Directional selection reduces developmental canalization against genetic and environmental perturbations in *Drosophila* wings

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Abstract

Natural selection may enhance or weaken the robustness of phenotypes against genetic or environmental perturbations. However, important aspects of the relationship between adaptive evolution and canalization remain unclear. Recent work showed that the evolution of larger wing size in a high altitude natural population of *Drosophila melanogaster* was accompanied by decanalized wing development – specifically a loss of robustness to genetic perturbation. But this study did not address environmental robustness, and it compared populations that may have numerous biological differences. Here, we perform artificial selection on this same trait in *D. melanogaster* (larger wing length) and directly test whether this directional selection resulted in decanalization. We find that in general, size-selected replicates show greater frequencies of wing defects than control replicates both after mutagenesis (genetic perturbation) and when subjected to high temperature stress (environmental perturbation), although the increase in defect frequency varies importantly among replicates. These results support the hypothesis that directional selection may result in the loss of both genetic and environmental robustness – offering a rare window into the relationship between adaptation and canalization.

Introduction

Canalization is the capacity of a biological trait to maintain a constant phenotype under genetic or environmental perturbation (Waddington 1942; Flatt 2005). It has several important implications in evolutionary biology. If a trait is under stabilizing selection, canalization of the trait can maintain the population close to the fitness optimum (Flatt 2005). By reducing phenotypic variation, canalization may constrain evolution, reducing the potential of adapting to future environmental changes (Charlesworth et al. 1982; Maynard Smith et al. 1985). On the other hand, canalization allows cryptic genetic variation to accumulate without phenotypic expression, which could contribute to future adaptation once the canalization is overcome by genetic/environmental perturbation (Gibson and Dworkin 2004; Masel 2005; Paaby and Rockman 2014).

While the phenomenon of canalization has been documented and conceptualized for a long time, its evolution and the forces shaping it remain elusive. If a trait facing environmental/

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mutational perturbations is under stabilizing selection for an optimal trait value, its developmental canalization should be maximized (Gavrilets and Hastings 1994; Wagner et al. 1998; Zhang and Hill 2008). However, if the trait or a correlated trait is under directional selection, the previous buffering mechanism may fail to buffer the development of the new phenotype as it approaches a novel optimum (Maynard Smith et al. 1985; Hill and Mulder 2010). This loss of coordination predicts that newly evolved traits will show reduction in canalization (decanalization) compared to the ancestral trait until compensatory evolution restores the canalization.

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Few empirical studies have examined the relation between novel directional selection and either canalization or developmental stability (the buffering of random developmental errors; Debat and David 2001; Flatt 2005). A classic example is from the Australian sheep blowfly, *Lucilia cuprina*, where developmental stability (as indicated by fluctuating asymmetry) in a novel insecticide resistance genotype was compromised relative to an ancestral susceptible genotype, and was restored by subsequent evolution of a modifier locus (Clarke and McKenzie 1987; McKenzie and Game 1987). Another example is an *in vitro* experimental evolution using an RNA enzyme (Hayden et al. 2012). By changing the substrate of the enzyme, a reduction of genetic and environmental robustness in enzyme activity resulted from the novel adaptation.

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Wing vein development in *Drosophila* provides a phenotypically tractable system to test hypotheses about developmental canalization because it has a multifaceted phenotypic readout which can be clearly and efficiently examined. Moreover, studies suggest that wing vein development may be under strong stabilizing selection within *Drosophila melanogaster* and across *Drosophila* species (Gilchrist and Partridge 2001; Houle et al. 2017). If so, the canalization of wing vein development should evolve to a maximum and recent evolution of the wing may weaken previous buffering mechanisms for the ancestral state. Indeed, in a *Drosophila melanogaster* population which recently colonized Ethiopian highlands, the evolved large wings have become more susceptible to mutagenesis than the small wings of the ancestral Zambia population, consistent with prediction that recent directional selection can lead to decanalization – at least in the context of genetic robustness (Lack et al. 2016). However, the exact agents of selection on the wing or a correlated trait are largely unknown in nature. Also, the level of decanalization might depend on specific history and the genetic background of the Ethiopian highland populations.

