Evolution of gene expression variance during adaptation to high temperature in *Drosophila*

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Abstract

Shifts in trait means are widely considered as evidence for adaptive responses, but the impact on phenotypic variance remains largely unexplored. Here, we studied gene expression variance of *Drosophila simulans* males before and after 100 generations of adaptation to a novel hot laboratory environment. In each of the two independently evolved replicate populations the variance of about 150 genes changed significantly (mostly reduction). Although different genes were affected in both replicates, these genes are related to digestion in the gut. This non-parallel selection response on the gene level in combination with a convergent response at a higher phenotypic level reflects genetic redundancy, a characteristic hallmark of polygenic adaptation. We propose that the constant and simple food source in the laboratory resulted in selection for reduced variance in digestive genes. In natural populations adaptation to diverse types of food may be beneficial, resulting in higher phenotypic variance. This empirical evidence of phenotypic variance being the direct target of selection during adaptation has important implications for strategies to identify selection signatures.

Introduction

Most studies of adaptation rely on shifts in trait mean as signal of selective response [1–6] . The variance of the trait in a population, which is the prerequisite for an adaptive response [7,8], has received considerably less attention. As a result, our understanding of the evolution of phenotypic variance is still rather limited. Probably most progress has been made in quantitative genetics, describing the evolution of phenotypic variance in response to a sudden shift in trait optimum [9–12]. For large populations and traits controlled by many unlinked loci

with equal effect, changes in trait optimum are not expected to affect the phenotypic variance [13,14]. In contrast, a much more complex picture is expected when the effect sizes are not equal, the population size is finite, or the traits have a simpler genetic basis [15–19].

In addition to these indirect effects on phenotypic variance, it is also possible that not trait mean, but the variance of a trait is the target of selection. For instance, stabilizing selection may reduce the variance of a trait [20]. Canalization, one potential consequence of stabilizing selection [21], describes the phenomenon that genetic and environmental perturbations can be buffered and henceforth reduce the phenotypic variance. A classic textbook example for a canalization factor is the heat shock protein Hsp70. Mutations of this chaperone gene result in increased phenotypic variance due to the unmasking of genetic variation [22]. Because canalization differs between populations, it has been proposed that it may also evolve [23,24].

We studied the evolution of gene expression variance after a shift in habitat and found that the variance of most genes remained unaffected, even for genes with a significant change in mean expression (Lai et al., 2021). Here, we focused on a small subset of genes, which changed their phenotypic variance during 100 generations of adaptation. We propose that selection operates directly on phenotypic variance. Because genes that evolved reduced variance were enriched in the gut and selection in natural populations may favor a high variance reflecting the diverse food sources, the constant and simple food source in the laboratory favored a single phenotype, resulting in a loss of phenotypic variance.

Results and discussion

Rapid changes in gene expression variance during adaptation

We measured the gene expression of ~20 whole body male individuals from two replicates of hot-evolved and reconstituted ancestral populations (Lai et al., 2021). After adapting for 100 generations to the high temperature regime, the transcriptomic response of hot-evolved populations was significantly diverged from their ancestors. Principle Component Analysis indicated that PC1 explained 11.9% of the total variation and separated the hot-evolved replicates from their ancestor which reflects the clear adaptive signatures to the novel, hot temperature regime (Lai et al., 2021). The variances of the expression of each gene were estimated and compared between the reconstituted ancestral populations and the two evolved populations. The usage of different lot numbers during the RNA-Seq library preparation

(Supplementary file 1), allowed to contrast only ancestral and evolved samples generated with the same lot number (See Materials and methods).

In both replicates, a small number of genes (166 and 148) significantly changed the expression variance after 100 generations of adaptation (F-test, FDR < 0.05; Figure 1; Supplementary file 2). Among the 166 genes with a significant change in variance in replicate 1, the variance of 125 genes decreased while only 41 genes showed a variance increase. This is a significant difference in the directionality of phenotypic variance evolution (χ^2=42.51, p-value < 7.0×10^{-11}). A similar difference was seen in replicate 2 (χ^2=14.30, p-value < 1.6×10^{-4}). 18 genes were shared between the two replicates. This suggests that the genes with significant changes in variance may be subjected to similar evolutionary processes.

Digestive genes in midgut rapidly decreased their transcriptional variance

In order to characterize plausible processes that could explain the significant changes in gene expression variance, we searched for gene ontology (GO) or tissue-specific expression enrichment. In both replicates genes with increased variance had no consistent enrichment in any biological processes or tissue-specific expression (Supplementary file 3 and 4). In contrast, despite mostly different genes had decreased variance in the two replicates, in both replicates a consistent enrichment for expression in the midgut was detected (Fisher's exact test, FDR < 0.05, Figure 2, Supplementary file 3). GO enrichment analysis identified also catabolism-related processes (e.g.: "organic substance catabolic process", "carbohydrate metabolic process" and "organonitrogen compound catabolic process") in both replicates (Supplementary file 4). In addition to the consistent enrichment in the midgut and catabolic processes, we also observed an enrichment for digestive enzymes [25] (Fisher's exact test, odds ratio = 4.21 and 3.53, p-value < 0.01), indicating that a different set of digestive genes in midgut rapidly decreased their transcriptional variance in the two replicates during 100 generations of adaptation. The enrichment in midgut and digestive genes persisted when we lowered the significance threshold of the F-test (FDR < 0.1, supplementary file 4), indicating that our result does not depend on a specific cutoff to define the genes with reduced gene expression variance.

