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The evolution of phenotypic plasticity in response to temperature stress

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Evolution of plasticity in *Drosophila*

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27 **Abstract**

28 Phenotypic plasticity is the ability of a single genotype to produce different
 29 phenotypes in response to environmental variation. The importance of
 30 phenotypic plasticity in natural populations and its contribution to phenotypic
 31 evolution during rapid environmental change is widely debated. Here, we show
 32 that thermal plasticity of gene expression in natural populations is a key
 33 component of its adaptation: evolution to novel thermal environments increases
 34 ancestral plasticity rather than mean genetic expression. We determined the
 35 evolution of plasticity in gene expression by conducting laboratory natural
 36 selection on a *Drosophila simulans* population in hot and cold environments.
 37 After more than 60 generations in the hot environment, 325 genes evolved a
 38 change in plasticity relative to the natural ancestral population. Plasticity
 39 increased in 75% of these genes, which were strongly enriched for several well-
 40 defined functional categories (e.g. chitin metabolism, glycolysis and oxidative
 41 phosphorylation). Furthermore, we show that plasticity in gene expression of
 42 populations exposed to different temperatures is rather similar across species.
 43 We conclude that most of the ancestral plasticity can evolve further in more
 44 extreme environments.

45 **Introduction**

46 Phenotypic plasticity is of great interest in ecology and evolution, because it
 47 describes the ability of single genotypes to produce distinct phenotypes in
 48 different environments (Pigliucci 2001). When populations encounter
 49 environmental change, plastic traits will result in phenotypic alterations without
 50 genetic response (Price et al. 2003). Of particular importance are those adaptive
 51 plastic responses where the altered phenotype is associated with higher fitness,
 52 because they provide a selective advantage in variable environments
 53 (Charmantier et al. 2008; Dey et al. 2016; Ghalambor et al. 2007; Nussey et al.
 54 2006; Suzuki et al. 2006) or during adaptation to a rapid environmental shift.
 55 Phenotypic plasticity is well documented for a broad range of phenotypes
 56 including morphological or life history traits (West-Eberhard 2003; Whitman et
 57 al. 2009). The technological advances in quantifying gene expression levels for
 58 entire transcriptomes have shifted the emphasis to gene expression patterns
 59 because many traits/phenotypes can be accurately quantified in a single
 60 experiment (Chen et al. 2015; Huang and Agrawal 2016; Zhao et al. 2015).
 61 Despite the conceptual appeal of adaptive plasticity in natural populations, our
 62 understanding of phenotypic plasticity in natural populations is still in its infancy
 63 (Forsman et al. 2015; Hendry et al. 2016; Merilä et al. 2014; Pigliucci 2005). In
 64 addition to adaptive plasticity, traits may be plastic in natural populations for
 65 other reasons: 1) neutral plasticity: variation in the trait has no fitness
 66 consequences (Via 1993) 2) deleterious plasticity: variation in the expression of
 67 the trait may be deleterious and selection operates to minimize it (Dewitt et al.
 68 1998; Ghalambor et al. 2007). The comparison of populations in a common
 69 garden experiment is an intuitive and popular approach to infer the selective

70 forces operating on plasticity (Merilä et al. 2014; Levis and Pfenning 2016).
71 Nevertheless, the link between plasticity and adaptation is only correlative and
72 may arise from other changes, not related to adaptation to the environmental
73 contrasts.

74 Experiments relying on standing genetic variation to study the evolution of
75 plasticity are well-placed in the framework of genetic accommodation (Braendle
76 and Flatt 2006): complex traits with multiple contributing loci can respond
77 quickly to environmental shifts. Hence, phenotypic plasticity could be rapidly
78 modulated in response to selection. Exposing natural populations to more
79 extreme environments provides clear predictions about the evolution of
80 plasticity (Chevin and Hoffmann 2017). While random changes in plasticity are
81 expected under neutrality, in the case of deleterious (costly) plasticity, reduced
82 plasticity is predicted (counter-gradient evolution). An increase in plasticity is
83 expected when plasticity is adaptive: genetic changes in the novel environment
84 will reinforce the ancestral plasticity (Ghalambor et al. 2007, Ho and Zhang
85 2018). No change in plasticity is difficult to interpret because it may reflect
86 absence of genetic variation, but also weak selection or neutral plasticity result
87 in the same outcome. Experimental evolution is a powerful approach to
88 distinguish between random and directed changes in plasticity because
89 environmental conditions can be tightly controlled and replicated experiments
90 provide more reliable results.

91 In *Drosophila*, the evolution of gene expression plasticity has been studied for a
92 range of different environmental stressors, ranging from alcohol to heavy metals
93 and temperature (Chen et al. 2015; Huang and Agrawal 2016; Zhao et al. 2015;
94 Clemson et al. 2016; Levine et al. 2011; Porcelli et al. 2016; Yampolski et al.

2012; Zhou et al. 2012). Natural *Drosophila* populations are exposed to daily and seasonal temperature fluctuations (Bergland et al. 2014; Machado et al. 2016), making this a particularly relevant abiotic factor in the context of phenotypic plasticity (Angilletta and Angilletta 2009). Measuring gene expression of a single heterozygous *D. melanogaster* genotype at four different temperatures showed that 83 % of the expressed genes exhibit a plastic expression pattern when exposed to a temperature gradient ranging from 13 to 29 °C (Chen et al. 2015). The variation in gene expression plasticity of natural *Drosophila* populations along latitudinal clines (Zhao et al. 2015; Porcelli et al. 2016) suggests that some of the plastic responses are driven by selection. We study the evolution of plasticity to infer the influence of high and low temperature regimes on the plasticity of gene expression in *Drosophila simulans* using laboratory natural selection (Fuller et al. 2005, see experimental design in Fig. 1). Specifically, we address the question how adaptation to more extreme temperatures modulates the plastic response of traits, which were already plastic in the founder population. We show that phenotypic plasticity does not prevent evolution. Rather, adaptation to more extreme temperature regimes increases the plastic response. In combination with clinal variation of gene expression in natural populations of both *D. simulans* and *D. melanogaster* (Zhao et al. 2015), our data provide convincing experimental evidence for adaptive phenotypic plasticity in a natural population.

Materials and Methods

Laboratory natural selection procedure

The laboratory natural selection setup is detailed in Mallard et al. (2018). In brief, 10 replicated *Drosophila simulans* populations were setup from 250 isofemale

lines collected in Northern Portugal in 2008. The replicated populations are maintained under two fluctuating temperature regimes (5 replicates in each): either a hot (mean temperature 23°C) or a cold treatment (mean temperature 15°C). In each environment, the temperature changed with a 10°C amplitude centered on the mean temperature synchronized on a 12/12 hours light/dark cycle. The same maintenance regime was used for populations in both temperature environments, only adjusting for the increased developmental time in the cold environment. Every generation, 1000 flies are sampled from the eclosed flies and distributed over 5 fresh bottles containing 70 ml standard *Drosophila* medium. After two egg layings for 48h and 72h in the hot and cold environment respectively, adults were frozen. We preferentially used the second egg collection for the next generation to avoid selection for early fecundity. We previously showed that the selection regime results in higher fitness of the evolved populations (Mallard et al. 2018).

