Restoring ancestral phenotypes by reduction of plasticity is a general pattern in gene expression during adaptation to different stressors in *Tribolium castaneum*

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Abstract

Plasticity and evolution are two processes enabling individuals to respond to environmental change, but how both are related and impact each other is still controversial. We studied plastic and evolutionary responses in gene expression of *Tribolium castaneum* after exposure to new environments that differed from ancestral conditions in temperature, humidity or both. Using experimental evolution with ten replicated lines per condition, we were able to demonstrate adaptation after 20 generations. We measured gene expression in each condition in adapted selection lines and control lines to infer evolutionary and plastic changes. We found more evidence for changes in mean expression (shift in the intercept of reaction norms) in adapted lines than for changes in plasticity (shifts in slopes). Plasticity was mainly preserved and was responsible for a large part of the phenotypic divergence in expression between ancestral and new conditions. However, we found that genes with the largest evolutionary changes in expression also evolved reduced plasticity and often showed expression levels closer to the ancestral stage. Results obtained in the three different conditions were similar suggesting that restoration of ancestral expression levels during adaptation is a general evolutionary pattern. We increased the sample size in the most stressful condition and were then able to detect a positive correlation between proportion of genes with reversion of the ancestral plastic response and mean fitness per selection line.

Introduction

Whenever facing environmental change, populations can adapt to new phenotypic optima by plasticity and evolution. Plasticity is the ability of a single genotype to produce multiple phenotypes as a function of the environment. It is often seen as an immediate response of individuals to changes in their environment. In contrast, evolution requires a change in allele

frequencies within a population. This process occurs over several generations and represents a more long-term response, which can result in local adaptation. It is still not well understood how these two processes are related and interact with each other (de Jong, 2005; Forsman, 2015; Ghalambor, McKay, Carroll, & Reznick, 2007; Price, Qvarnström, & Irwin, 2003; Wund, 2012).

Plasticity does not affect a population's genetic composition and should thus not have long-term consequences in the following generations. However, it changes the distribution of phenotypes on which selection can act (de Jong, 2005; Pfennig et al., 2010; Pigliucci, 2005; Price, Qvarnstrom, & Irwin, 2003). If plasticity perfectly matches the new phenotypic optimum, it prevents evolution since no genetic changes are required and selection is prevented (Ghalambor et al., 2007). On the other hand, plasticity is also crucial for a population's persistence and can reduce the costs of selection (Chevin, Lande, & Mace, 2010; Pavey, Collin, Nosil, & Rogers, 2010). It prevents extinction and protects populations from bottleneck effects, thereby maintaining a higher genetic variation on which subsequently selection can act (Fitzpatrick, 2012; Pfennig et al., 2010; Massimo Pigliucci, 2005). There is both, theoretical (Chevin et al., 2010; Draghi & Whitlock, 2012; Fierst, 2011) and empirical work (Schaum, Rost, Millar, & Collins, 2013), demonstrating that more plastic populations exhibit faster evolution. The benefits of plasticity for persisting in new habitats were also demonstrated in invasive species (Molina-Montenegro, Peñuelas, Munné-Bosch, & Sardans, 2012; Pichancourt & van Klinken, 2012; Yeh & Price, 2004).

The extent of plasticity can be represented as a reaction norm (Scheiner, 1993), which is the phenotypic trait value as a function of an environmental variable. Evolution can affect the reaction norm in two ways: The intercept can be shifted corresponding to a change in the mean phenotypic value. Alternatively, the slope of the reaction norm, i.e. the plasticity, can be

changed. Thus, plasticity itself can also be subject to evolution given that there is sufficient genetic variation (Garland & Kelly, 2008; Nussey, Postma, Gienapp, & Visser, 2005).