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Motivated by Lack et al. (2016), we used experimental evolution to directly test whether directional selection leads to developmental decanalization of both genetic and environmental perturbations. We artificially selected for large wing size for eight generations on replicate populations created from multiple Zambia inbred lines. We compared their canalization capacity to the control populations within the ancestral wing size range by imposing genetic (mutagenesis) and environmental (high temperature) stresses. The noted sensitivity of wing development to temperature in *Drosophila* (e.g., Debat et al. 2003; Powell et al. 2010) provided us with a clear motivation for using high temperature stress. Since Ethiopian *D. melanogaster* showed elevated wing vein defects under genetic perturbation (Lack et al. 2016), we tested for this same outcome from our wing size selection populations. We found that wing size selected populations exhibited higher rate of

wing vein abnormality (in both assays) as well as a greater diversity of abnormalities (in the mutagenesis assay), supporting the hypothesis that both genetic and environmental canalization can be disrupted by directional selection.

Methods and Materials

Artificial Selection.

The populations investigated in this study were created from about 200 Zambian isofemale lines (the “ZI” collection from Siavonga; Lack et al. 2016). A single non-virgin female fly from each line was collected and pooled into a base population. The individuals from the base population were partitioned into six populations, each was maintained 28×14×15-cm plastic cages with 14 vials with standard *Drosophila* medium (containing molasses, corn meal, yeast, agar, and antimicrobial agents). To produce the F₁ generation, the flies were allowed to oviposit for four days. Then the vials were incubated at 25 °C for twelve days, producing approximately 600 offspring per population. Starting at generation two, three populations were under selection treatments and the remaining three were assigned as control. For each selection population in each generation, 100 non-virgin female flies were assayed for wing size and the 20 largest-winged of these were taken to produce offspring. For each control population, 20 non-virgin female flies were taken randomly to produce offspring. The selected female flies were allowed to oviposit in vials for seven days before being discarded. Then the vials were incubated at 25 °C for ten days to produce the adults for next generation. The artificial selection started at generation two and continued for eight generations. After that, all the populations were maintained in the same enclosures at 18-20 °C without further artificial selection, with population sizes of ~500 individuals.

Phenotypic measurement.

CO₂-anesthetized female flies were visualized under an SM-4BX microscope and photographed using an AmScope MU1000 camera. Wings were isolated for imaging using a wing grabber (Houle et al. 2003). Images of flies taken during the selection process were analyzed and measured in ImageJ. “Wing size” was scored as the length of the wing from the middle of the posterior cross vein to the end of the third longitudinal vein. The measure for wing size has been inconsistent in previous literature (e.g., Imasheva et al. 1994; Gilchrist and Partridge 1999; Wolf et al. 2000; Debat et al. 2009). We defined our wing size trait based on convenient and consistently definable landmarks under our microscope. It is possible that we select for changes of wing vein position in addition to total wing size (Munoz-Munoz et al. 2016). But it did not compromise our ability to test the hypothesis, which is directional selection can lead to decanalization. 100 female flies from each selected population and 20 female flies sampled from each control populations were measured for right wing size under CO₂ anesthetization at each generation.

To assay decanalization, we used wing vein defects instead of variance in the selected trait itself, for multiple reasons. First, studies suggest that wing vein development might be under stronger stabilizing selection than wing size (Birdsall et al. 2000; Gilchrist and Partridge 2001), therefore wing vein traits might show greater canalization in non-evolved populations. Second, directional selection on wing size can alter the genetic variation of this

trait, obscuring comparisons of wing size variance under perturbation between control and selection populations. Third, observations from Ethiopian *D. melanogaster* gave us a strong prior suspicion that wing size evolution might lead to increased wing vein defects in the presence of perturbation (Lack et al. 2016).