Common garden experiment

Two parallel common gardens with identical experimental procedures were performed in a hot (23°C) and a cold (15°C) environment using eggs from the evolved populations at generation 39 (cold) and 64 (hot). Additionally, 5 replicates of the ancestral population were reconstituted from the founder isofemale lines. After two generations in the assayed environment, the second one with controlled larval density (300 eggs), we collected adults and separated the two sexes under shallow CO₂. Flies were frozen in liquid nitrogen after a 24h-36h recovery period at 2pm (approximately 6 hours after the start of the light cycle). During experimental evolution, the ancestral population was maintained at 18°C in the form of isofemale lines. The small population size in the isofemale

lines prevents adaptation to the culturing conditions and therefore, the reconstituted population reflects the ancestral population (Nouhaud et al. 2016).

Gene expression analysis

For all 15 populations from both common garden temperatures, we generated two RNA-Seq libraries, each from different sets of 25-30 males. We extracted total RNA-Seq using the Qiagen RNeasy Universal Plus Mini protocol (Qiagen, Hilden, Germany) with DNase I treatment according to the manufacturer's instructions. Quality control of the RNA was performed on agarose gels and the Qubit RNA HS or BR Assay kit (Invitrogen, Carlsbad, CA) for quantification. Strand-specific barcoded mRNA libraries were generated using the NEBNext® Ultra Directional RNA Library Prep Kit for Illumina with a protocol modified to allow for a larger insert size than the default 200bp. We purified polyA-mRNA from 3µg total RNA and fragmented for 8 min. The 42°C incubation step in the first-strand synthesis and the 16°C step in the second-strand synthesis were extended to 30 and 90 min., respectively. Size selection for a target insert size of 330bp was performed using AMPure XP beads (Beckman Coulter, Carlsbad, CA). PCR amplification followed the recommended protocol (NEB) with 12 PCR cycles and a 50 sec. extension step. The final libraries were bead-purified, quantified with the Qubit DNA HS Assay kit (Invitrogen, Carlsbad, CA) and pooled in equimolar amounts. To reduce batch effects, we combined libraries from ancestral, cold and hot evolved replicates and sequenced them in the same lane. Libraries were sequenced using a single-read 50bp protocol on a HiSeq2500. We trimmed the raw reads (quality threshold 20, minimum read length 40) using PoPoolation (Kofler et al. 2011). The trimmed reads were aligned to the *Drosophila simulans* reference genome (Palmieri et al. 2014) with GSNAP (Wu et

170 al. 2010) using a hadoop cluster. All subsequent analysis were performed in R
 171 (Team RC 2019) including read counts (Liao et al. 2013) and differential gene
 172 expression (Robinson et al. 2010). We normalized gene expression levels with
 173 the TMM method, restricting our analysis to the genes with an overall mean
 174 expression above one count per million (CPM) 11,200 genes). We used negative
 175 binomial GLMs to estimate the effect of selection regime, temperature, and their
 176 interaction on gene expression. We then computed ad hoc contrasts to find
 177 differentially expressed genes between groups of interest using likelihood ratio
 178 tests (*glmLRT* in edgeR). This allow us to determine for each gene whether the
 179 difference in expression either between two groups of samples (such as the
 180 effect of temperature on a given evolved population) or for a linear combination
 181 of these groups (such as the difference between the reaction norms of two
 182 populations) is statistically significant. The Benjamini-Hochberg procedure was
 183 applied to control for false discovery rate (Benjamini et al. 1995). All plasticity
 184 estimates as well as evolved differences between ancestral and evolved
 185 populations plotted in the manuscript are model fit values obtained from these
 186 contrasts.

187 When comparing the gene expression of evolved populations against the
 188 ancestral ones at a given temperature, we always used $FDR < 0.05$ (unless
 189 specified differently). We allowed a higher rate of false positive when testing for
 190 reaction norms between ancestral and evolved populations ($FDR < 0.1$). This was
 191 done because we restricted our analysis to genes that were already differentially
 192 expressed in at least one temperature with a stringent FDR. Once identified the
 193 genes showing a significant evolution of their reaction norms, we compared the
 194 absolute value of the ancestral and the evolved reaction norms to distinguish

195 between cases of reduced and increased plasticity. Gene ontology enrichment
196 was performed with Gorilla (Eden et al. 2009) using the complete list of retained
197 genes (n=11,200) as background data set and a FDR<0.05. We compared the
198 number of genes that evolved increased or decreased plasticity in the hot
199 evolved populations using a generalized linear model with a binomial
200 distribution. The estimated probability was compared to the 0.5 using a Wald
201 test.

202 In a second GLM, we analyzed the replicate specific evolutionary response. We
203 considered only the samples from the Ancestral and the Hot evolved populations
204 and each evolved population was treated as a different level of the “selection
205 regime” factor. The model formula was similar to the previous one but this latter
206 factor contained 6 levels (Ancestral and each of the 5 hot evolved replicate). We
207 processed as described above to detect genes with evolved differential
208 expression.

209 Detection of false positive genes with increasing plasticity

210 To avoid false positives, we restricted our set of candidate genes to those with a
211 significant change in expression in at least one of the two environments (15°C or
212 23°C) and a significant interaction effect. The rationale can be explained by
213 considering genes that evolved in expression in only one environment, but
214 remained unchanged in the second environment. Adding some minor random
215 noise could either result in a positive or negative correlation of the expression
216 changes in both temperatures. Because negative correlation increases the
217 significance in the interaction test, it may be possible that such random
218 fluctuations could bias our results towards the observed excess of genes with
219 increased plasticity. To rule out that such a potential bias affected our results, we

performed an additional test contrasting the ancestral plasticity and the plasticity of a hypothetical population that would have evolved its expression only at one temperature (i.e. replacing the expression levels of the evolved population in the second environment by the ancestral values). For all genes with a significant change in plasticity, we also detected a significant change in plasticity when we considered only the expression change in only one environment. We conclude that none of these genes are false detected due to a random measurement error in the second environment.

RNA-Seq quality control

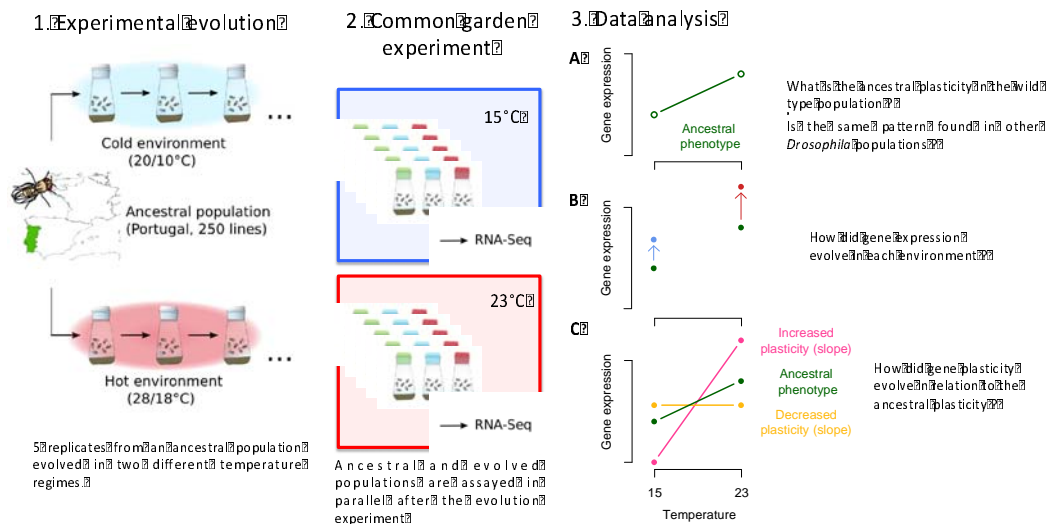
We performed several analyses to test the quality of each library. We first estimated heterogeneity in coverage (3' bias) of the 20% longest genes of the *D. simulans* annotation using the geneBody coverage tool implemented in the RSeQC package (Wand et al. 2012). Following Mallard et al. (2018), we removed strongly biased libraries (12 libraries in total). Additionally, we quantified the expression of 12 chorion and yolk protein genes to identify female contamination due to sexing mistakes or sample swap. We excluded libraries showing a total log₂ normalized expression of these genes higher than eight (4 libraries, see Fig. S6). These four libraries contained at least sixteen times the number of transcripts in the remaining libraries (see Fig. S6). After removing the biased and contaminated libraries, a total of 44 libraries remained for the analysis (less than 1.5 samples per population). Out of these 44 libraries, 16 combinations of populations and treatment had only one library left and 14 had 2 libraries. We retained only one measurement per population (n=30) by summing the gene counts of samples coming from the same population. Before pooling the libraries, we visually inspected the samples using multi-dimensional

245 scaling plots (Fig. S1). These plots inform about pairwise distance between
246 samples. While the replicates within a temperature regime were not well
247 separated, robust differences between the ancestral and the two groups of
248 evolved populations were seen. The number of mapped reads for each sample
249 can be found in Table S6.

