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**Research article**

**Genome-wide selection signatures reveal widespread synergistic effects of culture conditions and temperature stress in *Drosophila melanogaster***

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**KEYWORDS**

experimental evolution, *Drosophila melanogaster*, parallel evolution, local adaptation, G×E, temperature adaptation

23 **ABSTRACT**

24 Experimental evolution combined with whole-genome sequencing is a powerful approach to  
25 study the adaptive architecture of selected traits, in particular when replicated experimental  
26 populations evolving in opposite selective conditions (e.g. hot vs. cold temperature) are  
27 compared. Nevertheless, such comparisons could be affected by environmental effects shared  
28 between selective regimes (e.g. laboratory adaptation), which complicate the interpretation of  
29 selection signatures. Here, we used an experimental design, which takes advantage of the  
30 simplicity of selection signatures from founder populations with reduced variation, to study  
31 the fitness consequences of the laboratory environment (culture conditions) at two  
32 temperature regimes. After 20 generations of adaptation at 18°C and 29°C, strong genome-  
33 wide selection signatures were observed. About one third of the selection signatures can be  
34 either attributed to temperature effects, laboratory adaptation or the joint effects of both. The  
35 fitness consequences reflecting the combined effects of temperature and laboratory  
36 adaptation were more extreme in the hot environment for 83% of the affected genomic  
37 regions, fitting the pattern of larger expression differences between founders at 29°C. We  
38 propose that evolve and resequence (E&R) with reduced genetic variation allows to study  
39 genome-wide fitness consequences driven by the interaction of multiple environmental  
40 factors.

41

## 42 INTRODUCTION

43 Ecological genetics aims to characterize the interaction of organisms with their environment.  
44 Of particular interest is the characterization of adaptive responses, which are specific to a  
45 given habitat. Many approaches have been pursued to study the genetic basis of local  
46 adaptation (Savolainen et al., 2013, Tiffin & Ross-Ibarra, 2014, Whitlock, 2015, Hoban et al.,  
47 2016, Lorant et al., 2020). Allele frequency differences between populations from different  
48 environments are particularly powerful to test for correlation between genetic variation and  
49 environmental variables (Coop et al., 2010, Günther & Coop, 2013), and are widely applied  
50 to the analysis of clinal variation (Rako et al., 2007, Kolaczowski et al., 2011, Fabian et al.,  
51 2012, Bergland et al., 2016, Calfee et al., 2020). Despite being conceptually appealing, this  
52 approach faces several challenges. Historical demographic events, such as bottlenecks or  
53 admixture, may generate confounding signals, possibly resulting in false positives/negatives  
54 (Thornton & Jensen, 2007, Pavlidis et al., 2010, Lohmueller, 2014, Lotterhos & Whitlock,  
55 2015, Johri et al, 2020). Furthermore, estimating covariance between allele frequencies and  
56 environment is difficult as i) identifying and/or measuring the relevant environmental  
57 variables is challenging since many abiotic factors are correlated (Mittler, 2006, MacColl,  
58 2011) and ii) selection can vary over time (e. g. Bergland et al., 2014, Behrman et al., 2018,  
59 Grainger et al., 2021).

60

61 Experimental evolution, in particular laboratory natural selection, allows to study adaptive  
62 responses in a controlled laboratory environment (Burke & Rose, 2009, Garland & Rose,  
63 2009, Kawecki et al., 2012, Schlötterer et al., 2015). Exposing a mixture of genotypes to a  
64 monitored stressor, the adaptive response can be measured through time in replicate  
65 populations, combined with next-generation sequencing (Evolve and Resequence (E&R);  
66 Turner et al., 2011, Schlötterer et al., 2014, Long et al., 2015). While many experimental  
67 evolution studies rely on truncating selection to determine the genotypes contributing to the  
68 next generation (Turner et al., 2011, Turner & Miller, 2012, Griffin et al., 2017, Hardy et al.,  
69 2018, Gerritsma et al., 2019), laboratory natural selection builds on fitness differences  
70 between genotypes upon exposure (Garland & Rose, 2009) and hence provides a closer fit to  
71 adaptation and competition processes occurring in the wild (Hsu et al., 2021).

72

73 A major challenge for the interpretation of molecular selection signatures comes from the few  
74 recombination events during the laboratory experiment resulting in strong linkage  
75 disequilibrium (Nuzhdin & Turner, 2013, Tobler et al., 2014, Franssen et al., 2015). Strong

