Evolution of phenotypic variance in response to a novel hot environment

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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. Classic quantitative genetics provides a theoretical framework to predict how selection on phenotypic mean affects the variance. In addition to this indirect effect, it is also possible that the variance of the trait is the direct target of selection, but experimentally characterized cases are rare. 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 populations, the variance of 125 and 97 genes was significantly reduced. We propose that the drastic loss in environmental complexity from nature to the lab may have triggered selection for reduced variance. Our observation that selection could drive changes in the variance of gene expression could have important implications for studies of adaptation processes in natural and experimental populations.

Background

Most studies of adaptation rely on shifts in trait mean as signal of selective response (Jakšić et al., 2020; Lemos, Bettencourt, Meiklejohn, & Hartl, 2005; Mallard, Nolte, Tobler, Kapun, & Schlötterer, 2018; Nuzhdin, Wayne, Harmon, & McIntyre, 2004; Oleksiak, Churchill, & Crawford, 2002; Whitehead & Crawford, 2006) . The variance of the trait in a population, which is the prerequisite for an adaptive response (Bull, 1987; Falconer & Mackay, 1963), 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 (Bulmer, 1972; Chevalet, 1994; Kimura & Crow, 1964; Turelli, 1984). 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 (Hayward & Sella, 2019; Lande, 1976). 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 (Barton & Keightley, 2002; Barton & Turelli, 1987; Franssen, Kofler, & Schlötterer, 2017; Jain & Stephan, 2015; Keightley & Hill, 1989).

In addition to these indirect effects on phenotypic variance, it is also possible that it is not the trait mean, but rather the variance of the trait that is the target of selection. For instance, stabilizing selection may reduce the variance of a trait (Schmalhausen, Isadore, & Dobzhansy, 1951). Canalization, one potential consequence of stabilizing selection (Le Rouzic, Álvarez-Castro, & Hansen, 2013), 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 (Rutherford & Lindquist, 1998). Because canalization differs between populations, it has been proposed that it may also evolve (Flatt, 2005; Rice, 1998).

The selective forces operating after a shift in habitat can be characterized by the evolution of gene expression mean and variance using an experimental evolution setup. When stabilizing selection becomes stronger, even with no directional selection being involved, we expect a pronounced reduction in variance. When directional selection comes into place, for genes/traits with simple genetic architectures, we expect to observe associated changes of both mean and variance. In contrast, only mean evolution is expected when directional selection is operated

on polygenic traits/gene expression. We studied gene expression variance of *Drosophila simulans* males before and after 100 generations of adaptation to a novel hot laboratory environment. We found no detectable difference in variance evolution between the genes with significant changes in mean expression and those without, and thus discussed the polygenic basis of the expression evolution for most genes in a separate manuscript (Lai, Nolte, Jaksic, & Schlötterer, 2021). In this study, we focused on a small subset of genes, which changed significantly their phenotypic variance during 100 generations of adaptation. Genes which evolved a reduced expression variance were strongly enriched in the gut and food processing. We discuss how a potential increase in stabilizing selection pressure could reduce gene expression variance after a drastic change in environmental complexity from nature to the lab. Our results suggest that the shift in environment can drive the evolution of phenotypic variance, which requires further attention in the interpretation of phenotypic evolution.

Materials and methods

Experimental evolution

The setup of populations and evolution experiment have been described by (Barghi et al., 2019). Briefly, ten outbred populations seeded from 202 isofemale lines were exposed to a laboratory experiment on standard *Drosophila* medium (300g Agar + 990g sugar beet syrup + 1000g Malt syrup + 2310g corn flour + 390g soy flour +900g yeast in 37.5L water) at 28/18 °C with 12hr light/12hr dark photoperiod for more than 100 generations. Each population consisted of 1000 to 1250 adults at each generation.

Common garden experiment

The collection of samples from the evolution experiment for RNA-Seq was preceded by two generations of common garden (CGE) to control for transgenerational and environmental effects. Most phenotypic differences observed in a common garden experiment should reflect genetic differences. The common garden experiment was performed at generation 103 of the evolution in the hot environment and this CGE has been described in (Barghi et al., 2019; Hsu et al., 2019, 2020; Jakšić et al., 2020). In brief, an ancestral population was reconstituted by pooling five mated females from 184 founder isofemale lines. Each isofemale line was maintained at small population size (typically less than 50 individuals) at 18 °C for ~50 generations before the reconstitution on standard laboratory food. Potential adaptation to the lab environment with the residual heterogeneity or de novo mutation in each line could be possible. Nevertheless, as discussed (Barghi et al., 2019), given the small effective population size during the maintenance of each line, most variants/mutations are effectively neutral and thus adaptation is unexpected. This has also been experimentally tested by contrasting allele frequencies in populations which were reconstituted shortly after the establishment of the lines and after 50 generations, but no significant allele frequency differences were found. This supports the idea that no consistent adaptation pattern can be recognized in isofemale lines maintained in the laboratory (Nouhaud, Tobler, Nolte, & Schlötterer, 2016). Furthermore, it is important to keep in mind that selection on standing genetic variation could only occur until an isofemale line is inbred. The low Ne may, however, allow the accumulation of deleterious mutations within the isofemale lines. Nevertheless, since most mutations occurring in the laboratory are mostly recessive (unlike mutations spreading in natural populations) (Charlesworth & Charlesworth, 2010), we expect very little impact as the two generations of common garden resulted in heterozygous flies, where recessive deleterious mutations are masked. The reconstituted ancestral populations and two independently evolved populations at

107 generation 103 were reared for two generations with egg-density control (400 eggs/bottle) at
108 the same temperature regime as in the evolution experiment. Freshly eclosed flies were
109 transferred onto new food for mating. Sexes were separated under CO_2 anesthesia at day 3 after
110 eclosure, left to recover from CO_2 for two days, and at the age of five days, whole-body mated
111 flies of each sex were snap-frozen at 2pm in liquid nitrogen and stored at -80°C until RNA
112 extraction. In this study, more than 30 individual male flies from two reconstituted ancestral
113 populations (no. 27 and no. 28) and two randomly selected evolved populations (no. 4 and no.
114 9) were subjected to RNA-Seq. While it would have been desirable to sequence all 10 available
115 populations, due to budget constraints, we had to limit our analysis to two populations, since
116 this already involved the generation and sequencing of more than 120 independent libraries.
117
118 RNA extraction and library preparation
119 Whole bodies of individual male flies were removed from the -80°C freezer and immediately
120 homogenized in Qiazol lysis reagent (Qiagen, Hilden, Germany). The homogenate was treated
121 with DNase I followed by addition of chloroform, centrifugation and mixture of the upper
122 phase with 70% ethanol as described for the Qiagen RNeasy Universal Plus Mini Kit. The
123 mixture was subsequently loaded onto a RNeasy MinElute Spin column as provided by the
124 RNeasy Plus Micro Kit (Qiagen, Hilden, Germany), and all washing steps were performed
125 according to the instructions for that kit. All resulting total RNA was used to prepare stranded
126 mRNA libraries on the Neoprep Library Prep System (Illumina, San Diego, USA) following
127 the manufacturer's protocol: Neoprep runs were performed using software version 1.1.0.8 and
128 protocol version 1.1.7.6 with default settings for 15 PCR cycles and an insert size of 200bp.
129 All libraries for individuals of ancestral population no. 27 and evolved population no. 4 were
130 prepared with library cards of lot no. 20180170; all libraries for individuals of ancestral
131 population no. 28 and evolved population no. 9 were prepared with library cards of lot no.
132 20178099. 50bp single-end reads were sequenced on an Illumina HiSeq 2500.
133
134 RNA-Seq data processing and quality check
135 All RNA-Seq reads were trimmed using ReadTools (Gómez-Sánchez and Schlötterer, 2018)
136 with quality score of 20 and aligned to *Drosophila simulans* reference genome (Palmieri, Nolte,
137 Chen, & Schlötterer, 2015) using GSNAP (Wu, Reeder, Lawrence, Becker, & Brauer, 2016)
138 with parameter setting -k 15 -N 1 -m 0.08. The reference genome has been annotated using the
139 flybase gene IDs of *D. melanogaster* for all orthologous genes (Palmieri et al., 2015). Exon-
140 aligned reads were piped into Rsubread (Liao, Smyth, & Shi, 2019) to calculate read counts of