250

251 **Results**

252 We measured the gene expression patterns of our ancestral population and the
253 two evolved populations in two parallel common gardens at 15°C and 23°C. The
254 analysis of evolution of gene expression plasticity is complex and we followed a
255 three-step analysis as described in Figure 1.



256

257 Fig. 1: Experimental design. 1) We evolved two sets of five populations in either cold or hot
258 laboratory environments for 39 and 64 generations, respectively. 2) We measured gene
259 expression in two common gardens, where the evolved populations together with the ancestral
260 one were phenotyped at either 15°C or 23°C. 3) Gene expression analysis was done in three
261 successive steps. A) We first explored the plasticity of our ancestral population and compare it to
262 existing data sets. B) We investigated gene expression changes at 15°C and 23°C in the evolved
263 populations. C) We determined the evolved plasticity by measuring each of the evolved
264 populations in both temperature regimes. The evolved plasticity is compared to the ancestral one
265

266

Gene expression plasticity in the ancestral population (Fig. 1 – panel 3A)

267 We determined temperature mediated plasticity of gene expression by exposing
 268 the ancestral, hot evolved and cold evolved population to 15°C and 23°C. As
 269 expected from previous studies (Zhou et al. 2012; Chen et al. 2015), the
 270 expression of a large number of genes was modulated by temperature.
 271 Down-regulated genes, which are expressed at lower levels at 23°C than at 15°C
 272 in the ancestral population, are enriched for several GO categories including
 273 chitin-based cuticle and transmembrane transport genes (Table S1). Eighty-nine
 274 (83%) of the significant GO terms are also identified among the genes decreasing
 275 in expression at higher temperatures in *D. melanogaster* (out of 107 GO terms
 276 classified in Chen et al. (2015)). This overlap is probably conservative, because
 277 the sex of the flies analyzed and the temperature regimes differed between
 278 studies (Chen et al. (2015) measured females in 4 different temperatures).
 279 Interestingly Zhao et al. (2015) found that chitin genes were among the top
 280 plastic genes shared between *D. melanogaster* and *D. simulans*. In particular, the
 281 category “structural constituent of chitin-based cuticle” was consistently
 282 identified for genes decreasing with temperature across all three studies.
 283 Genes that are more highly expressed at 23°C than at 15°C in the ancestral
 284 population (up-regulated genes) are enriched for genes involved in translation,
 285 including a large number of ribosomal genes. Out of 21 GO terms, which were
 286 also enriched in Chen et al. (2015), 18 are classified as increasing in both analysis
 287 (Table S2). None of these categories were reported in Zhao et al. (2015).
 288 Such highly consistent gene expression changes across different experiments,
 289 suggest a highly robust pattern of plasticity, which is conserved not only among
 290 populations, but also between species.
 291 Evolution of gene expression in the focal temperature regime (Fig. 1 - panel 3B)

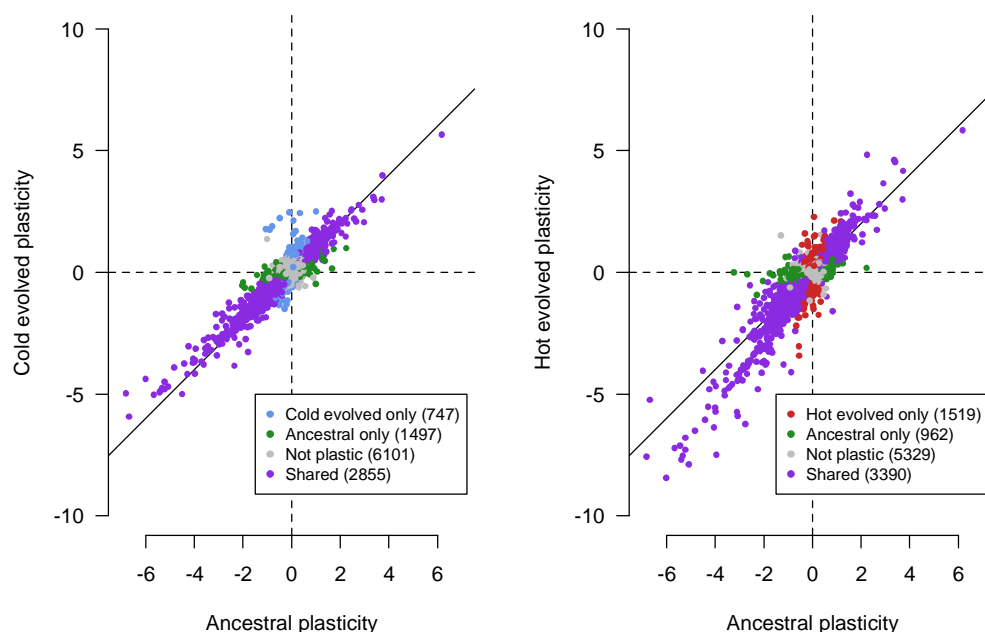
Only a small number of genes was differentially expressed in populations evolved in the cold environment when compared to the ancestral population (see Table S3 FDR<0.05; 42 genes at 15°C). A quite different pattern was observed for the hot-evolved populations. In the comparison to the ancestral population, 725 genes (see Table S4) were differentially expressed at 23°C. The small impact of adaptation to cold temperature may be the consequence of fewer generations in the new environment compared to the hot-evolved populations. But we cannot distinguish this effect from temperature-specific effects triggering a more pronounced evolution in the hot environment.

Evolution of gene expression plasticity (Fig. 1 – panel 3C)

With about 32% (n=3,602, FDR<0.05) of the expressed genes being differentially expressed between the two assaying temperatures the cold evolved population was slightly less plastic than the ancestral population (n=4,352, 39%, see Table S1). The hot evolved population had about 44% (n=4,909) plastic genes, which corresponds to about 15% more differentially expressed genes than the other two populations. These differences remain stable even when controlling for the overall library sizes by down sampling (see Fig. S3).

We evaluated the evolution of plasticity by correlating gene expression plasticity (\log_2FC between 15° and 23°C, i.e. the slopes shown in Fig. 1- panel 3C) in the ancestral population with the plasticity in the evolved populations. If the plasticity did not change during evolution, a high correlation is expected. Indeed, the plasticity was highly correlated between ancestral and evolved populations (Pearson correlation coefficients: 0.91 (cold evolved) and 0.89 (hot evolved), Fig. 2). Despite this overall conservation of gene expression plasticity, a closer inspection of Figure 2 (right panel) shows that for some genes plasticity changed

317 after evolution in the hot environment, but the direction of plasticity is not
318 affected (i.e.: the plasticity became more extreme).