76 linkage reduces the efficiency of natural selection as a consequence of Hill-Robertson effect  
77 (Hill & Robertson, 1966, Roze & Barton, 2006). Starting with many different founder  
78 genotypes (Baldwin-Brown et al., 2014, Kofler & Schlötterer, 2014, Kessner & Novembre,  
79 2015, Vlachos & Kofler, 2019) and using the selected haplotype blocks as the selective unit  
80 rather than individual SNPs (Franssen et al., 2017, Barghi et al., 2019, Otte & Schlötterer,  
81 2021) may partially overcome the lack of resolution. Nevertheless, increasing the number of  
82 founders will increase the pool of adaptive variants and consequently the number of  
83 beneficial genotypic combinations to reach the trait optimum (Yeaman, 2015, Barghi et al.,  
84 2019, Barghi & Schlötterer, 2020, Laruson et al., 2020). One proposed solution to study the  
85 selective response of highly polygenic traits builds on reducing the genetic variation in the  
86 founder population (Sachdeva & Barton, 2018, Burny et al., 2021, Langmüller et al., 2021).  
87 Assuming that even the use of only two haplotypes provides sufficient segregating variation  
88 to adapt to rapid thermal change, we focused on laboratory adaptation as an environmental  
89 factor common to two different temperature regimes. We used 18°C, a putatively non-  
90 stressful temperature regime since the two founder genotypes of our experiment showed very  
91 similar gene expression profiles at 18°C (Chen et al., 2015, Jaksic & Schlötterer, 2016). In  
92 contrast, 29°C is a very stressful temperature regime, close to the maximal temperature at  
93 which *D. melanogaster* populations can be maintained (Hoffmann, 2010). We observed a  
94 very strong selection response across the entire genome. About one third of the genomic  
95 regions responded either only to temperature, laboratory conditions, or exhibited a significant  
96 joint effect of both stressors. Our results demonstrate the importance of the combined effects  
97 of different environmental factors .

98

## 99 **MATERIALS AND METHODS**

100

101 **Experimental set-up** We used the Oregon-R and Samarkand strains inbred by Chen et al.  
102 (2015), and maintained since then at room temperature. The three replicates of both  
103 experimental evolution cages were set up in parallel, each with a census size of 1,500 flies  
104 and accidentally with a starting frequency of 0.3 for the Oregon-R genotype (0.7 for the  
105 Samarkand genotype) - rather than 0.5, as described in Burny et al, 2021. Briefly, all  
106 replicates were then maintained for 20 generations at either constant 29°C or constant 18°C  
107 in dark conditions before sequencing. 300 adults were transferred every generation to one of  
108 five bottles for two days of egg laying. After egg laying, all adults were removed and frozen.  
109 The egg lay resulted in a high density of larvae. Hence, we transferred a mixture of larvae

110 and food to two fresh food bottles. Adults collected 8-32 hours after eclosure of the first flies  
111 from all bottles were mixed to avoid population substructure. 300 adults from each vial  
112 started the next generation.

113

114 **DNA extraction, library preparation, sequencing** Whole-genome sequence data for the  
115 parental Oregon-R and Samarkand strains are available in Burny et al, 2021. The evolved  
116 replicates in generation F20 were sequenced using Pool-Seq: genomic DNA was prepared  
117 after pooling and homogenizing all available individuals of a given replicate in extraction  
118 buffer, followed by a standard high-salt extraction protocol (Miller et al., 1988). For the  
119 samples in the 29°C experiment, barcoded libraries with a targeted insert size of 480 bp were  
120 prepared using the NEBNext Ultra II DNA Library Prep Kit (E7645L, New England Biolabs,  
121 Ipswich, MA) and sequenced on a HiSeq 2500 using a 2×125 bp paired-end protocol. For the  
122 samples in the 18°C experiment, we used the same library preparation protocol, but with a  
123 target insert size of 280 bp, and 2×150 bp reads were sequenced on the HiSeq X Ten  
124 platform.

125

126 **Allele frequency tracking** We previously established a catalogue of parental SNPs (Burny et  
127 al., 2021). Briefly, a parental SNP was defined as a (nearly) fixed difference between parental  
128 lines with a 0/0 (1/1) genotype in the Samarkand parent and 1/1 (0/0) genotype in the  
129 Oregon-R parent at the marker position, conditioning for a frequency of the alternate allele  
130 lower than 0.05 (if 0/0) or higher than 0.95 (if 1/1) for a final list of 465,070 SNPs; 401,252  
131 and 63,818 SNPs on the autosomes and the X chromosome, respectively, equivalent to 1 SNP  
132 every 271 bp on the autosomes and 363 bp on X. The same processing and mapping steps  
133 were applied at 29°C and 18°C described in (Burny et al., 2021). The allele frequency have  
134 been obtained after converting processed BAM files from pileup (*samtools mpileup -BQ0 -*  
135 *dI0000*; version 1.10; (Li et al., 2009)) to sync files (using *PoPoolation2 mpileup2sync.jar*;  
136 (Kofler et al., 2011)). We then tracked the allele frequency at F20 of the Oregon-R allele in 3  
137 replicates at 29°C (replicates 1,2,3 in Burny et al, 2021) and 3 replicates at 18°C. The  
138 subsequent analyses have been performed with R (version 4.0.4; (R Core Team 2020)) and  
139 most panels have been generated with the ggplot2 R package (Wickham, 2016). We retained  
140 SNPs measured at both temperatures, leading to a total of 100,283, 89,929, 107,119, 103,760,  
141 72, 63,766 SNPs on 2L, 2R, 3L, 3R, 4 and X. Because the average coverage at the marker  
142 SNPs differs between both temperatures (12, 11, 9× at 18°C and 123, 107, 133× at 29°C), we  
143 down-sampled the 29°C coverage values to 12× by drawing the coverage at each locus from

