 53.1 wings for each replicate lineage/temperature. To examine changes in plasticity for wing shape among evolutionary lineages, we used trajectory.analysis in the geomorph package to analyze the trajectory of reaction norms for changes in

magnitude, shape, and correlation of the shapes of these reaction norms based on a multivariate linear model with treatment, rearing temperature and their interactions, with lineage nested within treatment.

Wings of both sexes were scored for presence of posterior crossveins in addition to other wing perturbations (anterior cross vein loss, wing margin perturbation, longitudinal vein loss, additional veins). A total of 6346 individuals were scored (~average 58.7 wings sex/lineage/temperature). Proportions of posterior crossvein loss was calculated for each vial replicate within sex/lineage/ temperature and modeled using a logistic mixed model with treatment, temperature, sex and their second-order interactions fit as fixed effects with the standard logit link. A random effect of replicate lineage for each temperature:sex term was included in the model. Because of extremely low observed numbers for qualitative wing defects other than posterior cross vein loss, all other phenotypes were grouped into a single category (essentially wild type VS non-wild type wing morphology). The proportion of wings with phenotypes were calculated and modeled as described above.

Results and Discussion

Perhaps the most contentious aspect of Waddington's explanation for genetic assimilation is that its evolution required changes in the extent of canalization of traits to genetic and environmental variation (Waddington 1942, 1961). Many researchers argued that the canalization model was unnecessary, with genetic assimilation being

explained as a simple threshold trait with underlying continuous genetic effects, i.e. a liability model (Landauer 1958; Stern 1958; Bateman 1959a) which is generally well supported, but which Waddington continued to reject as the likely explanation for genetic assimilation. Despite this, considerable work has examined conditions in which canalization can evolve, with several empirical examples clearly demonstrating variation in canalization (Lack et al. 2016; Groth et al. 2018). Nevertheless, to our knowledge the role that canalization plays for the evolution of genetic assimilation specifically (and for CVL in particular) has not been appropriately tested.

There's been many interpretations of the canalization model, but a common version is illustrated in **Fig. 1.3**. If genetic assimilation is due to changes in canalization, we would predict associated changes in sensitivity to perturbation. In our system, the constitutive classes (i.e. lab domesticated lineages with normal crossveins and genetically assimilated lineages with missing crossveins) should be the least sensitive compared with lineages with intermediate phenocopy frequencies during the evolutionary response (i.e. up-selection lineages). Waddington believed this should be true for both environmental and genetic effects, although these may in fact be independent evolutionarily (Masel and Siegal 2009). As such we used independent experiments to examine whether genetic and/or environmental canalization evolved in a manner consistent with Waddington's model.

With respect to genetic canalization, previous studies used release of CGV to infer canalization. Recent work suggests that examining the effects of new mutations is

a superior method of investigation (Hermisson and Wagner 2004; Geiler-Samerotte et al. 2019), which we employed here using mutagenesis. As the lineages were maintained as outbred populations, segregating rare mutations of large effect need to be accounted for. As such we optimized the crossing design to allow for observation of X linked mutations in hemizygous males, and F3s in females and males (to observe recessive allelic effect). We examined the focal (wing) and a control (eye) as targets of mutagenesis as these traits both have large mutational target size and easily measurable phenotypes. As the up-selection and genetic assimilation populations were selected for a wing phenotype (crossveinless), we expect the wing as a whole (i.e. all wing phenotypes collectively) would show more sensitivity to genetic changes if this system has gone through changes in canalization. The eye serves as a control since there has been no artificial selection for any phenotypes, and thus would have gone through no direct changes in canalization. Additionally, we also had non-mutagenized controls.

Inconsistent with Waddington's model, we did not observe an increase in mutational sensitivity in up-selection lineages compared with other evolved treatments. This is true if we examine the mutational sensitivity for both wings either looking at mutation rate in individuals (**Fig. 4.1, 4.5; Table 4.1, 4.5**) or the frequency of finding mutations among progeny in replicate vials from mutagenized males (**Fig. 4.2, 4.6; Table 4.2, 4.6**). Interestingly, the pattern we see among the evolutionary lineages in both mutagenesis experiments is that the genetic assimilation lineages have higher sensitivity to new mutations compared to up selection lineages. Although this is not significant, it is

the opposite relationship to what we would expect if the genetic assimilation populations were recanalized and the up selection populations decanalized (as suggested by Waddington's hypothesis of evolution by changes in canalization). Instead this suggests that possibly decanalization has occurred in these lineages, but that the genetically assimilation is not the result of the rapid evolution of re-canalization. Because this pattern is replicated in two separate mutagenesis experiments, we expect increasing sample sizes or repeating the experiment would result similarly in that the genetic assimilation lineages do not show evidence of being more canalized than the up-selection lineages. Potentially, repeating these experiments after many generations may show that canalizing mechanisms have evolved in the genetic assimilation lineages. However, this would not be relevant to the time scale of the evolution for genetic assimilation of the crossveinless phenotype. We also saw no differences among the control target (eye) mutations in either set of mutagenesis experiments (**Fig. 4.3, 4.4, 4.7, 4.8; Table 4.3, 4.4, 4.7, 4.8**). Additionally, the lineages exposed to heat stress during artificial selection did not show higher levels of new mutations (in the absence of chemical mutagenesis), inconsistent with TE mobilization having a substantial impact on increasing variation (i.e. new mutations) in these lineages (as seen in the controls for either mutant rate or probability of mutants).

We also examined whether these evolving lineages differed in robustness to environmental effects, in particular developmental temperature stress, examining changes in mean and variance for wing size and shape. Mean size decreased with

increasing rearing temperature as expected (**Fig. 4.9**), but with little evidence for differences in reaction norms among evolutionary treatments. While our experimental design provides a weaker test for differences in micro-environmental variances (as there are genetic confounds due to working with outbred populations), we also observed no differences (**Fig. 4.10**). We also saw no difference in mean shape for the selection lineages and most variation was explained by rearing temperature (**Fig. 4.11; Table 4.9**). We also looked at qualitative wing defects for the selection lineages across rearing temperatures. Missing posterior crossveins were of course common in up selection and genetic assimilation lineages due to artificial selection for this phenotype (**Fig. 4.12**). Overall, all other wing phenotypes scored were rare. Unlike in the mutagenesis assays for genetic canalization, where individuals were crossed back with siblings, for this experiment we were unlikely to observe any homozygotes for recessive deleterious variants. Due to rarity, all wing defects (besides missing posterior crossveins) were combined and when modeled, we observed no differences in the amounts of qualitative wing defects (**Fig. 4.13**).

These results demonstrate that neither genetic nor environmental canalization have evolved among our evolutionary treatments, despite the evolution of genetic assimilation. Thus, under these rapid time scales (less than 25 generations), changes in canalization are not necessary for the evolution of genetic assimilation nor is there a substantial role of new mutations of large effect in the genetic assimilation response. Although we have found that canalization does not play a role in genetic assimilation of

the crossveinless phenotype, canalization clearly can and has evolved in nature (Lack et al. 2016; Groth et al. 2018) and it is possible than canalization may have some role in other cases of genetic assimilation.

We have found that Waddington's changes in canalization model (1961) is not relevant in this system. However, the polygenic response from standing genetic variation we have found for this system (chapter 3) is consistent in the standard threshold liability model originally proposed by Waddington's student, Bateman (1959). Future work can look to confirm this model, by investigating the specific variants involved in the crossveinless phenocopy response and see if selection has acted to increase these alleles is aggregate for genetic assimilation.