141 each gene, and raw read counts of each gene were normalized for library size and subjected to
142 the TMM normalization in edgeR (Robinson, McCarthy, & Smyth, 2010). Samples with severe
143 3'-bias were removed based on the gene-body coverage plot (Jakšić & Schlötterer, 2016; Wang,
144 Wang, & Li, 2012). Additionally, we removed five individuals where the freezing process may
145 have led to detachment of body parts, such as eyes or heads. Specifically, we compared gene
146 expression between each sample and all other samples and identified the samples exhibiting
147 outlying expression pattern. Tissue enrichment analysis was performed for genes with at least
148 2-fold lower expression in the outlying samples to provide significant evidence of tissue
149 detachment. After quality control, each population remained approximately 20 individuals
150 (Supplementary file 1). Only genes with at least 1 count per million (CPM) were included in
151 the analyses to avoid extremely lowly expressed genes.
152
153 RNA-Seq data analysis
154 For all RNA-Seq data, we only compared samples which were prepared with library cards from
155 the same lot number to avoid batch effects (Comparison 1: evolved population no. 4 vs.
156 reconstituted ancestral population no. 27; Comparison 2: evolved population no. 9 vs.
157 reconstituted ancestral population no. 28).
158 For the analysis of the evolution of mean/variance in gene expression, we applied natural log
159 transformation (Heath, Bulfield, Thompson, & Keightley, 1995) to eliminate the strong
160 positive mean-variance correlation in RNA-Seq data due to the nature of the negative binomial
161 distribution (Supplementary figure 1) (Lai et al., 2021). The mean and variance of the
162 expression of each gene (lnCPM) were estimated in each population. The change of gene
163 expression mean was determined with the linear modeling framework implemented in limma
164 (Ritchie et al., 2015). The change of gene expression variance was determined with the F test
165 between the variance within the ancestral population and the variance within the evolved
166 population for each gene. P-value adjustment was performed using the Benjamini-Hochberg
167 false discovery rate (FDR) correction. To demonstrate concordant evolution in both replicates,
168 we tested whether more genes with significant variance/mean evolution in the same direction
169 were detected than expected by chance with Fisher's exact tests. In addition, we investigated
170 to which extent the heterogeneous response can be explained by stochastic variation due to the
171 experimental noise or sampling processes. Within the first comparison, we resampled twice 15
172 samples from both the evolved and reconstituted ancestral populations, performed two tests for
173 variance (contrasting the evolved and ancestral samples) and estimated the consistency

174 between the tests using the Jaccard Index as $\frac{n(A \cap B)}{n(A \cup B)}$, where A and B are the candidate gene sets
175 of the two tests. This process is repeated 100 times, generating the null distribution of Jaccard
176 Indices considering sole stochastic effects within an evolutionary replicate. We applied a
177 similar sampling scheme of 15 individuals to evaluate the consistency in evolution between
178 populations. We contrasted the evolved and ancestral samples for the two populations (rather
179 than for two samples of the same population, which was done to obtain the null distribution).
180 The observed Jaccard indices from 100 times of resampling were compared to the null
181 distribution.
182 We compared the proportion of genes with significant differences in mean in genes with and
183 without significant reduction in variance to evaluate whether the reduced variance is associated
184 with the selection on mean. Furthermore, we correlated the changes in gene expression
185 variance (ln(F)) with the magnitude of mean evolution (absolute value of mean difference) for
186 the genes with significant changes in either expression mean or variance.
187
188 Gene ontology and tissue enrichment analysis
189 We used ClueGO (Bindea et al., 2009) to perform gene ontology (GO) enrichment analyses of
190 the candidate genes have significant change on variance. To understand in which tissues the
191 genes of interest are expressed, we made use of tissue-specific expression profiles of adult
192 males of *Drosophila melanogaster* on flyatlas2 (Leader, Krause, Pandit, Davies, & Dow, 2018).
193 This data set includes 13 tissues in male flies. Genes that are expressed 4-fold higher in a given
194 tissue than in the whole body are identified. Fisher's exact test was performed to test if the
195 genes of interest are enriched for genes highly expressed in one tissue. P-value adjustment was
196 performed using the Benjamini-Hochberg false discovery rate (FDR) correction.
197
198 Microbiome diversity in ancestral and evolved populations
199 To explore the heterogeneity in gut microbiome, we performed 16S-rRNA amplicon
200 sequencing on three remaining individual males of the ancestral and evolved populations from
201 the same common garden experiment (Supplementary file 1).
202 We used primers designed to amplify the V3-V4 hypervariable regions of the 16S rRNA gene.
203 The primers had an overhang to match Nextera Index primers (Forward primer: 5'-
204 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3',
205 Reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-
206 GACTACHVGGGTATCTAATCC-3'). PCR products were amplified with 30 cycles at an

207 annealing temperature of 65°C, purified using AMPure XP beads (Beckman Coulter, Carlsbad,
208 CA) and subjected to a second PCR to introduce dual index sequences using Nextera XT Index
209 Kits (Illumina, San Diego, CA). In the second PCR, we used 6 cycles and an annealing
210 temperature of 55°C, and both PCRs were carried out in 5µl total volume using the NEBNext
211 Ultra II Q5 Mastermix (New England Biolabs, Ipswich, MA). The final amplicons were again
212 purified, quantified using the Qubit HS assay kit (Invitrogen, Carlsbad, CA), and 125 bp paired-
213 end reads were sequenced on an Illumina HiSeq 2500.
214 The 16S-rRNA sequence data were trimmed using ReadTools (Gómez-Sánchez & Schlötterer,
215 2018) with quality score of 20. Unpaired reads were removed. Owing to the variation in
216 sequencing depths between samples, all samples were down-sampled to the lowest depth. Each
217 bam file was converted into a fastq.gz file and analyzed with Kraken2 (Wood, Lu, & Langmead,
218 2019) following the recommended parameters and the estimation of genus abundance was
219 corrected by Bracken (Lu, Breitwieser, Thielen, & Salzberg, 2017).
220 Genus abundance of the microbiome community in each sample was obtained. With the
221 filtration (read number < 5), extremely lowly abundant genera were excluded. β-diversity
222 (Tuomisto, 2010) was then calculated to evaluate the heterogeneity of the microbiome
223 complexity among the three individuals from the same population.

224 $$\beta - diversity = \frac{\gamma - diversity}{\alpha - diversity}$$

225 where γ-diversity is the genera species richness in a population and α-diversity is the mean
226 richness within an individual.
227
228 Simulation study
229 We performed forward simulations with MimicrEE2 (Vlachos & Kofler, 2018) using the *qff*
230 mode to illustrate the influence of the genetic architecture on the evolution of phenotypic
231 variance during the adaptation to a new trait optimum. With 189 founder haplotypes (Barghi
232 et al., 2019), we simulated quantitative traits under the control of 20 numbers of loci with an
233 effective population size of 300. For each trait, we assume an additive model and the negative
234 correlation (r=-0.7) between the effect size ($\alpha \sim \Gamma(100,15)$) and starting frequency (Barghi et
235 al., 2019). We used *correlate()* function implemented in "fabricatr" R package (Blair et al.,
236 2019) to generate the effect sizes with negative correlation (r = -0.7) with starting frequency.
237 The sum of effect sizes of each trait was normalized to 1. We assumed heritability h^2 = 0.6. To
238 simulate strong stabilizing selection without trait optimum shift, we provided the fitness

239 function: $N\left(\bar{x}_A, 0.5\sqrt{V_A}\right)$, where $\bar{x}_A$ is the ancestral phenotypic mean and V_A is the ancestral
240 genetic variance. For the neutrality case, we assumed the same fitness for each individual. For
241 each trait under each scenario, the phenotypic variance was calculated at different generations
242 and normalized to the ancestral phenotypic variance at generation 1 to investigate the dynamic
243 of phenotypic variance during the evolution.
244
245 Transcription factor enrichment analysis
246 Transcription factor enrichment analysis among the genes with significant decreased variance
247 in the midgut was done with Rcistarget (version 1.0.2) (Aibar et al., 2017). First, enrichment
248 of cis-regulatory elements (CREs), 5kb upstream and intronic sequences, of the genes of
249 interest was identified. The motif-search database used here was based on the latest motif
250 ranking files of *Drosophila* species ("dm6-5kb-upstream-full-tx-11species.mc8nr.feather").
251 Parameter setting used in this analysis is as following: nesThreshold = 5 and aucMaxRank =
252 0.05. The predicted transcription factors (TFs) were considered as candidate TFs regulating the
253 genes of interest.
254

255 **Results**

256 ***Rapid changes in gene expression variance during adaptation***

257 We measured the gene expression of 19-22 whole body male individuals from two populations
258 independently evolved in a novel hot environment and reconstituted ancestral populations (81
259 samples in total). After adapting for 100 generations to the high temperature regime, the
260 transcriptomic response of hot-evolved populations was significantly diverged from their
261 ancestors. Principal Component Analysis indicated that PC1 explained 11.9% of the total
262 variation and separated the hot-evolved flies from their ancestor which reflects the clear
263 adaptive signatures to the novel, hot temperature regime (Lai et al., 2021). The variances of the
264 expression of each of the 10,583 genes were estimated and compared between the reconstituted
265 ancestral populations and the two evolved populations. Only samples from the same lot number
266 of library preparation (Supplementary file 1) were compared (See Materials and Methods).