Dependent on whether plastic responses are adaptive and increase fitness of an individual, different patterns describing the relationship between ancestral plasticity and evolution are possible. If plastic responses are adaptive, but not sufficient to reach the phenotypic optimum, evolution should work in the same direction as the plastic response (referred to as Baldwin effect (Crispo, 2007) or cogradient variation (Conover, Duffy, & Hice, 2009). In this case, selection may favour the most plastic individuals, causing evolved populations to exhibit a higher plasticity than their ancestors (Crispo 2007; Lande 2009). Another possible outcome is genetic assimilation: An initially environmentally induced phenotype can get fixed by a loss of plasticity and becomes continuously expressed even in the ancestral environment (Levis & Pfennig, 2016; Pigliucci, Murren, & Schlichting, 2006). In contrast, if plastic responses are maladaptive, we expect to observe evolutionary changes opposite to plasticity (countergradient variation ((Conover et al., 2009) or genetic compensation (Grether 2005)). Maladaptive plasticity was proposed as a possible mechanism promoting evolution since it moves phenotypes further away from the optimum and thereby increases the strength of selection on the phenotype (Ghalambor et al., 2007). Both co-gradient (Barton, Sunnucks, Norgate, Murray, & Kearney, 2014; Conover et al., 2009) and counter-gradient (Conover et al., 2009; Ghalambor et al., 2015; Laugen, Laurila, Räsänen, & Merilä, 2003) evolutionary changes have been found indicating that adaptive and maladaptive plasticity are common. It was even argued that reversion of ancestral plasticity occurs more frequently (Ho & Zhang, 2018) and is often not beneficial for long-term adaptation.

Plastic responses in physiology, behaviour or morphological traits are often initiated by changes in gene expression (Hodgins-Davis & Townsend, 2009; Wray, 2007). The transcriptome

represents a direct link between genotype and phenotype making it particularly interesting to study the interplay between plasticity and evolution. Transcription is highly plastic and modulating expression levels is an important part of an organism's physiological adjustment to environmental change (Gibson, 2008; McCairns & Bernatchez, 2009). On the other hand, there are also many studies demonstrating evolutionary divergence in gene expression between locally adapted populations (Alvarez, Schrey, & Richards, 2015; Guo et al., 2016; Romero, Ruvinsky, & Gilad, 2012; Townsend, Cavalieri, & Hartl, 2003; Whitehead & Crawford, 2006). Gene expression may even evolve more rapidly than changes in proteins since mutations affecting magnitude of expression are less likely to be deleterious than changes in protein structures (Carroll, 2005; Wray, 2007). However, it is not clear how fast plasticity in gene expression can change. Some studies reported changes in plasticity in some genes after adaptation to new conditions (Morris et al., 2014; Passow et al., 2017; von Heckel, Stephan, & Hutter, 2016), whereas others found only limited evolution of plasticity (Yampolsky, Glazko, & Fry, 2012) or less than expected (Huang & Agrawal, 2016).

In our study we used whole transcriptomes to understand the interplay between plasticity and evolution at the gene expression level using the model organism *Tribolium castaneum* (red flour beetle) and to quantify their contribution to the total phenotypic divergence between populations inhabiting different environments. More specifically, we were interested to test whether evolutionary changes in a new environment affected the same genes and were in the same direction as the ancestral plasticity. This can help us to understand how plasticity affected evolution, i.e. whether it can prevent evolution, or whether maladaptive plasticity increased strength of selection and promote evolution. Next, we tested how evolution affected gene expression reaction norms, i.e. whether adaptation to new conditions was achieved by changes in slopes or intercepts.