144 a Poisson distribution of mean 12 and then applying binomial sampling with a sample size set  
145 to the sampled coverage to mimic Pool-Seq sampling noise (Taus et al, 2017). In order to  
146 both limit noise in allele frequency measurements and to take linkage into account, the allele  
147 frequency values are averaged in non-overlapping windows of size  $w=50, 250$  or  $500$  SNPs  
148 for a total of  $8,021, 1,603, 801$  measurements on the autosomes (2 and 3) and  $1,275, 255, 127$   
149 on X for each window size respectively, where the last window of each chromosome,  
150 containing fewer than  $w$  SNPs. Windows of size  $w=50, 250$  or  $500$  SNPs correspond to  $13.6$   
151  $[12.8; 14.4], 67.8 [59.7; 76.0]$  and  $135.6 [115.8; 155.4]$ kb on average for the autosomes and  
152  $18.2 [16.6; 19.7], 90.5 [81.4; 99.5]$  and  $180.9 [162.1; 199.8]$ kb for X. The 95% confidence  
153 intervals have been obtained by the mean  $\pm 1.96$  SE, with SE standard error. The main  
154 results are represented at 250-bp level. A window position  $i$  is defined by its center ((right-  
155 left)/2). By convention, if the Oregon-R allele frequency at F20 is higher (lower) than its  
156 initial frequency of 30% (70%), the Oregon-R (Samarkand) allele increased in frequency and  
157 the allele frequency change (AFC) is positive (negative).

158 **Comparing the response between the 18°C and 29°C selection regimes** We classified the  
159 AFC of each window after 20 generations as non-significantly deviating from neutrality or  
160 presenting a selection signal. In order to test deviation from neutrality, we performed 100  
161 neutral simulation runs using MimicrEE2 (Vlachos & Kofler, 2018). The neutral simulations  
162 mimic the experimental set-up, *i.e.* starting with 30% of Oregon-R flies over 1,500 flies,  
163 using three replicates and the same marker SNPs providing the *D. melanogaster*  
164 recombination map (Comeron et al., 2012) updated to version 6 of the reference genome  
165 using the Flybase online Converter (<https://flybase.org/convert/coordinates>; accessed in July  
166 2020). For each simulation run, we computed the average AFC over the three replicates per  
167 window. Per temperature and per chromosome, an empirical p-value per window  $w$  ( $p_w^{18^\circ\text{C}}$   
168  $^{\text{neutral}}$  or  $p_w^{29^\circ\text{C}}$   $^{\text{neutral}}$ ) is calculated as the fraction of AFC values higher (lower) than the  
169 empirical AFC when the observed AFC is positive (negative) divided by the total number of  
170 average AFC values. We finally applied a Benjamini-Hochberg correction per chromosome  
171 ( $p.\text{adj}_w^{18^\circ\text{C}}$   $^{\text{neutral}}$  and  $p.\text{adj}_w^{29^\circ\text{C}}$   $^{\text{neutral}}$ ). If a window presents a selection signal, it either favors  
172 the same parental allele at both temperatures (with a change in magnitude or not) or different  
173 alleles - for example the Oregon-R allele at 29°C ( $\text{AFC}_w^{29^\circ\text{C}} > 0$ ) and the Samarkand allele at  
174 18°C ( $\text{AFC}_w^{18^\circ\text{C}} < 0$ ). To check which scenario is more likely, we fitted a simple linear model  
175 (LM) for each window  $w$ , with AFC as response and temperature as fixed categorical  
176 explanatory factor, where  $\alpha_w^{\text{intercept}}$  corresponds to 18°C-reference level and  $\alpha_w^{\text{temperature}}$  is the

177 contrast between 29°C and 18°C. We extracted the corresponding p-value ( $p_w^{LM}$ ) and applied  
178 a Benjamini-Hochberg correction per chromosome on the non-neutral windows ( $p_{adj_w}^{LM}$ ). A  
179 significant window is classified as displaying a change in magnitude with the temperature  
180 favoring the same parental allele ( $\alpha_w^{intercept}$  and  $\alpha_w^{temperature}$  of same sign) or a different allele  
181 ( $\alpha_w^{intercept}$  and  $\alpha_w^{temperature}$  of different sign). For a given False Discovery Rate (FDR)  
182 threshold, a genomic window  $w$  is then classified in one of the following 6 classes: “drift  
183 only”, “change 18°C only”, “change 29°C only”, “no temperature effect”, “different  
184 magnitude” and “different direction” (see Table SI 1 for logical conditions on windows  
185 affection to each class). We then recorded the fraction of windows affected in each of the 6  
186 classes for different values of FDR (5%, 10%, 15%) per chromosome and averaged genome-  
187 wide (GW). We also computed the autocorrelation per chromosome and per replicate using  
188 the *acf* R function; the autocorrelation at a given step  $k$  is defined as the correlation between  
189 windows at positions  $i$  and  $i+k$ , where  $k$  is called the lag. We eventually recorded the distance  
190 where a significant decrease in autocorrelation at a 5% threshold (below  $1.96/\sqrt{n}$ ,  $n$  the  
191 number of windows), *i.e.* a rough proxy of linkage equilibrium, is reached.