267

268 In both hot evolved populations, a small number of genes (166 and 148) significantly changed
269 in expression variance after 100 generations of adaptation (F-test, FDR < 0.05; Figure 1;
270 Supplementary file 2). Among the 166 genes with a significant change in variance in
271 population 1, the variance of 125 genes decreased while only 41 genes showed a variance
272 increase. This is a significant difference in the directionality of phenotypic variance evolution
273 (χ^2=42.51, p-value < 7.0×10^{-11}). A similar difference was seen in population 2 (97 decreased
274 and 51 increased; χ^2=14.30, p-value < 1.6×10^{-4}). 14 genes with a significant variance change
275 in the same direction (11 decreased and three increased) were shared between the two
276 independently evolved populations, which is significantly more than expected by chance
277 (Fisher's Exact Test, odds ratio = 7.07, p-value < 7.52×10^{-8}). Hence, the same selection
278 pressure was likely to drive the loss of expression variance in the two populations evolving
279 independently in the same novel hot environment. This parallel variance change persisted when
280 we lowered the significance threshold of the F-test to FDR < 0.1 that yield 274 and 229 genes
281 with significant changes in variance in both populations (Fisher's Exact Test, odds ratio = 4.49,
282 p-value < 2.49×10^{-8}).

283

284 ***Reduction in expression variance is not associated with changes in mean expression levels***

285 Previous analyses of the same evolved populations identified many genes with a significant
286 shift in mean expression and associated biological processes in response to adaptation to the
287 new temperature regime in the laboratory (Barghi et al., 2019; Hsu et al., 2020; Jakšić et al.,
288 2020). Consistent with these results, the comparison of ancestral and evolved populations in

289 this study identified 2,775 genes in the first replicate and 2,677 genes in the second replicate
290 which significantly changed mean expression (Supplementary file 3). Most of them were
291 shared with those detected in previous studies (Jakšić et al., 2020) (Fisher's exact test, odds
292 ratio = 4.1 and 3.0, FDR < 2.2×10^{-16}). 94.1% of the genes with a significant mean expression
293 change in both evolved populations changed in the same direction, significantly more than
294 expected by chance ($\chi^2 = 898.03$, p-value < 2.2×10^{-16}). This concordance suggests that
295 most of the altered expression means are mainly driven by selection, rather than by drift. If
296 these changes in mean expression are driven by selection either on *cis*-regulatory variation or
297 few *trans*-acting factors, it is expected that changes in mean are also associated with reduced
298 expression variance. For genes with a significant decrease in variance, the proportion of genes
299 with significant change in mean was slightly higher compared to genes without variance
300 change, but the difference is not significant (Figure 2, $\chi^2 = 3.96\ and\ 1.96$, FDR > 0.05).
301 Further support for the independence of variance and mean changes in expression comes from
302 the observation that, out of 11 genes with reduced variance in both populations, only one gene
303 showed a significant change in mean in both populations. Furthermore, the magnitude of mean
304 changes was basically not correlated with the changes in variance among genes with
305 significantly altered means or variances (Supplementary figure 2, Spearman's rho = 0.047 and
306 -0.001). These results suggest that mean and variance are evolving independently, probably
307 because of different selection pressures.
308
309 ***Digestive genes in midgut rapidly decreased their transcriptional variance***
310 In order to characterize processes that could explain the significant changes in gene expression
311 variance, we searched for gene ontology (GO) or tissue-specific expression enrichment. In both
312 populations, genes with increased variance had no consistent enrichment in any biological
313 processes or tissue-specific expression (Supplementary file 4 and 5). In contrast, for the genes
314 with decreased variance, the enrichment for expression in the midgut was significant for both
315 populations (Fisher's exact test, FDR < 0.05, Figure 3, Supplementary file 4). We note that
316 allometric changes during adaptation cannot explain this pattern since all genes expressed in
317 the affected tissues would be modified. We found, however, that different sets of midgut-
318 expressed genes changed their expression variance in the two populations (Supplementary file
319 6). GO enrichment analysis identified also catabolism-related processes (e.g.: "organic
320 substance catabolic process", "carbohydrate metabolic process" and "organonitrogen
321 compound catabolic process") in both populations (Supplementary file 5). In addition to the

consistent enrichment in the midgut and catabolic processes, we also observed an enrichment for digestive enzymes (Lemaitre & Miguel-Aliaga, 2013) (Fisher's exact test, odds ratio = 4.21 and 3.53, FDR < 0.01, Supplementary file 6), indicating that different sets of digestive genes in midgut rapidly decreased their transcriptional variance in the two populations 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.

Next, we focused on the 11 genes that showed parallel variance reduction in both populations. We note that 11 genes are not sufficient for a robust enrichment analysis despite several of them are relevant to digestion itself or feeding preference. *CG7025* (*FBgn0031930*), which is highly expressed in midgut (Leader et al., 2018). It was annotated as a digestive enzyme (Lemaitre & Miguel-Aliaga, 2013) and is part of the environment/microbiome-induced response in gut (Broderick, Buchon, & Lemaitre, 2014; Li et al., 2009; MacMillan et al., 2016). *CG1440* (*FBgn0030038*) is a peptidase involved in proteolysis (Larkin et al., 2021). It is expressed in digestive system (Larkin et al., 2021) and is related to the hedgehog signaling in response to dietary limitation (Çiçek et al., 2016). *Unc-119* (*FBgn0025549*), *Miga* (*FBgn0030037*), *CG12581* (*FBgn0037213*) and *CG32017* (*FBgn0052017*) are involved in olfactory memory formation (Walkinshaw et al., 2015), which may contribute to feeding preference.

Discussion

Change in gene expression variance is a selective response

Contrasting the gene expression of individual flies from ancestral and evolved populations, we studied the evolution of gene expression variance, a key parameter to understand adaptation which has not received sufficient attention in empirical studies. More than 100 genes experienced a significant change in variance during 100 generations of evolution in a novel hot environment. We consider this a conservative estimate because with a sample size of 20 individuals only rather pronounced variance shifts are significant. The analysis of more individuals probably would have identified even more genes with a significant change in variance. The significant enrichment of catabolism related GO categories indicates that the reduction in variance did not affect a random set of genes, as expected for stochastic changes occurring during 100 generations of evolution. While unlikely, it is conceivable that the enrichment for specific GO categories and tissues in a single population could be the outcome of genetic drift affecting a master regulator for these processes. Nevertheless, we observed that in two independent populations, not only the same GO categories and tissue enrichment was observed, but also more genes with a reduced variance were shared between the two populations than expected by chance. We conclude that the observed pattern of variance change reflects a shared selection response across independently evolved populations.

The lack of significant association between the genes with significantly reduced variance and the genes with significant mean changes indicates the operation of an independent stabilizing selection pressure reducing the variance of gene expression. Given that number of genes with significant differences in mean expression was much larger (>2500) than the number of genes with significant variance change (~100), it might appear that selection on mean is stronger than selection on variance. Nevertheless, the power to detect changes in mean and variance differs and contributes to the discrepancy in the number of significant genes in the two categories. A larger sample size provides more power, but the difference in power to detect changes in mean and variance change in gene expression would persist.

Potential selection pressures for the reduction in expression variance in gut

Based on the functional enrichment of catabolism related GO categories and enrichment for genes expressed in the gut, we considered different hypotheses that could explain the altered expression variance in the gut – microbiome, temperature 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 (Kokou et al., 2018). To investigate whether 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 population and the reconstituted ancestral population (Supplementary file 1). The β-diversity, which quantifies the heterogeneity in microbiome complexity within a population, was very similar for evolved and ancestral populations (Supplementary figure 3 and Table 1). Despite the limitations of a very reduced sample size, our result is consistent with previous studies (Wong, Ng, & Douglas, 2011). The microbiome composition cannot explain the reduced variance as we observed high heterogeneity in composition among individuals from both the ancestral and evolved populations (Supplementary figure 3).

The experimental evolution study has been designed to study the impact of a shift in mean temperature, which remained constant across the entire experiment (Barghi et al., 2019). Natural populations experience substantial environmental heterogeneity – either seasonal or, due to microclimatic variation. As temperature modulates feeding preference and possibly digestion in *Drosophila* (Brankatschk et al., 2018), it is conceivable that some of the reduced expression variance might be explained by a more homogeneous temperature regime. Nevertheless, it is noteworthy that mean expression in several tissues are strongly affected by temperature – such as neural system and fat body (Hsu et al., 2020; Jakšić et al., 2020), but the involved genes do not experience a reduction in expression variance. In contrast, the reduction in gene expression variance in the gut implies specific selection pressure on the transcriptional variation of a subset of genes.

