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1 Gene regulatory evolution in cold-adapted fly populations neutralizes plasticity and may
2 undermine genetic canalization

3

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11

12

13 **Abstract**

14 The relationships between adaptive evolution, phenotypic plasticity, and canalization remain
15 incompletely understood. Theoretical and empirical studies have made conflicting arguments on
16 whether adaptive evolution may enhance or oppose the plastic response. Gene regulatory traits
17 offer excellent potential to study the relationship between plasticity and adaptation, and they can
18 now be studied at the transcriptomic level. Here we take advantage of three closely-related pairs of
19 natural populations of *Drosophila melanogaster* from contrasting thermal environments that reflect
20 three separate instances of cold tolerance evolution. We measure the transcriptome-wide plasticity in
21 gene expression levels and alternative splicing (intron usage) between warm and cold laboratory
22 environments. We find that suspected adaptive changes in both gene expression and alternative
23 splicing tend to neutralize the ancestral plastic response. Further, we investigate the hypothesis that
24 adaptive evolution can lead to decanalization of selected gene regulatory traits. We find strong

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25 evidence that suspected adaptive gene expression (but not splicing) changes in cold-adapted
26 populations are more vulnerable to the genetic perturbation of inbreeding than putatively neutral
27 changes. We find some evidence that these patterns may reflect a loss of genetic canalization
28 accompanying adaptation, although other processes including hitchhiking recessive deleterious
29 variants may contribute as well. Our findings augment our understanding of genetic and
30 environmental effects on gene regulation in the context of adaptive evolution.

31

32 **Keywords**

33 adaptive evolution, transcriptomic plasticity, genetic canalization, *Drosophila melanogaster*

34

35 **Significance Statement**

36 It is unclear whether adaptive evolution is concordant or discordant with regulatory plasticity,
37 especially for splicing plasticity which is rarely studied. Here we analyzed RNA-seq data from
38 three pairs of natural fly populations that represent separate adaptive evolution to cold climate.
39 We found that adaptive evolution is generally discordant with the ancestral plasticity between
40 cold and warm temperatures for gene expression abundance and splicing. We also investigate the
41 hypothesis that adaptation leads to decanalization of the selected traits. By comparing the
42 expression variance between inbred and outbred samples, we found evidence that adaptation may
43 lead to genetic decanalization for expression abundance but not for splicing. Our study reveals
44 the relationship between adaptation, plasticity and canalization in three instances in nature.

45

46 **Introduction**

47 For organisms to cope with environmental changes, two important strategies are adaptive
48 evolution and phenotypic plasticity (Meyers & Bull, 2002). Phenotypic plasticity is the

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49 phenomenon of a single genotype producing different phenotypes under different environmental
50 conditions. Producing different phenotypes from a genotype often requires an intermediate step,
51 such as gene expression change (Ghalambor et al., 2007; Pfennig et al., 2010). It has also been
52 shown in many cases that expression evolution contributes to adaptive evolution (e.g., Fraser et
53 al., 2010; Nourmohammad et al., 2017). Studying the interactions between expression plasticity
54 and adaptive expression evolution can generate insights on how these two processes help
55 organisms respond to environmental changes.
56
57 Adaptation to a new environment may change gene expression in the same direction as the initial
58 plasticity, which suggests the initial plasticity is beneficial, shifting the phenotype toward the
59 optimal phenotypic values in the selective environment (Chevin et al., 2010; Via, 1993).
60 Alternatively, a plastic response can be deleterious if it shifts the phenotype away from the
61 optimum under environmental perturbation and adaptive evolution should restore the phenotype
62 toward the ancestral state (Scheiner, 1993; von Heckel et al., 2016). The latter scenario predicts a
63 negative relationship between the direction of plastic change and that of evolutionary change,
64 resulting in a "counter-gradient" pattern (Conover & Schultz, 1995). Finally, environmentally
65 induced expression change may have little effect on fitness. In that case, evolutionary change is
66 determined by drift, showing no relation with the plasticity in terms of direction. Many recent
67 laboratory and field studies have observed a "counter-gradient" pattern for plasticity and
68 adaptive evolution for gene expression (e.g., Dayan et al., 2015; Fischer et al., 2021; Ghalambor
69 et al., 2015; Ho & Zhang, 2018; Huang & Agrawal, 2016; Koch & Guillaume 2020; Levine et al.,
70 2011; Ragland et al., 2015) while some other studies found that adaptive plasticity is more
71 common (Bittner et al., 2021; Josephs et al., 2021; Kenkel & Matz, 2017; Mallard et al., 2020).

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72 However, few of them have studied multiple natural population pairs that reflect separate
73 adaptation events. Moreover, the relationship between plasticity and adaptive evolution for other
74 regulatory traits such as splicing is unexplored.
75
76 On the other hand, adaptation to a new environment might have a significant consequence on the
77 level of canalization of the selected trait. Canalization refers to the capacity of a trait to maintain
78 constant phenotype under genetic or environmental perturbation (Waddington 1942; Flatt, 2005).
79 This concept has been defined operationally in different ways (Dworkin 2005). Environmental
80 canalization has been described as the opposite of phenotype plasticity (Nijhout and Davidowitz
81 2003), as indicated by the constancy of a trait across environments. Hence, plasticity can be
82 viewed as a type of environmental de-canalization. However, environmental canalization can
83 alternatively be defined based on the trait variation observed among individuals in one
84 environment compared to others (Stearns and Kawecki 1994). Similarly, genetic canalization can
85 be described as the constancy of the phenotype under heritable perturbations, such as mutations
86 (de Visser et al. 2003). It can be measured as the inverse of the variance caused by mutations
87 (Flatt, 2005).
88
89 Canalization can potentially constrain evolution by reducing phenotypic variation (Charlesworth
90 et al. 1982; Maynard Smith et al. 1985), or it may facilitate evolution by allowing cryptic genetic
91 variation to accumulate (Gibson & Dworkin, 2004; Masel, 2005; Paaby & Rockman, 2014). If a
92 trait is under stabilizing selection in the ancestral environment, its developmental canalization
93 may be favoured by selection (Gavrilets & Hastings, 1994; Wagner et al., 1997). However, when
94 adaptive evolution shifts a trait to a new optimum, the previous canalization mechanism may be

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95 undermined. This possibility, that adaptive evolution could result in decanalization, was hinted at
96 when blowflies with newly-evolved insecticide resistance were found to have bristle asymmetry
97 and prolonged development (Clarke & McKenzie, 1987; McKenzie & Game, 1987), and also
98 when in vitro selection on an enzyme resulted in reduced robustness to both genetic and
99 environmental perturbations (Hayden et al. 2012). A natural example of decanalization of the
100 same trait that evolved adaptively was then provided by Lack et al. (2016b), who found that
101 highland Ethiopian *D. melanogaster* with derived large wing size had greatly reduced genetic
102 robustness of wing development. One subsequent study found that environmental decanalization
103 may not have occurred for these Ethiopian fly wings (Pesevski & Dworkin 2020), highlighting
104 the potential distinctness of genetic and environmental canalization. A further study showed that
105 when wing size in the same species was artificially selected, both genetic and environmental
106 decanalization of wing development ensued (Groth et al. 2018). Whereas, longer-term lab
107 selection of *D. melanogaster* for rapid development was suggested to increase canalization of
108 that trait (Ghosh et al. 2019). Whether a decanalizing effect of directional selection exists for a
109 substantial proportion of adaptive gene regulatory traits has not previously been investigated, and
110 this question represents one focus of the present study.
111
112 In this study, we take advantage of African and European populations of *Drosophila*
113 *melanogaster* that have experienced parallel adaptation to colder climates, to study the
114 interaction between adaptation and plasticity as well as canalization in gene expression.
115 Originating from a warm sub-Saharan range (Lachaise et al., 1988; Pool et al., 2012;
116 Sprengelmeyer et al., 2020), populations have independently occupied colder environments at
117 least three times: in higher latitudes of Eurasia (here represented by the France FR population),

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118 and in the highlands of Ethiopia (EF population) and South Africa (SD population). Flies from
119 these three locations were paired with genetically similar populations from warm regions: Egypt
120 (EG), Ethiopia lowland (EA), and South Africa lowland (SP), respectively, together representing
121 three different population pairs: Mediterranean (MED), Ethiopian (ETH), and South African
122 (SAF). These cold-adapted populations have evolved separately from the warm-adapted ones for
123 ~1,000-2,000 years (~15,000-30,000 generations) (Sprengelmeyer et al., 2020). They show
124 parallel changes in cold tolerance, as measured by recovery after prolonged 4°C cold exposure
125 (Pool et al., 2017) and greater egg-to-adult survival at 15°C (Huang et al., 2021). These cold-
126 adapted populations also show a genome-wide excess of parallel allele frequency shifts (Pool et
127 al. 2017).
128
129 In our related study (Huang et al. 2021), we identified evolutionary changes in gene expression
130 abundance and alternative splicing (intron usage) between cold- and warm-adapted populations
131 for each pair at a low temperature, approximating the derived cold environment. Here, we rear
132 the same crosses from these populations at a warm temperature, approximating the ancestral
133 environment, and analyze transcriptomes of adult females. First, we compare the directions of
134 gene regulatory plasticity between cold and warm environments and the directions of
135 evolutionary changes between cold and warm populations for both expression abundance and
136 intron usage. We find adaptive evolution tends to counter plasticity by restoring the ancestral
137 regulation (neutralizing the ancestral plasticity). Second, since we previously analyzed gene
138 regulation from some parental inbred lines of the outbred crosses we otherwise analyze (Huang
139 et al. 2021), we use inbreeding as a form of genetic perturbation (Réaale & Roff, 2003; de Visser
140 et al., 2003) to study regulatory canalization in the cold and warm populations and its potential

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141 disruption due to adaptive evolution. We use the ratio of regulatory trait variance among inbred
142 samples to that among outbred samples as a potential signal of genetic decanalization. We indeed
143 find transcriptome-wide evidence consistent with adaptive evolution leading to decanalization
144 for expression abundance traits, although other processes such as hitchhiking deleterious variants
145 many contribute to this signal as well.
146
147