Material and Methods

Animal rearing, experimental evolution

We used the *Tribolium castaneum* Cro1 strain (Milutinović, Stolpe, Peuß, Armitage, & Kurtz, 2013), collected from a wild population in 2010 and adapted to lab standard conditions (32°C, 70% relative humidity (r.h.)) for more than 20 generations. Beetles were kept in 24h darkness on organic wheat flour mixed with 10% organic baker's yeast. We sterilized flour and yeast by heating them for 12h at 80°C before use. To test for adaptation to new environmental conditions we used replicate lines and exposed them to three treatment and control (CT) conditions. The conditions in the treatments were: Dry (D): 33°C and 30% r. h.; Hot (H): 37°C and 70% r. h.; Hot-Dry (HD): 37°C and 30% r. h. To generate replicate lines, we used 120 individuals (60 females and 60 males in the pupal stage) and placed them into a vial containing 80g medium. We produced six lines per selection regime (treatments plus control), resulting in a total of 24 lines. For each new generation, we randomly collected 120 pupae and placed them into a new vial. After seven to ten days, in which the pupae became adults, mated and laid eggs, adult beetles were removed by sieving the medium. We waited until the next generation (eggs/larvae in the medium) had reached the pupal stage and again collected 120 pupae per line to establish the next generation. This is similar to natural selection since individuals, depending on their fitness, do not contribute equally to the next generation. In generation 15 we produced additional mixed lines to prevent loss of genetic diversity by gene drift and inbreeding, which might impede adaptation: We mixed the six replicate lines of each selection regime in equal proportions (20 individuals from each replicate line) four times, resulting in four mixed lines with 120 individuals each. In total we had 39 lines: six normal and four mixed lines per selection regime (one line in D

125 became extinct). The transplant experiment to test for adaptation was conducted in generation
126 22.
127
128 *Reciprocal transplant and fitness assay*
129 Before testing for adaptation, all lines stayed for two generations in the same condition to
130 remove potential maternal or epigenetic effects (Figure 1): Beetles of generation 20 from all
131 selection lines were transferred to control conditions, in which they stayed for one week to mate
132 and lay eggs. After removal of the adults, we waited until their offspring had reached the pupal
133 stage and separated males and females. These individuals (generation 21) developed completely
134 in control conditions. When they reached the adult stage, we created 13 full-sib families per
135 selection line by transferring one virgin male with one virgin female of the same selection line in
136 15mL tubes with 1g of medium. After four days, in which the beetles could mate and lay eggs, 9g
137 of medium was added to provide food for the developing offspring and each mating pair was
138 transferred to a new vial. We repeated this three times, resulting in four vials per mating pair
139 containing medium and eggs. Immediately after removal of the mating pair, vials of each mating
140 pair were randomly assigned to the four different conditions, resulting in full-sib families split
141 across all conditions. As soon as offspring in these vials had reached the pupal stage, males and
142 females (four females and four males per family and condition) were separated and transferred
143 to 15 mL tubes with 5 g of medium and remained there until they were used for the fitness assay
144 two weeks later. These beetles were transferred to the treatments at the egg stage. They
145 developed completely in treatment conditions. We then assessed their performance in each
146 condition by estimating their fitness to test for adaptation. A virgin male and a virgin female of
147 the same selection line from the same condition but from different families were again placed
148 into a 15 mL tube with 1 g medium. After four days, the mating pair was removed. Males and
149 females were transferred to 1 mL Eppendorf tubes (one individual per tube), immediately frozen

150 in liquid nitrogen and stored at -80°C to use them for gene expression measurements. 9 g
151 medium was added to the mating tube. After four weeks (in CT and H) or five weeks (D and HD),
152 all offspring had reached the adult stage and were counted. We used the number of adult
153 offspring as an estimate of the fitness of a mating pair.
154
155 *Statistical analysis*
156 To test whether selection regime significantly influenced number of offspring produced and test
157 whether 20 generations in the treatments resulted in adaptation, we compared offspring
158 numbers of selection lines in their native condition to CT-lines transferred to the same condition.
159 We applied linear mixed models using the R-packages *lme4* (Bates, Mächler, Bolker, & Walker,
160 2015), and *lmertest* (Kuznetsova, Brockhoff, & Christensen, 2017) and *lsmeans* (Lenth, 2016) to
161 obtain *p*-values and confidence intervals. We included line and family as random factors,
162 selection and line type (mixed/normal) and their interaction as fixed effects. To test whether the
163 selection regime influenced how lines responded to the treatments, we used a linear mixed
164 model with offspring number in CT and treatment conditions as response variable, condition,
165 selection regime, line type (normal/mixed) and interactions as fixed effects and line, family and
166 interaction between line and condition as random effects. A significant interaction between
167 condition and selection regime indicates a significant effect of the selection regime on the
168 response to the conditions.
169
170 *RNA extraction, library preparation and sequencing*
171 208 female beetles (Table 1) p stored at -80°C were homogenized in Tri-Reagent® (Zymo
172 Research, California, USA) using an electric bead mill. RNA was extracted with the RNA Mini Prep
173 kit (Zimo Research, California, USA) following the instructions of the manufacturer. RNA-quality
174 was checked on a TapeStation (Agilent, Waldbronn, Germany) and concentrations were