Potential selection pressures for the reduction in expression variance in gut

Given the consistent enrichment for the same tissues and GO categories in two independent replicates, we conclude that random genetic drift is an unlikely explanation for the pronounced reduction of expression variance in these genes. Rather, we propose that the reduction in expression variance is a response to selection imposed by an altered environmental factor. Based on functional enrichment, we consider two different hypotheses that could explain the altered expression variance in the gut – microbiome and diet.

It is well-established that the microbiome has a pronounced effect on gene expression in the gut, but without a strong taxon-specific signal [26]. To rule out that heterogeneity in microbiome complexity explains the evolution of gene expression variance, we used all remaining flies of the same common garden experiment from one evolved replicate and the corresponding ancestral population (Supplementary file 1) to investigate the microbiome diversity. The β-diversity, which quantifies the heterogeneity in microbiome complexity within a population, was very similar for evolved and ancestral populations (Figure 3 and Table 1). Despite the limitations of a very reduced sample size, our result is consistent with previous studies [27]. Similarly, the microbiome composition cannot explain the reduced variance, as we observed high heterogeneity in composition among individuals from the ancestral and evolved populations (Fig. 3).

Alternatively, strong selection pressure on the phenotypic variance may have been imposed by the monotonic laboratory food. Natural *Drosophila* populations are feeding from different food sources in different microhabitats, that may require a broad gene expression diversity in digestive genes. We reason that such gene expression heterogeneity may be either deleterious in a simple laboratory environment or specific expression patterns may be optimal on the laboratory food (Figure 4). Either scenario imposes a strong stabilizing selection on phenotypic variance with no apparent directional effect on phenotypic mean in the novel environment for the focal populations.

To illustrate our hypothesis, we simulated a quantitative trait experiencing strong stabilizing selection over 200 generations and compared the dynamic of phenotypic variance with neutrality. Our results showed substantial decrease in phenotypic variance when strong stabilizing selection is imposed (Figure 5). This provides an illustrative support that the strong stabilizing selection caused by monotonic lab food could alter the transcriptomic variation in midgut digestion rapidly.

Genetic redundancy and its regulatory basis

A particularly interesting observation was that both replicates had different sets of genes with reduced variation, but both sets were enriched for genes expressed in the gut and the digestive enzymes. Such genetic redundancy is a hallmark of polygenic adaptation[28] and adaptation in replicated small populations provides an excellent opportunity to study it (Figure 6a). While in large populations more parallel selection responses are expected, genetic drift, in particular during the early stage, affects the selection outcome across replicate populations of moderate size. With genetic drift, the expression variance of digestion-related genes can be pushed in either direction. Henceforth, selection will favor genes for which drift acts synergistically with selection, leading to a heterogeneous outcome across replicates if there is sufficient polygenicity (i.e. more contributing loci than needed to reach the new trait optimum).

While we demonstrated genetic redundancy for genes involved in digestive function, the regulatory basis of the reduced variation is not yet clear. Gene expression can be regulated either in *cis* or in *trans*. *Cis*-regulation implies that independent regulatory variants are favored for each gene contributing to the selected phenotype (Figure 6b). It appears unlikely that each of the genes is independently targeted by selection. Rather, a more parsimonious explanation would be that several transcription factors (TFs) which cooperatively regulate these genes are the target of selection and reduced the expression variance of downstream genes. We explored this hypothesis and searched for *trans*-acting TF binding sites shared among genes with decreased expression variance and high expression in the midgut. We identified 18 and 8 TFs in replicate 1 and 2, some of which evolved their mean expression (Supplementary file 5), but none evolved a significant change in expression variance. The lack of significant variance evolution in these candidate targets of selection suggests a more complex regulation of transcriptional variance. We consider it highly likely that the expression of each redundant gene is in turn regulated by several trans-acting factors – providing a second layer of possible genetic redundancy (Figure 6b). Clearly, more work is needed to study the regulatory architecture of genetic redundancy, but the experimental framework introduced here provides an excellent starting point.

Concluding remarks

Previous studies on adaptive phenotypic evolution mainly focused on “population means”, to explain adaptation to different environments. Nevertheless, selection altering “phenotypic

variance within a population" (e.g.: stabilizing selection, disruptive selection…) has been largely neglected. To our knowledge our study provides the first empirical evidence that phenotypic variance can be the direct target of selection during adaptation. This has important consequences for future research on phenotypic evolution, rather than searching for changes in mean phenotype as a response to selection, it may also be important to consider that phenotypic variance may be subject to selection and can contribute to our understanding of adaptation processes in natural and experimental populations.