148 **Results**

149 **Adaptive evolution and naïve plasticity in expression and splicing**

150 We collected RNA-seq data from whole female adult samples representing the three warm/cold
151 population pairs described above, raised at temperatures intended to reflect either an ancestral
152 warmer environment (25°C) or a derived cooler environment (15°C). For each of these six
153 populations, the transcriptomes from eight unique outbred crosses were analyzed. We first
154 performed PCA on the normalized expression read counts across samples from different
155 environments and populations. There are signals of temperature environment on transcriptome
156 variation, separating the samples from the cold environment from the respective samples from
157 the warm environment along the PC1 (Fig. S1). Then we characterized the genes/introns that
158 showed consistent plastic expression/splicing changes between 15°C and 25°C rearing
159 environments in warm-adapted populations (*P*). Formally, we required the direction of plasticity
160 for at least seven out of the eight crosses to be consistent with the average plastic change
161 (Materials and Methods). We refer to this pattern from warm-adapted populations as ancestral or
162 naïve plasticity, hypothetically representing the initial influence of derived cooler environments
163 on expression/splicing prior to any cold adaptation. Across the six populations (three warm/cold-

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164 adapted population pairs), 14-65% of all gene expression traits met the consistent plasticity
165 criterion (at least seven out of eight crosses showing the same direction of plastic change), as did
166 24-36% of intron usage traits (Table 1A). The Mediterranean pair had the greatest proportions of
167 consistently plastic traits for both expression and splicing. Across all populations and traits, an
168 average 29.9% genes/introns show consistent plasticity by our criteria. In light of our expected
169 false positive rate (7%), we estimate that approximately 77% of the identified genes are true
170 positives for plasticity, which provides a substantially enriched set of plasticity candidates for the
171 transcriptome-wide analyses described below.
172
173 We then set out to assess the general relationship between a warm-adapted population's naïve
174 plasticity and the evolved difference between the populations in the derived environment. Here,
175 we are asking whether evolutionary changes are concordant or discordant with naïve plasticity,
176 as opposed to asking how plasticity itself has evolved. We compared the directions of initial
177 plasticity (P) in warm-adapted populations and evolved change (E), which is the difference in
178 expression between cold and warm-adapted populations at 15°C. If P and E were in the same
179 direction (i.e., P □ $E > 0$), the evolved change was defined as "concordant" (Fig. 2). Considering
180 all plastic genes for gene expression abundance, the proportion of "concordant" changes was 53%
181 in MED, 55% in ETH, and 39% in SAF. For intron usage, the proportion of "concordant"
182 changes was 41% in MED, 53% in ETH, and 38% in SAF.
183
184 We further partitioned those traits where P and E were in opposite directions ("discordant", P □
185 $E < 0$). If the evolved change moved expression/splicing farther from ancestral levels than the
186 ancestral plasticity did, (the magnitude of evolved change was more than twice that of plasticity,

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187 $|E| > |2P|$), we defined it as "reversing". If instead, the evolved change brought the cold
188 population at 15°C back closer to the warm population at 25°C, then we defined the evolved
189 change as "neutralizing". These categories may have distinct evolutionary interpretations, since
190 neutralizing changes are consistent with a single trait optimum across thermal environments,
191 whereas for reversing changes, the observation that evolution increased trait differentiation
192 between the populations in their home environments could indicate distinct adaptive optima in
193 cold vs. warm environments. We found that among "discordant" changes, "reversing" was a
194 relatively small fraction across population pairs (Fig. 2; on average 11% for expression
195 abundance and 1.7% for intron usage). It is worth noting that the Ethiopia pair appears to have a
196 much higher proportion of "reversing" changes (32% for expression and 3.6% for intron usage)
197 than the other two pairs, suggesting distinct evolution in the Ethiopia pair.
198
199 To examine whether putative adaptive evolution in expression/splicing in cold populations
200 followed the initial plastic changes, we focused on genes showing elevated population
201 differentiation in gene expression abundance or intron usage between warm- and cold-adapted
202 populations based on Huang et al. 2021 (about 339 outlier genes and 351 outlier intron junctions
203 for each population pair). These candidates for adaptive gene regulatory evolution were
204 identified using a P_{ST} outlier approach, focusing on the top 5% of genes/introns for each
205 population pair separately (Huang et al. 2021; Materials and Methods). Among these outliers (an
206 average of 339 genes per population pair for expression and 351 introns for splicing), the
207 numbers of expression abundance genes also passing the cutoff for plasticity were in the range of
208 93 to 205 across population pairs; the numbers of divergent intron usage traits passing the
209 plasticity cutoff were in the range of 77 to 116 (Table 1B).

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210

211 For both expression abundance and intron usage, we observed a general pattern that plastic P_{ST}
212 outliers had lower proportions of "concordant" naïve expression plasticity than plastic non-
213 outliers across all population pairs (Fig. 3). In other words, putatively adaptively evolved
214 divergence tended to oppose the naïve plasticity. For expression abundance, we found the
215 difference in the "concordant" proportion was significant for the ETH (χ^2 = 8.3, df = 1, p =
216 0.0039) and SAF pairs (χ^2 = 15.1, df = 1, p = 9.9e-05) but only marginally significant for the
217 MED pair (χ^2 = 3.6, df = 1, p = 0.058). For intron usage, the difference in the "concordant"
218 proportion was significant for the MED (χ^2 = 6.7, df = 1, p = 0.0096) and the SAF pair (χ^2 = 21.7,
219 df = 1, p = 3.2e-06) but not the ETH pair (χ^2 = 0.24, df = 1, p = 0.62). Mirroring transcriptome-
220 wide patterns, a large majority of P_{ST} outliers with "discordant" changes were "neutralizing"
221 rather than "reversing"; the average proportion of "reversing" is 4% for gene expression and 9%
222 for intron usage. Hence, the largest share of putatively adaptive regulatory changes served to
223 mitigate ancestral plasticity.

224

225 Because the same data from the warm-adapted population in the cold environment was used to
226 estimate both plastic and evolutionary changes, it is possible that random deviations in this
227 estimate could yield a bias (Mallard et al., 2018; Ho & Zhang, 2019). For example, if this
228 quantity was underestimated, then plasticity would become more negative but evolution would
229 become more positive. This potential bias toward discordant changes should be more
230 pronounced for non-outliers in light of their more uncertain direction of evolution (whereas
231 outliers tend to have greater magnitudes of evolution, for which the direction is less likely to be
232 altered by measurement error). This suggestion assumes that outliers reflect true evolution

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233 (adaptive or otherwise) and were not generated via measurement error; we note that each
234 population's extreme high and low values were excluded from P_{ST} calculations as a strategy to
235 reduce the influence of measurement error (Huang et al., 2021). In contrast to the above
236 prediction based on bias, we observed above that P_{ST} outliers had more discordant changes than
237 non-outliers (Figure 3).
238
239 The potential bias toward discordant changes should only exist if overlapping data are used to
240 estimate plasticity and evolution. Therefore, we repeated the above analysis using non-
241 overlapping subsets of the data to estimate plasticity and evolution. We randomly split the shared
242 data (the warm-adapted population in 15°C, $Z_{warm_15°C}$), with four samples being used to estimate
243 plasticity ($P_{warm} = Z_{warm_15°C}' - Z_{warm_25°C}$) and the other four being used to estimate evolution (E
244 $= Z_{cold_15°C} - Z_{warm_15°C}'$) and identify P_{ST} outliers, for all possible subdivisions of the data. The
245 results were qualitatively similar to those reported above (Figure S3 versus Figure 3). All three
246 population pairs again showed an excess of discordant changes among P_{ST} outliers for expression.
247 For splicing, the MED and SAF pair showed a significant excess of discordant changes among
248 outliers (Fig. S3).
249

250 **GO functional enrichment for plastic genes/splicing**

251 Functional analysis (GO enrichment testing) of all plastic genes (regardless of P_{ST} outlier status)
252 with expression/splicing showing either concordant, neutralizing or reversing evolved changes is
253 shown in Table S4. For gene expression, we found more significant GO categories for genes
254 showing "neutralizing" changes among three population pairs, compared to "concordant" or
255 "reversing". While for splicing, most of the significant GO categories were found for those

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256 showing "concordant" in the MED pair (Table S4). Further, we considered the candidates for
257 adaptive differentiation between warm- and cold-adapted populations (Huang et al. 2021) and
258 performed similar GO enrichment tests. Power for these analyses may be reduced because only
259 71 - 203 adaptation candidates with consistent plasticity were assigned among our three
260 categories (see above). We ran separate GO enrichment analyses based on whether the potential
261 adaptive evolution is "concordant" or else "neutralizing" the initial plasticity (there were fewer
262 than 10 genes/introns showing "reversing" changes and therefore this category was not
263 considered). We only found significant GO terms for the MED pair. For "concordant", there is
264 one significant term, "lipid particle"; For "neutralizing", the significant terms are "coenzyme
265 binding", "cofactor binding", "ion transmembrane transporter activity", "magnesium ion
266 binding", "NAD binding" and "transferase activity, transferring acyl groups". Interestingly,
267 "lipid particle" is also found in the GO terms for P_{ST} outliers at the adult stage in MED pair
268 (Huang et al., 2021).
269
270 **Gene regulatory evolution and genetic canalization**
271 We then explored how selection history may influence gene regulatory canalization. Our primary
272 interest was to test whether transcriptomic evolution supported the hypothesis that adaptive
273 evolution may be accompanied by a loss of canalization, which was suggested in the case of *D.*
274 *melanogaster* wing size evolution in nature and in the laboratory (Lack et al., 2016; Groth et al.,
275 2018). Here, we leveraged previously-collected RNA-seq data (Huang et al., 2021) from a subset
276 of the parental inbred strains of the outbred crosses that all other data and analyses were derived
277 from. Inbreeding reflects a broad genetic perturbation due to recessive deleterious variants being
278 made homozygous. Comparisons between inbred and outbred data can therefore illuminate the