Analysis of defective wing vein architecture was performed and imaged similarly with both left and right wings. The vein defects were identified based on whether it was incomplete, bent, forked, split or marked by ectopic vein material (see Fig. 1 for sample photo for each category). Flies with mildly damaged wings (i.e. torn or folded wings with no veins affected) were still considered in analysis except cases where defects are equivocal due to damage. Because various genes and pathways are often responsible for different abnormality phenotypes in different wing veins (Biehs et al. 1998; Crozatier et al. 2004; Terriente-Felix et al. 2010), we further categorized the defects based on the phenotype and location to qualitatively understand the variety of genes and pathways affected by decanalization. For the incomplete, bent, forked and split abnormalities, location was defined based on the affected wing vein. For ectopic vein material, it was located to one of the seven regions divided by the wing veins (Fig. 1). Similar abnormalities in different locations were categorized as distinct defect types.

Mutagenesis treatment.

At generation 10, male flies collected from each population were mutagenized by exposure to ethyl methane sulfonate solution (25 μ M in 1% sucrose) for 24 hours. For two out of the three populations of selected and control groups, we had enough individuals to create a replicate assay. For each population/replicate, 17 of these males were mated with 17 virgin females from the same population for five days and then the males were discarded. This timing means males should only pass on postmeiotic mutations, which should be unique to different gametes and not be shared among F₁ siblings. Females were transferred to new medium to oviposit for another five days to increase the sample sizes. To produce flies for the wing phenotyping, F₁ individuals were transferred to new medium to oviposit for about eight days. Eight days after the F₁ female were removed, the F₂ flies were collected and separated by sex after mating for five days. The males were used for wing phenotyping. The F₂ females were transferred to new medium to oviposit for about eight days. Then the F₃ females were collected for phenotyping, starting eight days after the removal of the F₂ females. Note that in the case of recessive *de novo* mutations, F₂ males may manifest X-linked mutations, while F₃ females may be affected by mutations on any chromosome because homozygous mutations can be generated at F₃ (see Fig. 3A of Lack et al. 2016 for illustrations of the design and outcomes of the mutagenesis assay).

Environmental treatment.

At generation 17 (eight generations after the size selection), we performed the environmental canalization assay using temperature stress. For each of the three size-selected populations and two available control populations, ~200 male and female flies were collected and placed in 10 vials (~10 females and 10 males per vial) at 31°C heat stress or 23°C as benign treatment. The flies were allowed to oviposit for five days and then transferred to new medium at the same temperature to continue producing offspring for five

days. After two rounds of transfers, the adults were removed, resulting in 30 vials in total per population. Offspring (separated by sex) were collected for wing phenotyping 14 and 18 days after oviposition for heat and benign treatment respectively due to different development rates at each temperature. Because the temperature treatment produced lower frequencies of defects generally, we did not attempt to classify unique defect phenotypes.

Although there were eight generations of relaxed selection before the assay for environmental canalization, we think this is unlikely to affect our interpretation of the results. During the relaxed selection period, both the control and selected populations were maintained under the same procedure with relatively large population sizes (~500). Therefore, differences in phenotypic robustness should still reflect the populations' history of directional selection. Drift during relaxed selection is not expected to have a large impact because the relevant time interval is only $\sim 0.016N$ generations. Although selection might conceivably act to reverse some of our size selection, selection coefficients (and hence trait changes) should be far lower than during our eight generations of artificial selection. Wing vein canalization is unlikely to be under strong selection because defects rarely occur in the benign lab environment and hence the wing defect trait is essentially invisible to selection during these intervening generations. As shown below, the results of our environmental robustness assay indicated that canalization was not restored during relaxed selection.

Analysis

An unpaired t-test was used to examine whether the wing sizes are significantly different between the populations after eight generations of size selection and the controls. A paired t-test was used to examine whether the size changes between generation three and generation ten were significantly different from 0.