192 **Ancestral gene expression re-analysis** We used ancestral gene expression values at 18°C  
193 and 29°C for each genotype (Chen et al., 2015). The parental gene expression is reported as  
194 the log2-transformed fold change of expression of the Samarkand genotype relative to the  
195 Oregon-R genotype expression used as a reference, noted logFC S/O. In order to correlate  
196 parental gene expression and allele frequency changes, we computed the AFC per gene as the  
197 average of AFC of parental markers located within the gene. To that aim, we needed to  
198 convert the genes position to the updated version of the *D. melanogaster* GTF annotation  
199 (v6.36). We downloaded the gene conversion IDs from FlyBase using “[wget  
200 ftp://ftp.flybase.net/releases/current/precomputed\\_files/genes/fbgn\\_annotation\\_ID\\_\\*.tsv.gz](ftp://ftp.flybase.net/releases/current/precomputed_files/genes/fbgn_annotation_ID_*.tsv.gz)”  
201 the 25<sup>th</sup> November 2020. Over 7,853 gene expression values, remained 7,844 genes for which  
202 the conversion was possible. We then computed per gene the average AFC of all SNPs within  
203 the entire genic region (exons, introns and UTRs) over a total of 7,751/7,844 genes due to the  
204 sparse distribution of marker SNPs with on average 36 markers (median of 12) per gene. We  
205 first searched for the presence of any genome-wide correlations between the logFC S/O  
206 differential (logFC S/O 29°C - logFC S/O 18°C) and the AFC differential (AFC 29°C - AFC  
207 18°C) paired by gene, measured by the Spearman correlation coefficient  $\rho$ . Assuming that  
208 correlation, if it exists, might be caused by a subset of genes, we also computed  $\rho$  coefficients  
209 for an increased number of top genes (by subsets of 50 genes) either ranked by the absolute

210 logFC S/O differential or by the absolute AFC differential. To assess if the obtained trend, an  
211 exponential decrease of  $\rho$  with an increasing number of genes was more often seen than  
212 under a random ordering of the genes, we computed for each set of top  $x$  genes and for each  
213 ranking, the 95<sup>th</sup> quantile of 100 randomly chosen set of  $x$  genes. Eventually we performed a  
214 transcription factor binding sites (TFBS) enrichment analysis until 5kbp up-stream of each  
215 gene for the top 50 genes either ordered by decreasing logFC S/O at 18°C (48 genes present  
216 in the motifs database) or by logFC S/O at 29°C (44 genes present in the motifs database)  
217 using the RcisTarget bioconductor package (version 1.6.0; (Aibar et al., 2017)). The motifs  
218 database was downloaded from  
219 [https://resources.aertslab.org/cistarget/databases/drosophila\\_melanogaster/dm6/flybase\\_r6.02](https://resources.aertslab.org/cistarget/databases/drosophila_melanogaster/dm6/flybase_r6.02/mc8nr/gene_based/dm6-5kb-upstream-full-tx-11species.mc8nr.feather)  
220 [/mc8nr/gene\\_based/dm6-5kb-upstream-full-tx-11species.mc8nr.feather](https://resources.aertslab.org/cistarget/databases/drosophila_melanogaster/dm6/flybase_r6.02/mc8nr/gene_based/dm6-5kb-upstream-full-tx-11species.mc8nr.feather) the 20<sup>th</sup> November  
221 2020. Enrichment was defined using the default enrichment score of 3 and the number of  
222 motifs associated to a TFs was reported for each analysis.

223

## 224 **RESULTS**

225 We exposed two genotypes, Samarkand and Oregon-R, to two different environmental  
226 stressors, laboratory adaptation and temperature. Two E&R experiments shared the same  
227 laboratory environment, but differed in temperature regime. Three replicate populations were  
228 maintained for 20 generations at either 18°C or 29°C. Genome-wide allele frequencies of  
229 genotype-specific marker SNPs were determined by Pool-Seq (Schlötterer et al., 2014).  
230 Because genotype-specific alleles start at the same frequency in all replicates and only few  
231 recombination events were expected during the experiment, we averaged the allele  
232 frequencies in non-overlapping windows of 250 consecutive SNPs to obtain reliable allele  
233 frequency estimates. This strategy is supported by the high autocorrelation of neighboring  
234 SNPs, up to a distance of 6.7Mb (Fig SI 1). We inferred selection by contrasting the allele  
235 frequencies of the Oregon-R genotype at the start of the experiment (30%) to those after 20  
236 generations, relative to simulated frequency changes under neutrality. A positive allele  
237 frequency change (AFC) indicates that the Oregon-R allele increased in frequency.