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279 relative strength of genetic canalization between different groups (Réaale & Roff, 2003; de
280 Visser et al., 2003). For example, we would predict that for a gene expression abundance trait, a
281 population with relatively lower canalization for this gene would have greater deviations in
282 expression level in the inbred samples compared to the outbred samples, whereas a population
283 with greater canalization for this gene would have relatively less difference in the variability of
284 inbred and outbred samples (less effect of the perturbation). We therefore calculated, for each
285 relevant regulatory trait in each population, the variance of this trait among inbred (V_{inbred}) and
286 outbred ($V_{outbred}$) samples. Inbred data was available, and hence these comparisons were made,
287 for the colder 15°C environment only.
288
289 Although we had a particular interest in decanalization involving potential adaptive changes
290 showing regulatory novelty, we began by comparing V_{inbred} and $V_{outbred}$ across all expression and
291 splicing traits, many of which may be evolving neutrally. For each population, the V_{inbred} and
292 $V_{outbred}$ are highly correlated (Fig S4). Compared between populations, warm-adapted
293 populations had much lower average $V_{outbred}$ than cold-adapted populations (Table 2), in spite of
294 having somewhat higher genetic diversity than their cold-adapted counterparts (Lack et al.,
295 2016a). The reasons for this pattern are unclear; it is possible that the stressed transcriptomic
296 profiles of warm-adapted flies made them relatively more similar. In contrast, warm-adapted
297 populations had higher V_{inbred} than cold-adapted populations, except the SAF pair (Table 2).
298 Concordantly, we found that warm-adapted populations consistently had more genes and introns
299 showing $V_{inbred} > V_{\text{outbred}}$ than cold-adapted populations (Fig. 4A&B). The much greater increase
300 in V_{inbred} relative to $V_{outbred}$ in warm-adapted populations may reflect the relatively greater stress
301 experienced by these flies at 15°C, compared with those from populations better adapted to such

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302 conditions (in other words, the effects of genetic and environmental perturbation may be
303 synergistic). Alternatively, the warm-adapted populations may be expected to hold a somewhat
304 greater number of recessive deleterious variants to be exposed by inbreeding (see Discussion).
305
306 Regardless of the forces shaping transcriptome-wide patterns of inbred and outbred variance, we
307 were interested in asking whether genes potentially under recent adaptive regulatory evolution
308 (P_{ST} outliers) show distinct patterns of inbred versus outbred trait variance consistent with
309 decanalization, compared to other genes. If adaptive evolution has resulted in decanalization, we
310 predict that P_{ST} outliers will have elevated ratios of V_{inbred} to $V_{outbred}$ in cold-adapted populations
311 compared with their warm-adapted counterparts. However, one alternative explanation for such
312 a result is the hitchhiking of deleterious recessive regulatory variants, since any given selected
313 haplotype may have linked harmful variants that increase in frequency alongside the favored
314 variant (e.g. Chun & Fay 2011; Good & Desai 2014). Fortunately, these competing hypotheses
315 make contrasting predictions with regard to adaptive changes that produce “novel” states distinct
316 from those observed in ancestral range populations versus those “neutralizing” naïve plasticity to
317 maintain ancestral regulatory states. The decanalization hypothesis hinges on adaptation
318 producing new phenotypes that are not fully buffered by ancestral canalization mechanisms.
319 Therefore, this hypothesis would predict that novel adaptive regulatory changes should have
320 greater potential to undermine ancestral buffering mechanisms than neutralizing changes, and
321 therefore novel outliers should have greater ratios of inbred versus outbred variance. Whereas,
322 the deleterious hypothesis depends only upon the hitchhiking process itself, and not the
323 magnitude or direction of the adaptive regulatory change, and therefore it predicts no difference
324 between novel and neutralizing changes.

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325

326 In testing the above predictions, we first classified all expression and splicing traits based on
327 whether their evolution was novel or "neutralizing" in the cold environment. Similar to the
328 plasticity section, we used the two values P_{warm} and E to characterize each regulatory trait (Fig.
329 S2). To classify novel changes, we required that the evolved change took the expression value
330 out of the ancestral plasticity range, which included both cases where P □ $E > 0$ ("concordant")
331 and those where P □ $E < 0$ and $|E| > |2P|$ ("reversing"). The remaining traits showing P_{warm} □ E
332 < 0 and $|E| < |2P|$ were again classified as neutralizing. Averaged across three population pairs,
333 37% of all expression abundance and 45% of all intron usage traits were deemed novel.

334

335 We then compared novel/neutralizing results from all genes to those from P_{ST} outliers. For each
336 of our P_{ST} outlier genes showing elevated differentiation between a warm and cold population
337 (Huang et al., 2021) that also showed evidence for regulatory novelty, we therefore tested
338 whether the V_{inbred} / V_{outbred} ratio (r) was higher in the cold population (r_{cold}) or the warm
339 population (r_{warm}). If directional selection has had important decanalizing effects on the evolved
340 regulatory traits, we would predict that novel P_{ST} outliers would be more likely to have a larger
341 r_{cold} than r_{warm}, compared with non-outlier genes classified as novel. For gene expression
342 abundance, we found that the fraction of genes with $r_{cold} > r_{warm}$ was significantly higher for
343 outliers than the nonoutliers for all three population pairs (Fig. 4C. MED pair: 29% vs 20%, $\chi^2 =$
344 6.7, df = 1, p = 0.0098; ETH pair: 47% vs 13%, $\chi^2 = 182$, df = 1, p < 2.2e-16; SAF pair: 45% vs
345 24%, $\chi^2 = 33$, df = 1, p = 8.0e-9.). The result is consistent with the hypothesis that directional
346 selection on expression abundance led to decanalization on these same traits.

347

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348 Alternatively, the above difference between novel outliers and novel non-outliers is also
349 compatible with the hitchhiking of deleterious variants. Furthermore, a qualitatively similar
350 excess of $r_{cold} > r_{warm}$ genes was observed for neutralizing expression changes as well (Fig. 4E .
351 MED pair: 36% vs 22%, $\chi2 = 12$, df = 1, p = 0.0005; ETH pair: 54% vs 36%, $\chi2 = 13$, df = 1, p =
352 0.0003; SAF pair: 40% vs 27%, $\chi2 = 11$, df = 1, p = 0.0008.). Since the deleterious model
353 predicts a greater probability of $r_{cold} > r_{warm}$ for all types of outliers, this result for neutralizing
354 changes might indicate a role for this hitchhiking process.
355
356 Since the decanalization model does make a stronger prediction of inbreeding disruption for
357 novel outliers, and the deleterious model does not, we conducted a more formal test asking if
358 novel outliers showed a more enhanced $r_{cold} > r_{warm}$ probability (compared to novel non-outliers)
359 than neutralizing outliers (compared to neutralizing non-outliers). We performed a regression
360 GLM analysis to model the above probability as a function of outlier status, novel/neutralizing
361 category, and we tested for a significant interaction between those two variables. The MED and
362 SAF returned non-significant results, with different directions as indicated by Figure 4. However,
363 the ETH pair yielded a highly significant interaction indicating a greater decanalization signal for
364 novel than neutralizing outliers (p = 2.6e-5). In addition, the outlier enrichment of $r_{cold} > r_{warm}$
365 was stronger for genes where neutralizing evolutionary changes “overcompensated” for plasticity
366 ($|P| < |E| < |2P|$) versus genes where evolutionary changes “undercompensated” for plasticity ($|E|$
367 $< |P|$; Fig. S5), which is not predicted by the deleterious model. Based on a similar regression
368 GLM analysis as described above, this excess was significant for the MED pair specifically (Fig.
369 S5; p = 0.0053). Under the decanalization hypothesis, it is possible that evolved expression
370 changes that do not generate substantially novel states might still disrupt ancestral buffering

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371 mechanisms, particularly and seem more likely to do so if their magnitude of change is greater.
372 Hence, we find some support for the decanalization hypothesis specifically.
373
374 In contrast to the results for gene expression, for intron usage, the fraction of genes with $r_{cold} >$
375 r_{warm} was not significantly different between outliers and non-outliers for any population pair or
376 partition of outlier introns. Two pairs showed a qualitatively similar pattern for novel changes
377 (Fig. 4D) but just one did so for neutralizing changes (Fig. 4F). Hence, there was no clear
378 support for decanalization or deleterious hitchhiking for the putatively adaptive alternative
379 splicing traits examined.
380

381 **Discussion**

382 Gene regulation can play a key role in mediating complex interactions between genotype,
383 environment, and fitness. In this study, we use parallel cold-adapted natural fly populations to
384 investigate the interplay between gene regulatory evolution, plastic effects of the environment on
385 a trait, and canalization which may buffer against such effects. Regulatory plasticity may be
386 beneficial if it is aligned with a trait's fitness-environment gradient (*e.g.* if a gene contributing to
387 cold tolerance is upregulated under cold conditions). In other cases, environmental stresses may
388 trigger unfavorable shifts in gene regulation.
389
390 Here, focusing on outliers that may reflect adaptive regulatory evolution, we observe evidence of
391 adaptive gene expression divergence opposing the initial plasticity for gene expression and
392 splicing across three pairs of natural populations that evolved in parallel (Fig. 3). These patterns
393 are consistent with several previous studies, which found similar "counter-gradient" patterns in

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394 expression (Levine et al., 2010; Dayan et al., 2015; Ghalambor et al., 2015; Huang & Agrawal,
395 2016; Ho & Zhang, 2019; Koch & Guillaume 2020). Unlike past studies, we also examine
396 splicing evolution and find that it shows a similar pattern, suggesting this "counter-gradient"
397 result may be general for transcriptomic traits. Further, among the expression/splicing traits
398 showing evolution opposing the initial plasticity, the majority of them are neutralizing the initial
399 plastic response, i.e., restoring the expression toward the ancestral state, rather than reversing
400 changes generating novel regulatory states.
401
402 There are at least two potential reasons why adaptive evolution may neutralize the initial
403 plasticity. First, the initial plasticity may have represented a maladapted state induced by the
404 perturbation of a cold environment. Therefore, subsequent adaptation to cold will restore the
405 expression/splicing back to the ancestral optimal level. On the other hand, some of the initial
406 plasticity may be beneficial in the short-term. The naïve populations may respond to a new
407 environment by altering expression/splicing immediately, e.g., stress response, which allows
408 them to persist in the environment with certain costs. Once better mechanisms of coping with the
409 environment have evolved, the stress response may no longer be induced. Without stress
410 response activation, regulation for the cold-adapted population in the cold environment could
411 revert toward that observed for the warm-adapted population in the warm environment. From the
412 GO enrichment test (Table S2), we do find significant enrichment for GO terms "response to
413 stimulus" and "response to stress" among the genes that show naïve plasticity, although they are
414 not limited to genes showing "neutralizing" changes. The presence of stress response genes
415 among the "neutralizing" category could make sense if warm-adapted flies in the cold
416 environment manifest a stressed transcriptional state, then it could make sense for (less-stressed)