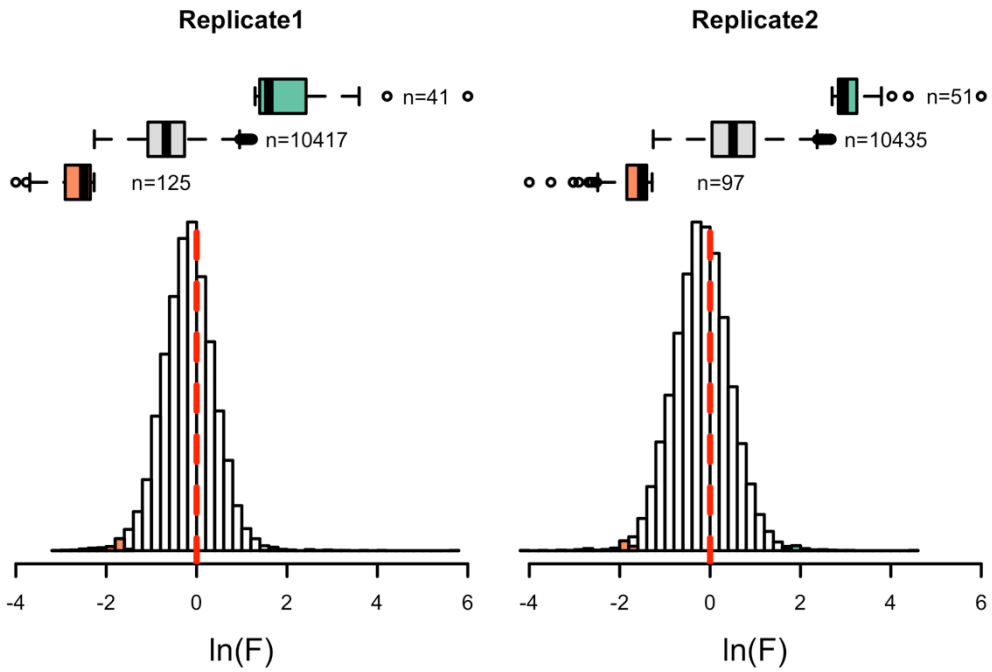


Figure 1. Evolution of gene expression variance. The distribution of the change in gene expression variances (ln(F)) during the evolution experiment in the 1st (left panel) and 2nd (right panel) replicate. Boxes in salmon indicate the genes with decreased variance in both replicates (n=125 and 97) and boxes in green represent genes with increased variance (n=41 and 51). Boxes in grey include the other genes without significant change in variance (n=10417 and 10435).