Figures

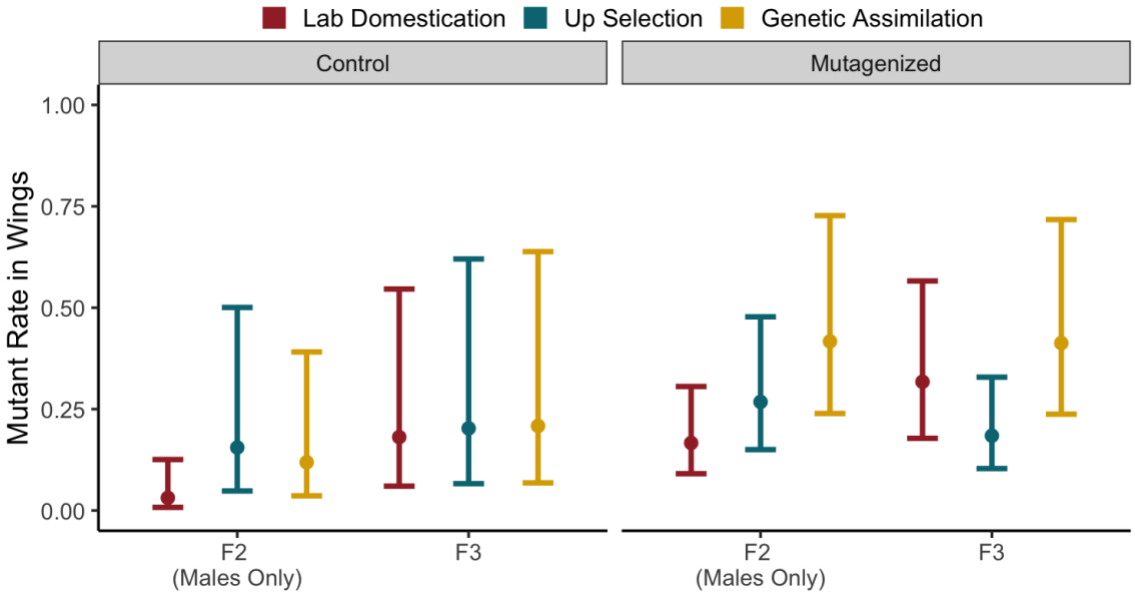

Figure 4.1: No differences in sensitivity to mutagenesis by way of mutant rate in wings (Mutagenesis Part 1). Mutant rate is the estimated rate of observed phenotypes per individual. $n_{Mutagenized}$ = 69-138 and $n_{Control}$ = 24-30 replicate vials sorted for each selection lineage. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model.

Table 4.1: ANOVA for the mutant rate in wings (Mutagenesis Part 1). Lineage refers to selection lineage, treatment refers to mutagenized/control, and generation refers to progeny counted in F2 or F3.

	X^2	df	p
Lineage	9.29	2	0.01
Treatment	8.26	1	0.004
Generation	2.10	1	0.147
Lineage x treatment	3.06	2	0.217
Lineage x generation	20.75	2	<0.001
Treatment x generation	8.53	1	0.003
Lineage x treatment x generation	0.70	2	0.705

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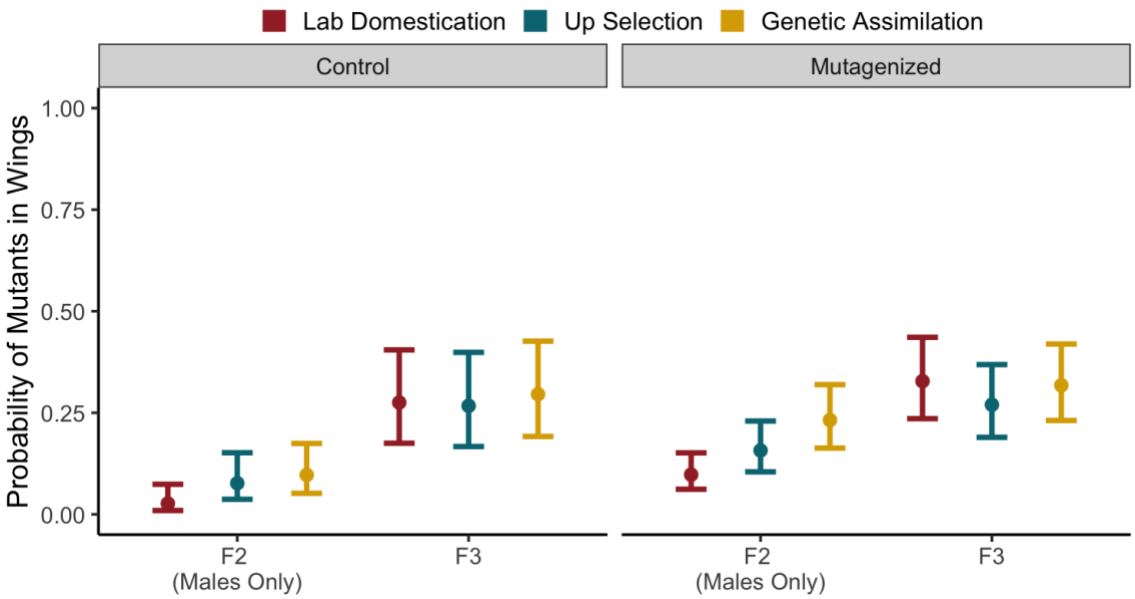

Figure 4.2: No differences in sensitivity to mutagenesis by way of probability of mutants in wings (Mutagenesis Part 1). Probability of mutants is the estimated number of phenotypes expected per vial sorted. $n_{Mutagenized}$ = 69-138 and $n_{Control}$ = 24-30 replicate vials sorted for each selection lineage. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model.

Table 4.2: ANOVA for the probability of mutants in wings (Mutagenesis Part 1). Lineage refers to selection lineage, treatment refers to mutagenized/control, and generation refers to progeny counted in F2 or F3.

	X^2	df	p
Lineage	1.93	2	0.381
Treatment	10.81	1	0.001
Generation	101.92	1	<0.001
Lineage x treatment	0.98	2	0.613
Lineage x generation	25.02	2	<0.001
Treatment x generation	13.60	1	<0.001
Lineage x treatment x generation	0.20	2	0.904

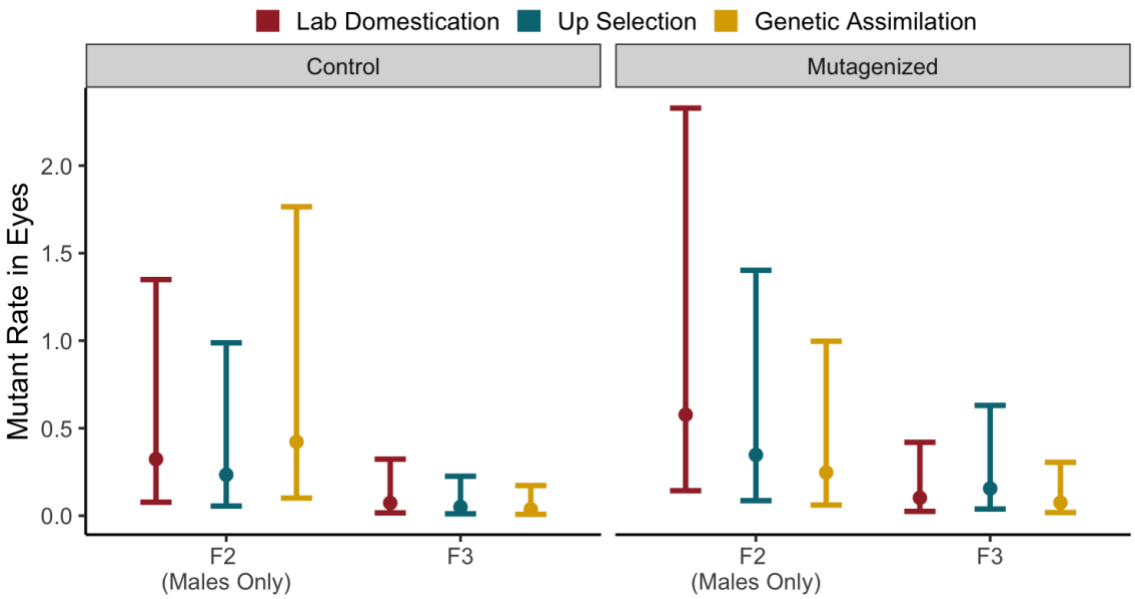

Figure 4.3: No differences in sensitivity to mutagenesis by way of mutant rate in eyes (Mutagenesis Part 1). Mutant rate is the estimated rate of observed phenotypes per individual. $n_{Mutagenized}$ = 69-138 and $n_{Control}$ = 24-30 replicate vials sorted for each selection lineage. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model.