319

320 Fig. 2: Evolution of gene expression plasticity after selection in cold (left) or hot environments
321 (right). Plasticity is measured as the \log_2 fold change of gene expression at 15°C and 23°C. We
322 compare the plasticity of the ancestral population (x-axis) plotted against the plasticity of the
323 evolved populations. Overall, the pattern of gene expression plasticity is conserved for many
324 genes (purple). Genes that are significantly plastic in only one population have lower \log_2 fold
325 changes in other population (green, blue and red dots). Despite this overall conservation of
326 plasticity, highly plastic genes tend to deviate from the solid line in the hot evolved populations
327 (slope=1) indicating an increased plasticity.

328

329 In the cold evolved replicate populations, only a small subset of the genes that
330 evolved a change in expression at 15°C or 23°C displayed a significant difference
331 in the plasticity relative to the ancestral population (2 at 15°C and 2 genes at
332 23°C, FDR < 0.1).

333 Among the genes that evolved gene expression differences in the hot-evolved
334 populations either at 15°C or at 23°C (n=930), we distinguished three different
335 classes: 1) genes with significant change in plasticity (325 genes, FDR<0.1); 2)

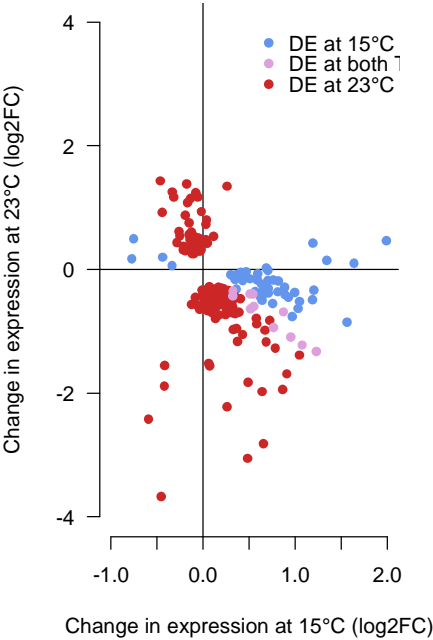
genes with small differences in the magnitude of gene expression differences (log₂FC) between the evolved and the ancestral population in each environment - here, a reliable detection of changes in plasticity or constitutive expression differences is not possible ; 3) genes with no change in plasticity, but constitutive expression differences (i.e. a change in the same direction at both temperatures, FDR<0.05, n=50). This third class of genes was enriched for oxido-reduction processes suggesting a global down-regulation of detoxification genes (FDR<0.1, 6 cytochrome p450 genes, 2 UDP-glucuronosyltransferases). Because some of these genes were also down-regulated in the cold evolved populations (23 genes using FDR<0.1 including 6 p450 genes, see Table S3), we conclude that their constitutive change in expression is not directly related to absolute temperature but a response to either temperature stress or to adaptation to shared environmental conditions.

Among the 325 genes with a significant evolution of plasticity, we noticed significantly more genes with increased plasticity (n=241) than with decreased plasticity (n= 84, p<0.001). This result is not biased by ancestrally non-plastic genes that cannot decrease plasticity: the ratio of genes with increased plasticity to genes with decreased plasticity does not change when only ancestrally plastic genes are analyzed (log₂FC > 1 in the ancestral population, n=62 and 20 respectively, p<0.001). No GO categories were enriched for genes with reduced phenotypic plasticity. In contrast, genes with increased phenotypic plasticity were enriched for several GO terms (116 processes, 34 functions and 28 components). Because the different number of genes in both categories may have affected the enrichment tests, we randomly selected multiple sets of 85 genes among the 242 significant ones and performed the GO analysis for each set. We

361 obtained significantly more enriched processes genes that evolved an increased
362 plasticity (20 bootstrap iterations, $p < 0.0004$; mean number of enriched
363 processes 18.3). Two particularly prominent classes of GO terms were either
364 related to cuticle formation and chitin production or metabolism including the
365 electron transport chain and glucose metabolic processes.

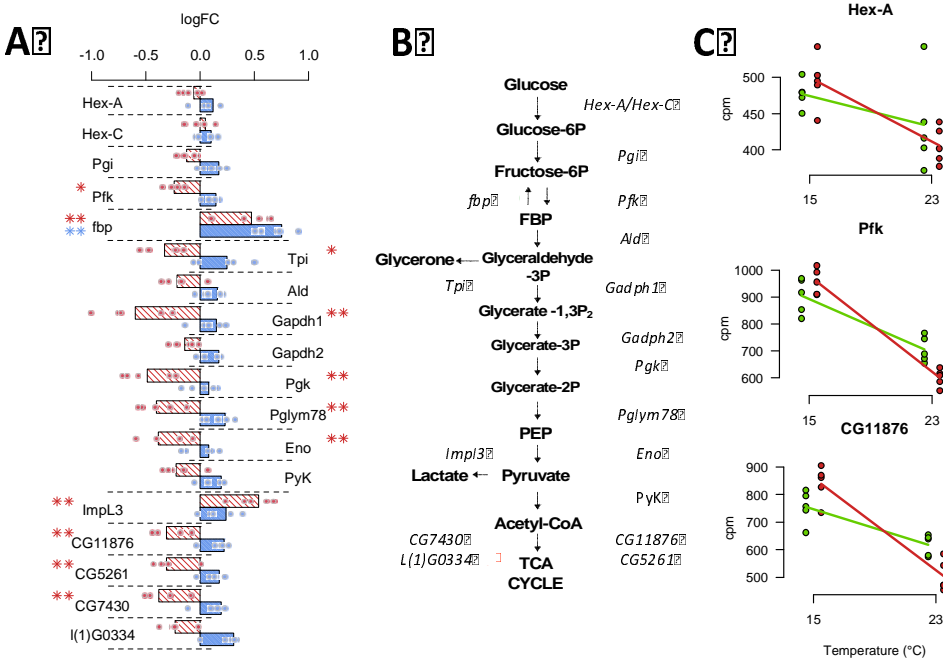
366 For most of the genes that evolved a difference in gene expression between
367 ancestral and evolved populations, there is a significant change at only one
368 temperature ($FDR < 0.05$). Nevertheless, we noted a strong negative correlation
369 for the sign of the expression differences between hot evolved and founder
370 populations at 15°C and 23°C (Fig. 3, $\chi^2_{1,241} = 133$, $p < 0.0001$, see also Fig. S4 for a
371 complementary test), suggesting that evolution modulated the temperature
372 sensitivity of gene expression. This negative correlation is particularly
373 pronounced for genes involved in energy production (see Figs. 4 and S2 for
374 glycolysis and oxidative phosphorylation) but also for chitin related genes.

375 On the other hand, genes that evolved a decreased plasticity show a much
376 weaker correlation of the sign of expression change between temperatures
377 ($\chi^2_{1,84} = 2.8$, $p = 0.09$).