238

239 After 20 generations marked allele frequency changes occurred at both temperature regimes  
240 (Fig 1A). The three replicate populations of each temperature regime showed a strikingly  
241 parallel selection response as indicated by the shaded area corresponding to +/- one standard  
242 deviation around the mean of the 3 replicates (Fig 2A). Overall, Oregon-R alleles were more



243 likely to increase in frequency than Samarkand alleles, with 90% and 80% of the windows  
244 displaying positive AFC at 18°C and 29°C respectively.

245

246 Given that the *Drosophila* populations were adapting to two different environmental  
247 stressors, laboratory environment and temperature, it is possible to evaluate their individual  
248 and joint effect on the selection response across the entire genome. We characterized the  
249 selection response by classifying windows changing more in frequency than expected under  
250 neutrality in each of the temperature regimes (Fig 2A,2B, Table SI 1). On the one hand, the  
251 direction of the selection response, *i.e.* the increase in frequency of the Oregon-R or  
252 Samarkand alleles, differed for 10% of the windows between the two temperatures (Fig 2,  
253 black). 8% (Fig 2, light blue) and 14% (Fig 2, purple) of the windows displayed a significant  
254 allele frequency change relative to drift, only at either 18°C or 29°C respectively. On the  
255 other hand, a similar allele frequency change was observed for 38% (Fig 2, dark green) of the  
256 windows in the two experiments, which we attribute to laboratory adaptation only. In total,  
257 roughly 2/3 of the genome responded only to one of the two environmental stressors.  
258 Nevertheless, a remarkable large fraction of windows showed a significant combined effect  
259 of the two environmental stressors. 28% (Fig 2, light green) changed in the same direction,  
260 but at a different magnitude between temperatures. This pattern of frequency change  
261 indicates that temperature modulates the adaptive response to the selective force, common to  
262 both experiments. For most of these windows (83%), the most extreme allele frequency  
263 change was observed at 29°C, which may suggest that the two stressors, temperature and  
264 laboratory environment, act synergistically. Only a small fraction (1%, Fig 2, yellow) of  
265 windows did not change in frequency beyond what is expected by drift in either treatment.  
266 Qualitatively similar results were obtained when the comparison between the two  
267 temperature regimes was performed for single SNPs or averaged across windows of 50, 250  
268 and 500 SNPs as well as with different False Discovery Rate (FDR) thresholds (Fig SI 3).

269

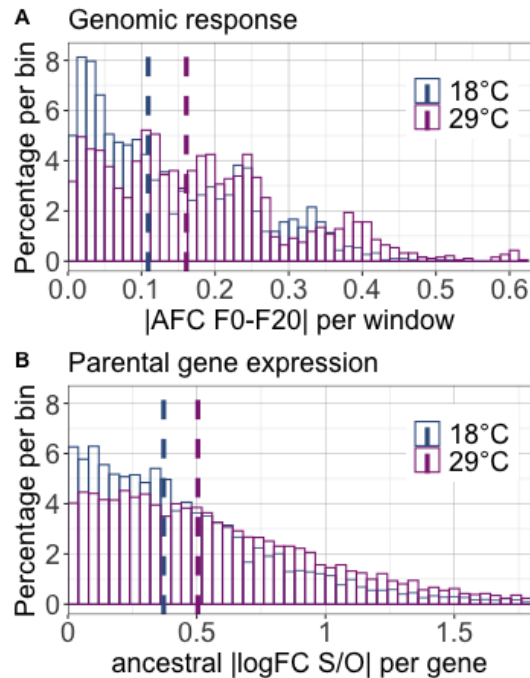
270 The elevated selection response to a high temperature laboratory environment may indicate  
271 that temperature stress increases the phenotypic variance on which selection can operate. We  
272 scrutinized this hypothesis by re-analyzing RNA-Seq data from the two parental genotypes  
273 exposed to 18° and 29°C (Chen et al., 2015). Although the difference in gene expression  
274 between the two genotypes was much more pronounced at 29°C than at 18°C, we found a  
275 positive correlation of the differences in gene expression between the two genotypes between  
276 18°C and 29°C (Fig 2C, Spearman  $\rho = 0.68$ ). This confirmed that the hot temperature

277 environment amplifies phenotypic differences between the two genotypes. Since traits with a  
278 higher phenotypic variance are responding more strongly to selection, we compared the  
279 genomic response at the two temperatures and found that the correlated expression changes  
280 are mirrored by the parallel selection response of genomic windows at the two temperatures  
281 (Fig 2A,2B,2D, Spearman  $\rho = 0.49$ ). Similar to the parental gene expression with a 36%  
282 increase of the median absolute logFC S/O at 29°C, the median absolute AFC increased by  
283 48% at 29°C relative to 18°C.