Table 4.3: ANOVA for the mutant rate in eyes (Mutagenesis Part 1). Lineage refers to selection lineage, treatment refers to mutagenized/control, and generation refers to progeny counted in F2 or F3.

	X^2	df	p
Lineage	5.23	2	0.073
Treatment	2.28	1	0.131
Generation	257.83	1	<0.001
Lineage x treatment	2.88	2	0.237
Lineage x generation	15.94	2	<0.001
Treatment x generation	5.54	1	0.019
Lineage x treatment x generation	7.30	2	0.026

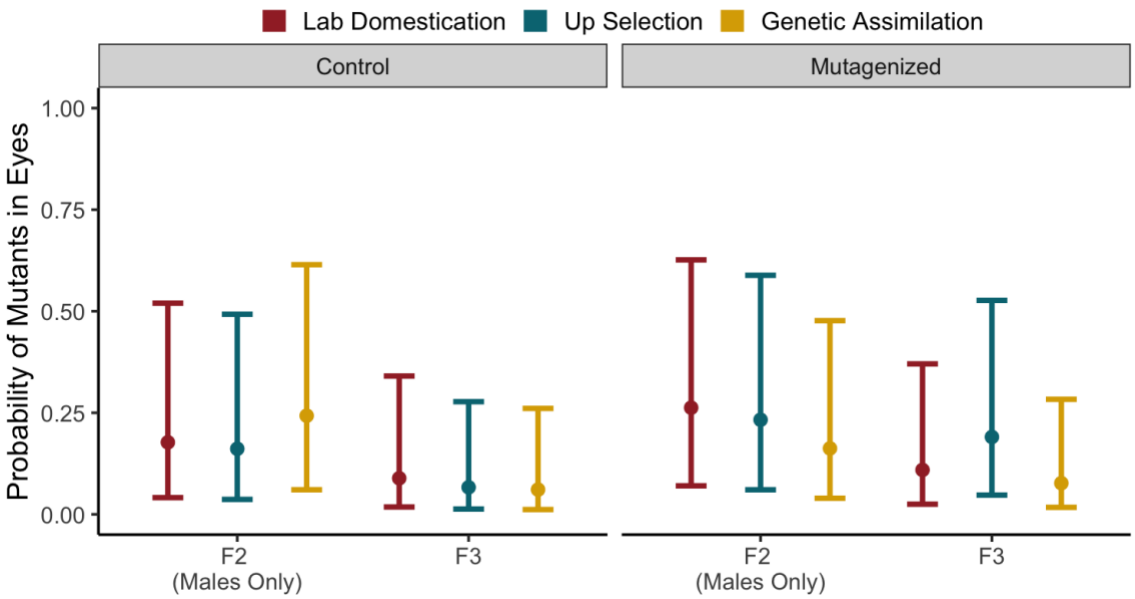

Figure 4.4: No differences in sensitivity to mutagenesis by way of probability of mutants in eyes (Mutagenesis Part 1). Probability of mutants is the estimated number of phenotypes expected per vial sorted. $n_{Mutagenized}$ = 69-138 and $n_{Control}$ = 24-30 replicate vials sorted for each selection lineage. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model.

Table 4.4: ANOVA for the probability of mutants in eyes (Mutagenesis Part 1). Lineage refers to selection lineage, treatment refers to mutagenized/control, and generation refers to progeny counted in F2 or F3.

	X^2	df	p
Lineage	5.04	2	0.081
Treatment	4.06	1	0.044
Generation	64.41	1	<0.001
Lineage x treatment	9.65	2	0.008
Lineage x generation	10.34	2	0.006
Treatment x generation	2.35	1	0.125
Lineage x treatment x generation	3.44	2	0.179

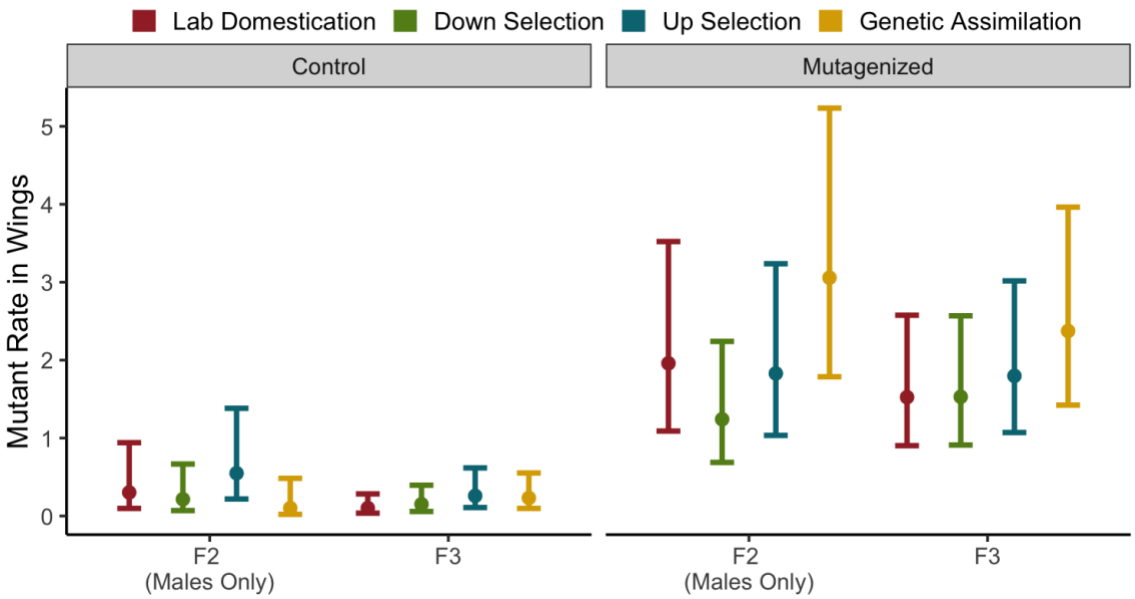

Figure 4.5: No differences in sensitivity to mutagenesis by way of mutant rate in wings (Mutagenesis Part 2). Mutant rate is the estimated rate of observed phenotypes per individual. $n_{Mutagenized}$ = 27-30 and $n_{Control}$ = 15 replicate vials sorted for each selection lineage. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model.

Table 4.5: ANOVA for the mutant rate in wings (Mutagenesis Part 2). Lineage refers to selection lineage, treatment refers to mutagenized/control, and generation refers to progeny counted in F2 or F3.