378

379 Fig. 3: Log₂FC in gene expression of genes increasing in plasticity during evolution in the hot
380 environment and being differentially expressed in at least one assayed temperature. X- and Y-
381 axis show the impact of adaptation to a hot environment on the gene expression at 15°C and 23°C
382 relative to the ancestral population. Most of these genes evolved a change in expression in the
383 opposite direction ($p < 0.0001$) and are therefore located in the top-left and bottom-right quarters
384 of the plot.
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387



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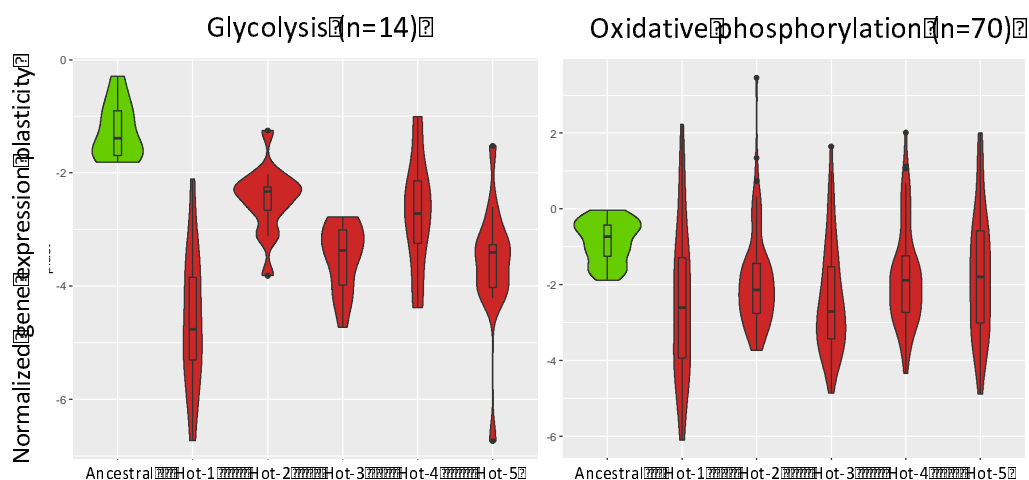
Fig. 4 Evolution of plasticity in the glycolysis pathway **A)** Bar chart of the \log_2FC of gene expression evolution from Fig. 2. The difference between the ancestral and the hot evolved populations measured at 15°C (blue) and 23°C (red) are shown. Single dots superimposed on the bar show the divergence of 5 hot-evolved replicates from the mean ancestral expression. **B)** Glycolysis pathway with the main regulatory enzymes from panel A. Most genes involved in glycolysis are significantly down regulated at 23°C (** FDR<0.05 , * FDR<0.1). Even for comparisons with no statistically significant difference, most of the genes down regulated at 23°C are up-regulated at 15°C. **C)** Expression plasticity is highly reproducible across replicates. Three enzymes of the glycolysis pathway illustrate the highly consistent response across all five replicates. The ancestral replicate populations are indicated by green dots and the hot evolved populations by red dots (all genes are shown in a Supplementary File). Lines indicate plasticity based on the mean expressions values of the five replicates.

Replicate specific evolution

Our previous analysis is looking for significant changes in expression across five independently evolved populations. Yet, it does not inform us about the parallel evolution of each population. We addressed this by analyzing each replicate independently to detect genes evolving increased or decreased phenotypic plasticity.

In each evolved population, we detected genes that evolved plasticity (range: 85-249, mean =134, 409 genes in total). Only 18 genes were significant in all 5 replicates and 272 in only a single population. Similarly to what we found in the main analysis, more genes displayed increased plasticity (ranging 63-175,n=318) than decreased plasticity (ranging 26-41, n=101). This observation is very consistent across populations: a gene that evolved plasticity in one replicate is found significant in another one with the same frequency (41% and 45% for genes decreasing and increasing plasticity respectively). For changes in reaction norm the consistency across replicates is highly dependent on the direction of change. We observed a low correlation for genes that evolved decreased plasticity (mean $r^2= 0.03$, see Fig. S5) while genes that increased plasticity were highly correlated among replicates (mean $r^2=0.77$, see Fig. S5).

420 A GO enrichment analysis at the replicate level showed that only chitin related
421 gene ontologies were significantly enriched in all 5 evolved populations (see
422 Table S5). The increased plasticity of the metabolism related genes were only
423 significantly overrepresented in the first replicate. Nevertheless, we attribute
424 this mainly to a lack of statistical power: the increase in plasticity for the genes
425 involved in glycolysis and oxidative phosphorylation is consistent across all
426 replicates (Fig. 5).
427



428
429 Fig. 5: Highly consistent down-regulation of glycolysis and oxidative phosphorylation plasticity
430 across all 5 hot-evolved replicates. The difference in expression between 15°C and 23°C of the
431 ancestral population (green) is higher than in each of the evolved populations (red) indicating
432 more negative reaction norms. We only show genes from the glycolysis and oxidative pathways
433 that were ancestrally down-regulated (right panel, n=14, left panel, n=70).
434

435 Discussion

436 Only limited counter-gradient evolution

437 Only very few studies were able to address the evolution of gene expression over
438 short evolutionary time scales. The adaptation of *Drosophila melanogaster* to salt
439 and cadmium-enriched medium (Huang and Agrawal 2016) showed that gene
440 expression plasticity evolved, but in the opposite direction to the plasticity seen

441 in the ancestral populations. These food supplements are novel environmental
 442 conditions, which are very rarely encountered by fruit flies in their natural
 443 environments. The authors proposed that this counter-gradient evolution could
 444 be explained by the selection on phenotypes that are only beneficial under these
 445 extreme conditions, but not in the environments typically encountered by
 446 *Drosophila*: the plastic response would correspond to a “stress” that is no longer
 447 expressed when population are adapted to this new environment. This is in
 448 sharp contrast to the experimental design of this study. Temperature is one of
 449 the most important environmental factors driving local adaptation in ectotherms
 450 (Angilletta and Angilletta 2009; Fuller and al. 2005). This applies also to
 451 *Drosophila* (Zhao et al. 2015; Bergland et al. 2014; Machado et al. 2016;
 452 Klepstatel et al. 2013) where significant clinal variation is seen on the genomic
 453 and transcriptomic level (Zhao et al. 2015; Machado et al. 2016; Hoffmann and
 454 Weeks 2007). Only a moderate fraction of genes (25% of all genes with evolved
 455 plasticity) that experienced counter gradient evolution, i.e. a decrease in the
 456 slope of the reaction norm. Interestingly, these genes were not enriched for
 457 functional categories and did not evolve consistently across our replicates. Thus,
 458 we failed to find biological processes for which gene expression plasticity would
 459 be strongly maladaptive. More likely, the gene expression of these genes is not
 460 well-adapted, possibly due to pleiotropic functional requirements, which are
 461 relaxed in the laboratory environment. The large fraction of genes for which the
 462 ancestral plasticity evolved to more extreme values suggests that the laboratory
 463 conditions match many ecologically relevant forces encountered by natural
 464 *Drosophila* populations.

In our study, we contrasted whole organisms gene expression across environments - a common practice in the study of gene expression evolution in *Drosophila* (Chen et al. 2015a, 2015b; Huang and Agrawal 2016; Zhao et al. 2015; Clemson et al. 2016; Levine et al. 2011; Porcelli et al. 2016; Yampolski et al. 2012; Zhou et al. 2012). Nevertheless, a potential problem is that during evolution allometric changes may occur - this is that the relative abundance of some cell types changes (Montgomery and Mank 2016). In fact, a recent study showed that females adapting to a new temperature regimes also evolved allometric changes, while males were much less affected (Hsu et al. 2020). While such allometric changes could affect gene expression means, the impact on plasticity is not clear. If the evolved allometric changes do not change with assaying temperature, no influence on the analysis of phenotypic plasticity is expected. On the other hand, if allometric changes are modulated by assaying temperature, this could be considered as an extended evolved phenotypic plasticity and will not affect our conclusions.