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417 cold-adapted flies in that same cold environment to show stress response gene regulation more
418 aligned with that of the warm-adapted population in its home environment. In other cases, novel
419 regulatory states at stress response genes might protect against the challenges of the cold
420 environment.
421
422 It is worth noting that, although the neutralizing cases are enriched in P_{ST} outliers relative to non-
423 outliers, ~20% – 50% of the outliers show "concordant" changes (Fig. 3). For GO enrichment
424 tests for those showing "concordant" plasticity evolution, only the MED pair has significant
425 terms. These significant GO terms in MED are related to the synapse and behavior, which is
426 consistent with other findings that synapse-associated genes are associated with cold tolerance
427 (Mackay et al., 2012; Pool et al. 2017).
428
429 Notably, the ETH pair has many more genes/introns showing reversing changes than the other
430 two pairs. The ETH highland population exhibits distinct phenotypic evolution such as darker
431 pigmentation (Bastide et al., 2014), larger body size (Pitchers et al., 2013; Lack et al., 2016b),
432 and reduced reproductive rate (Lack et al., 2016b), raising the possibility that the strong
433 plasticity-reversing expression change in EF may contribute to the unique phenotypic evolution
434 of this high altitude (3050 m) population. We note that the ETH pair was previously found to
435 have unique regulatory evolution in other respects (Huang et al., 2021): a large majority of its
436 adult (and larval) P_{ST} outliers had increased rather than decreased expression, and it showed a
437 particular importance of trans-regulatory evolution compared with the other population pairs.
438 Hence, while multi-population comparisons like this one may illuminate evolutionary patterns of

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439 general significance, the specific ecological and evolutionary context of each population may
440 importantly influence transcriptome-wide patterns of change.
441
442 Plasticity can be reduced by environmental canalization (Debat & David, 2001; Liefting et al.,
443 2009), which may or may not share a common basis with genetic canalization (Wagner et
444 al.,1997; Meiklejohn & Hartl, 2002; Flatt 2005; Pesevski & Dworkin 2020). Here we explored
445 whether putatively adaptive regulatory evolution may disrupt genetic canalization, using
446 inbreeding as a broad genetic perturbation. We first observed a transcriptome-wide pattern of
447 warm-adapted populations showing greater vulnerability to the genetic perturbation of
448 inbreeding than cold-adapted populations in all pairs for both expression abundance and intron
449 usage (Fig. 4A&B). One explanation is that a low temperature is a form of environmental stress
450 to the warm-adapted populations but not so much to the cold-adapted ones, and that this stress
451 induces a relatively uniform transcriptomic response. In combination, the environmental
452 perturbation of cold stress may have compromised the ability of warm-adapted organisms to
453 buffer the genetic perturbation of inbreeding and led to a greater reduction in canalization (higher
454 $V_{inbred}/V_{outbred}$ ratio) than the less-stressed cold-adapted organisms (Chen et al., 2015). Although
455 this inbreeding-environment interaction has been observed before, the underlying mechanisms
456 are not understood (Kristensen et al., 2006; Reed et al., 2012). Another potential contributor to
457 the apparently greater transcriptome-wide effects of inbreeding on warm-adapted populations is
458 a potentially larger number of recessive deleterious variants. All three of our cold-adapted
459 populations have slightly lower genomic diversity than their warm-adapted counterparts and
460 appear to have gone through mild population bottlenecks after the population pairs diverged
461 (Lack et al. 2016a; Sprengelmeyer et al. 2020). After the initial generations of such a bottleneck,

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462 recessive load is expected to be lower in the bottlenecked population than in the non-
463 bottlenecked counterpart (Kirkpatrick and Jarne 2000), and hence our warm-adapted populations
464 would be predicted to harbor greater recessive load than their cold-adapted partners.
465
466 Aside from the above transcriptome-wide differences in canalization, adaptation could reduce
467 canalization if selection shifts gene regulation outside of the buffered ancestral range. Previously,
468 evidence of decanalization linked to adaptive evolution in nature was limited to transient
469 developmental stability in insecticide-resistant blowflies (Clarke & McKenzie, 1987; McKenzie
470 & Game, 1987) and wing abnormalities in large-winged Ethiopian *D. melanogaster* (Lack et al.
471 2016b). Here, we find that genes with putatively adaptive expression changes are more
472 vulnerable to inbreeding in all three population pairs (Fig. 4C; Fig. 4E). These results are
473 consistent with the adaptation-decanalization hypothesis, but they could also be generated if
474 linked deleterious recessive regulatory variants hitchhiked along with favored haplotypes. We
475 reasoned that only the decanalization model predicts that stronger and especially "novel"
476 regulatory changes should result in greater vulnerability to inbreeding, and we found some
477 evidence in support of this prediction. Specifically, we found that (1) particularly for the ETH
478 population pair, outlier genes with novel regulatory changes were significantly more vulnerable
479 to inbreeding, and (2) for the MED and ETH population pairs, neutralizing changes with greater
480 magnitudes showed stronger evidence for decanalization (Fig. S5). It is intriguing that
481 decanalization evidence was strongest for the ETH pair, given that wing size evolution in the
482 cold-adapted member of this pair was the morphological inspiration for the adaptation-
483 decanalization hypothesis (Lack et al. 2016b).
484

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485 Our findings involving large numbers of putatively adaptive expression traits could hint at a
486 much broader relationship between adaptive evolution and decanalization, at least for gene
487 expression. However, variation in the strength of evidence for decanalization among our
488 population pairs suggests that evolutionary context may be an important factor modulating the
489 relationship between adaptation and canalization. Further work is needed to understand this
490 relationship more clearly. For example, to what extent do adaptive changes by themselves break
491 down canalization, versus decanalization occurring separately as a prerequisite for adaptive trait
492 change? And to the extent that decanalization occurs concurrently with adaptation trait evolution,
493 how often does it occur as a pleiotropic byproduct of the adaptive change itself, versus arising
494 from linked deleterious variants?
495
496 In contrast, evidence for decanalization was not observed to a significant degree for putatively
497 adaptive intron usage changes (Fig. 4D). It is possible that our splicing P_{ST} outliers may be less
498 enriched for targets of strong adaptive evolution than our expression outliers. Alternatively, there
499 may be a meaningful difference in how the two traits are buffered. While our plasticity data
500 suggest that expression and splicing are similarly susceptible to environmental plasticity (Table
501 1), it is possible that intron usage traits are less vulnerable to the genetic perturbation of
502 inbreeding, perhaps due to differences in the properties of biological networks governing
503 expression and splicing.
504
505 In this study, we leveraged parallel adaptive divergence among natural populations of a model
506 organism to broadly examine the relationship between adaptive evolution, thermal environment,
507 and gene regulation. However, much work remains to be done. Our finding that gene expression

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508 evolution tends to neutralize ancestral plasticity is consistent with many although not all previous
509 studies (see Introduction), and suggests that many plastic expression changes may be
510 maladaptive in novel environments. For the first time, we also showed a similar pattern for
511 alternative splicing, in the context of intron usage. It will therefore be of interest to assess the
512 degree to which a broader range of traits (gene regulatory and otherwise) show counter-gradient
513 patterns of evolution and plasticity. Our investigation of the relationship between adaptive gene
514 regulatory evolution and decanalization particularly calls out for further research, in light of this
515 topic's lack of prior study. It was only relatively recently that an instance of morphological
516 evolution in nature was found to yield decanalization of the same structure's development (wing
517 size in our highland ETH population; Lack et al. 2016b). We find some evidence that putatively
518 adaptive gene expression traits are linked to (genetic) decanalization on a transcriptome-wide
519 scale, which raises the question of whether a more general role for adaptive evolution in
520 undermining canalization may exist. However, there is a considerable need to investigate this
521 relationship across a broader range of organisms and traits.
522

523 **Materials and Methods**

524 *RNA sample collection and sequencing*

525 As described in previous publications (Lack et al., 2016a; Pool et al., 2016; Huang et al., 2021),
526 we have three *Drosophila melanogaster* population pairs, each representing cold-adapted and
527 warm-adapted populations from the same region: a Mediterranean pair (France FR and Egypt
528 EG), an Ethiopian pair (EF, EA) and a South African pair (SD, SP). Within each of these six
529 populations, we selected 16 strains and assigned them into eight crosses. These strains had been
530 inbred for eight generations. To reduce within-cross variance, pairs of strains for a cross were

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531 chosen based on minimal overlapping heterozygosity genome-wide. Each cross was conducted
532 concurrently at both 25°C, the control warm condition, and 15°C, the derived cold condition (Fig.
533 1A). Twenty virgin females and 20 males were collected from maternal and paternal lines
534 respectively for each cross and allowed to mate and lay eggs for a week in half-pint bottles. Each
535 bottle contained standard *Drosophila* medium (containing molasses, cornmeal, yeast, agar, and
536 antimicrobial agents). To collect samples for RNA-seq, 30 female F1 offspring were collected 4-
537 5 days after eclosion and shock-frozen in liquid nitrogen immediately. To compare the effects of
538 inbred and outbred on expression variation after adaptive evolution, we also reared four inbred
539 lines at 15°C for each population (Fig. 1A) and collected 30 females 4-5 days after eclosion.
540 15°C represented the derived environment where the cold-adapted populations underwent
541 selection.
542
543 For RNA extraction, 30 females for each sample were homogenized using TissueLyser II
544 (Qiagen, Hilden, Germany). Total mRNA was isolated using the Magnetic mRNA Isolation Kit
545 (New England Biolabs, Ipswich, MA) and cleaned up using RNeasy MinElute Cleanup Kit
546 (Qiagen, Hilden, Germany). Strand-specific libraries were prepared using the NEBNext mRNA
547 Library Prep Reagent Set for Illumina. Libraries were size-selected for approximately 150 bp
548 inserts using AMPureXP beads (Beckman Coulter, CA, USA). The libraries were quantified
549 using Bioanalyzer and manually multiplexed for sequencing. All libraries were sequenced on a
550 HiSeq2500 (V4) with 2 ⮾ 75bp paired-end reads in two flow cells. Numbers of paired-end reads
551 generated for different libraries can be found in Table S1. Data from parental outbred samples,
552 parental inbred samples, and all F1 samples were each largely sequenced on the same flow cell