175 measured with aQubit® Fluorometer (Life Technologies, California, USA). Libraries were created
176 with 500 ng RNA for each individual separately with the LEXOGEN mRNA-Seq Library Kit
177 following the manual (LEXOGEN GmbH, Vienne, Austria). Library quality was checked on a
178 TapeStation (Agilent, Waldbronn, Germany) and concentrations were determined by qPCR.
179 Libraries were diluted to the same molarity and pooled (33-36 libraries per pool). All treatments
180 and selection regimes were randomized during RNA-extraction, library preparation, and
181 sequencing. Single-end sequencing was performed in five runs on the Illumina NextSeq 500
182 (Illumina, Inc, California, USA) using the 75 cycles High Output Kit. After quality control using
183 FastQC (www.bioinformatics.bbsrc.ac.uk/projects/fastqc) reads (adaptors were trimmed and the
184 first 10 bases were hard trimmed, minimum average quality Q10, minimum tail quality 10,
185 minimum read length 20) were mapped against the reference genome
186 (ftp://ftp.ensemblgenomes.org/pub/release30/metazoa/gtf/tribolium_castaneum/Tribolium_ca
187 staneum.Tcas3.30.gtf.gz) with STAR v.2.5 (Dobin et al., 2013). We then used FeatureCounts (Liao,
188 Smyth, & Shi, 2014) to count the number of reads that mapped to each gene in the reference
189 genome. Mapping as well as read counting was performed within the data analysis framework
190 SUSHI (Hatakeyama et al., 2016).
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196 *Gene expression analysis*
197 Gene expression analysis was done in R (R Core Team, 2017). We used the R package *edgeR*
198 (Robinson, McCarthy, & Smyth, 2010) for normalizing (method: TMM) expression data to cpm
199 (counts per million) after filtering lowly expressed genes (minimum of one cpm in at least two

200 samples). For subsequent differential expression analysis we used the R package *limma* (Law,
201 Chen, Shi, & Smyth, 2014; Ritchie et al., 2015). From the differential expression analysis, we
202 obtained the number of differentially expressed genes (DE genes) within lines between
203 conditions (plastic changes, see Figure 1) or between lines of different origins (CT vs. selection)
204 within conditions (evolutionary changes, see Figure 1). The total phenotypic divergence in gene
205 expression between CT and treatments (i.e. total change TC) is the differential expression (log2-
206 fold change) between CT-lines in CT and selection lines in the treatments (Figure 1 and 2). The
207 ancestral plasticity (PC_{CT}) is the differential expression of CT-lines between CT and treatment
208 conditions, while the evolved plasticity (PC_{Sel}) is the same difference measured in selection lines.
209 The evolutionary changes are EC_T when measured as differential expression between CT and
210 selection lines in the treatments and EC_{CT} when measured in CT (Figure 2). Finally, differences
211 between plastic responses of CT- and selection lines (the interaction between treatment and
212 selection regime) give the evolutionary change in plasticity. To partition TC into changes
213 explained by ancestral plasticity (PC_{CT}) and evolutionary changes (EC_T, see Figure 2 and 3), we
214 calculated the relative contribution of each component to the total. We obtained the log2-fold
215 changes of each transcript to evaluate and compare the magnitude of the plastic and
216 evolutionary changes. We accounted for non-independence among individuals from the same
217 line by using the *duplicateCorrelation* function (Smyth et al. 2005) and added sequencing runs as
218 batch effect. A gene is classified as differentially expressed (DE) with a FDR ≤ 5% after adjusting
219 for multiple testing (Benjamini and Hochberg 1995).
220 In a second step, we used the normalized read counts (cpm, TMM-normalized) corrected for
221 batch effects (sequencing runs) using the *removeBatchEffect* function in the *limma* R package
222 (Ritchie et al., 2015). These counts were used to quantify original levels Lo, plastic levels Lp and
223 adapted levels La of selection lines. This allowed us to conduct an analysis for each selection
224 lines separately as was described by (details see below)