To examine the effect of selective history on canalization of mutagenesis, we analyzed the wing vein results using a generalized linear mixed model (*glmer* function) from the “lme4” package in *R* v3.3.0 (R Development Core Team, 2016):

$$Z = H + G + H \times G + Pop + Rep$$

where *Z* is the phenotype with or without defect, which was modeled as a binary trait using “binomial” family with “logit” link. *H* denotes whether the fly is from a size-selected population or a control (selective history). *G* denotes whether it is F₂ male or F₃ female (generation/sex). The random effect replicate (*Rep*) is nested within a higher level of random effect population (*Pop*). We first examined whether there is an interaction effect between selective history and generation/sex; the model above was compared with a reduced model without the interaction *H*×*G* using likelihood ratio test. As the interaction *H*×*G* was significant ($p = 0.041$), the data were analyzed for each sex separately. For each sex, a full model is:

$$Z = H + Pop + Rep$$

and was compared against a model without the H term to test the effect of selective history on abnormal wing veins. To examine the generation/sex effect for genetic robustness, the data were analyzed for each history group separately. For the control or selection populations, the full model is:

$$Z = G + Pop + Rep$$

and compared with a model without the sex effect (G) using a likelihood ratio test.

For the diversity of wing defects, we standardized the number of distinct defect types by dividing the total flies scored for each population. The standardized defect diversity (D), the proportion of individuals yielding a novel wing defect type, for each population/replicate was analyzed by a mixed model using the *lmer* function:

$$D = H + G + H \times G + Pop$$

where D is treated as a continuous trait with family as “null” by default. As the interaction $H \times G$ was not significant, we removed this term to test the history effect using a full model $D = H + G + Pop$ compared with a reduced model without H term for the combined F_2 male and F_3 female data. We also performed a similar analysis for data of each sex separately, using a full model $D = H + Pop$ compared with a reduced model without the history H term. To test the generation/sex effect on defect diversity, the full model $D = H + G + Pop$ was used to compared with a reduced model without the sex effect (G) for the combined data of both history groups.

To examine the effect of selection on environmental canalization, the wing phenotype of each individual fly was analyzed by a generalized linear mixed model:

$$Z = H + T + H \times T + Pop$$

where Z is again whether the phenotype is abnormal or not. T denotes temperature treatment (heat stress or benign temperature). We found no generation/sex effect nor other interaction effects involving sex, therefore we kept the model without terms involving sex. As we are interested in whether the effect of temperature stress on wing vein phenotype depends on the selection history, the model above was compared with a reduced model without the interaction $H \times T$ using likelihood ratio test. We did not explicitly model density (adults per vial, which was not found to differ significant between control and selection treatments), individual parentage, or parental age.

Results

After eight generations of size selection, the wing sizes of the selected populations were significantly larger than those of the control populations ($t = 5.3$, $p = 0.014$; Fig. 2) and those near the beginning of the selection at generation three ($t = 15.1$, $p = 0.004$; suitable measurements were not available from generation two). The control populations showed no

significant change on wing size ($t = 1.4$, $p = 0.3$). These results suggest that the wing sizes responded to artificial selection within eight generations.

Genetic robustness

After male flies were mutagenized at generation ten, the F_2 male offspring of the mutagenized males from the selected populations yielded significantly higher wing vein defect frequency than those from the control populations ($\chi^2 = 6.7$, $df = 1$ here and below, $p = 0.009$; Fig 3A). The F_3 female offspring also showed an average defect frequency from the selected populations of more than twice as that from the control populations, but this is not statistically significant ($\chi^2 = 1.8$, $p = 0.18$; Fig 3B), potentially in part due to smaller F_3 sample size (1936 F_2 males versus 1091 F_3 females measured). Comparing between generations/sexes, the F_2 male offspring showed a significantly greater decanalization to mutagenesis than the F_3 female offspring (For the data combining both males and females, the interaction of history and generation/sex is significant ($\chi^2 = 4.2$, $p = 0.041$)). It is worth noting that without mutagenesis, the defect frequency was very low for both control and selection populations (inferred from data collected at benign temperature, Fig. 4) and no significant difference existed between the two groups ($\chi = 0.07$, $p = 0.79$). Therefore, the difference in defect frequency between control and selection populations after mutagenesis should reflect increased sensitivity to new mutations.