	X^2	df	p
Lineage	6.20	3	0.102
Treatment	103.10	1	<0.001
Generation	0.87	1	0.35
Lineage x treatment	3.06	3	0.382
Lineage x generation	1.08	3	0.782
Treatment x generation	0.76	1	0.383
Lineage x treatment x generation	2.74	3	0.434

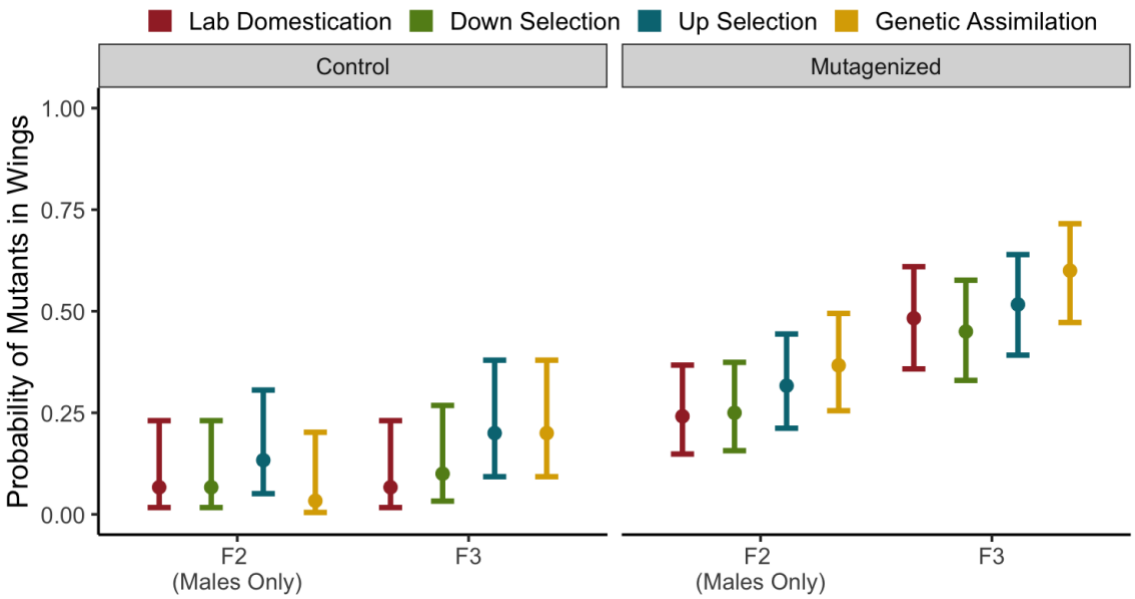

Figure 4.6: No differences in sensitivity to mutagenesis by way of probability of mutants in wings (Mutagenesis Part 2). Probability of mutants is the estimated number of phenotypes expected per vial sorted. $n_{Mutagenized}$ = 27-30 and $n_{Control}$ = 15 replicate vials sorted for each selection lineage. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model.

Table 4.6: ANOVA for the probability of mutants in wings (Mutagenesis Part 2). Lineage refers to selection lineage, treatment refers to mutagenized/control, and generation refers to progeny counted in F2 or F3.

	X^2	df	p
Lineage	7.56	3	0.056
Treatment	53.21	1	<0.001
Generation	24.87	1	<0.001
Lineage x treatment	1.71	3	0.635
Lineage x generation	0.33	3	0.954
Treatment x generation	0.30	1	0.582
Lineage x treatment x generation	1.79	3	0.617

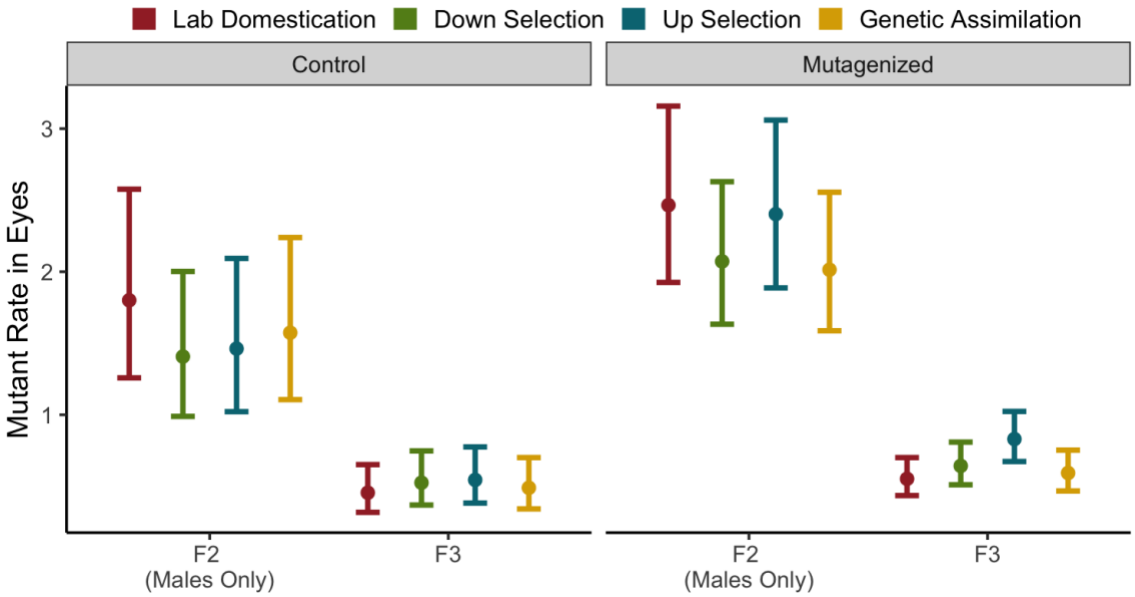

Figure 4.7: No differences in sensitivity to mutagenesis by way of mutant rate in eyes (Mutagenesis Part 2). Mutant rate is the estimated rate of observed phenotypes per individual. $n_{Mutagenized}$ = 27-30 and $n_{Control}$ = 15 replicate vials sorted for each selection lineage. Model was adjusted with +1 to account for complete separation. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model.

Table 4.7: ANOVA for the mutant rate in eyes (Mutagenesis Part 2).Lineage refers to selection lineage, treatment refers to mutagenized/control, and generation refers to progeny counted in F2 or F3.

	X^2	df	p
Lineage	4.65	3	0.199
Treatment	16.03	1	<0.001
Generation	288.18	1	<0.001
Lineage x treatment	1.46	3	0.69
Lineage x generation	4.94	3	0.176
Treatment x generation	0.51	1	0.476
Lineage x treatment x generation	0.11	3	0.991

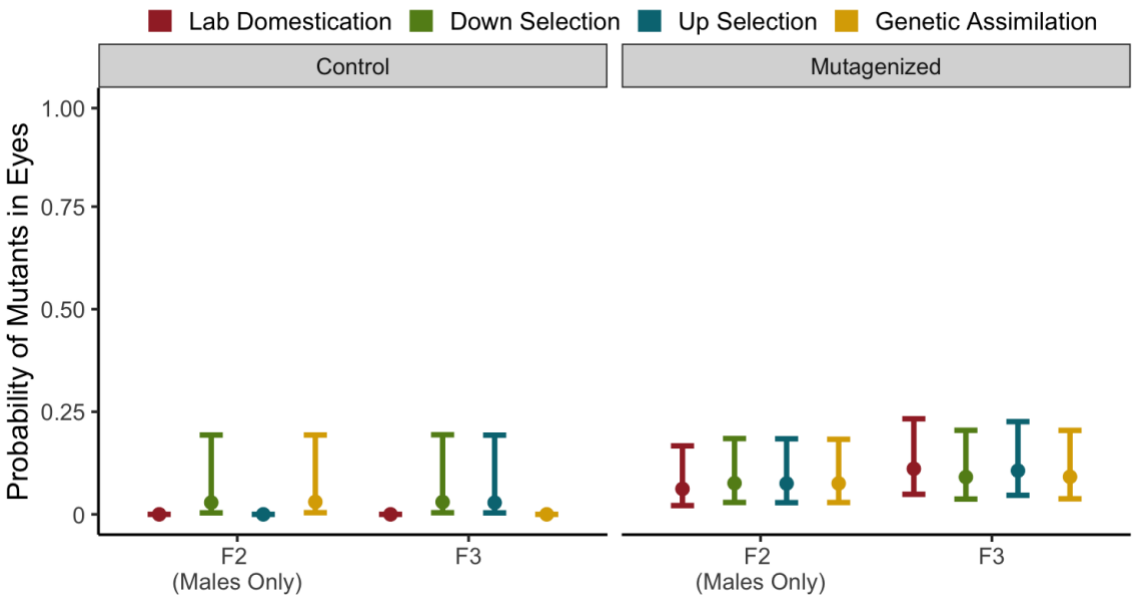

Figure 4.8: No differences in sensitivity to mutagenesis by way of probability of mutants in eyes (Mutagenesis Part 2). Probability of mutants is the estimated number of phenotypes expected per vial sorted. $n_{Mutagenized}$ = 27-30 and $n_{Control}$ = 15 replicate vials sorted for each selection lineage. Lab domestication F2 & F3, up selection F2, and genetic assimilation F3 had no individuals with defects. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model.