225

226

227 *Evolution of reaction norms*

228 Reaching a new phenotypic optimum can be achieved by changing the mean (intercept of the
229 reaction norm) or by changing the plasticity of a trait (slope of reaction norm) (Figure 3). We
230 were interested to assess which of these patterns was more prevalent in gene expression
231 evolution. To test for an evolutionary change in plasticity (slope), we first evaluated the similarity
232 in plasticity between CT- and selection lines by calculating the correlation between PC_{CT} and PC_{Sel}
233 (Figure 3A) among all genes, independent of the significance. Second, to test for an evolutionary
234 change in mean expression (intercept), we calculated the correlation between the evolved
235 differences in CT (EC_{CT}) and in treatment conditions (EC_T). A significant correlation indicates that
236 the overall mean has changed (Figure 3A). Further, for all genes with significant EC_T, we
237 quantified how much of this difference could be explained by a shift in the mean or by a change
238 in plasticity. A shift in the mean corresponds to EC_{CT}, whereas the remaining difference (EC_T-EC_{CT})
239 gives the evolutionary changes in plasticity (see also Stoks et al. 2016). Relative contributions of
240 each component were obtained by dividing them by the total EC_T. To test for significant effects
241 of the selection regime on differential expression (i.e., on number of DE genes, log2-fold change,
242 and correlations), we used a permutation test. We randomly assigned samples and their
243 transcriptomes to either CT selection or treatment (D, H, HD) selection (number of samples for
244 each selection was not changed) and repeated the DE analysis. We kept the original assignment
245 to lines and conditions and repeated the DE analysis for each permuted data set. If observed
246 values (e.g. number of DE genes, correlations) obtained from the original dataset were higher
247 than the most extreme 5% of the distribution calculated from permutations, they were
248 considered significant.

249

250

251 *Comparing plastic and evolutionary responses*

252 To infer the relationship between ancestral plasticity and evolution we compared the direction
253 of PC_{CT} to the direction of EC_T. Evolution may *reinforce* the plastic response when PC_{CT} is in the
254 same direction as EC_T (Figure 2B). If EC_T is in opposite direction, it *reverses* PC_{CT} (Figure 2A). To
255 test which of these patterns was more prevalent, we followed Ho and Zhang 2018 (Ho & Zhang,
256 2018). Expression levels (cpm) of CT-Lines in CT conditions represented the original stage (*Lo*),
257 CT-Lines in treatment the plastic stage (*Lp*), and selection lines in their respective condition the
258 adaptive stage (*La*) (Figure 2). For subsequent analysis we used genes with appreciable PC_{CT} ($|Lp$
259 $- Lo| > 0.2\ Lo$) and EC_T ($|La - Lp| > 0.2\ Lo$) (Ho & Zhang, 2018) and calculated the proportion of
260 plastic genes with reinforced or reversed changes.

261 An alternative classification is to assess whether PC_{CT} brings expression levels closer to *La*. In this
262 case, the total change TC would be larger than EC_T (Figure 2C). Such a pattern can occur even if
263 PC_{CT} and EC_T are in opposite directions and classified as reversion (Figure 2C). To confirm that
264 our results were not sensitive to the applied cutoff (20% *Lo*), we repeated the analysis with a
265 cutoff of 50% *Lo* and without any cutoff (Supporting information, Figure S1.3 A-H). Recently, it
266 was pointed out (Mallard et al. 2018; Ho and Zhang 2019) that an excess of reversions relative to
267 reinforcements is expected to be observed due to a statistical artefact that cannot be completely
268 removed by permutation tests. We therefore applied an additional parametric bootstrapping
269 test as proposed by Ho and Zhang (Ho and Zhang 2019), to compare proportion of reversions
270 and reinforcements (Supporting information, Figure S1.3 J-L).