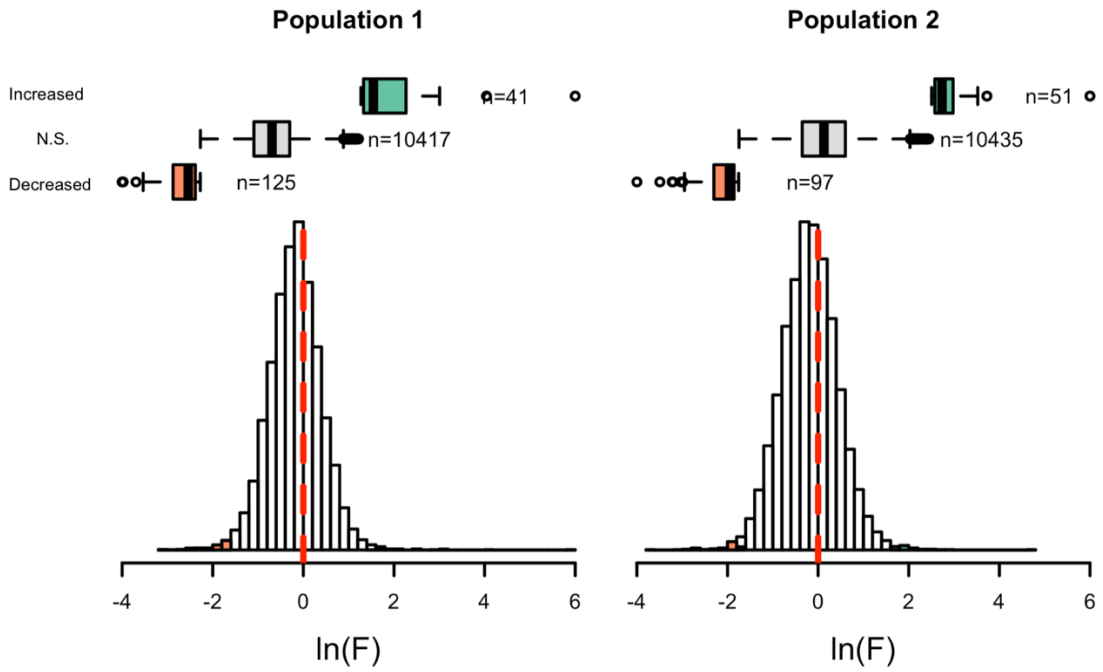
Alternatively, the reduced variance in midgut and digestion could also be caused by the unvarying diet in the laboratory. Natural *Drosophila* populations are feeding from different food sources in different microhabitats, that will result in a broader distribution of phenotypes in the ancestral population compared to laboratory evolved ones. This implies that such gene expression heterogeneity would be associated with fitness costs in a simple laboratory environment or specific expression patterns may be optimal on the laboratory food. Either scenario is consistent with polygenic adaptation driven by a narrower fitness function.

Given the experimental challenge to functionally validate putative selection pressures modifying expression variance, we performed computer simulations to illustrate our hypothesis

412 that gene expression variance could be the direct target of selection during adaptation (Figure
413 4). We simulated a quantitative trait experiencing stabilizing selection over 200 generations
414 and compared the dynamic of phenotypic variance with neutrality. Our results showed
415 substantial decrease in phenotypic variance when stabilizing selection is imposed
416 (Supplementary figure 4). This provides an illustrative support that the stabilizing selection
417 caused by monotonic environment could alter the transcriptomic variation rapidly.
418
419 Nevertheless, we acknowledge that canalization may have evolved and stabilizing selection
420 could also act on the environmental component (i.e. expression noise of the genes) to reduce
421 the phenotypic variance within population. Nevertheless, it is important to note that in this case
422 canalization would act only on a subset of functionally related genes. Further work on
423 canalization is required to determine the plausibility of this interpretation.
424
425 ***Genetic redundancy and its regulatory basis***
426 Another interesting observation was that both populations have different sets of genes with
427 reduced variation (though the overlap is significantly more than random expectation), but both
428 sets were enriched for genes expressed in the gut and the digestive enzymes (Supplementary
429 file 6). Since we relied on a significance threshold to identify genes with a significant change
430 in expression variance, the heterogeneity of the response in two independent evolved
431 populations may (partly) be explained by stochastic effects reducing the power to detect the
432 same genes in both replicates. To address this, we used a less stringent significance threshold
433 (FDR of 0.1), but the heterogeneity in genes with significant expression differences between
434 the two replicates persisted. Moreover, we show that resampling within the same replicate
435 results in significantly more consistency than observed between the two populations (see
436 material and method; Supplementary figure 5). This suggests that the stochastic effects cannot
437 explain the heterogeneity observed between replicates. Rather, the heterogeneity also reflects
438 the genetic redundancy during polygenic adaptation (Figure 5a; Barghi, Hermisson, &
439 Schlötterer, 2020), which has been previously documented at the genomic level in the studied
440 populations (Barghi et al., 2019). Our results are consistent with redundancy not only at the
441 genomic but also at the gene expression level. If more contributing loci are segregating in the
442 founder population than needed to reach the new trait optimum, the choice of redundant
443 adaptive routes (variants/genes) is stochastic. This is particularly relevant during the early stage
444 where drift affects the selection outcome (both for mean and variance) across replicate
445 populations of moderate size (Bolnick, Barrett, Oke, Rennison, & Stuart, 2018; Langmüller &

446 Schlötterer, 2020). With such initial stochasticity, the expression variance of digestion-related
447 genes can be pushed in either direction. Henceforth, selection for variance reduction will favor
448 genes for which drift acts synergistically with selection, leading to a heterogeneous outcome
449 across populations (i.e. variance is reduced for different genes in the two replicates).
450
451 While we discussed the genetic redundancy for genes involved in digestive function, the
452 regulatory basis of the reduced variation is not yet clear. Gene expression can be regulated
453 either in *cis* or in *trans*. *Cis*-regulation implies that independent regulatory variants are favored
454 for each gene contributing to the selected phenotype (Figure 5b). It appears unlikely that each
455 of the genes is independently targeted by selection. Rather, a more parsimonious explanation
456 would be that several transcription factors (TFs) which cooperatively regulate these genes are
457 the target of selection and reduced the expression variance of downstream genes. We explored
458 this hypothesis and searched for *trans*-acting TF binding sites shared among genes with
459 decreased expression variance and high expression in the midgut. We identified 18 and 8 TFs
460 in the two populations, some of which evolved their mean expression (Supplementary file 7),
461 but none evolved a significant change in expression variance. The lack of significant variance
462 evolution in these candidate targets of selection suggests a more complex regulation of
463 transcriptional variance. We consider it highly likely that the expression of each redundant
464 gene is in turn regulated by several trans-acting factors – providing a second layer of possible
465 genetic redundancy (Figure 5b). We attempted to incorporate previously documented genomic
466 signatures of selection (Barghi et al., 2019) into the regulatory basis of the evolution of gene
467 expression variance. Interestingly, we found that ~50% of the candidate genes/TFs covered at
468 least a significant SNP 5kb up-/down-stream the genic regions. However, given the large
469 number of selection targets, this number is not significantly more than random expectation
470 (Permutation test, p-value > 0.05). Hence, no conclusive inference on the complex regulatory
471 basis of redundant gene expression can be achieved from the genomic data. Clearly, more work
472 is needed to study the regulatory architecture of genetic redundancy, but the experimental
473 framework introduced here provides a starting point.
474
475 **Concluding remarks**
476 Previous studies on adaptive phenotypic evolution mainly focused on "population means", to
477 explain adaptation to different environments. Nevertheless, also selection on "phenotypic
478 variance within a population" (e.g.: stabilizing selection, disruptive selection…) has been
479 studied, but the possibility that environmental changes affect the strength of stabilizing

selection has not yet been sufficiently explored. In a recent commentary (Harpak & Przeworski, 2021) highlighted that environmentally triggered changes in the strength of stabilizing selection may have important implications for the understanding of polygenic traits. To our knowledge, our study provides the first empirical evidence that the shift from a complex natural environment to a simple laboratory environment could result in drastic changes in the expression variance of certain genes during adaptation. This has important consequences for future research on adaptive phenotypic evolution: In addition to searching for changes in mean phenotype as a response to selection, it is also 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.