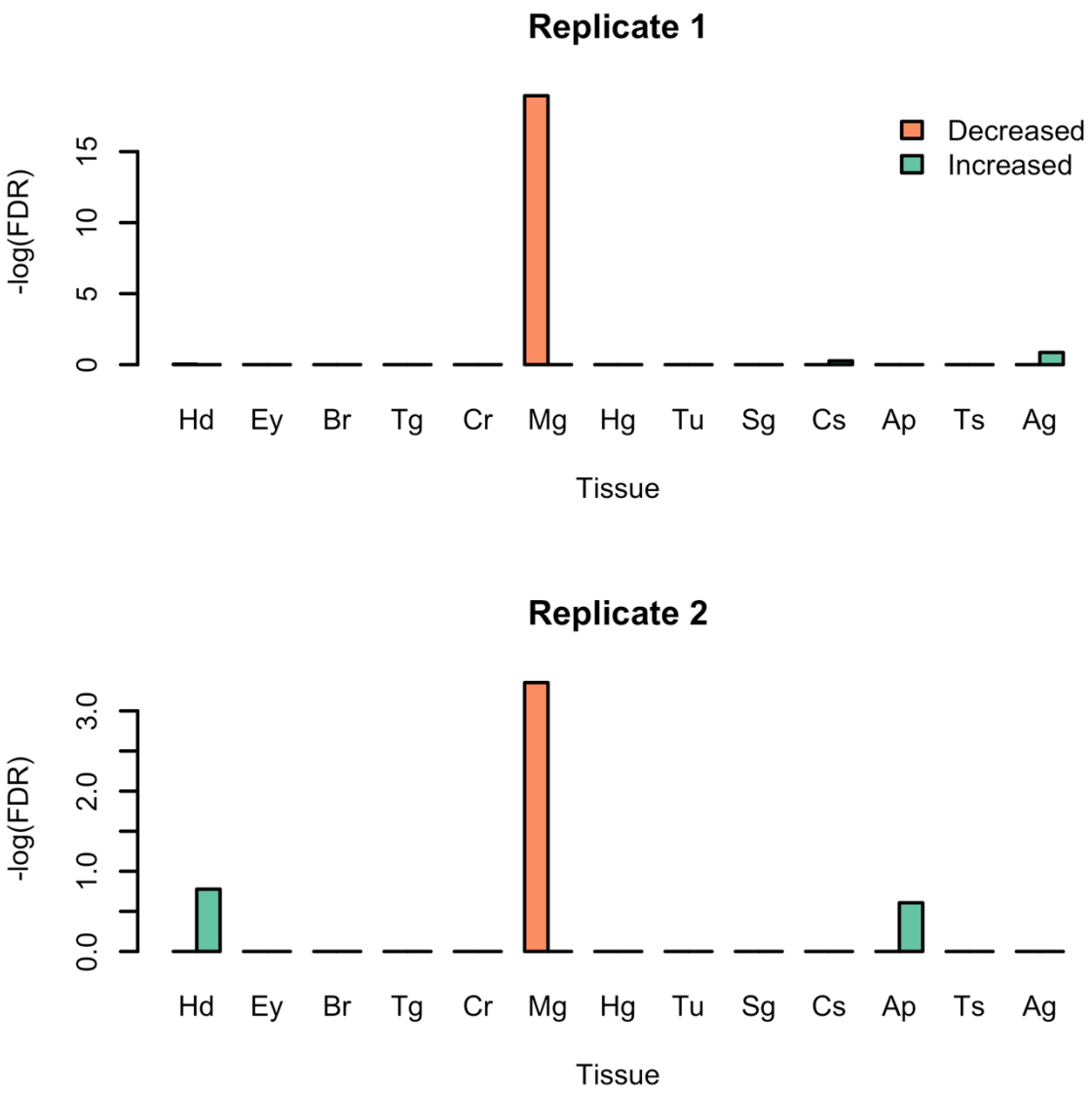


Figure 2. Tissue enrichment of genes with significant changes in expression variance. The bars indicates the significance (-ln(FDR)) of enrichment for genes with significant variance changes (orange: genes with decreased variance; green: genes with increased variance) among genes with tissue-specific gene expression pattern (Br-brain, Hd-head, Cr-crop, Mg-midgut, Hg-hindgut, Tb-malpighian tubule, Tg-thoracoabdominal ganglion, Cs-carcass, Sg-salivary gland, Fb-fat body, Ey-eye and Hr-heart). In both replicates a highly significant enrichment can be found in the midgut for genes with reduced expression variance.

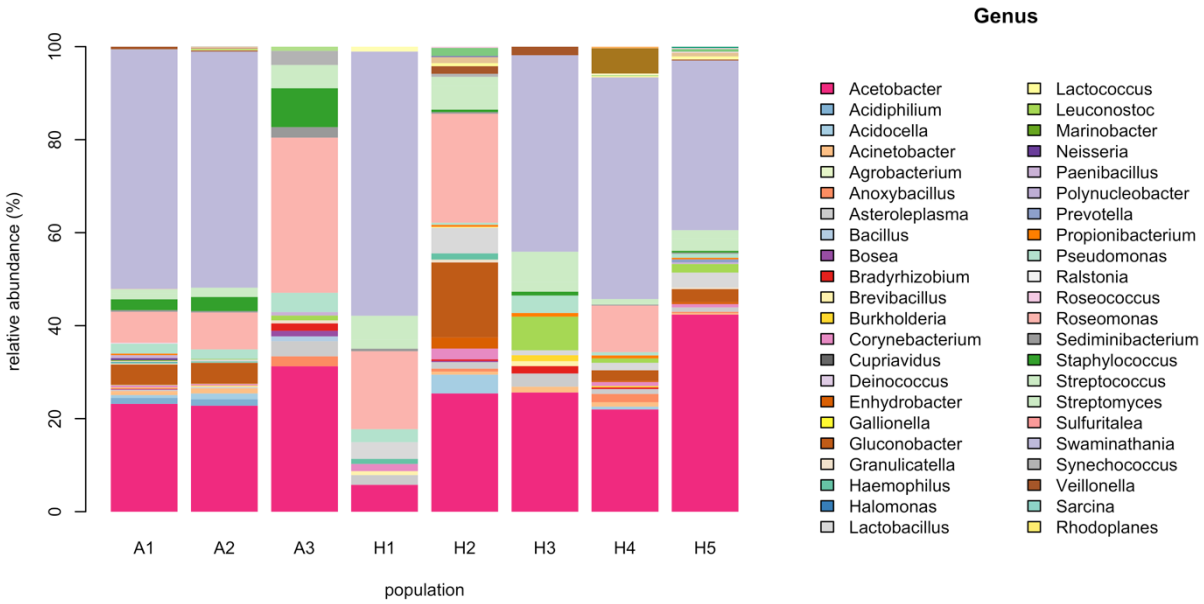


Figure 3. Microbiome composition in ancestral and evolved flies.

Microbiome composition on the genus level for three individuals from the ancestral population (A1-A3) and five individuals from a hot-evolved population (H1-H5).