For the diversity of unique defects, both F_2 and F_3 offspring from the selected populations showed significantly higher defect diversity than those from the control populations ($\chi^2 = 4.2$, $p = 0.041$ for F_2 male; $\chi^2 = 6.8$, $p = 0.009$ for F_3 female; $\chi^2 = 5.8$, $p = 0.016$ for data combining both generations/sexes; Fig. 3C & D). The results above are consistent with directional selection on wing size leading to a loss of genetic robustness of wing development.

To examine the sex/generation effect on the mutational sensitivity, the F_3 female offspring showed higher defect frequency as well as higher defect diversity than the F_2 male offspring regardless of the population history (defect frequency, $\chi^2 = 24.1$, $p = 9e^{-7}$ for control populations, $\chi^2 = 12.2$, $p = 0.0005$ for selection populations; defect diversity, $\chi^2 = 12.2$, $p = 0.00047$ for control, $\chi^2 = 8.7$, $p = 0.003$ for selection populations). This difference may reflect the contribution of recessive *de novo* mutations on all chromosomes in the F_3 females, rather than the X chromosome specifically as in the F_2 males.

Environmental robustness

To test whether size selection also leads to decanalization under environmental stress, we measured the wing defect frequency of the size-selected populations and control populations at benign temperature (23°C) and high temperature (31°C). There is a significant interaction effect between the selective history and temperature treatment ($\chi^2 = 6.5$, $p = 0.011$). On average, the increase in wing defect frequency due to temperature stress was about 14-fold higher for the size-selected populations than the control populations. There is notable variability among replicates of the selection populations, with the S2 population showing much stronger increase in defect frequency than the other two (Fig. 4). However, after excluding the data from S2, the interaction effect between history and temperature treatment

is still significant ($\chi^2 = 4.8$, $p = 0.028$), suggesting the other two selection populations also increased in sensitivity to heat stress. This pattern suggests that selection for large wings can lead to their loss of canalization against environmental stress.

Discussion

In this study, we have leveraged *D. melanogaster* as an experimental model system and its wing veins as a readout of phenotypic robustness, seeking to examine the effect of directional selection on trait canalization. A weakened robustness of wing vein phenotypes would be expected if ancestral canalization mechanisms fail to buffer evolved developmental processes. After eight generations of selection for larger wings, there was evidence for increased frequencies of wing vein corruption following mutagenesis and high temperature stress. To our knowledge, the study provides the first direct experimental evidence of the loss of canalization against both genetic and environmental perturbation after the same tissue experienced directional selection.

The loss of both environmental and genetic robustness in a single selection experiment suggests a possibility that both types of decanalization are linked to the same mechanism. More broadly, this is related to whether the same processes are responsible for the evolution of genetic canalization and environmental canalization. Theory suggests that if a trait is under stabilizing selection, environmental canalization may evolve more readily than genetic canalization because stabilizing selection reduces the amount of genetic variance for the trait and thus compromises the benefit of genetic canalization (Wagner et al. 1997). But if the mechanism buffering against environmental perturbation can also buffer against genetic perturbation, genetic robustness can evolve as a byproduct of the populations adapting to fluctuating environments (Meiklejohn and Hartl 2002; Visser et al. 2003; Masel and Siegal 2009). For example, heat shock proteins are temperature-induced and able to buffer the phenotypic effects of genetic variation (Rutherford and Lindquist 1998; Fares et al. 2002; Queitsch et al. 2002; Casanueva et al. 2012; but see Geiler-Samerotte et al. 2016). Intriguingly, population S2 shown highest sensitivity to both mutations and heat, suggesting that genetic and environmental decanalization may share the same genetic causes. It is also worth noting that temperature and other stresses may introduce *de novo* somatic mutations, but these environmentally-induced changes still represent an aspect of environmental perturbation and are distinct from germline mutations.