Table 4.8: ANOVA for the probability of mutants in eyes (Mutagenesis Part 2). Lineage refers to selection lineage, treatment refers to mutagenized/control, and generation refers to progeny counted in F2 or F3.

	X^2	df	p
Lineage	0.05	3	0.997
Treatment	4.12	1	0.042
Generation	1.16	1	0.282
Lineage x treatment	0.03	3	0.999
Lineage x generation	0.3	3	0.961
Treatment x generation	0.01	1	0.929
Lineage x treatment x generation	0.06	3	0.996

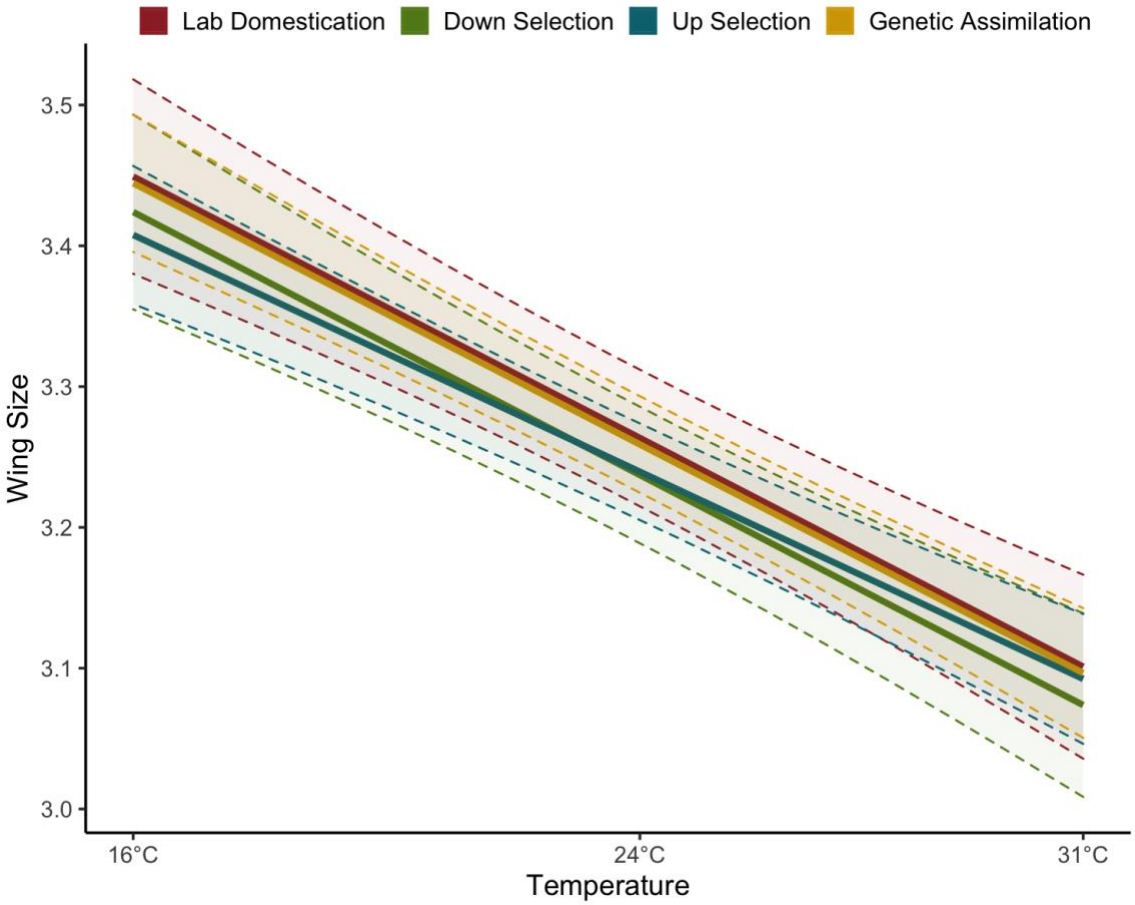

Figure 4.9: No differences in macro-environmental canalization measured as the slope of the temperature induced reaction norm for wing size among evolutionary lineages. Slopes are very similar with overlapping confidence bands.

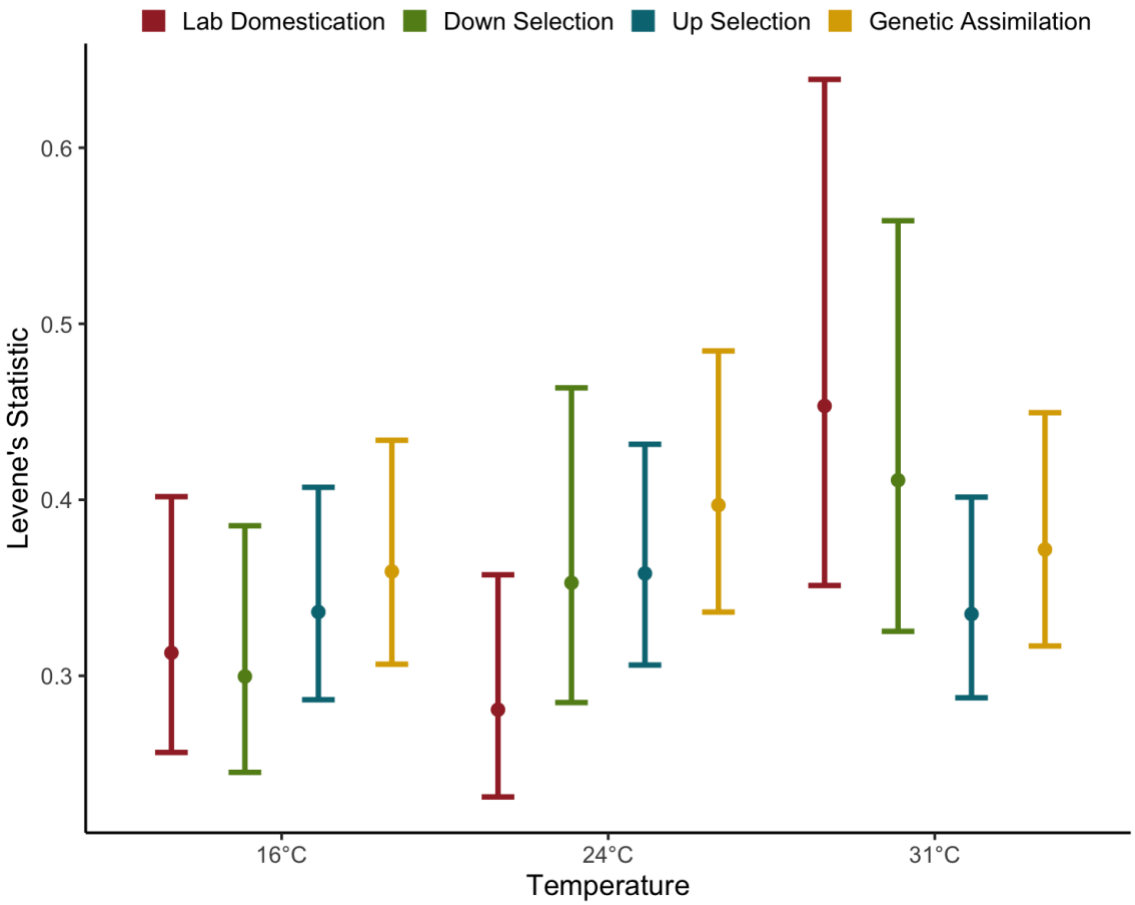

Figure 4.10: No differences in micro-environmental canalization for wing size among evolutionary lineages. Micro-environmental canalization measured as variation (using Levene's statistic) within selection lineages for different rearing temperatures. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model. ANOVA shows treatments do not differ from each other ($p>0.7$).