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553 as other samples of the same type, with a minority of samples having initially low depth of
554 coverage subsequently topped off by further sequencing on the same machine.
555
556 *Quantifying gene expression and intron usage frequency*
557 The paired-end sequence reads from each of the F1 samples were mapped to the transcribed
558 regions annotated in *D. melanogaster* (release 6, BDGP6.84) using STAR with parameters from
559 ENCODE3's STAR-RSEM pipeline (Dobin et al., 2013; Li & Dewey, 2011). Numbers of
560 mapped read pairs for different samples can be found in Table S2. For gene expression, the
561 numbers of reads mapped to each gene were quantified using RSEM (Li & Dewey 2011). Reads
562 mapped to the rRNA were excluded in the analysis. Table S3 provides the read counts for each
563 gene in each sample. The expression abundance for each gene was the number of reads mapped
564 to the gene per million reads (standardized by total reads mapped to the transcriptome).
565
566 To visually describe the transcriptome variation among samples, we first performed principal
567 component analysis (PCA) for the F1 samples from different temperature conditions across
568 populations. We used DESeq2 (Love et al. 2014) to construct the data object from the matrix of
569 count data output from RSEM and performed variance stabilizing transformation (vst). The top
570 5000 genes with highest variance across samples at the transformed scale were used for PCA
571 with the function *prcomp* in R.
572
573 To quantify intron usage, we used Leafcutter (Li et al., 2018) to estimate the excision frequencies
574 of alternative introns. Leafcutter took the alignment files generated by STAR as input to quantify
575 the usage of each intron. Then Leafcutter formed clusters that contained all overlapping introns

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576 that shared a donor or acceptor splice site. The default parameters were used: > 50 reads
577 supporting each intron cluster and < 500kb for intron length. The numbers of intron excision
578 events for different clusters in each sample can be found in Table S3. The intron usage frequency
579 is the number of intron excision events divided by the total events per cluster. It is worth noting
580 that Leafcutter only detects exon-exon junction usage and it is unable to quantify 5' and 3' end
581 usage and intron retention, which may be confounded by differential bias among libraries.
582
583 *Comparison of naïve plasticity and evolutionary changes*
584 The detailed methods of identifying candidates for adaptive evolution between cold and warm
585 populations can be found in Huang et al. (2021). Briefly, that study used P_{ST} statistics to quantify
586 gene regulatory divergence (for both expression abundance and intron usage) between cold- and
587 warm-adapted populations in each pair. That approach was used because the goal was to identify
588 transcriptomic traits with unusually high population differentiation relative to within-population
589 variation (representing candidates for local adaptation), as opposed to simply testing whether any
590 significant population difference in expression/splicing existed at all. The upper 5% of P_{ST}
591 quantile was used to identify outliers that are more likely to have been under adaptive evolution.
592 (evaluated separately for each population pair). While this threshold is necessarily arbitrary, in
593 light of our present study's focus on transcriptome-wide patterns, it is not essential that every P_{ST}
594 outlier be a true product of local adaptation. Instead, we simply need a group of genes that is
595 meaningfully enriched for true local adaptation targets, and that contains enough such genes to
596 provide reasonable power for the analyses described below. Given that we are studying
597 population pairs that have occupied contrasting environments for tens of thousands of
598 generations (Sprengelmeyer et al. 2020) and the differ in multiple traits (e.g. Bastide et al. 2016;

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599 Lack et al. 2016b; Pool et al. 2017), it seems reasonable to propose that P_{ST} outliers are enriched
600 for targets of local adaptation, and that differences between outliers and non-outliers are
601 informative regarding the characteristics of genes under adaptive transcriptomic evolution.
602 However, we note that factors such as population differences in organ size could also influence
603 our whole-organism RNA-seq data, as further discussed in Huang et al. 2021.
604
605 To quantify plasticity, we used the difference in gene expression between rearing conditions for
606 each cross. For expression abundance, we calculated the change of expression abundance from
607 cold to warm condition for each of the eight crosses (where expression in both conditions was
608 above 0). To identify genes showing naïve plasticity in warm-adapted populations, we required
609 consistent expression changes (consistently higher or lower expression for the cross in cold
610 condition than that in warm condition) in at least seven out of eight crosses following the
611 direction of average change among the eight crosses. Similarly for intron usage, we calculated
612 the between-environment differences in intron usage proportion for each of the eight crosses.
613 The criterion to identify plastic splicing in warm-adapted populations was consistent differences
614 in at least seven out of eight crosses showing the same direction as the average difference. In the
615 absence of true plasticity, only 7% of genes should meet this criterion by chance (two-sided
616 binomial calculation). Null simulations in which all 16 values were normally distributed
617 confirmed that the false positive rate should be close to 7%. While a 7% false positive rate is
618 slightly higher than the conventional 5%, it represents a reasonable compromise in light of the
619 data available and the goals of our study. Here, our need is to define a set of plasticity-enriched
620 genes of adequate size to offer power for the analyses described below.
621

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622 To examine whether adaptation to cold environments tends to enhance or oppose the naïve
623 expression plasticity (Fig. 1B), we first calculated the expression value for every gene in each
624 population at each condition. For expression abundance, the median abundance among eight F1
625 samples was used as the expression value for a population in a certain condition (Z). For intron
626 usage, the corresponding value was the median of intron usage proportion among the eight F1
627 samples. To determine whether a regulatory trait evolved to enhance or oppose ancestral plastic
628 regulation (Fig. 1C-E), we first examined "naïve plasticity" (warm-adapted population in cold
629 environment, $Z_{warm_15°C}$, compared to the warm environment, $Z_{warm_25°C}$; Fig. 1B). We then asked
630 whether evolution in the cold-adapted population enhanced or opposed the ancestral naïve
631 plasticity. Formally, we asked whether the differences in trait values between cold- and warm-
632 adapted populations in the cold environment, $E = Z_{cold_15°C} - Z_{warm_15°C}$, were in the same
633 direction as the plastic response of the warm population, $P_{warm} = Z_{warm_15°C} - Z_{warm_25°C}$. If E ⍰
634 $P_{warm} > 0$, the evolved regulation was assigned as "concordant" (Fig. 1C). If E ⍰ $P_{warm} < 0$, the
635 evolved regulation was assigned as "discordant", and these discordant traits were further
636 partitioned into two categories. We defined as "neutralizing" traits in which the cold population's
637 "home" value ($Z_{cold_15°C}$) evolved to be closer to the warm population's "home" value ($Z_{warm_25°C}$)
638 than predicted by the warm population's plastic response, and therefore $|E| < 2|P_{warm}|$ (Fig. 1D).
639 In contrast, we defined as "reversing" traits in which an evolved change E was both opposing the
640 direction of plasticity P and took $Z_{cold_15°C}$ farther from $Z_{warm_25°C}$ than predicted by the warm
641 population's plastic response ($|E| > 2|P_{warm}|$; Fig. 1E). For both categories where evolved
642 expression was farther from the warm population's "home" value range (either concordant or
643 reversing), we defined them as "novel".
644

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645 The differences in "concordant" to "discordant" ratio between P_{ST} outliers and non-outliers were
646 tested based on the chi-squared test in R version 3.3.0. for both expression abundance and
647 splicing. It is worth noting that the test assumes that regulation is independent among genes and
648 introns. However, it is possible that the expression or splicing of different genes is causally
649 related because of the shared regulatory network.
650
651 Because the calculations of P_{warm} and E included a common value, $Z_{warm_15°C}$, any random
652 measurement error on this value may generate an artifact of a negative relationship between
653 P_{warm} and E (Mallard et al. 2018; Ho & Zhang, 2019). We therefore repeated this analysis while
654 subdividing the data for estimating $Z_{warm_15°C}$: four random crosses were used to estimate P_{warm}
655 and the other crosses were used to estimate E. We used the latter four crosses of warm-adapted
656 populations at 15°C for measuring E and the eight crosses of cold-adapted populations at 15°C to
657 identify P_{ST} outliers and non-outliers. Then we used the same criteria to categorize the
658 evolutionary changes into the "concordant" or "discordant" and repeated the same analysis for
659 all sets of subdivided data (70 sets). Since we used $Z_{warm_15°C}$ estimated from different data sets to
660 calculate P_{warm} and E, the potential artifact of a negative relationship between P_{warm} and E should
661 be removed. The fraction of the subdivided data (70 sets) showing an excess of concordant
662 changes in P_{ST} outliers vs. non-outliers was the p-value for testing whether P_{ST} outliers had a
663 significantly lower concordant proportion than non-outliers.
664
665 *GO enrichment test for expression plasticity*
666 Gene Ontology (GO) enrichment tests were performed using the R package "clusterProfiler" (Yu
667 et al., 2012) based on the fly genome annotation (Carlson 2019). For expression abundance, the

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668 gene ID for concordant, neutralizing or reversing outliers were input as the focal gene list while
669 all the genes used in studying expression abundance divergence were input as the universe gene
670 list. For intron usage, they were mapped to gene regions to generate the universe gene list. Genes
671 including concordant, neutralizing or reversing outlier intron junctions were used in the focal
672 gene list. The types of GO terms being tested contained all three sub-ontologies: Biological
673 Process (BP), Cellular Component (CC) and Molecular Function (MF). Selection of over-
674 represented GO terms was based on adjusted p-value < 0.1 using "BH" FDR method (Benjamini
675 & Hochberg, 1995) for each sub-Ontology. Based on the description of the GO terms, only the
676 enriched GO terms with distinct functions were reported. If multiple GO terms were over-
677 represented by the same set of candidate genes, only the term annotated with fewest genes was
678 reported.
679