491

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

498

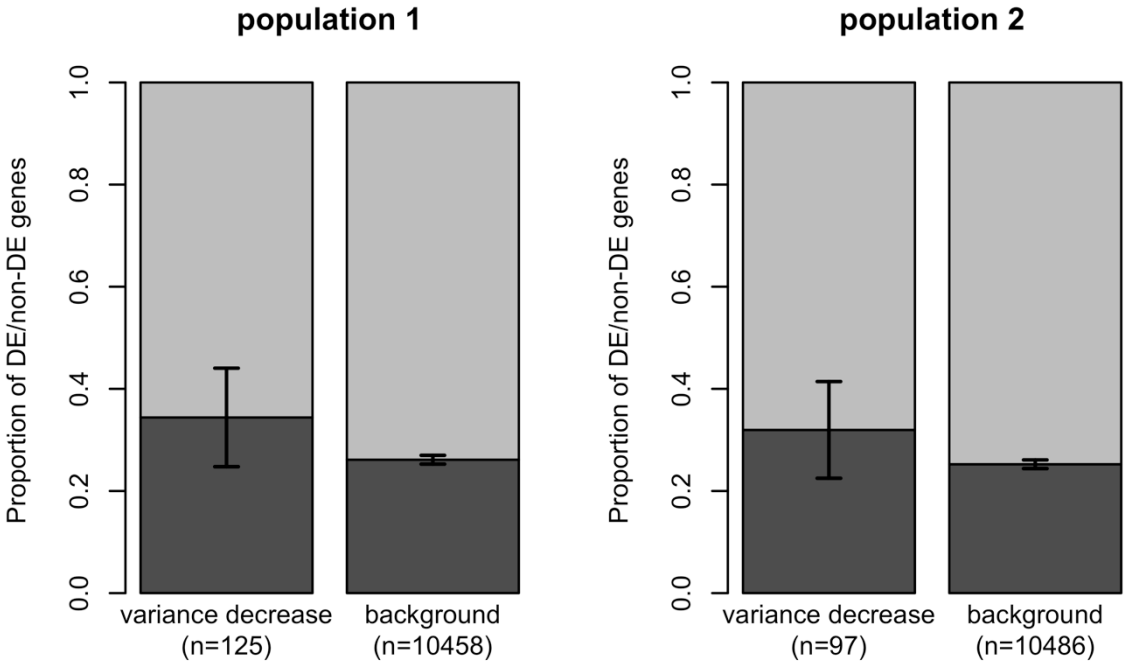


Figure 2. No significant enrichment of DE genes for the genes with significant variance change. Despite a trend for a slightly higher fraction of genes with a change in expression mean among genes with a significant change in expression variance, considering both populations jointly, no significant difference was noticed compared to genes without significant variance decrease ($\chi^2 = 3.96\ and\ 1.96$, FDR > 0.05). The y-axis denotes the proportion of DE (dark grey)/non-DE (light grey) genes in the genes with significant variance decrease and without (background) for two populations. The error bar indicates the 95% confidence interval.

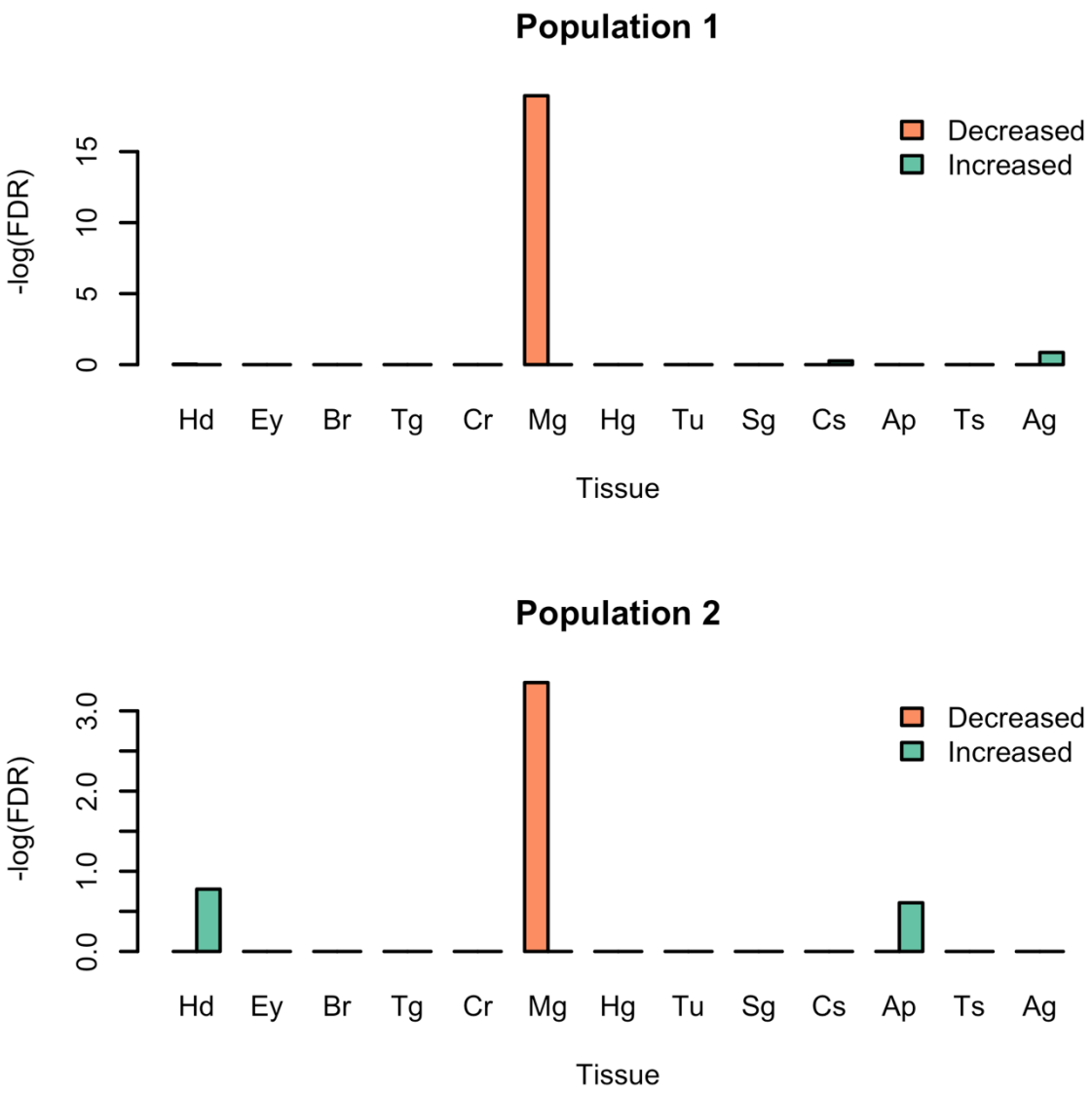


Figure 3. Tissue enrichment of genes with significant changes in expression variance. The bars indicates the significance (-ln(FDR)) of enrichment for genes with significant variance changes (orange: genes with decreased variance; green: genes with increased variance) among genes with tissue-specific gene expression pattern (Hd-head, Ey-eye, Br-brain, Tg-thoracoabdominal ganglion, Cr-crop, Mg-midgut, Hg-hindgut, Tu-malpighian tubule, Sg-salivary gland, Cs-carcass, Ap-rectal pad, Ts-testis and Ag-accessory glands). In both populations, a significant enrichment (Fisher's exact test, FDR < 0.001) can be found in the midgut for genes with reduced expression variance.