284

285 Given that both gene expression differences and selective responses are correlated between  
286 temperatures, we were interested whether they are actually functionally linked. We asked if  
287 the genes with the largest temperature-specific expression differences between the two  
288 parental genotypes also display the largest temperature-specific selection response. We  
289 measured the correlation of the parental gene expression and AFC differentials between 29°C  
290 and 18°C ( $|(\text{Sam-Or})_{18^\circ\text{C}} - (\text{Sam-Or})_{29^\circ\text{C}}|$ ). Neither for the full set of genes nor the top genes  
291 (ranked by decreased differential of logFC S/O or AFC), the AFC differential was  
292 significantly correlated with the parental expression differential (Fig SI 4). We conclude that  
293 the allele frequency changes in the experimental evolution are not primarily driven by  
294 parental expression differences. Thus, either parental gene expression differences have  
295 limited implication for fitness or the observed gene expression differences are driven by  
296 trans-acting factors rather than by cis-regulatory variation. We studied the potential of  
297 transcription factors driving the parental expression differences and found that the 50 genes  
298 with the strongest expression differences between the parents (ranked by decreasing absolute  
299 logFC) at 18°C and at 29°C (17 genes in common) were enriched for many rather than a few  
300 transcription factor binding sites (152 at 18°C and 133 at 29°C). We conclude that the  
301 temperature-specific gene expression differences between Samarkand and Oregon-R could be  
302 driven by many transcription factors, consistent with gene expression having a polygenic  
303 architecture.

304



305

306 **Figure 1.** Differences between the parental genotypes at 18°C (blue) and 29°C (purple).

307 Histograms of absolute allele frequency change of the Oregon-R allele between F0 and F20

308 (|AFC F0-F20|) for non-overlapping windows of 250 SNPs (A) and the absolute log2-fold

309 difference of expression between Samarkand and Oregon-R genotypes (|logFC S/O|) per gene

310 (B). The percentage of windows (A) and genes (B) in each of the 50 equally-spaced bins (bin

311 size: 0.0125 (A), 0.04 (B)) is reported on the y-axis. The dashed lines represent the median

312 absolute allele frequency change at 18°C and 29°C (0.11 at 18°C and 0.16 at 29°C, A; paired

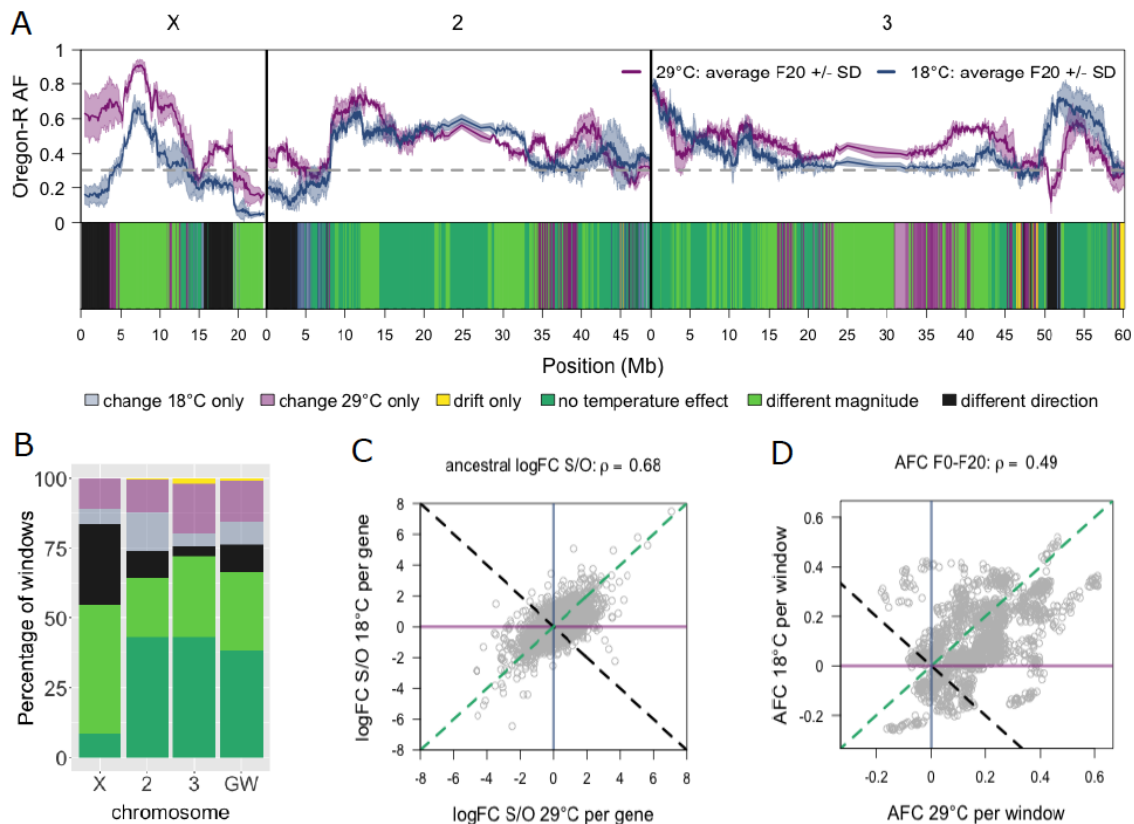
313 Wilcoxon one-sided test  $p$ -value= $1.3 \times 10^{-40}$ ) and median absolute logFC S/O (0.37 at 18°C