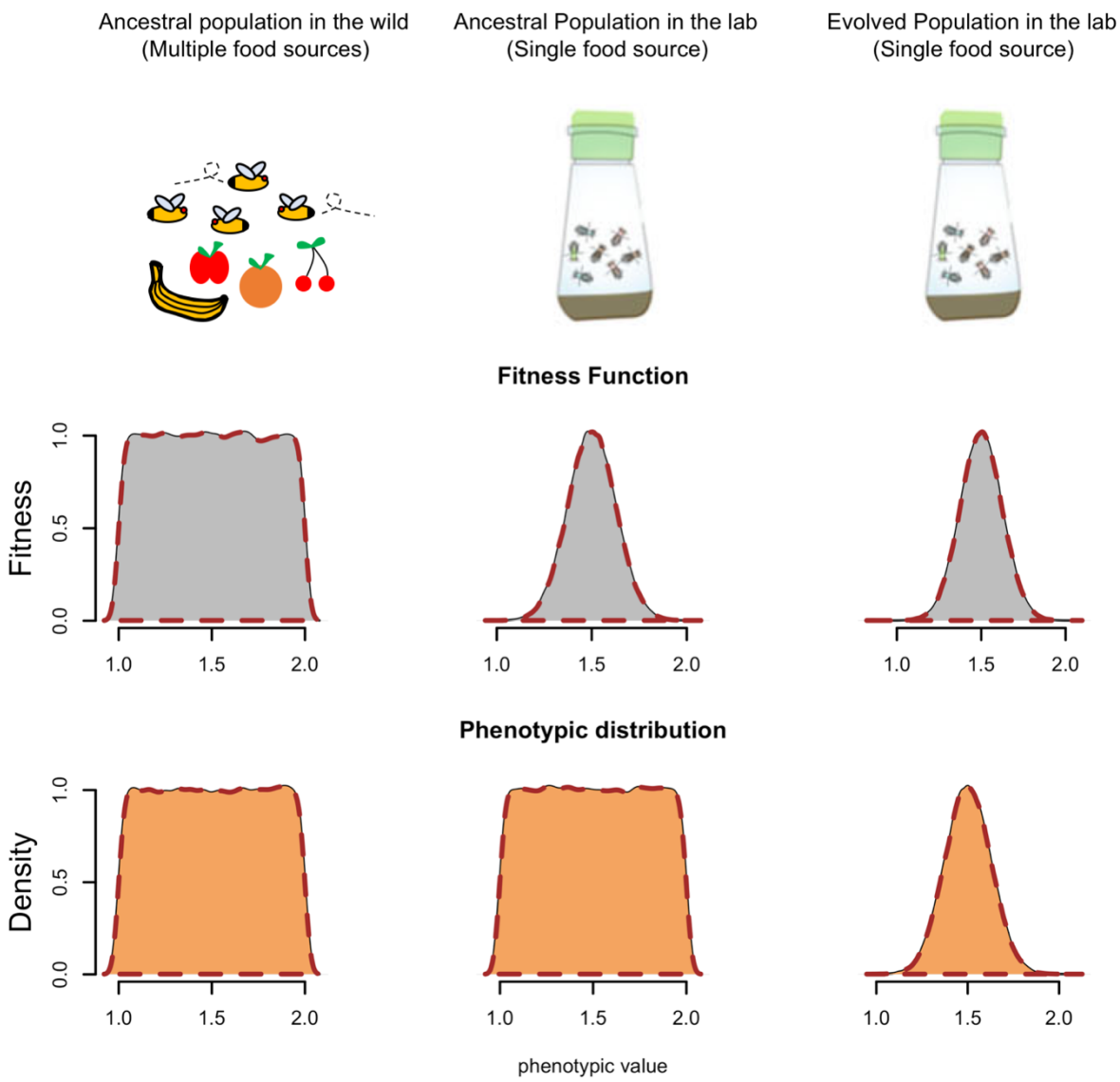


Figure 4. Hypothesis of a simpler lab food selecting for decreased gene expression variance in the midgut. A proposed model for potential selection for decreased expression variance in midgut imposed by the drastic change in food supply. Food sources change dramatically when we bring these files from the wild into the lab. The distributions of fitness landscapes and expression value of the genes encoding digestive enzymes may change as the food sources switched. After 100 generations on a single food source, the genes encoding digestive enzymes decreased their expression variance.