Plasticity in gene expression suggests adaptive plasticity

The evolutionary implication of phenotypic plasticity is a controversial topic with two extreme perspectives. With the same genotype expressing different phenotypes in response to the environment, it is often assumed that these phenotypes provide a higher fitness to their carriers (Via et al. 1995). If phenotypic plasticity results in a good match of phenotype and environment, this could even make genetic adaptation expendable (Charmantier et al. 2008). On the other hand, phenotypic plasticity of many traits may not contribute to fitness and reflects pleiotropic responses to environmental changes. This uncertainty about the evolutionary consequences has not yet been settled because of the

490 difficulty to link plasticity with fitness advantage. Our study links the
 491 evolutionary response in a laboratory natural selection experiment to plasticity
 492 in the founder population. Out of 3,605 genes with plastic gene expression
 493 pattern after exposure to two temperatures, 327 genes (9%) changed plasticity
 494 after 59 generations. Reasoning that the hot laboratory environment is more
 495 extreme than the habitat of the founder population, genes with adaptive
 496 plasticity for temperature should evolve towards increased plasticity (Lande
 497 2009; Garland and Kelly 2006). Consistent, with this expectation, 75% of the
 498 genes with evolved plasticity increased their environmental sensitivity. Genes
 499 with increased thermal sensitivity showed functional enrichment and were more
 500 consistent in their change across replicates. Our gene expression results are in
 501 line with the prevalence of genetic variation for thermal plasticity in natural
 502 *Drosophila* populations (Zhao et al. 2015; Levine et al. 2011, but see Clemson et
 503 al. 2016). The parallel evolution in plasticity suggests a selective advantage of
 504 populations with evolved plasticity, but our experiment cannot decide whether
 505 the evolved plasticity is providing the fitness advantage or it is a pleiotropic
 506 effect caused by the true target of selection. Finally, most of the genes with
 507 increased plasticity showed an opposite evolutionary response at 15°C and 23°C
 508 leading to the reinforcement of the ancestral plasticity which is expected in the
 509 case of adaptive plasticity (Ghalambor et al. 2007, Ho and Zhang 2018).
 510 Future experiments, measuring individual flies gene expression would allow us
 511 to study the evolution of the trait gene expression plasticity. Comparing the trait
 512 distribution in the ancestral and evolved populations after the new trait
 513 optimum has been reached will provide further insights in the underlying
 514 adaptive architecture.

515 Evolution of plasticity is more frequent than constitutive expression changes

516 In hot evolved populations, only 7% (52 out of 729) of the genes, which evolved
517 a significant response relative to the founder population at 23°C, showed a
518 constitutive expression difference rather than an evolutionary change of
519 plasticity. It is not clear if this predominance of plastic response reflects the
520 design of the laboratory natural selection experiment, which involved daily
521 temperature fluctuation or a correlated response to directional selection
522 (Garland and Kelly 2006).

523 While the flies evolved in a novel temperature regime with daily fluctuations, we
524 measured gene expression in constant temperature regimes to avoid
525 confounding effect of development at different temperatures. Classic examples
526 for the persisting effects of short-term exposure to high temperatures are
527 phenocopies. Short (<5h) sensitive periods of *Drosophila* pupae result in
528 different phenotypes depending on the developmental stage during exposure
529 (Mitchell and Lipps 1978). Hence, even small differences in developmental
530 timing could result in large phenotypic variation within or between populations.
531 Thus, we opted for a constant temperature common garden. This strategy
532 assured phenotypic measurements insensitive to daily temperature fluctuations,
533 reflecting fixed temperature effects that are comparable to existing phenotype
534 data. Given that the expression of most genes changes monotonically with
535 temperature (Chen et al. 2015a), we anticipated that observed differences in
536 reaction norm at 15 and 23°C can be extrapolated to more extreme temperatures,
537 such as 10 and 28°C.

538 While it is possible that the observed gene expression changes are not the direct
539 target of selection, it would not challenge our claim that ancestral plasticity is

likely to be adaptive: even if the evolution of the gene expression in our experiment is only correlated with the selected trait(s), the ancestral plasticity we observed at the gene expression level remains an indicator of adaptive plasticity because the direction of change is, by definition, the same between these correlated traits. The validity of our conclusions would only be challenged if during evolution these phenotypic correlations across temperature were broken. We consider this, however, unlikely as the temperature response is conserved across populations and various *Drosophila* species (see also Zhao et al. 2015).

We previously identified SNF4Ay and Sestrin as targets of selection in the same hot evolved populations (Mallard et al. 2018). Both genes are associated with activity of AMPK, a key enzyme in metabolism regulation. Interestingly, the role of AMPK in thermal plasticity have been highlighted in marine invertebrates such as mussels and rock crabs that are regularly subjected to temperature variation during tides (Frederich et al. 2009 ; Jost et al. 2014). Moreover, mussels experience seasonal variation in thermal plasticity of AMPK activity (Jost et al. 2014), which is comparable to the evolution of plasticity in our evolved populations. In addition to metabolism, chitin synthesis was found to be plastic, which is shared with *D. melanogaster* (Chen et al. 2015) and in the North American cline (both *D. melanogaster* and *D. simulans* (Zhao et al. 2015)). Chitin is involved in exoskeleton morphogenesis and its decreased synthesis may be associated with the temperature-induced size reduction in *Drosophila*. However, we did not find any evolution of body size during our experiment (data not shown). Alternatively, chitin is also essential for trachea formation (Moussian et

564 al. 2005), and the evolution of its synthesis in our experiment could be linked
565 with the decrease in metabolism gene expression.

566 Previous experimental evolution studies in *Drosophila* have found inconsistent
567 results regarding the evolution of gene expression plasticity (Huang and Agrawal
568 2016; Yampolski et al. 2012) and it is not clear if this inconsistency can be
569 explained by different environmental stressors. On the other hand, it has been
570 proposed that plasticity increases during the initial phase of adaptation to novel
571 environments, followed by genetic assimilation (Lande 2009). In this theoretical
572 scenario, also called “plasticity first” (Levis and Pfenning 2016; Levis et al. 2018),
573 the genomic variation which encodes phenotypic plasticity is favored as a rapid
574 phenotypic response. As a consequence, selection signatures are expected for
575 genes modulating plasticity, rather than in cis-regulatory variation of genes with
576 modified gene expression patterns.

577 In the context of the current ongoing climate change, the role of phenotypic
578 plasticity has been widely discussed - does plasticity favor or limit genetic
579 adaptation (Merilä and Hendry 2014; Sgrò et al. 2016; Vázquez et al. 2017;
580 DeBiasse et al. 2018)? As recently stated by Kelly (2019), if plasticity is a major
581 contributor of adaptation to climate change, then the amount of available genetic
582 variation for plasticity could be a reliable predictor of a population vulnerability.

583 In particular, for *Drosophila*, the potential of plasticity for attenuating the impact
584 of climate change has been challenged. Thermal plasticity does not correlate with
585 latitude (Sørensen et al. 2016) and did not respond to laboratory natural
586 selection in higher order phenotypes when submitted to stable or fluctuating
587 environments (Fragata et al. 2016; Manenti et al. 2015). Our experiments
588 provide some important insights into this debate. The highly parallel response in

replicated populations demonstrates that genetic variation in thermal plasticity is a reservoir for adaptation in novel thermal environments. Because we studied plasticity after only a moderate number of generations, our study is not informative for more long-term evolutionary processes. Recently, it has been shown that on the long term this evolutionary response could lead to extinction unless a small number of genetic loci are involved (Nunney 2016). Whether a phase of genetic assimilation will follow this initial increase in plasticity will depend on the availability of the relevant variation. If such variants are still segregating, it could be informative to test our experimental populations at later generations. If new mutations are required, experimental evolution in *Drosophila* may not be well-suited to address this question because the spread of new mutations is rare (Burke et al. 2010).