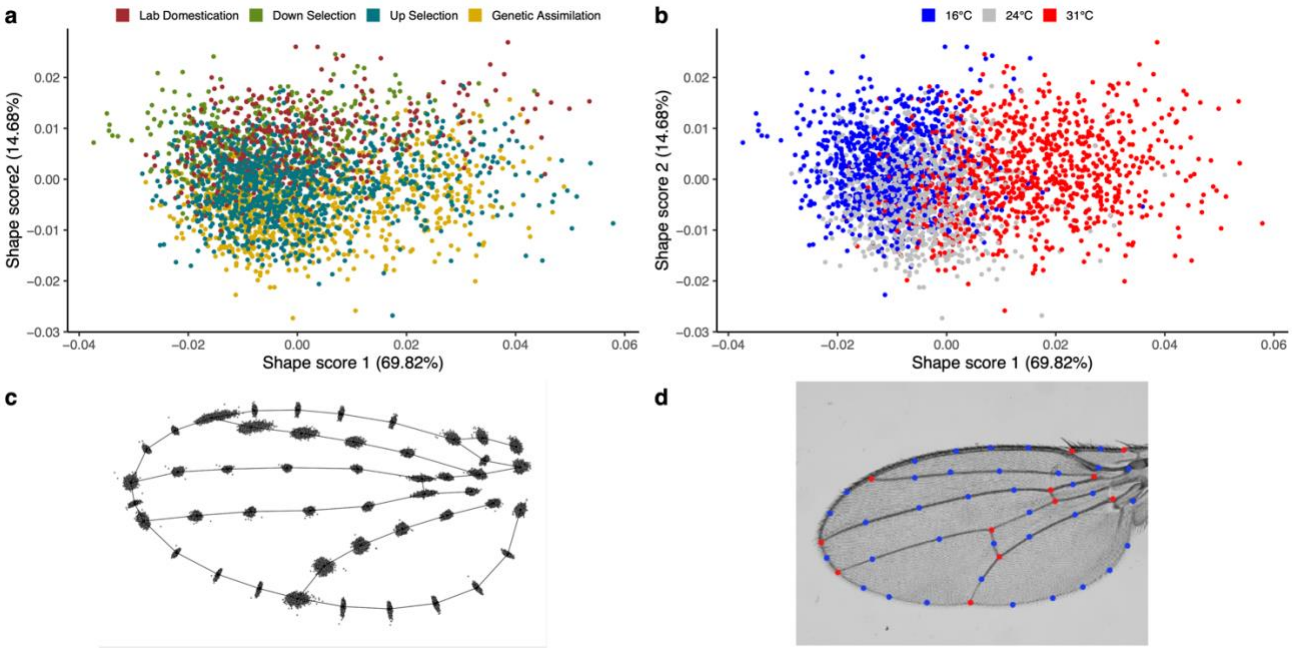

Figure 4.11: No differences in environmental canalization for wing shape among evolutionary lineages. a) Shape scores for selection lineages and b) same scores colored by rearing temperature show much of the variation in wing shape is due to temperature. Shape scores are projections of observed data onto vectors defined by PCs of fitted values. c) All specimens' landmark data plotted on a mean shape wing show small variation in distribution. d) Depicts landmark positions on wing image.

Table 4.9: Pairwise comparisons of trajectory analysis for macroenvironmental changes in wing shape. Table shows Procrustes distances of "shape" of reaction norms between evolutionary lineages, p-value under permutation. Magnitude and correlation of reaction norms for evolutionary lineages showed similar, nonsignificant results.

	distance	Z	p
Lab domestication:Genetic assimilation	1.019	0.189	0.450
Lab domestication:Down selection	0.357	0.191	0.410
Lab domestication:Up selection	0.788	0.116	0.453
Genetic assimilation:Down selection	0.723	-0.034	0.542
Genetic assimilation:Up selection	0.434	-0.43	0.673
Down selection:Up selection	0.449	-0.38	0.656

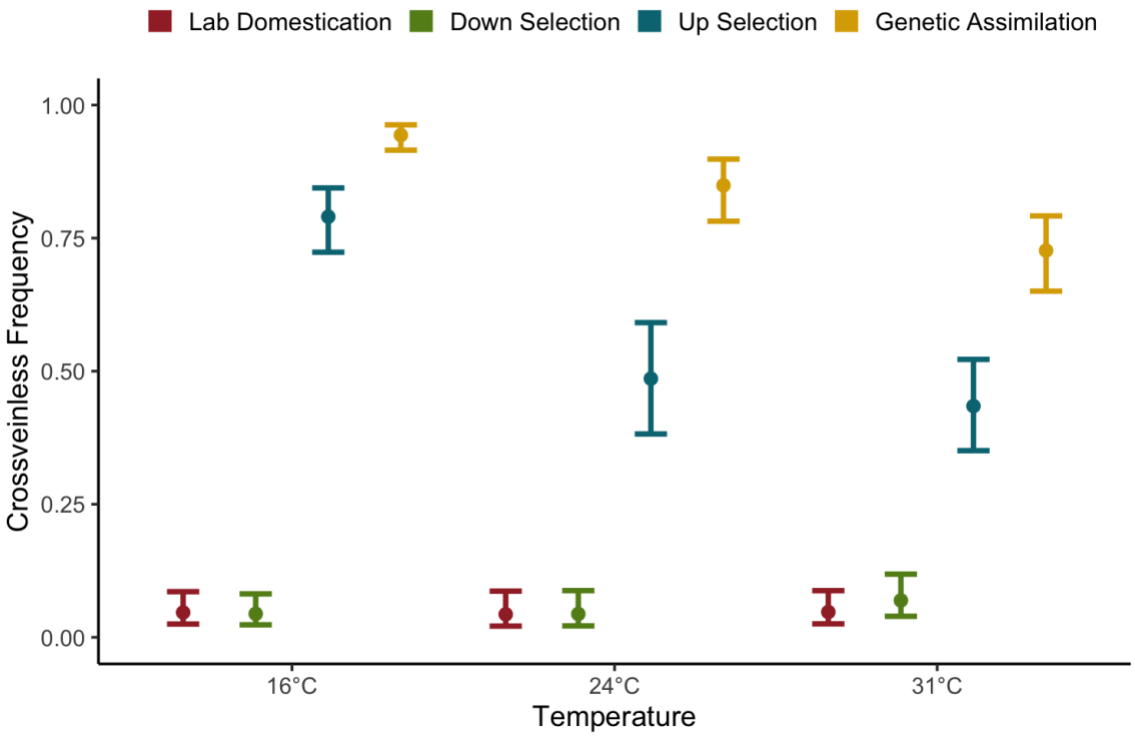

Figure 4.12: Up selection and genetic assimilation lineages have more posterior crossvein defects. As a test for sensitivity to rearing temperature, all wing defects for selection lineages were counted and up-selection and genetic assimilation lineages displayed more posterior crossvein defects. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model.