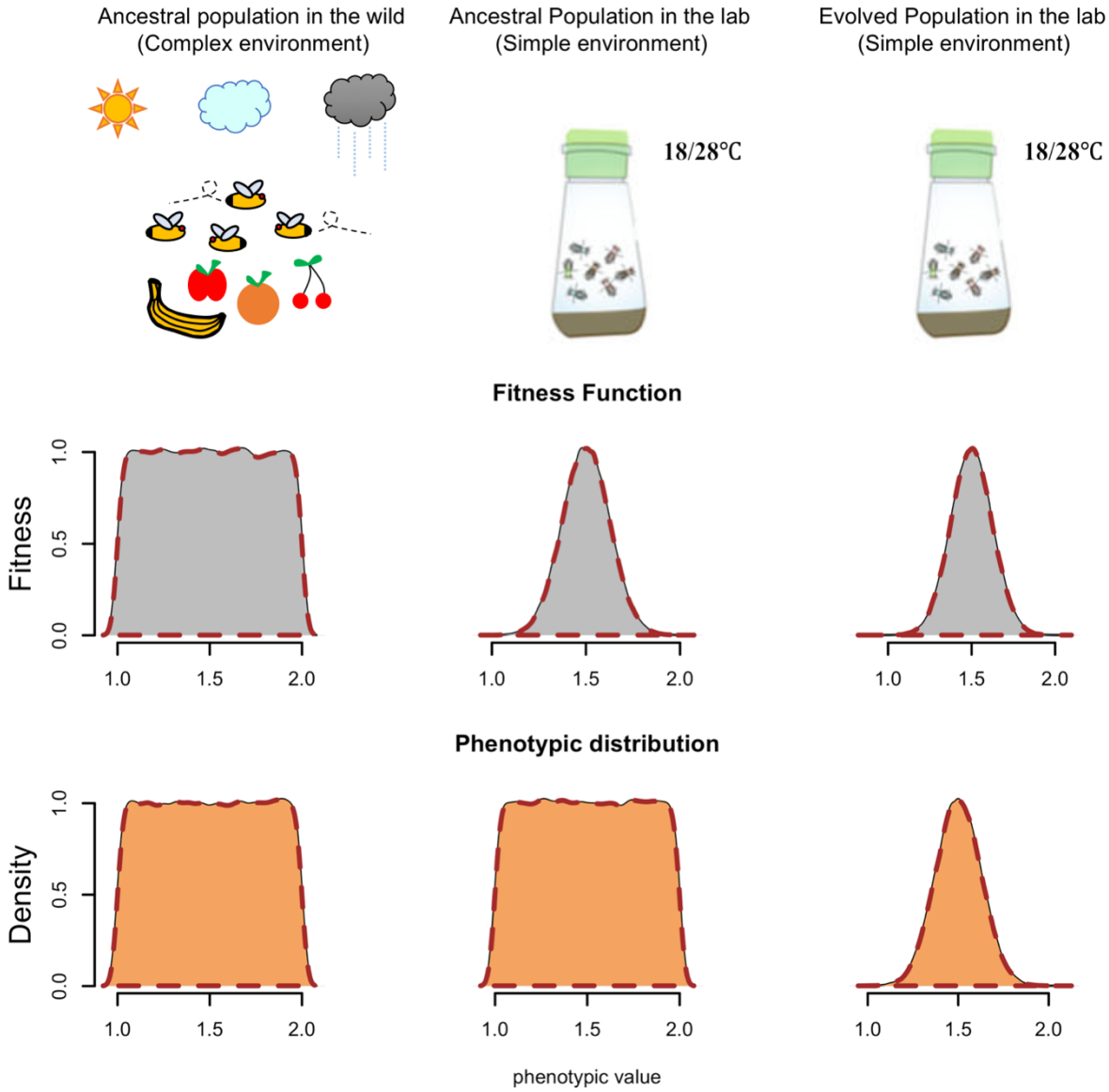
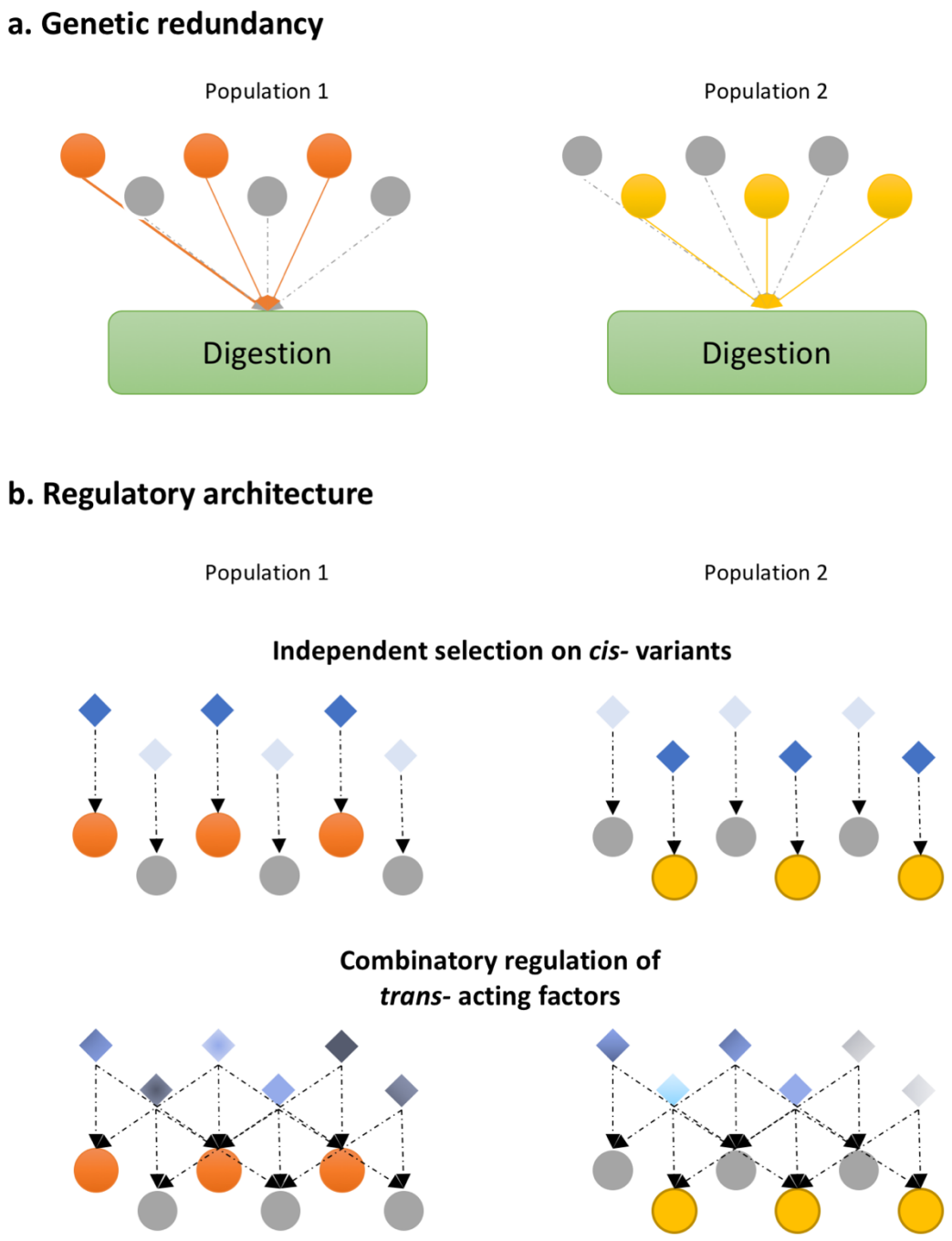


Figure 4. Hypothesis of a simpler lab environment selecting for decreased gene expression variance in the midgut. A proposed model for potential selection for decreased expression variance in midgut imposed by the drastic change in the environmental complexity. Food sources/fluctuation of temperature 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 environmental shift. After 100 generations in a simpler environment, the genes encoding digestive enzymes decreased their expression variance.



528
529 **Figure 5. Schematic illustration of genetic redundancy at gene level with two possible**
530 **regulatory architectures explaining the reduction in expression variance. a.** Genetic
531 redundancy: six genes contribute to digestion (higher-level phenotype) and the new phenotypic
532 optimum could be reached by expression changes of three genes. Stochastic effects result in
533 different gene sets (orange/yellow) responding to selection in the two populations. **b.** Two
534 hypotheses about a regulatory architecture that allows for the rapid decrease in variance of

535 digestion-related genes. Either selection acts independently on the *cis*-regulatory variants of
536 each gene or combinatorial changes of several TFs reduce the expression variance.
537
538

539 **Table 1. Microbiome diversity in the reconstituted ancestral and hot-evolved population**
540 **based on 16S-rRNA amplicon sequencing.** [a] indicates the 95% confidence interval obtained
541 from 100 bootstrap replicates.

	Ancestral	Evolved
α-diversity	33.84±0.90[a]	27.60±0.57
β-diversity	1.42±0.04	1.37±0.03
γ-diversity	47.74±1.39	37.87±1.08

542

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Author contribution

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

Competing interests

The authors declare no competing interests.

Data accessibility statement

All sequencing data are available from the European Nucleotide Archive (ENA) under the accession number PRJEB37011. Original data and scripts for the analysis are available on the GitHub repository of this study (https://github.com/cloudweather34/variance).

Correspondence and requests for materials should be addressed to C.S.