680 *Testing the decanalizing effect of adaptation on gene expression*

681 We examined the level of canalization for gene expression under inbreeding as a genetic
682 perturbation. For each population, we selected four outbred F1 samples from which we also had
683 data from one of the parental inbred lines. For gene expression abundance, we calculated the
684 variance among four inbred samples and that among four outbred samples for each population.
685 For intron usage, we re-ran leafcutter based on all inbred samples (24 samples in total for six
686 populations) and all outbred samples (24 samples). Then we calculated the variance for inbred
687 samples (V_{inbred}) and for outbred samples ($V_{outbred}$) for each population. While variance estimates
688 from only four values are expected to be noisy, our focus is exclusively on transcriptome-wide
689 correlations and not conclusions about individual genes. Only those expression abundance or
690 intron usage traits with variance above 0 for both inbred and outbred samples in both populations

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691 for each pair were used in this comparison. We first compared the transcriptome-wide fractions
692 showing $V_{inbred} > V_{outbred}$ between cold and warm populations for both expression abundance and
693 intron usage. Because the hypothesis is about recent adaptive change to novel states resulting in
694 decanalization, we focused on the set of genes/introns that evolved expression novelty (identified
695 in the previous section). To test the role of selection on canalization, we compared the fractions
696 showing higher V_{inbred} to $V_{outbred}$ ratio (r) in the cold population (r_{cold}) than that in the warm
697 population (r_{warm}) for P_{ST} outliers with that for non-outlier controls using a chi-squared test. We
698 note that we are only comparing variances between inbred and outbred samples of the same gene
699 from the same population, and not between genes that may have different average expression
700 levels. The comparisons between the P_{ST} outliers and non-outliers were done for "novelty"
701 category and for "neutralizing" category. For those in the "neutralizing" category, we further
702 classified them into undercompensating ($|E| < |P_{warm}|$) or overcompensating ($|P_{warm}| < |E| <$
703 $2|P_{warm}|$) and performed similar analysis for each category.
704
705 To distinguish between decanalization model and deleterious model, we performed a generalized
706 linear model (GLM) regression analysis on expression abundance across genes for each
707 population pair:
708 $$Pr \sim Status + Category + Status \times Category$$
709 where Pr is whether a gene showed $r_{cold} > r_{warm}$ as opposed to $r_{cold} > r_{warm}$. *Status* is whether a
710 gene was a P_{ST} outlier or non-outlier. *Category* is whether a gene was classified as "novelty" or
711 "neutralizing". We tested for a significant interaction between *Status* and *Category* variables by
712 comparing the above full model against a reduced model without the interaction term. A
713 likelihood ratio test was used to determine significance. We repeated the analysis on genes

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714 classified as "neutralizing" to test the effect of overcompensating/undercompensating category
715 on the enrichment of $r_{cold} > r_{warm}$ in outliers vs. non-outliers.

716 **Data Availability**

717 The raw RNAseq reads for samples in the cold condition (15°C) are available from the Sequence
718 Read Archive (SRA) under BioProject PRJNA720479, and those from the warm condition (25°C)
719 are under BioProject PRJNA758705. Table S3 and relevant codes are available at
720 https://github.com/YuhengHuang87/ExpressionPlasticityCanalization
721

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726

727 **Figure legends**

728 **Fig. 1. Illustrations of the experimental design and different expectations for adaptative**
729 **changes regarding the naïve plasticity.** (A) Experimental design is shown for one population (EG,
730 a warm population) as an example. Crosses generated outbred offspring at 15°C (derived condition)
731 and 25°C (ancestral condition) to study transcriptome plasticity. Inbred strains were reared at 15°C to
732 study genetic canalization by comparing the variance of trait value for inbred vs. outbred samples at
733 15°C. Whole female adults were collected from the offspring. (B-E) Conceptual illustrations of
734 different expectations for evolved changes and naïve plasticity. (B) Naïve plasticity for a warm
735 population. The difference between trait values at 15°C vs. 25°C is a measure of plasticity (P_{warm}). (C)
736 The evolved change (E, solid arrow) is the difference between trait values between cold (blue dot)

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737 and warm population (orange square) at 15°C. If the evolved change is in the same direction as the
738 naïve plasticity, the evolved change is regarded as “concordant”. (D) If the evolved change is in the
739 opposite direction as the naïve plasticity (E ⍰ $P_{warm} < 0$) and moves the trait closer to the warm
740 population’s trait value at 25°C ($|E| < 2|P_{warm}|$), it is regarded as “neutralizing”. The orange dashed
741 line indicates the level of plastic change but in the opposite direction. (E) If the evolved change
742 moves the trait further from the warm population’s trait value at 25°C with a magnitude greater than
743 the initial plasticity in the opposite direction (E ⍰ $P_{warm} < 0$; $|E| < 2|P_{warm}|$), it is regarded as
744 “reversing”. The “concordant” and “reversing” categories are both regarded as “novelty” for the
745 canalization analysis, because they each take the cold-adapted population’s trait value in its home
746 environment farther away from the warm-adapted population’s trait value in its own home
747 environment than naïve plasticity would have done on its own.
748
749 **Fig. 2 The plastic responses of the warm-adapted population (P_{warm}, x-axis) and the evolved**
750 **differences in expression between populations in the derived cold environment (E, y-axis)**
751 **for all genes (upper panel) and introns (lower panel) that showed consistent naïve plasticity.**
752 Dots located in the lower left and upper right quadrants indicate that the plasticity responses and
753 evolutionary changes are concordant.
754
755 **Fig. 3 The proportion of genes/introns classified as “concordant” of naïve plasticity for P_{ST}**
756 **outliers and non-outliers.** * indicates the proportion is significantly different between P_{ST}
757 outliers and non-outliers ($p < 0.01$).
758
759 **Fig. 4. Genetic decanalization measured by V_{inbred} relative to $V_{outbred}$ (r).** A & B: The
760 transcriptome-wide fraction of regulatory traits for which $r > 1$ for each warm-adapted and cold-

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761 adapted population is shown for expression abundance (A) and intron usage (B). C – F: The
762 fraction of traits with novel regulatory changes (C & D) or with neutralizing changes (E & F) for
763 which $r_{cold} > r_{warm}$ for P_{ST} outliers and non-outlier controls is shown for expression abundance
764 (left) and intron usage (right). * indicates that the fractions are significantly different (all with p
765 < 0.05).
766

767 **Table legends**

768 Table 1. Numbers of gene expression abundance and intron usage traits showing plasticity in
769 warm- and cold-adapted populations for each population pair. Panel A shows all the
770 genes/introns and panel B shows those with elevated expression/splicing differentiation between
771 adult females from cold- and warm-adapted populations at 15°C (P_{ST} outliers identified in Huang
772 et al. 2021). The percentage in parentheses indicates the proportion showing consistent plasticity.
773

774 Table 2. Transcriptome-wide mean variance of expression or intron usage among inbred strains
775 or outbred crosses within a population, averaged across all genes/intron usages.
776

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957
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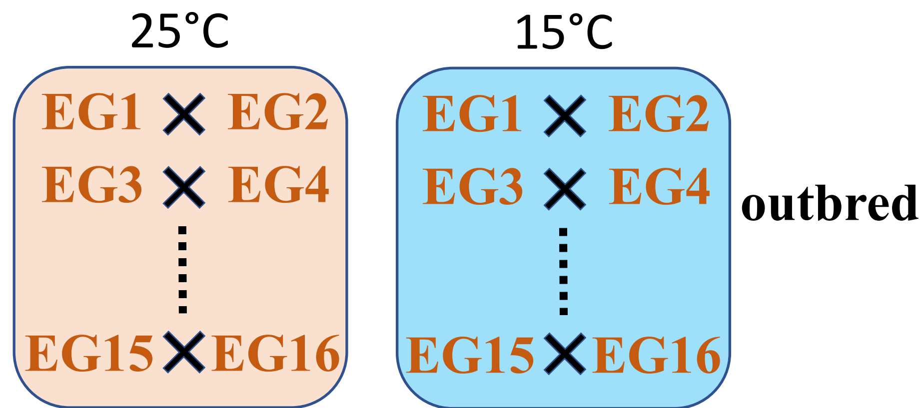
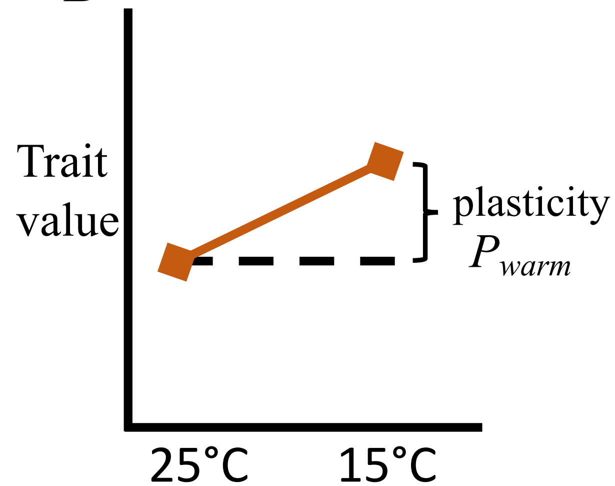
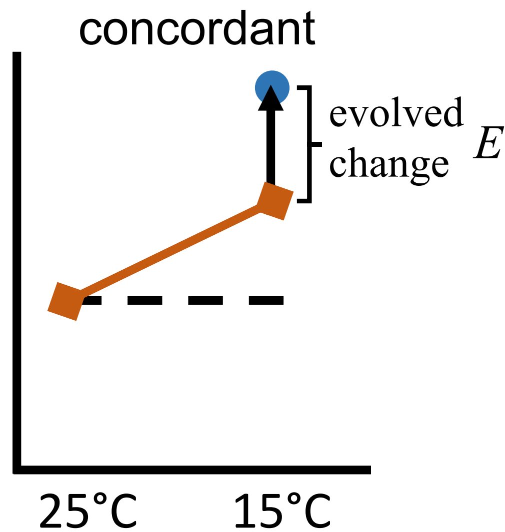
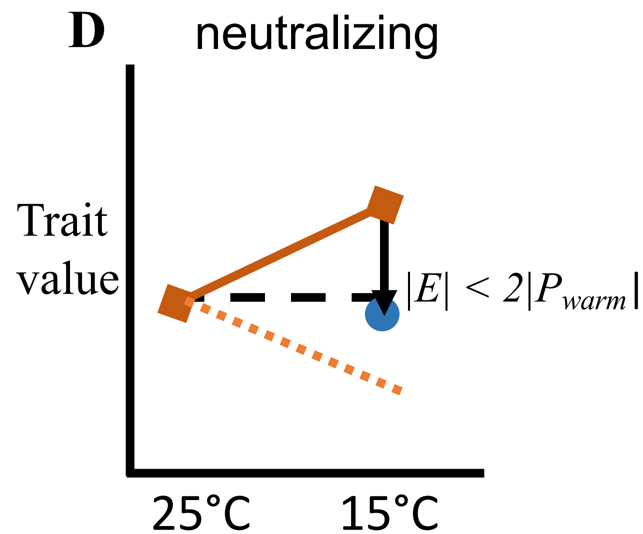
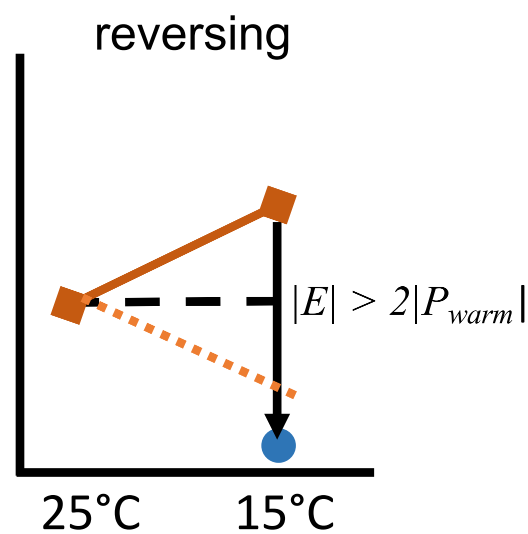