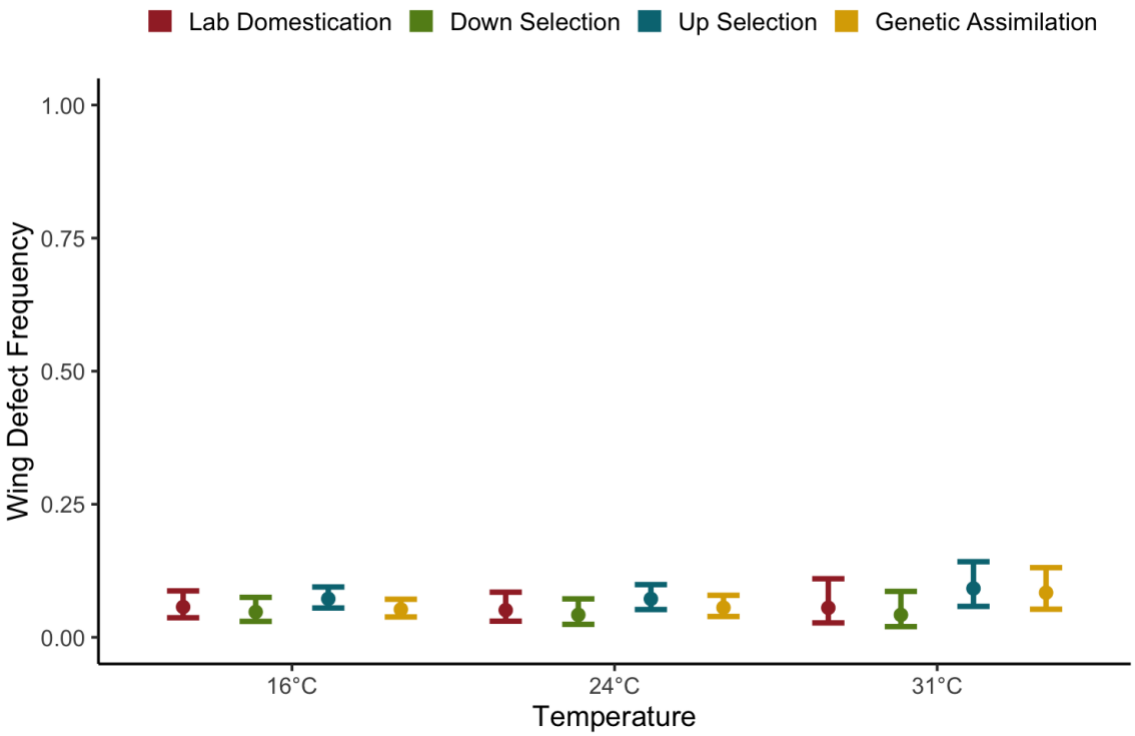

Figure 4.13: No differences in sensitivity to rearing temperature among evolutionary lineages. Wing defects include anterior crossvein, wing margin, and longitudinal vein defects. All defects were combined for modeling due to rarity of defects. Error bars are 95% confidence intervals on estimated effects from a generalized linear mixed model; none of the treatments show significant differences from each other at either density ($p>0.1$).

Chapter 5: Conclusion

In readdressing Waddington's classic experiment, we have found that the CGV contributing to the CVL phenotype response may not be entirely phenotypically "cryptic" and may contribute to variation in fitness in natural populations under some circumstances. This matches with recent work that has shown that in some instances, CGV may have previously unmeasured pleiotropic effects (Duveau and Félix 2012). While we demonstrate that the variation influencing the CVL phenotype may not be phenotypically "cryptic" in all circumstances, we note that the fitness effects we observe may be due to allelic combinations rarely occurring in nature. Since we observed aggregate effects of a polygenic CGV response, we cannot disentangle the individual phenotypic effects. Thus, it is possible are underestimating phenotypic effects because variants may cancel each other out if these effects differ in sign. It is also possible that in looking at the effects of these alleles in aggregate, we are also counting the effects of deleterious hitchhiking variants that may be linked to the selected CVL variants. Although as the experiment stands, we cannot separate the contribution of CVL and linked variants, we think it is unlikely that the linked variants are solely responsible for the deleterious effects we see in relaxed selection and the viability assays. From chapter 3 we see from signatures of selection that the CVL alleles are not solely new or rare. Relatively common alleles occur on multiple backgrounds which means that any linked deleterious variants (which would be rare) are less likely to increase dramatically in

frequency, meaning there may not be a large contribution of linked deleterious variants on fitness in these lineages. Additionally, it is possible that the deleterious effects we see are due a relative decrease in effective population size (and thus localized regions of increased inbreeding) given the much stronger selection for the up selection lineages than either the down selection or lab domestication lineages. Using the genomic data from chapter 3, we will also estimate the effective population size on each set of evolutionary lineages to examine this possibility. Another future direction for this work is to address if the crossveinless alleles, which in aggregate influence traits such as viability, are maintained due to either density or frequency dependent selection. One example of this could be *foraging* alleles which are known to be maintained by density-dependent selection (Sokolowski et al. 1997) and which we saw had variants selected on in our genetic assimilation lineages (see '*for*' in **Fig. 3.5**). *Foraging* has been shown to interact with posterior crossvein development (Schleede and Blair 2015). There were also plans for additional work to be done in understanding the maintenance of the crossveinless alleles in natural populations by looking at both experimentally evolved and naturally collected populations along a latitudinal cline. We originally planned to complete this work but constrained by restrictions put in place due to COVID-19. As of publication of this thesis, we have obtained natural populations along a latitudinal cline and have completed a year and a half of experimental evolution for a single natural population split into three sub-populations raised at differing temperature regimes. This work is aimed to look for any correlation between maintenance of crossveinless alleles

and population temperature history (both mean and variance), and if natural selection is acting on the crossveinless alleles. We expect that if the alleles influencing the crossveinless phenotype are deleterious, they would be selected out by natural selection, and thus the populations experiencing higher temperatures may have fewer crossveinless alleles. This work is being completed by future students.

Based on the small reductions in diversity and increases in F_{ST} spanning many parts of the genome, we suggest that the genetic basis of the assimilation of the CVL phenotype response is polygenic, spanning much of the genome. We cannot determine how many variants are contributing to variation for this trait (many small-effect alleles or relatively fewer, somewhat larger-effect alleles) because we have not identified specific selected variants versus linked variants or artefacts. However, based on several lines of evidence we argue this trait is influenced by many smaller effect alleles. The experimental design we have is perhaps biased towards selecting for moderate-effect alleles because of our effective population size (at most, our effective population size is 200) and thus we are biased in detecting variants with a higher selection coefficient. By principle, alleles with a larger effect for the trait would be selected on more strongly, and so we are likely to see large-effect alleles rise in frequency before smaller-effect alleles. In contrast, if this work was done with a much larger effective population size, we could potentially be selecting on many variants of much smaller effect and still see the same phenotypic response to selection for the crossveinless phenotype; this may not result in large allele frequency changes in only 25 generations, but still result in

strong phenotypic response. Put another way, our experimental design might actually underestimate the actual number of segregating genetic variants that could contribute, because our effective population size limits the detectable response to variants with a higher selection coefficient. Yet through both genetic and genomics scans, we still see many regions of the genome have been selected. Considering all of this, we think it is likely that many alleles of small-effect are contributing to the crossveinless phenotype in natural populations. That said, we do recognize there is a strong sweep (both in high F_{ST} and reduced nucleotide diversity) found at the end of chromosome 2R that may represent an allele of relatively large effect as we see a strong signature of selection, and we have plans to further investigate what genes are in this region that could contribute. However, no matter the number of genes influencing this trait, the response still stems from standing genetic variation and is not consistent with single, large-effect mutations for the evolution of genetic assimilation. It is likely that the different replicate up-selection lineages have many of the same alleles, because hybrid progeny do not have a high reduction in crossveinless frequency. It is possible that the higher CVL frequency among genetic assimilation hybrids (relative to up selection lineages) is due to the different genetic assimilation lineages having a higher aggregate number of the same CVL alleles. This is what we would expect under a model where the same alleles responsible for the CVL phenotype response accumulate in populations during selection and lead to genetic assimilation (i.e. threshold liability model). As seen from genetic tests, we know all genetic assimilation lineages have some overlap in alleles that