314 and 0.50 at 29°C, B; paired Wilcoxon one-sided test  $p$ -value= $1.8 \times 10^{-152}$ ). For the sake of

315 clarity, the x-axis of panel B is bounded at 1.8, which corresponds to 1.5 times the maximum

316 inter-quartile range of the gene expression data. The full histogram is shown in Fig SI 2.

317



318

319 **Figure 2.** A) *Top.* Genome-wide allele frequencies after evolving for 20 generations at two  
 320 temperature regimes. The frequency of the Oregon-R allele is averaged for non-overlapping  
 321 windows of 250 SNPs (solid line) +/- one standard deviation (shaded area) at 18°C (blue) and  
 322 29°C (purple). *Bottom.* Each window is classified (see Methods) in one of the 6 color-coded  
 323 classes depending of the AFC between 18°C and 29°C: change at 18°C only (light blue),  
 324 change at 29°C only (light purple), drift only (yellow), no temperature effect (dark green),  
 325 different AFC magnitude but same direction of effect (light green), opposite alleles increase  
 326 at 18°C and 29°C (black). B) Percentage of the genomic windows per class (defined for an  
 327 FDR threshold of 10% per chromosome and averaged genome-wide (GW)). Scatterplots of  
 328 the logFC S/O (C) and AFC (D) at 18°C (y-axis) and 29°C (x-axis). We reported the  
 329 Spearman  $\rho$  correlation coefficients.

330

331 **DISCUSSION**

332 We studied the selective impact of two different environments on a genomic scale by  
333 combining laboratory and temperature adaptation. Contrary to the recommended design for  
334 E&R studies (Kofler & Schlötterer, 2014), which facilitate the identification of a moderate  
335 number of selection targets occurring at sufficiently high starting frequencies, we did not use  
336 a large number of founder genotypes. Rather, we restricted the variation to only two different  
337 founder genotypes, as also done in experimental evolution with yeast (e.g. Kosheleva &  
338 Desai, 2018). The advantage of this experimental design is that all selection targets have the  
339 same starting frequency and a more parallel selection response is expected because polygenic  
340 traits have fewer selection targets contributing to a new trait optimum (Barghi & Schlötterer,  
341 2020, Höllinger et al., 2019).

342

343 We found pronounced selection responses, which fall into two classes – temperature-specific  
344 (change in the direction of the allele frequency change) and laboratory adaptation (parallel  
345 selection with similar intensities in the two temperature regimes). In addition, 28% of the  
346 genomic windows responded in the same direction, but to a different extent, indicating the  
347 joint contribution of both environmental factors.

348

349 Temperature-specific adaptation implies that temperature uncovers fitness differences  
350 between genotypes. 14% of the genomic windows responded only at 29°C and 8% were  
351 private to 18°C, a pattern consistent with conditional neutrality (Schnee & Thompson, 1984).  
352 The selection responses private to 18°C indicate that even at an assumed benign temperature,  
353 selection occurs - highlighting the challenge of performing control experiments for  
354 temperature adaptation. In 10% of the windows, different alleles were favored at each  
355 temperature regime. Such temperature-specific selection responses provide an excellent  
356 starting point for the identification of causative variants driving temperature adaptation.  
357 Nevertheless, the broad genomic regions responding to selection preclude the distinction  
358 between causative variants and neutral hitchhikers (Franssen et al., 2015, Nuzhdin & Turner,  
359 2013, Tobler et al., 2014) after 20 generations. Additional generations as well as a larger  
360 population size could facilitate the uncoupling of the causative variants from the passenger  
361 alleles and improve resolution (Langmüller et al., 2021, Phillips et al., 2020).

362

363 Laboratory adaptation is an umbrella term for stressors that can be attributed to the  
364 experimental laboratory setup (Matos et al., 2002, Matos et al., 2000, Simoes et al., 2007).

365 Examples of such factors are adaptation to high larval density / early fertility (Hoffmann et  
366 al., 2001, Mueller, 1997), sexual selection (Fricke & Arnqvist, 2007) and adaptation to the  
367 laboratory food (Bochdanovits & de Jong, 2003, Lai & Schlötterer, 2021, Vijendravarma et  
368 al., 2012). With about one third of the genomic windows showing a parallel selection  
369 response at both temperature regimes, laboratory adaptation was an important factor in this  
370 study.