A
between-temperature
difference is a measure of
plasticity for a cross
25°C
15°C
EG1 × EG2
EG3 × EG4
EG15 × EG16
EG1 × EG2
EG3 × EG4
EG15 × EG16
outbred

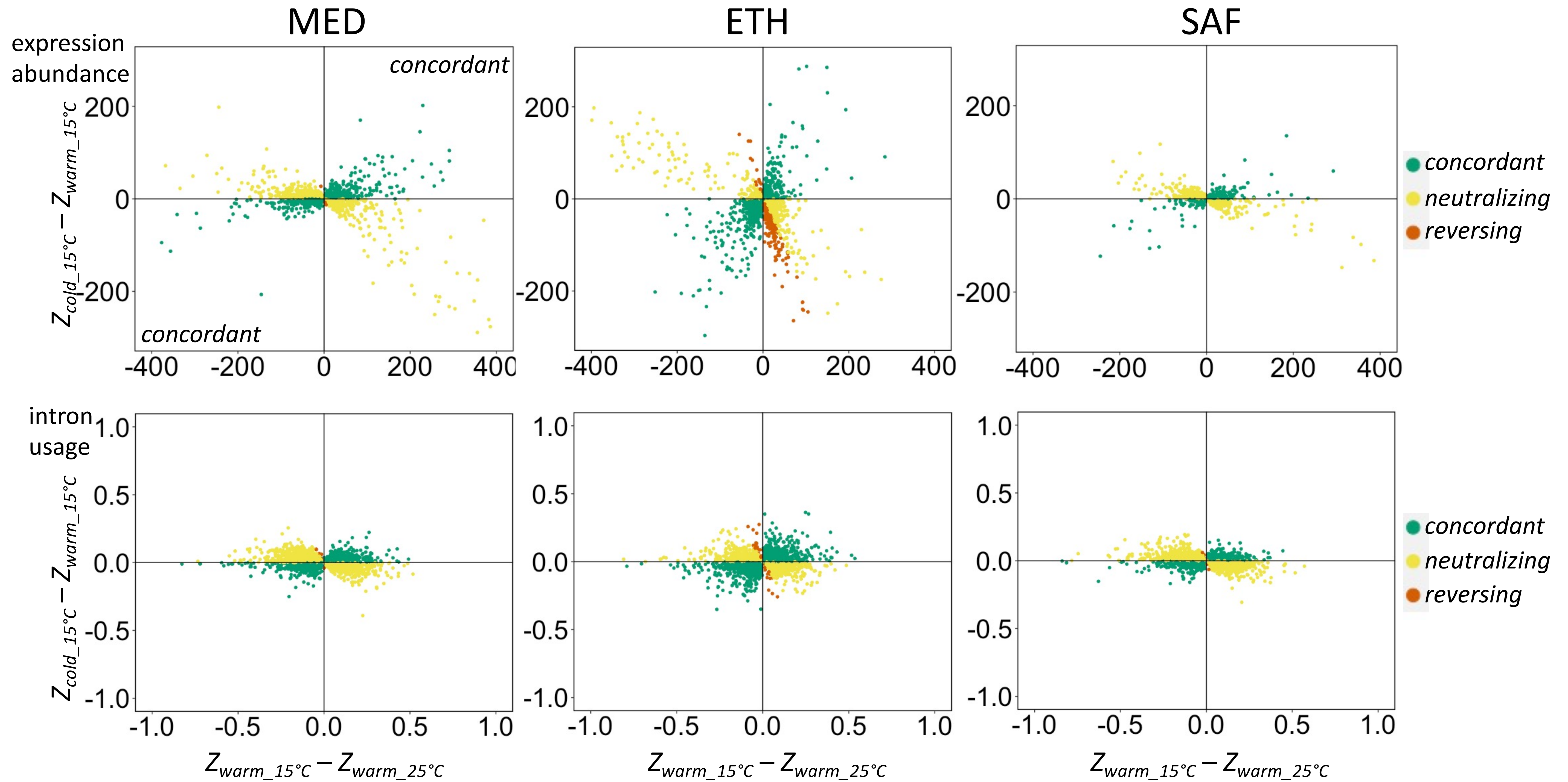
EG1 EG2
EG3 EG4
inbred
inbred vs. outbred
variance is a measure
of genetic canalization