The decanalization of a trait after reaching a new phenotypic value by selection can be viewed as a cost of adaptation. Such cost could arise from pleiotropic decanalizing effects of the selected alleles themselves (such as alleles that determine large wing size in our study) or from the deleterious effects of variants linked to the targets of selection (Knott and Haley 2000; Paaby and Rockman 2013). Similarly, for *D. melanogaster* wing size evolution in nature, Lack et al. (2016) found evidence that a loss of genetic robustness was either due to wing size alleles themselves or nearby linked variants: wing size and decanalization cosegregated in the offspring of crosses between large-winged and small-winged populations. If so, directional selection may predictably reduce canalization for this trait until it is restored by subsequent modifier alleles (which might be a relatively slow and nuanced evolutionary process). However, the replicate selection populations from the same

ancestral population shown various degrees of decanalization (Fig. 3; Fig. 4). This suggests that different sets of alleles can respond to the directional selection and their decanalizing effects (or effects from linked variants) vary. Or, the same sets of alleles were selected consistently among replicates, but their decanalizing effects depend on interactions with other alleles, which can be different among replicates because of drift.

To further understand the mechanisms of decanalization caused by the directional selection, it will be important to know the genes underlying wing size evolution and wing vein defects (which could be the same genes) and how they interact to shape wing development. As a threshold trait, wing vein defects can be controlled by genes from different pathways, such as Notch, EGF (epidermal growth factor) and Dpp (Decapentaplegic) signaling pathways (De Celis 1998; Johannes and Preiss 2002; Crozatier et al. 2004). Wing size, as a quantitative trait, is shown to be influenced by many loci, each with relatively small effect size (Vonesh et al. 2016). Some of these candidate genes were also in the EGF and Dpp pathways, indicating the changes in wing size might influence wing vein development via these pathways. Wing vein development is sensitive to the expression dosage of these pathways (Biehs et al. 1998; Johannes and Preiss 2002), and so canalization might evolve to buffer relevant expression levels. If directional selection then increases wing size, it may alter the expression of genes in these pathways closer to the limit of their buffering. Perturbations may then alter expression beyond this buffering limit, resulting in abnormal wing veins. While this represents one plausible general scenario, at present the relevant molecular mechanisms of wing size evolution and decanalization remain unknown.

In conclusion, our experiment serves as a case study on the relationship between adaptation and canalization. We found that directional selection on wing size results in the decanalization of wing vein development with respect to both genetic and environmental perturbations. It will be necessary to study a broad range of traits to test whether this side effect of adaptation is a general phenomenon. Further studies on the genetic and physiological basis of the selected traits and the diagnostic traits will be important for understanding the mechanisms of decanalization.

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Fig. 1. Sample photos for the normal wing (A) and different wing vein defect categories. In the normal wing photo (A), the major wing veins used to identify location of the defect are labeled in red. The five main categories of wing vein defects are incomplete (B), bent (C), forked (D), split (E) and marked by ectopic vein material (F). For photos B-E, the defects are located to specific veins; for F, the defect is located between L2 and L3 veins.

Fig. 2.

The response of wing size to artificial selection ($\pm$ standard derivation). Generation 3 is after a single generation of selection. At generation ten, the selected (*S*) populations show larger wing size than the control (*C*) populations ($p = 0.014$) and the earlier populations at generation three ($p = 0.004$).

Fig. 3.

The wing vein defect frequency (A & B) and the standardized defect types (C & D) for each population after mutagenesis treatment. Each dot represents the mean for each population. The bar for each history represent the mean across the three replicates for each history group. The * between the control and selection history groups indicates their means are significantly different ($p < 0.05$).

Fig. 4.

The wing vein defect frequency under different temperature treatment (benign and heat) for each history (control and selection population). The changes in defect frequency by heat (shown by the slopes) are significantly different between the control and selection history groups ($p = 0.011$).