influence crossveinless frequency. We also observed through genomic scans that the evolution of genetic assimilation in Waddington's classic experiment is due to a polygenic response largely from segregating alleles. This is resonant with past work showing the CVL phenotype response and genetic assimilated lineages are polygenic (Milkman 1965b; Mohler 1965b, 1967). Recent work suggested that new mutations of large effect caused by temperature-stress induced transposon activity could explain the genetic assimilation seen by Waddington (Fanti et al. 2017). However, the results from each experiment described in chapter 3 (crosses among lineages, quantitative complementation mapping and genome scan) are each independently inconsistent with the role of a *de novo* variant of large effect contributing to the response. Additionally, the control crosses from the mutagenesis also demonstrate no differences between those populations that evolved with heat stress (and predicted from studies like Fanti et al. 2017 to elevate mutation rates) and the lab domestication lineages that evolve without heat stress. Even Waddington and Bateman thought in some cases that large-effect mutations were responsible for the genetic assimilation of certain phenotypes (Bateman 1959b; Waddington 1961). However, this was not the case for venation phenotypes, specifically crossveinless (Waddington 1953a, 1961; Bateman 1959a). It should be noted that mutations occurring during selection could still contribute to genetic assimilation, but likely the majority of the phenotypic response it is due to segregating alleles from the ancestor population. While new mutations of large effect due to mobilization of transposable elements is not relevant for this system, their role in

adaptive response in nature has been demonstrated (Lanciano and Mirouze 2018; Schrader and Schmitz 2019). Although our results do not suggest that large-effect mutations are responsible for the evolution of genetic assimilation, we would still like to address if there is any evidence of transposable element mobilization in the lineages, possibly with the pool-seq data already on hand (if we can utilize tools such as PoPoolationTE2 (Kofler et al. 2016a) which is meant for pooled data sets) or with some additional long-read sequencing (which will allow us to better map specifically where these transposable element fall, as we may not be able to accurately map with the short-read data we have). Future work plans to address the polygenic nature of this response and identify some of the specific causal variants and possible genetic interactions amongst them that contribute to genetic assimilation.

We found little evidence for changes in either genetic or environmental canalization contributing to the evolution of genetic assimilation. Genetic assimilation of the crossveinless phenotype occurs in less than 25 generations which may be too short to evolve new canalizing mechanisms. There is some discussion that although decanlization can occur in one to two generations, canalization evolves over many more generations (Gibson and Lacek 2020). However, it is possible that the evolution of (re)canalization could occurs on much shorter time scales. For example, blowflies that evolved insecticide resistance initially showed increased fluctuating asymmetry relative to susceptible blowflies. Yet with continued selection for resistance, the resistant individuals evolved back to levels similar to that of the susceptible blowflies (in less than

10 generations) (Clarke and Mckenzie 1987). Thus canalization can sometimes occur within such a time frame as matches the genetic assimilation of the crossveinless phenotype. Regardless, we do not see changes in canalization among our artificial selection lineages. Our tests for environmental evolution did have some confounds in that we captured both the effects of environmental and genetic variation in our measure of phenotypic variation as we are working with outbred populations. Although we did not explicitly test models such as the liability threshold model (originally proposed for this system by Bateman in 1959), we have shown that genetic assimilation is largely due to a response of standing genetic variation in the ancestor which is broadly consistent with the predictions of this model. Future work in identifying specific casual variants contributing to genetic assimilation may confirm whether the variants contributing to genetic assimilation are naturally segregating and that through selection, they accumulate in populations. Despite canalization not seemingly to be necessary for genetic assimilation, it clearly occurs in natural systems (Lack et al. 2016; Groth et al. 2018). As such it still remains possible that it is still relevant to genetic assimilation in other contexts. Alternatively, the interesting hypothesis of canalization and the interesting phenomena of genetic assimilation, both worth studying in their own right, may be largely independent of one another.

As with other seemingly puzzling phenomena, extraordinary claims about mechanisms contributing to genetic assimilation have been proposed, but often with tenuous evidence. In particular the contribution of epigenetics, canalization, and high

rates of transposable elements activity and mutation have been recently over-emphasized as alternatives to cryptic genetic variation and the standard quantitative genetic model of threshold liability. In the case of genetic assimilation of CVL, both current and past evidence is consistent with a “typical” polygenic response on an underlying threshold trait primarily due to standing genetic variation. While for a lab based artificial selection experiment addressing questions of genetic assimilation and canalization this may seem largely academic. However, given the increasing need to evaluate how populations and species may survive rapid anthropogenic changes to the environment, any potential roles of CGV and genetic assimilation in contributing to evolutionary rescue become increasingly important. These mechanisms may not only be less parsimonious but also detrimental to our understanding in how to care for populations responding to climate change and undergoing rapid evolution.

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Appendices

Table A1: Heat-stress pilot experiments. All individuals are from the ancestral populations used to start artificial selection experiment.

Heat Stress	Age (hrs, ±2)	Replicate Trials	Total Flies Counted	Adult Survival (%)	CVL Frequency (%)
41.5°C for 30 min	21	2	151	90.75	3.45
	22.5	2	127	97.15	1.9
	24	2	127	52.65	5.5
37.5°C for 4hrs	21	4	380	96.72	5.625
	22.5	4	386	98.35	7.25
	24	4	445	98.88	12.4

Table A2: All crosses between up-selection replicate lineages in round-robin design.

R1♂ x R2♀	R2♂ x R1♀
R2♂ x R3♀	R3♂ x R2♀
R3♂ x R4♀	R4♂ x R3♀
R4♂ x R5♀	R5♂ x R4♀
R5♂ x R6♀	R6♂ x R5♀
R6♂ x R1♀	R1♂ x R6♀

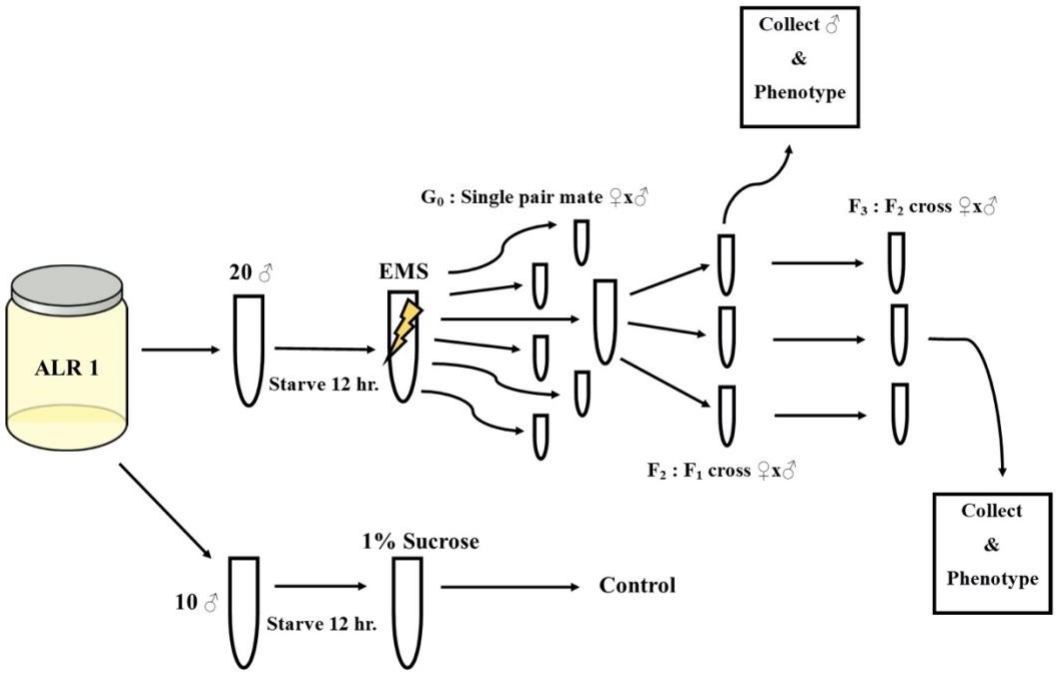

Figure A1: Sample flowchart for mutagenesis experimental procedure.