B
Trait
value
plasticity
P_{warm}
25°C
15°C

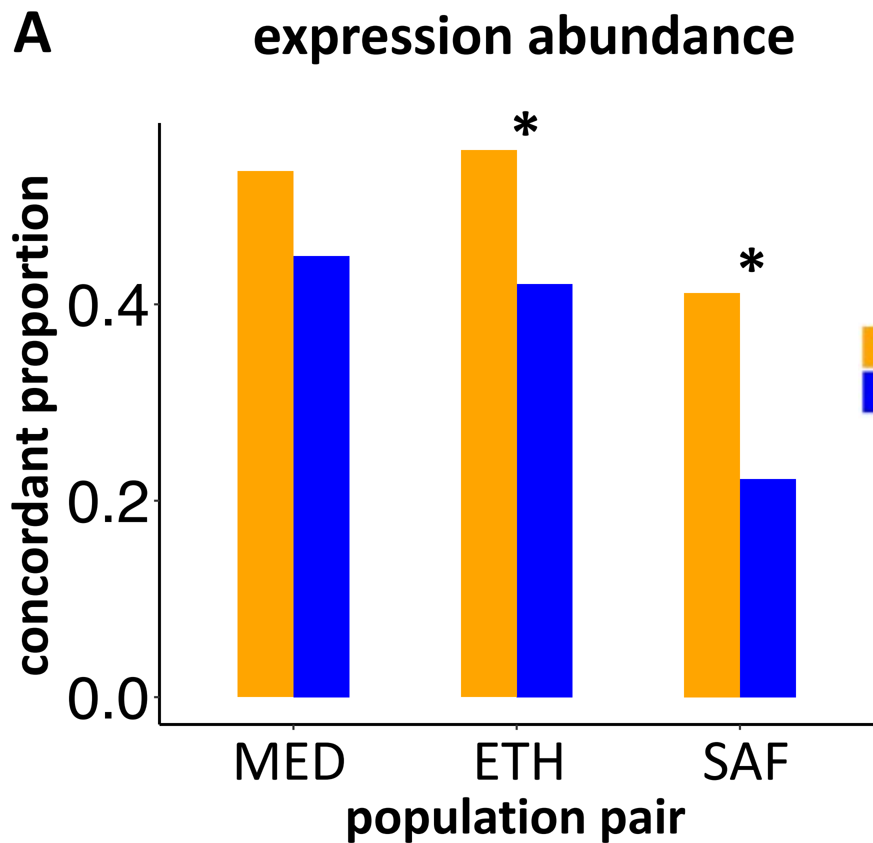

C
concordant
evolved
change
E
25°C
15°C

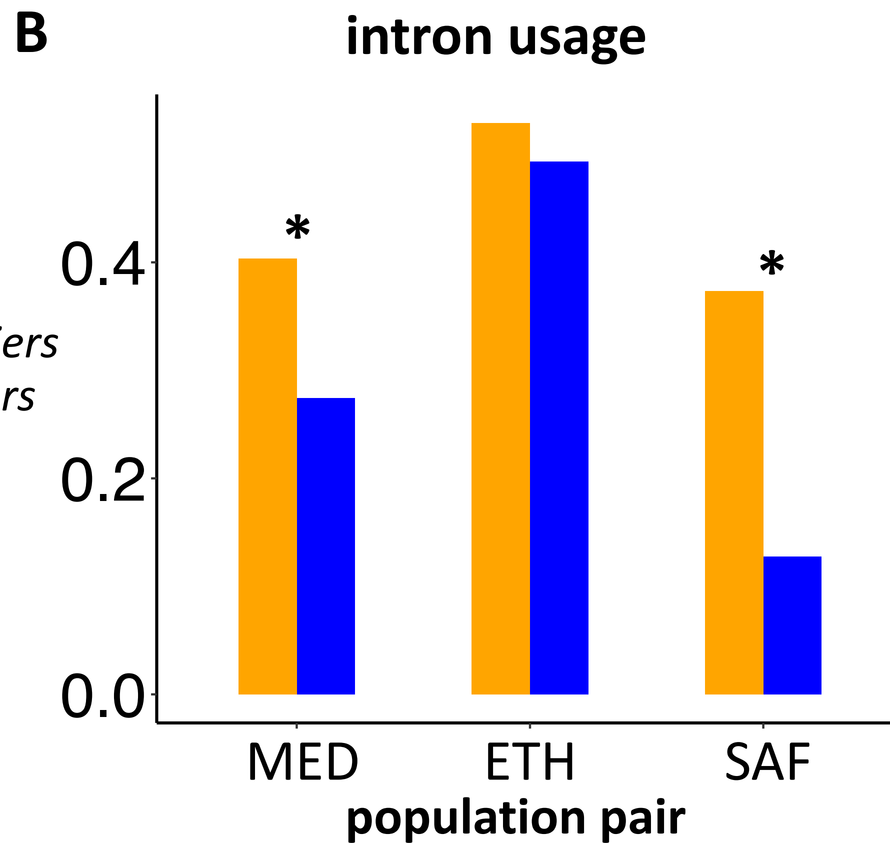

D
neutralizing
Trait
value
$|E| < 2|P_{warm}|$
25°C
15°C


E
reversing
$|E| > 2|P_{warm}|$
25°C
15°C



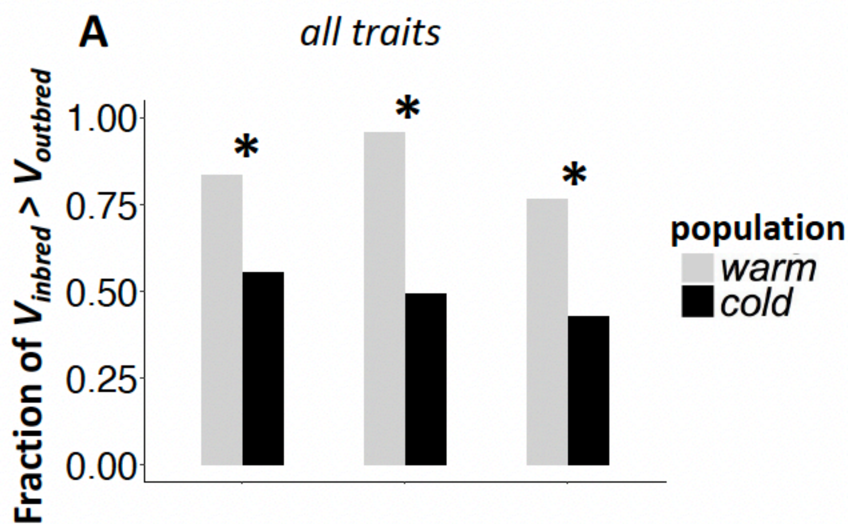
MED
ETH
SAF
expression abundance
intron usage
concordant
concordant
neutralizing
reversing
$Z_{cold_15°C} - Z_{warm_15°C}$
$Z_{warm_15°C} - Z_{warm_25°C}$

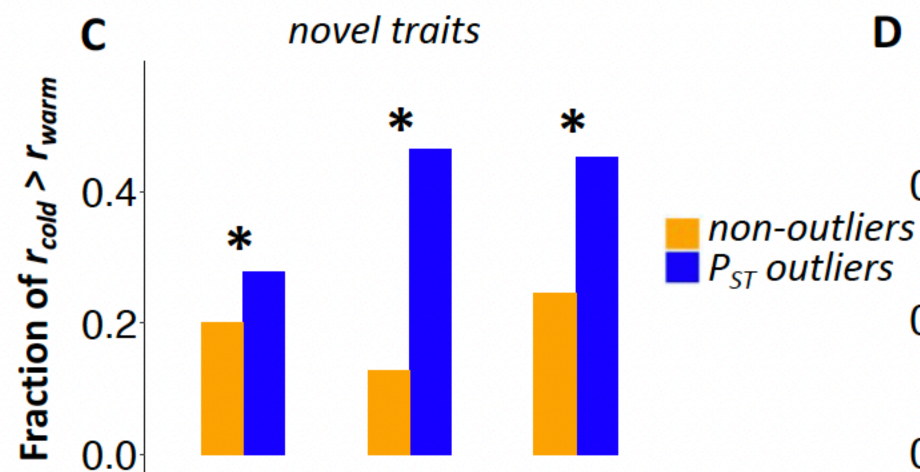

A
expression abundance
concordant proportion
0.0
0.2
0.4
*
*
MED
ETH
SAF
population pair
non-outliers
P_{ST} outliers

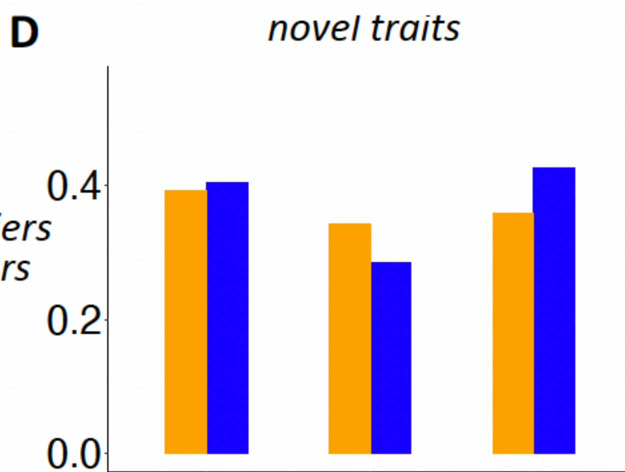

B
intron usage
0.0
0.2
0.4
*
*
MED
ETH
SAF
population pair

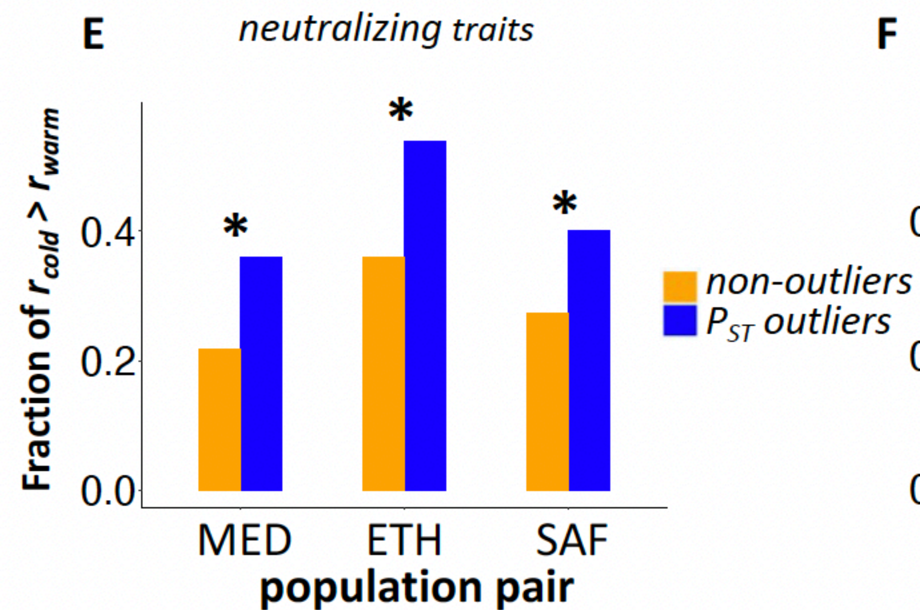
expression abundance

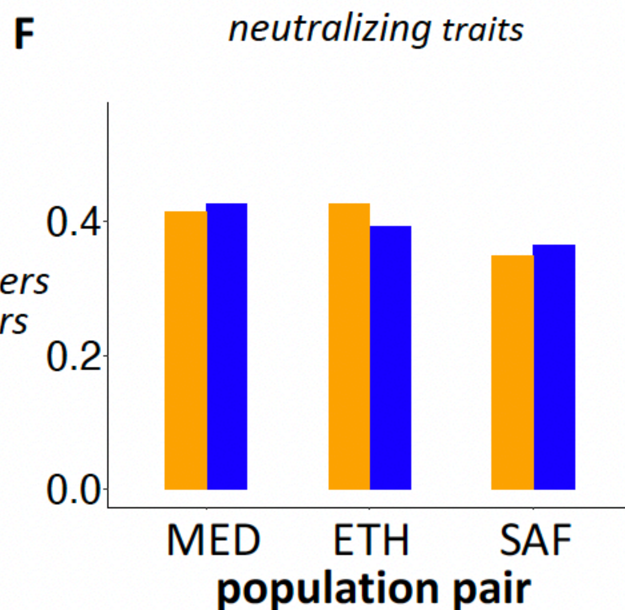
intron usage











A

All population	MED		ETH		SAF	
population	warm	cold	warm	cold	warm	cold
Expression abundance	4890 (43%)	7453 (65%)	3286 (28%)	1627 (14%)	2195 (19%)	2303 (20%)
Intron usage	3219 (32%)	3658 (36%)	2884 (29%)	2358 (24%)	2504 (25%)	2399 (24%)

B

P_{ST} **outliers**	MED		ETH		SAF	
population	warm	cold	warm	cold	warm	cold
Expression abundance	138 (41%)	205 (60%)	126 (37%)	93 (27%)	117 (35%)	118 (35%)
Intron usage	113 (32%)	116 (33%)	77 (22%)	114 (32%)	94 (27%)	78 (22%)

Table 1. Numbers of gene expression abundance and intron usage traits showing plasticity in warm- and cold-adapted populations for each population pair. Panel A shows all the genes/introns and panel B shows those with elevated expression/splicing differentiation between adult females from cold- and warm-adapted populations at 15°C (P_{ST} outliers identified in Huang et al. 2021). The percentage in parentheses indicates the proportion showing consistent plasticity.

	pair	MED		ETH		SAF	
	population	warm	cold	warm	cold	warm	cold
Expression abundance	V_{inbred}	23438	15965	61003	37384	12847	34075
	$V_{outbred}$	5132	10412	5083	25574	3125	61581
Intron usage	V_{inbred}	0.0071	0.0067	0.0113	0.0095	0.0064	0.0073
	$V_{outbred}$	0.0039	0.0059	0.0033	0.0049	0.0042	0.0089

Table 2. Transcriptome-wide mean variance of expression or intron usage among inbred strains or outbred crosses within a population, averaged across all genes/intron usages.