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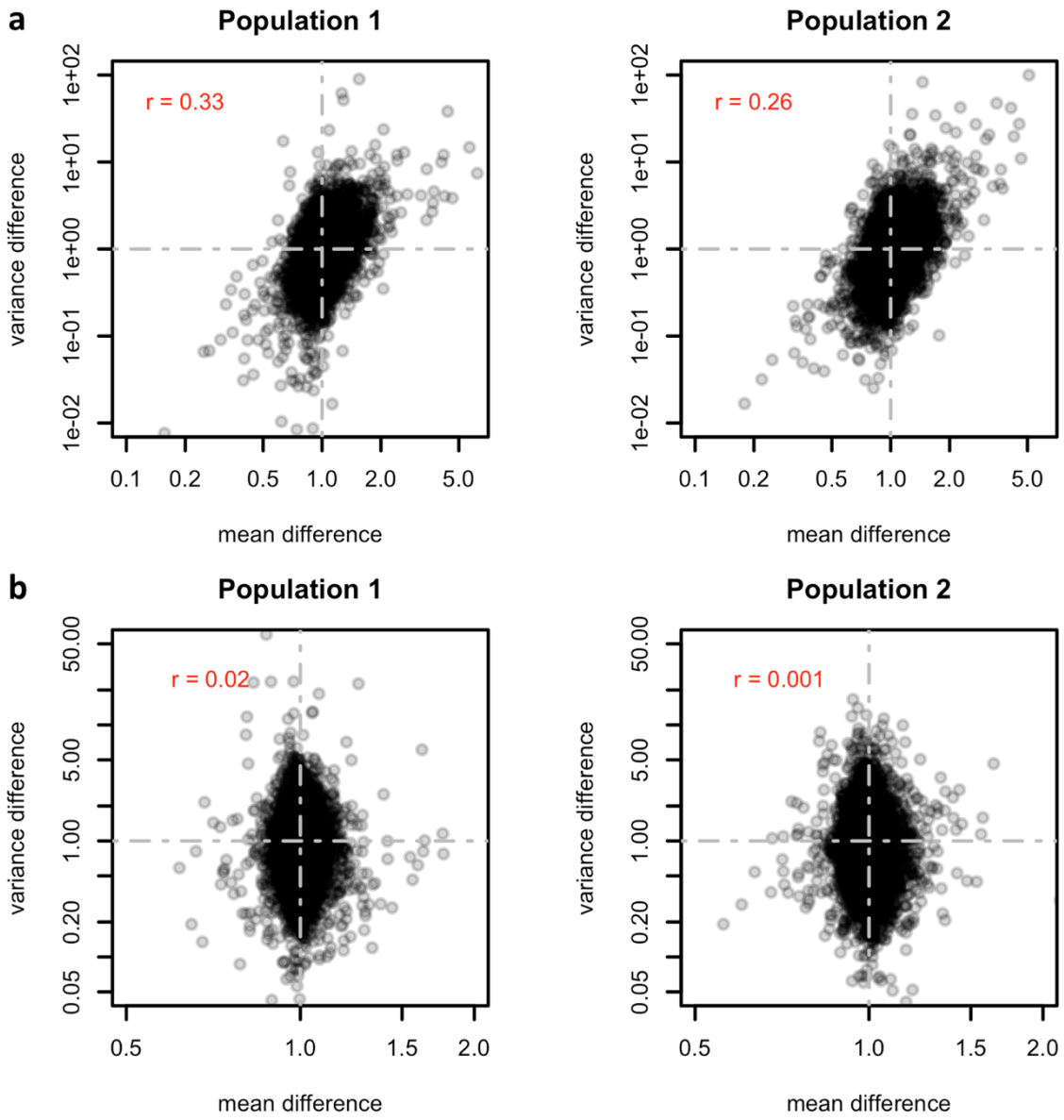
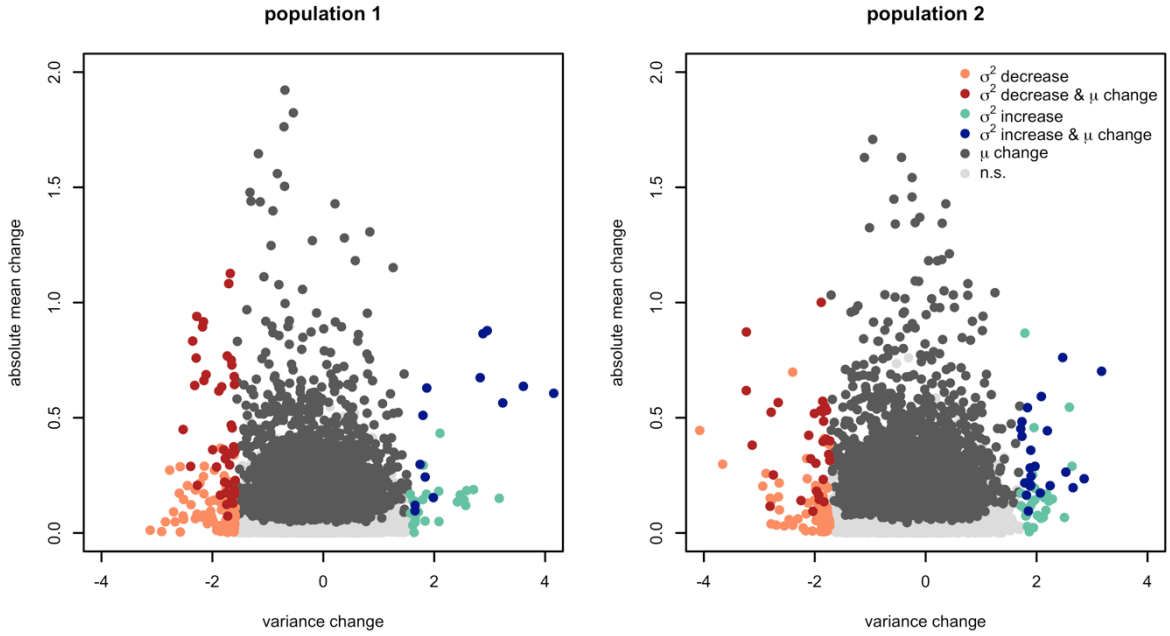
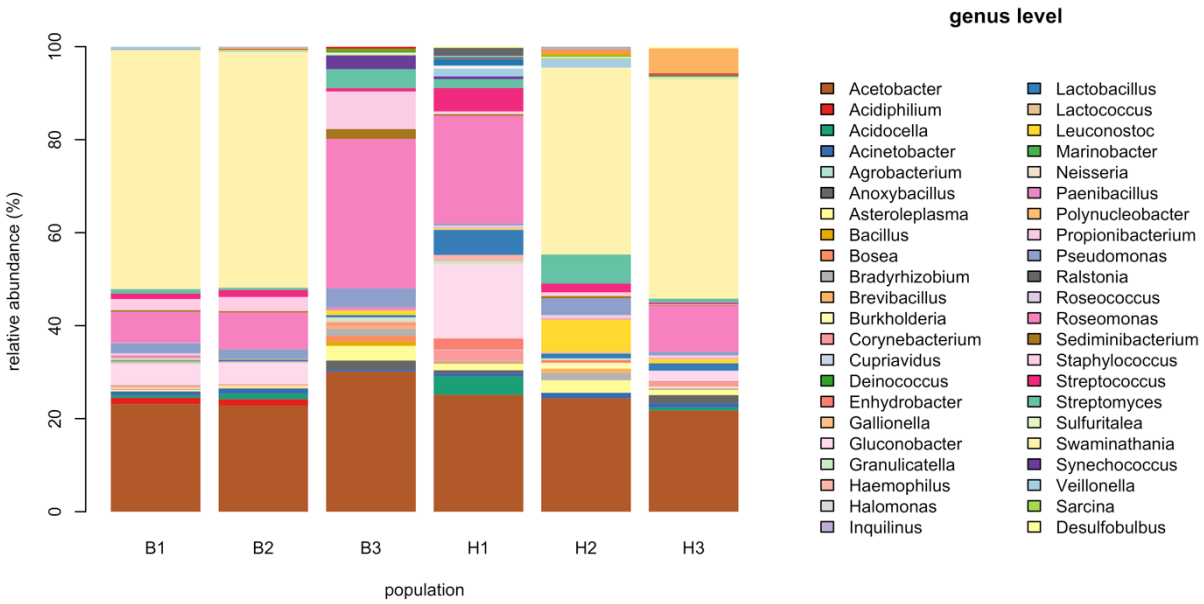