371

372 Of particular interest is the significant difference in allele frequency change for 28% of the  
373 windows with parallel selection signatures, because it suggests an interaction between  
374 laboratory adaptation which drives the parallel response and temperature which modulates  
375 the strength of selection. Adaptation to larval density may be an excellent candidate driving  
376 this laboratory adaptation because we maintained the populations at high, but not well-  
377 controlled, larval densities. Higher larval density does not only increase competition  
378 (Mueller, 1988) but also interactions between larval density and heat stress survival (Arias et  
379 al., 2012) as well as body size (James & Partridge, 1998) and locomotor activity (Schou et  
380 al., 2013) were previously detected.

381

382 Because laboratory experiments cannot fully match natural conditions, it is not possible to  
383 conduct these experiments in a full factorial design - we can only modulate the temperature  
384 under laboratory conditions, but not in the natural environment. This implies that our design  
385 cannot distinguish between additive and interaction effects of temperature *per se* and  
386 laboratory adaptation. Selection responses driven by multiple selection factors can be  
387 problematic for the interpretation of the selection signatures. Experiments contrasting  
388 ancestral and evolved populations cannot distinguish between laboratory adaptation and  
389 selection driven by the focal factor (temperature in our study). When populations are  
390 compared, which evolved towards two different focal environments (here, different  
391 temperatures), the influence of laboratory adaptation is less severe: selection responses with  
392 the same direction and magnitude will not be seen in this contrast. Parallel selection  
393 responses that differ in magnitude will be interpreted as a pure temperature effect. An  
394 experimental design, which does not only include populations evolved in two different focal  
395 conditions (*i.e.* different temperatures), but also the ancestral founder populations, similar to  
396 this study, can distinguish between laboratory adaptation, adaptation to focal factor and  
397 combined effects. Nevertheless, if laboratory adaptation interacts with temperature (or other  
398 focal factors), it is possible that small differences in laboratory environment (e.g. food recipe)

399 may result in a different selection response. We propose that this may contribute to the  
400 difficulties to replicate temperature-associated effects.

401

402 An alternative explanation for the shared directional selection response at 18°C and 29°C is  
403 the presence of genotype-specific deleterious mutations. Since the two parental strains were  
404 maintained at small effective population size for many generations, it is conceivable that the  
405 influence of deleterious alleles is more pronounced than for genotypes freshly collected from  
406 wild. The selection signatures may thus also reflect fitness disadvantage of deleterious  
407 combinations of parental alleles that can be detected when the two competing genotypes are  
408 maintained at large population size. The observation that temperature stress can both increase  
409 and decrease the selection response is consistent with previous studies on deleterious  
410 mutations (Agrawal & Whitlock, 2010). While frequently the selection response was found to  
411 be positively correlated with stress level (e.g. Shabalina et al., 1997, Chu & Zhang, 2021),  
412 also the opposite pattern has been observed (Elena & de Visser, 2003, Kishony & Leibler,  
413 2003). Since we cannot determine how much of the parallel selection response can be  
414 attributed to deleterious mutations, it is important to realize that we probably overestimate the  
415 influence of laboratory adaptation.

416

417 One important limitation of this study is the pronounced linkage disequilibrium in the  
418 founder population. During 20 generations, too few recombination events occur to break the  
419 association between neighboring windows. This is indicated by autocorrelation of allele  
420 frequency up to 8Mb. Thus, even though our analyses are based on windows of 250  
421 neighboring SNPs, neighboring windows are still highly correlated. This implies that  
422 neighboring windows may exhibit a selection response due to linkage, rather than due to an  
423 independent selection target. Different selection intensities will also determine the size of the  
424 genomic region affected, leading to a complex interplay between linkage, direction of  
425 selection and selection strength. Therefore, the number of windows showing a given selection  
426 response may not be an accurate reflection of the number of selection targets with a given  
427 behavior. Nevertheless, the prevailing effects of temperature and laboratory adaptation on  
428 fitness should be robust against the effects of linkage.

429

430 We conclude that E&R experiments starting with strongly reduced genetic variation can  
431 provide a powerful approach to study adaptation, in particular when experiments are  
432 performed on an environmental gradient (*i.e.* multiple different temperatures). This setup

433 provides new insights into adaptation, in particular when the E&R experiments are performed  
434 for more than only 20 generations, since additional generations provide more opportunity for  
435 recombination and the selection targets can be characterized with a higher resolution.  
436



## 437 DATA AND SCRIPTS AVAILABILITY

438 The sequencing data underlying this article are available in the European Nucleotide Archive  
439 (ENA) at <https://www.ebi.ac.uk/ena/browser/view/>, and can be accessed with PRJEB46805  
440 from Burny et al, 2021 (29°C) and XXX for new data (18°C) specifically generated for this  
441 study. All scripts (command lines and data analysis) and final files underlying this article are  
442 available in Zenodo at <https://dx.doi.org/10.5281/zenodo.5614819>. Additional table and  
443 figures underlying this article are available in its online supplementary material.

444

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451

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