601

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609 **Data Accessibility**

Sequence reads from this study will be available at the European Sequence Read Archive (<http://www.ebi.ac.uk/ena/>). Additionally, we will provide our gene expression count table and all our R code on dryad.

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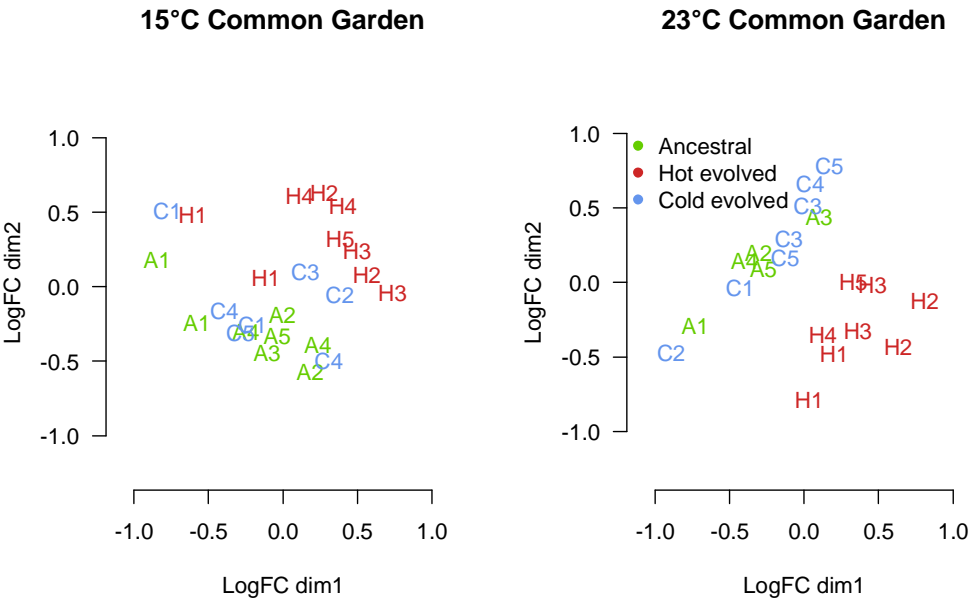
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802 **Supplemental Figures**

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Fig. S1: Multi-dimensional scaling plots of the different samples. Ancestral, Cold evolved and Hot evolved population are respectively labeled A[1-5], C[1-5] and H[1-5] and colored in green, blue and red. Left and right panels respectively show the samples of the 15°C and 23°C common garden experiments. In both experiments, we did not detect any outliers.

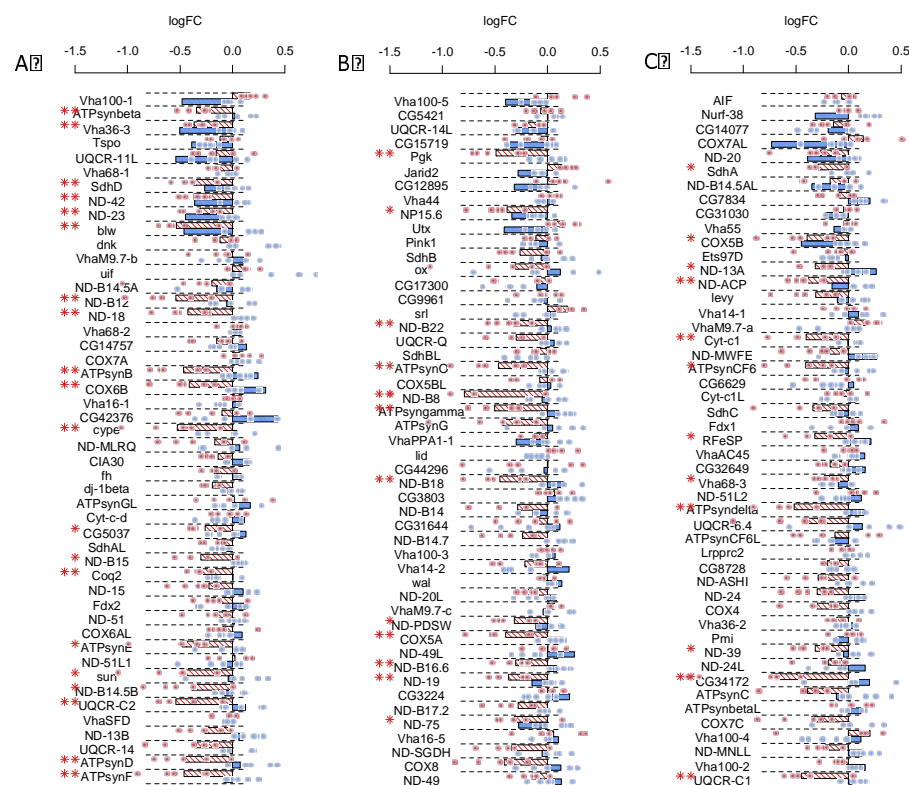
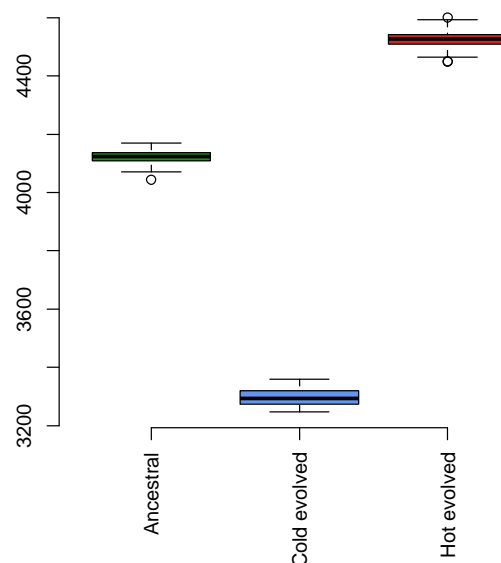


Fig. S2: Log₂FC of gene expression between the ancestral and the hot evolved populations at 15°C (blue) and 23°C (red) of the 146 genes involved in the oxidative phosphorylation. Most of these genes are significantly down regulated at 23°C (n=45, ** FDR<0.05, * FDR<0.1). Even though most comparison are not statistically significant, most of the down regulated genes at 23°C are up-regulated at 15°C. We produced independent plot for each gene showing the variability across our five replicates in a Supplementary File.