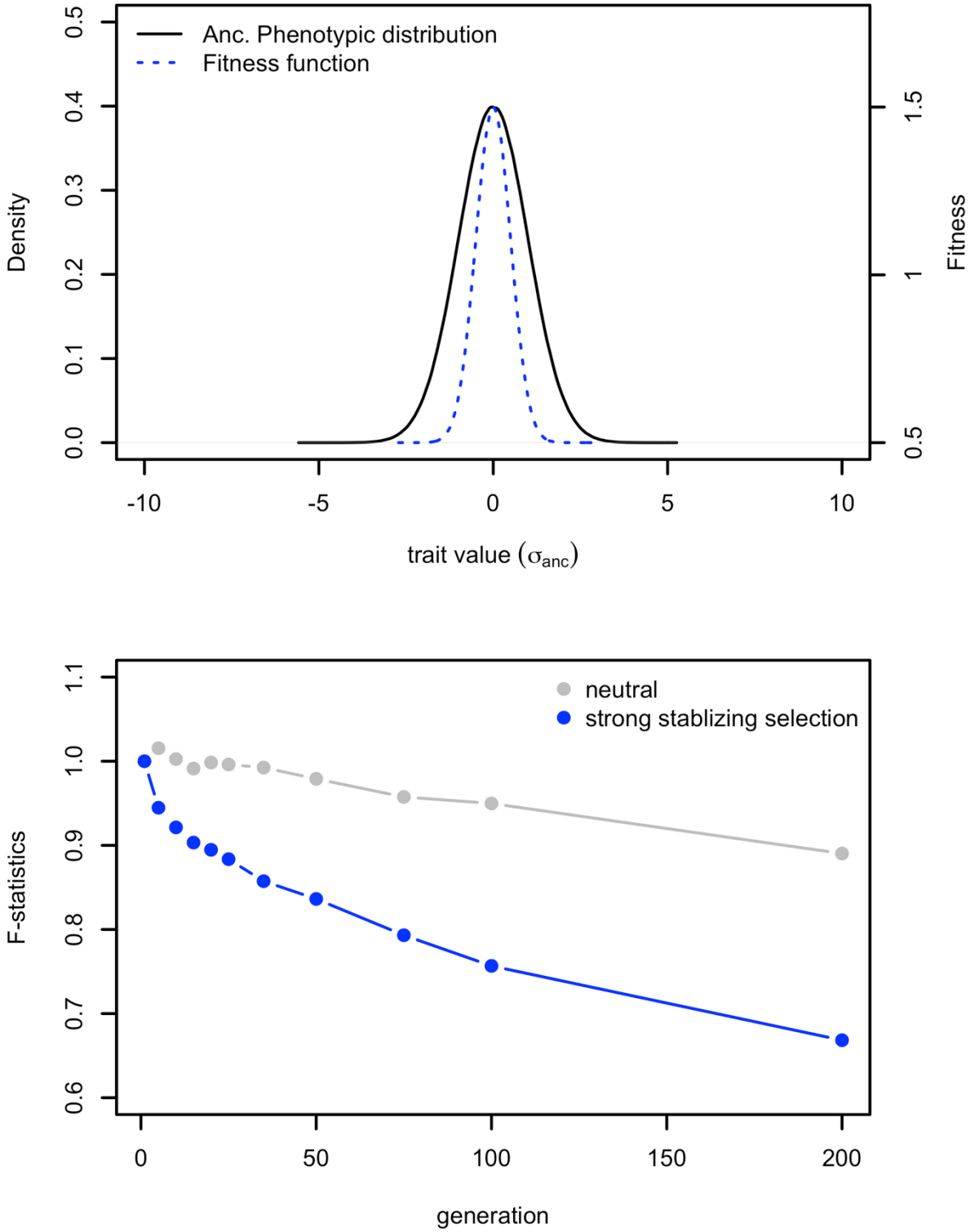
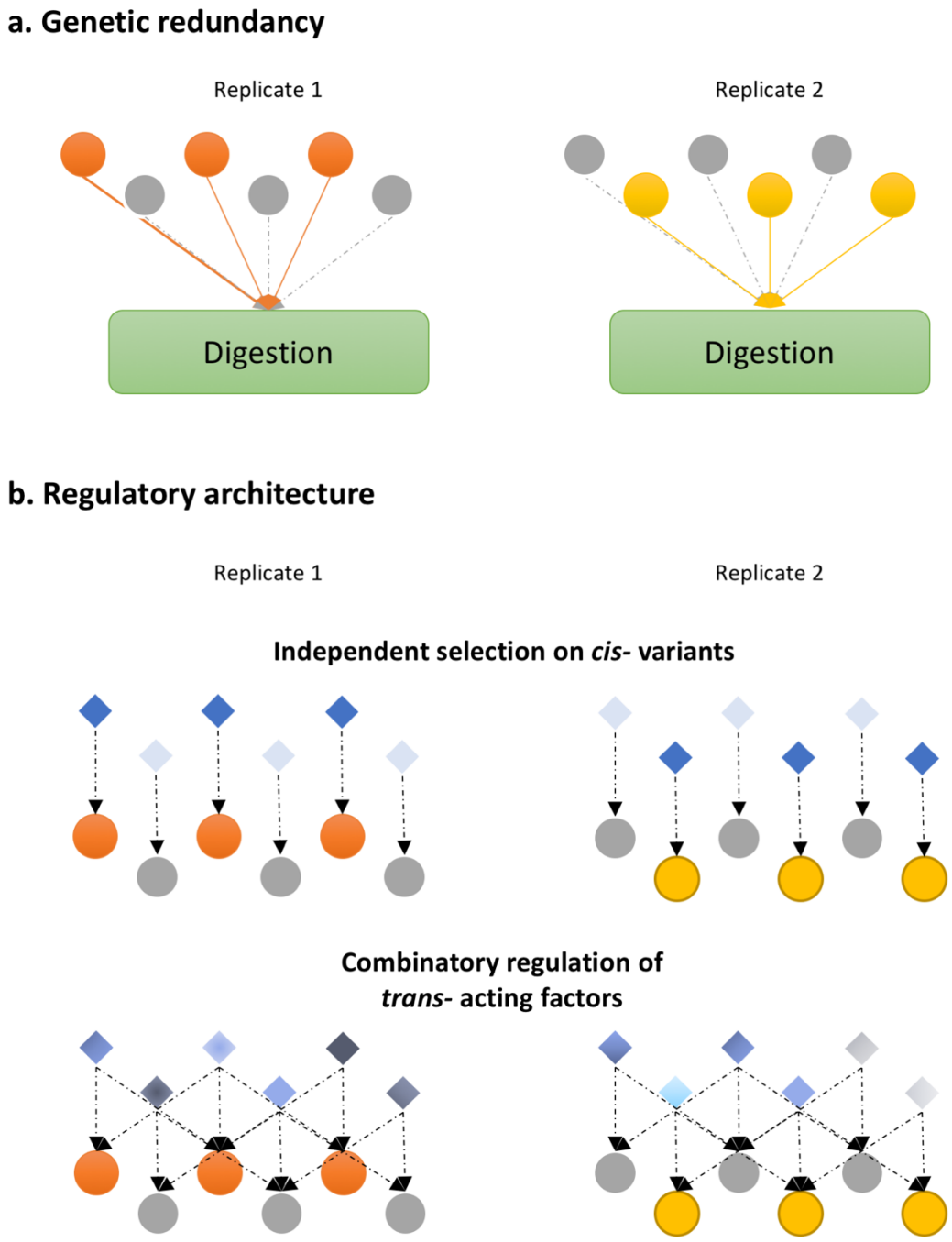


Figure 5. Reduction in variance by strong stabilizing selection.

a. Computer simulations of a scenario where the shift to a simpler environment results in stronger stabilizing selection. The ancestral phenotypic distribution of quantitative trait under stabilizing selection before the population was introduced to the simple environment (black).

The fitness function after the habitat shift is shown in blue. The variance of the fitness function is set to 0.5 standard deviation of the ancestral trait distribution. **b.** The changes in phenotypic variance under strong stabilizing selection (blue) and neutrality (grey). The change in phenotypic variance (F) is calculated as the ratio between the evolved and ancestral phenotypic variance at each generation ($\sigma^2_{gen.}/\sigma^2_0$) for each scenario. For each scenario, 1000 runs of simulations have been performed.