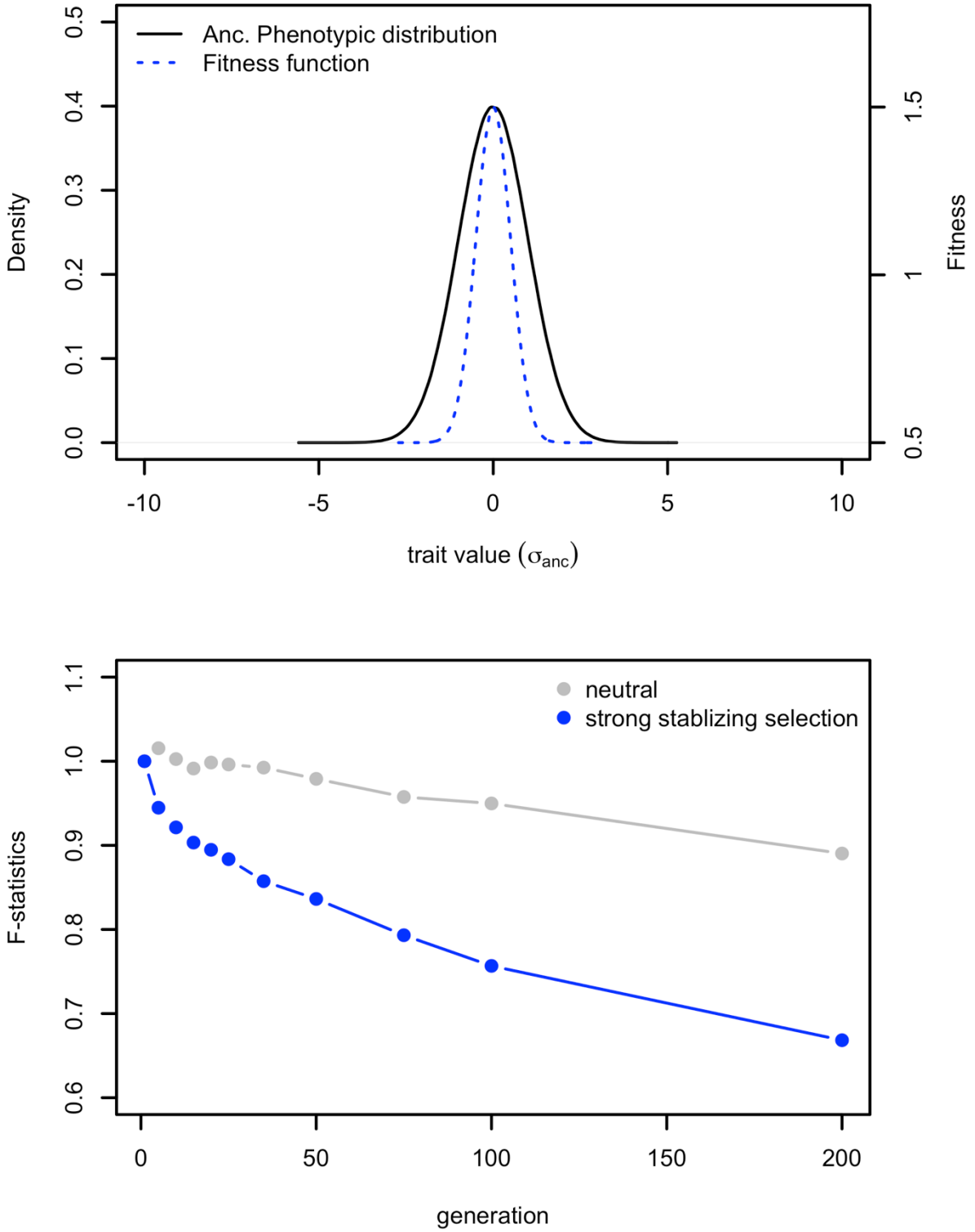
Supplementary figure 1. Log-transformation eliminates the positive relationship between the changes in mean and variance of gene expression. In each panel, the difference in mean expression($\frac{\overline{y_{evo.}}}{\overline{y_{anc.}}}$) and the difference in variance ($\frac{var(y_{evo.})}{var(y_{anc.})}$) before **(A)** and after **(B)** the natural log-transformation of each gene were visualized. The systematic positive correlation in the differences due to the positive mean-variance dependency of negative binomial distribution is removed by the log-transformation on gene expression level.



Supplementary figure 2. No correlation between the evolutionary response of variance and mean: evidence from genes which changed significantly in mean or variance. Each gene is plotted for the two populations with the change in variance ($\ln(\frac{\sigma^2_{evo}}{\sigma^2_{anc}})$) on the X-axis and absolute mean change ($|\mu_{evo} - \mu_{anc}|$) on the Y-axis. Genes with significant changes in variance (depicted in color) fall into both categories-with and without significant changes in mean expression. Only a negligible correlation was detected in a correlation analysis of the changes in variance and mean experiment in the 1st (left panel; rho = 0.047) and 2nd (right panel; rho = -0.001) population among the genes with significantly altered means or variances. The negligible correlation suggests an independent selection response for mean and variance.



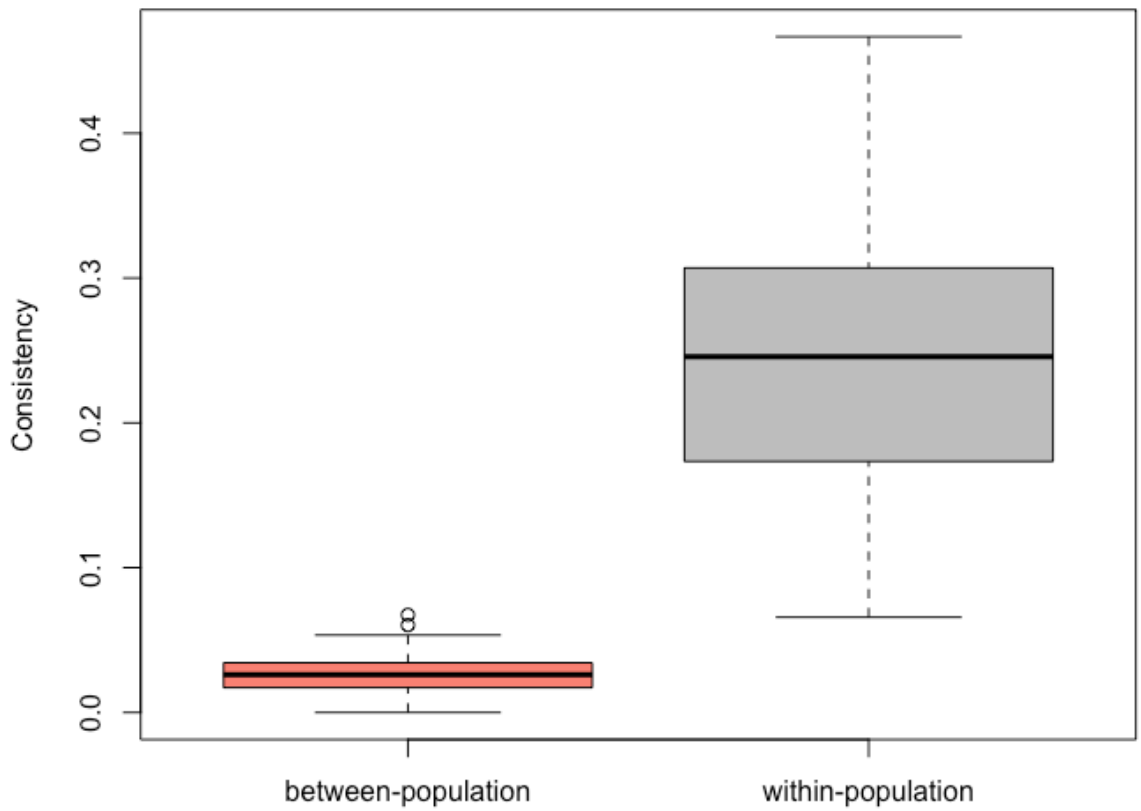
Supplementary figure 3. Microbiome composition in ancestral and evolved flies. Microbiome composition on the genus level for three individuals from the ancestral population (A1-A3) and five individuals from a hot-evolved population (H1-H3).



Supplementary figure 4. 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 simulation runs were performed.



780

781 **Supplementary figure 5. Heterogeneous selection response between the two populations**
782 **cannot be explained by sampling variation.** To demonstrate that the observed heterogeneity
783 between two evolved populations is not due to the stochastic effect, within the first comparison
784 (ancestral population 1 vs. evolved population 1), we subsampled two sets of 15 individuals
785 from the ancestral and evolved population and performed tests for variance (contrasting the
786 evolved and ancestral samples) on this two sets independently. We then estimated the
787 consistency between tests using the Jaccard Index. This process is repeated 100 times,
788 generating the null distribution of Jaccard Indices considering sole stochastic effects within an
789 evolutionary replicate (grey). Similar sampling scheme for 15 individuals was applied to the
790 comparison between the two populations 100 times. The observed Jaccard indices between two
791 populations are shown in red box and falls clearly outside of the null distribution. The
792 significant higher consistency in the resampling analysis within the same replicate (Wilcoxon's
793 test, W = 9999, p-value < 2.2×10^{-16}) suggests that the stochastic effects along cannot explain
794 the heterogeneity observed between replicates.