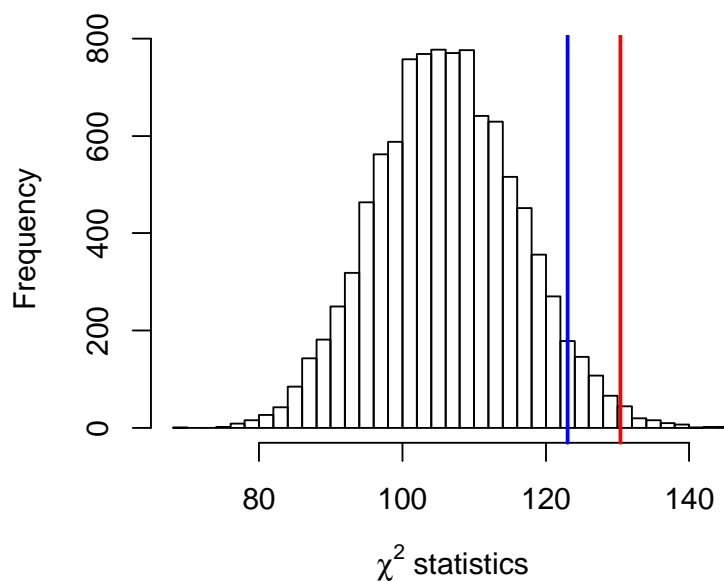
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828 Fig. S3: The number of genes statistically plastic in the ancestral and the two
829 groups of evolved populations after down-sampling each library to the same
830 number of reads (14,464,603). These distribution result from 100 independent
831 sampling of the complete libraries. Although the total number of plastic genes is
832 smaller after the down-sampling, the differences between groups are similar.
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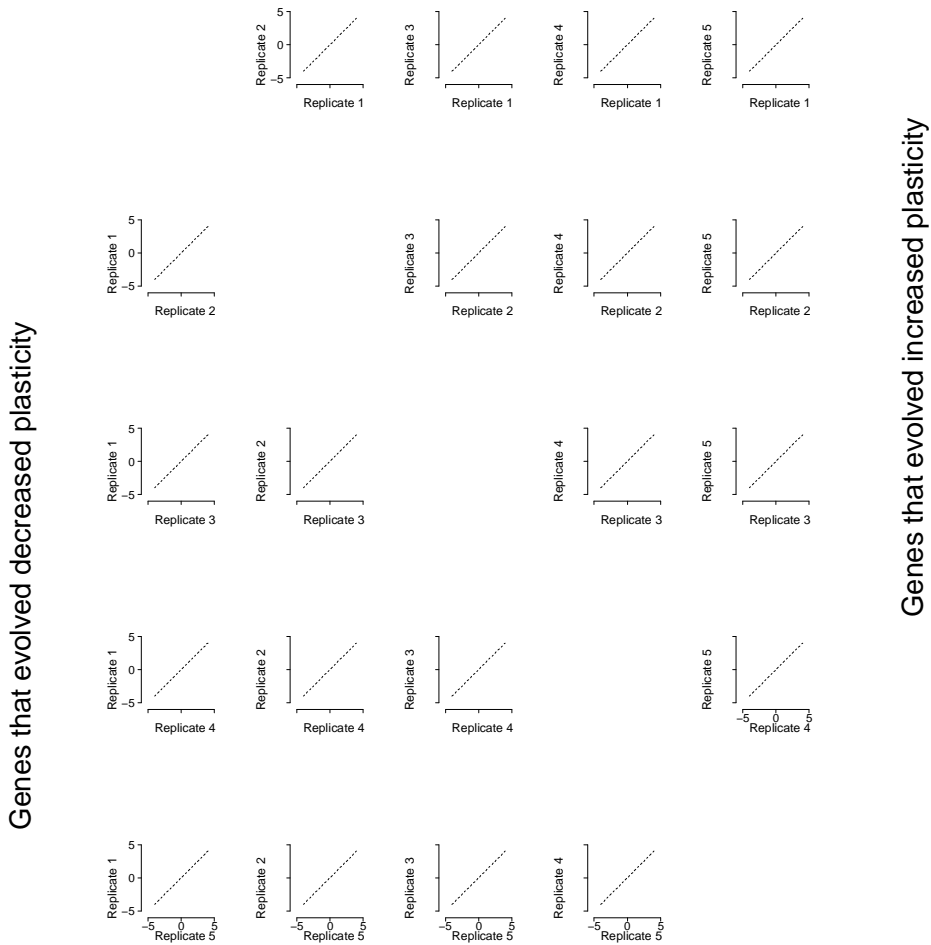
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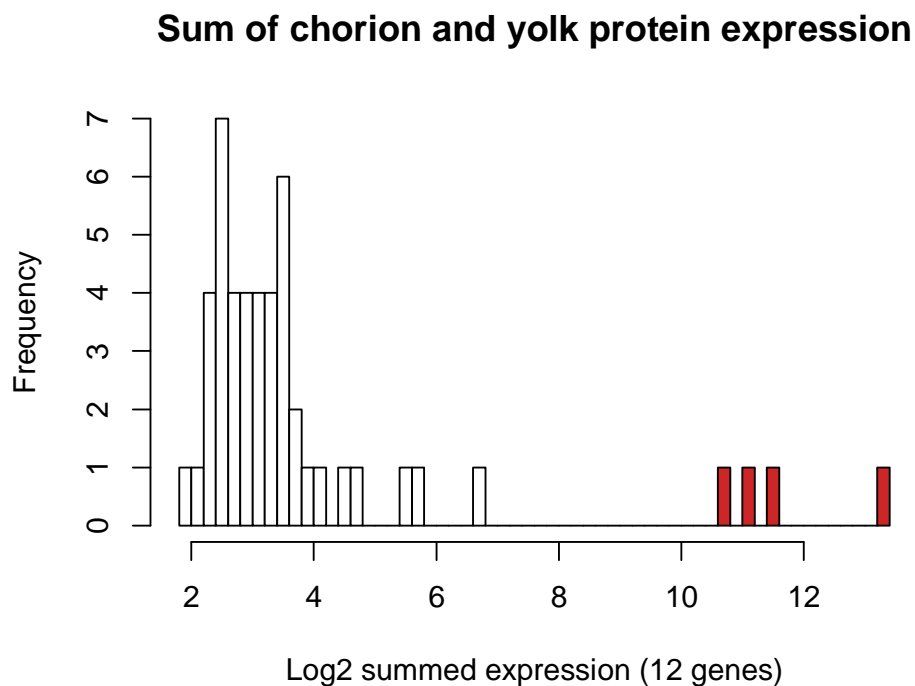
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Fig. S4: The direction of the evolved gene expression change is highly correlated at 15°C and 23°C. Because the genes are unlikely to evolve independently, the χ^2 assumptions are violated. To overcome this limitation, we generated an empirical distribution of χ^2 statistics by bootstrapping the genes that had a significant change in plasticity during evolution in the hot environment and still found a more significant correlation for the sign of gene expression change at 15°C and 23°C than expected by chance. This empirical distribution of χ^2 statistics was obtained by bootstrapping 241 genes 10,000 times from the list of 417 genes that evolved a significant change in plasticity (FDR<0.1, here we did not condition on a significant evolution at 15°C or 23°C). For each list of 241 genes, a χ^2 test of independence was computed between the direction of the evolved expression change at 15°C and 23°C. The statistic obtained using the genes that evolved increased plasticity (observed data, red line) is larger than 99% of the statistics obtained by bootstrapping (the blue line shows the 95% threshold). This test indicates that the negative correlation observed for the list of genes showing increasing plasticity could not be obtained from a random sampling of genes showing overall plasticity evolution. Here we assume that both lists of genes have equal modularity: the evolution of a subset of these genes is due to a similar number of causative events.

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859 Fig. S5: Pairwise log2FC of reaction norm slopes between five evolved replicates
860 (as rows and columns). The bottom half displays the genes with decreased
861 plasticity during evolution and the top half the genes with increased plasticity
862 during evolution (see Fig 1-3C with matching color code). There is a strong
863 correlation of the plasticity evolution across all 5 hot-evolved replicates for
864 genes with increased plasticity (top) but not for genes with decreased plasticity
865 (bottom).
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871 Fig. S6: Identification of libraries with female contamination. We summed the
872 expression of the nine chorion genes (CP15 to 19, CP36, CP38) and three yolk
873 proteins (YP1 to 3). We excluded four outlier libraries (in red) with > 1769
874 counts per million reads for the 12 indicator genes. The retained libraries had <
875 111 counts per million reads.
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