221



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224 **Figure 6. Schematic illustration of genetic redundancy at gene level with two possible**
225 **regulatory architectures explaining the reduction in expression variance. a.** Genetic
226 redundancy: six genes contribute to digestion (higher-level phenotype) and the new
227 phenotypic optimum could be reached by expression changes of three genes. Stochastic

effects result in different gene sets (orange/yellow) responding to selection in the two replicates (replicate1/replicate2). **b.** Two hypotheses about a regulatory architecture that allows for the rapid decrease in variance of digestion-related genes. Either selection acts independently on the *cis*-regulatory variants of each gene or combinatorial changes of several TFs reduce the expression variance.

236 **Table 1. Microbiome diversity in the reconstituted ancestral and hot-evolved population**
237 **based on 16S-rRNA amplicon sequencing**

	Ancestral	Evolved
α-diversity	23	16.7
β-diversity	1.83	1.8
γ-diversity	42	30

238

239 **Materials and methods**

240 Experimental evolution

241 The setup of populations and evolution experiment have been described by [29]. Briefly, ten
242 outbred populations seeded from 202 isofemale lines were exposed to a laboratory
243 experiment at 28/18 °C with 12hr light/12hr dark photoperiod for more than 100 generations.
244 Each replicate consisted of 1000 to 1250 adults at each generation.

245

246 Common garden experiment

247 The collection of samples from the evolution experiment for RNA-Seq was preceded by two
248 generations of common garden (CGE). The common garden experiment was performed at
249 generation 103 of the evolution in the hot environment and this CGE has been described in
250 [5,29–31]. In brief, an ancestral population was reconstituted by pooling five mated females from
251 184 founder isofemale lines [32]. Two replicates of the reconstituted ancestral population and
252 two independently evolved populations at generation 103 were reared for two generations
253 with egg-density control (400 eggs/bottle) at the same temperature regime as in the evolution
254 experiment. Freshly eclosed flies were transferred onto new food for mating. Sexes were
255 separated under CO2 anesthesia at day 3 after eclosure, left to recover from CO2 for two
256 days, and at the age of five days whole-body mated flies of each sex were snap-frozen at 2pm
257 in liquid nitrogen and stored at -80°C until RNA extraction. In this study, more than 30
258 individual male flies from two reconstituted ancestral populations (replicate no. 27 and no.
259 28) and two evolved populations (replicate no. 4 and no. 9) were subjected to RNA-Seq.

260

261 RNA extraction and library preparation

262 Whole bodies of individual male flies were removed from the -80°C freezer and immediately
263 homogenized in Qiazol lysis reagent (Qiagen, Hilden, Germany). The homogenate was
264 treated with DNase I followed by addition of chloroform, centrifugation and mixture of the
265 upper phase with 70% ethanol as described for the Qiagen RNeasy Universal Plus Mini Kit.
266 The mixture was subsequently loaded onto a RNeasy MinElute Spin column as provided by
267 the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany), and all washing steps were
268 performed according to the instructions for that kit. All resulting total RNA was used to
269 prepare stranded mRNA libraries on the Neoprep Library Prep System (Illumina, San Diego,
270 USA) following the manufacturer's protocol: Neoprep runs were performed using software
271 version 1.1.0.8 and protocol version 1.1.7.6 with default settings for 15 PCR cycles and an
272 insert size of 200bp. All libraries for individuals of ancestral replicate no. 27 and evolved

replicate no. 4 were prepared with library cards of lot no. 20180170; all libraries for individuals of ancestral replicate no. 28 and evolved replicate no. 9 were prepared with library cards of lot no. 20178099. 50bp single-end reads were sequenced on an Illumina HiSeq 2500. All sequencing data will be available in European Nucleotide Archive (ENA) under the accession number PRJEB37011 upon publication.

RNA-Seq data processing and quality check

All RNA-Seq reads were trimmed using ReadTools (Gómez-Sánchez and Schlötterer, 2018) with quality score of 20 and aligned to *Drosophila simulans* reference genome [33] using GSNAP [34] with parameter setting -k 15 -N 1 -m 0.08. Exon-aligned reads were piped into Rsubread [35] to calculate read counts of each gene, and raw read counts of each gene were normalized with the TMM method implemented in edgeR [36]. Samples with severe 3'- bias were removed based on visual inspection of the gene-body coverage plot [37,38]. We observed some outlier individuals and suspected that the freezing process may have led to detachment of body parts, such as eyes or heads, in these individuals. We compared gene expression between such outliers and all other samples and performed tissue enrichment analysis for genes with at least 2-fold lower expression in the outlier samples. Samples with evidence of tissue detachment were excluded. After filtering, each population remained approximately 20 individuals (Supplementary file 1). Only genes with at least 1 count per million base (CPM) were included in the analyses to avoid extremely lowly expressed genes.

RNA-Seq data analysis

For all RNA-Seq data we only compared samples which were prepared with library cards from the same lot number to avoid batch effects (Replicate 1: evolved replicate 4 vs. reconstituted ancestral population replicate 27; Replicate 2: evolved replicate 9 ss. reconstituted ancestral population replicate 28).

For the analysis of expression variance evolution, we applied natural log transformation [39] to eliminate the strong mean-variance dependency in RNA-Seq data due to the nature of the negative binomial distribution (Lai et al., 2021). The variance of the expression of each gene (lnCPM) was estimated in each population. The change of gene expression variance was determined with the F test between the variance within the ancestral population and the variance within the evolved population of each gene. P-value adjustment was performed using the Benjamini-Hochberg false discovery rate (FDR) correction.

307 Gene ontology and tissue enrichment analysis

308 We used ClueGO [40] to perform gene ontology (GO) enrichment analyses of the candidate
309 genes have significant change on variance. To understand in which tissues the genes of
310 interest are expressed, we made use of tissue-specific expression profiles of adult males of
311 *Drosophila melanogaster* on flyatlas2 [41]. This data set includes 13 tissues in male flies.
312 Genes that are expressed 2-fold higher in a given tissue than in the whole body are identified.
313 Fisher's exact test was performed to test if the genes of interest are enriched for genes highly
314 expressed in one tissue. P-value adjustment was performed using the Benjamini-Hochberg
315 false discovery rate (FDR) correction.

316

317 Microbiome diversity in ancestral and evolved populations

318 To explore the heterogeneity in gut microbiome, we performed 16S-rRNA amplicon
319 sequencing on three remaining individual males of the ancestral and evolved populations
320 from the same common garden experiment (Supplementary file 1).

321 We used primers designed to amplify the V3-V4 hypervariable regions of the 16S rRNA
322 gene. The primers had an overhang to match Nextera Index primers (Forward primer: 5'-
323 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3',
324 Reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-
325 GACTACHVGGGTATCTAATCC-3'). PCR products were amplified with 30 cycles at an
326 annealing temperature of 65°C, purified using AMPure XP beads (Beckman Coulter,
327 Carlsbad, CA) and subjected to a second PCR to introduce dual index sequences using
328 Nextera XT Index Kits (Illumina, San Diego, CA). In the second PCR, we used 6 cycles and
329 an annealing temperature of 55˚C, and both PCRs were carried out in 5µl total volume using
330 the NEBNext Ultra II Q5 Mastermix (New England Biolabs, Ipswich, MA). The final
331 amplicons were again purified, quantified using the Qubit HS assay kit (Invitrogen, Carlsbad,
332 CA), and 125 bp paired-end reads were sequenced on an Illumina HiSeq 2500.

333 The 16S-rRNA sequence data were trimmed using ReadTools [42] with quality score of 20.
334 Unpaired reads were removed. Owing to the variation in sequencing depths between samples,
335 all samples were down-sampled to the lowest depth (66,625 pairs/sample, Supplementary file
336 1). Each bam file was converted into a fastq.gz file and analyzed with Kraken2 [43] following
337 the recommended parameters and the estimation of genus abundance was corrected by
338 Bracken [44].

339 Genus abundance of the microbiome community in each sample was obtained. With the
340 filtration (read number < 5), extremely lowly abundant genera were excluded. β-diversity [45]
341 was then calculated to evaluate the heterogeneity of the microbiome complexity among the
342 three individuals from the same population.

343 $$\beta - diversity = \frac{\gamma - diversity}{\alpha - diversity}$$

344 where γ-diversity is the genera species richness in a population and α-diversity is the mean
345 richness within an individual.
346
347 Simulation study
348 We performed forward simulations with MimicrEE2 [46] using the *qff* mode to illustrate the
349 influence of the genetic architecture on the evolution of phenotypic variance during the
350 adaptation to a new trait optimum. With 189 founder haplotypes [29], we simulated quantitative
351 traits under the control of 20 numbers of loci with an effective population size of 300. For
352 each trait, we assume an additive model and the negative correlation (r=-0.7) between the
353 effect size ($\alpha \sim \Gamma(100,15)$) and starting frequency (Barghi et al., 2019). We used *correlate()*
354 function implemented in "fabricatr" R package [47] to generate the effect sizes with negative
355 correlation (r=-0.7) with starting frequency. The sum of effect sizes of each trait was
356 normalized to 1. We assumed heritability h^2 = 0.6. To simulate strong stabilizing selection
357 without trait optimum shift, we provided the fitness function: $N\left(\bar{x}_A, 0.5\sqrt{V_A}\right)$, where $\bar{x}_A$ is the
358 ancestral phenotypic mean and $\sqrt{V_A}$ is the ancestral genetic variance. For the neutrality case,
359 we assumed the same fitness for each individual. For each trait under each scenario, the
360 phenotypic variance was calculated at different generations and normalized to the ancestral
361 phenotypic variance at generation 1 to investigate the dynamic of phenotypic variance during
362 the evolution.
363
364 Transcription factor enrichment analysis
365 Transcription factor enrichment analysis among the genes with significant decreased variance
366 in the midgut was done with Rcistarget (version 1.0.2) [48]. First, enrichment of cis-regulatory
367 elements (CREs) 5kb upstream and intronic sequences of the genes of interest
368 (Supplementary file 5) was identified. The motif-search database used here was based on the
369 latest motif ranking files of Drosophila species ("dm6-5kb-upstream-full-tx-
370 11species.mc8nr.feather"). Parameter setting used in this analysis is as following:

371 nesThreshold = 5 and aucMaxRank = 0.05. The predicted transcription factors (TFs) were
372 considered as candidate TFs regulating the genes of interest.
373

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381

382 **Author contribution**

383 W.Y.L and C.S. conceived the study. W.Y.L performed the data analysis. W.Y.L. and C.S.
384 wrote the manuscript.
385

386 **Competing interests**

387 The authors declare no competing interests.

388 **Correspondence and requests for materials** should be addressed to C.S.
389
390

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