271 To better understand the relationship between the within-line proportions of reversed genes
272 and proportions of genes with *La* closer to *Lo* with adaptation, we calculated the Spearman
273 correlation between the proportions of reversed expression changes (or *La* closer to *Lo*
274 respectively) and mean offspring number in seven selection lines in HD. We focused on HD

because it was the most extreme environment with the strongest decline in offspring number. To test for significance, we used permutations: Mean offspring numbers were randomly assigned to lines and correlation was calculated again. Proportion of permutations with a correlation coefficient exceeding the observed value gave the respective *P*-value.

280 **Results**

281 *Fitness assay showed evolutionary adaptation*

282 We found that selection lines had a higher offspring number in their native condition compared
283 to CT-lines (Dry: $F_{1,14}$= 10.04, P=0.007; Hot: $F_{1,16}$= 4.78, p=0.044; Hot-Dry: $F_{1,16}$= 23.85, p=1.660E-
284 04), confirming that adaptation had occurred (Figure 4). In contrast to treatment conditions,
285 there was no difference in offspring number between CT- and selection lines under CT conditions
286 (CT-lines: 64.89 [61.49, 68.29]; D-lines: 65.76 [61.26, 70.25], H-lines: 67.41 [63.76, 71.06], HD-
287 lines: 65.56 [62.17, 68.94]) (Figure4). Using three additional mixed models, we compared how
288 lines from different selection regimes responded to treatments. We found significant negative
289 effects for all stress treatments (D: $F_{1,14}$= 222.10, p = 5.21E-10; H: $F_{1,16}$= 615.10, p = 3.37E-14; HD:
290 $F_{1,16}$= 714.39, p = 8.664e-15) (Figure4). Interaction between selection and treatments, i.e.
291 whether the response to the treatment was different dependent on selection regime, was
292 significant for HD-lines ($F_{1,16}$= 12.12, p=0.003051) and for D ($F_{1,14}$= 13.22, p= 0.002682), but not
293 for H ($F_{1,16}$= 0.6879, p= 0.42). Interaction between selection regime, treatment and line type
294 (normal/mixed) was not significant in any treatment indicating that mixing lines did not have an
295 effect on adaptation.

296

297 *Evolution of gene expression*

298 We found many more genes with expression divergence relative to Control (TC) than genes with
299 evolved changes in the treatments (EC_T) or in control conditions (EC_{CT}) (Table 2). Increased
300 temperature caused expression divergence in a larger number of genes than reduced humidity
301 (Table 2). Most DE genes with significant TC also had significant ancestral plasticity PC_{CT} (Dry:
302 33.9%; Hot: 72.9%, Hot-Dry: 82.2%), but only a minority showed significant evolutionary change
303 EC_T (Dry: 4.9%; Hot: 1.1%; Hot-Dry: 1.0%, see also Figure S1.1). About half of the DE genes with

304 EC_T in Hot and Hot-Dry then did not diverge significantly from their ancestral expression levels in
305 Control (TC ~ 0). Overall, TC had a larger log2-fold change contribution from plastic changes
306 (PC_{CT}) than evolved changes in Hot and Hot-Dry (median proportions PC_{CT}/TC; Hot: 71.47%; Hot-
307 Dry: 86.30%), but not in Dry (42.90%). Many genes had PC_{CT} > TC, especially in HD (Figure 5). In
308 these cases, EC_T was negative and reduced TC (Figure 5).

309 *Evolution of reaction norms*

310 We were then interested to examine whether EC_T were due to a change in mean expression
311 (shift in the intercept of reaction norms) or to a change in plasticity (different slopes of reaction
312 norms, see Figure 3). First, we found a significant correlation between EC_{CT} and EC_T, in all three
313 treatments (permutation tests: $p < 0.0001$) (Table 4, Figure 5). This indicates that for a majority
314 of genes, evolution in the past 20 generations affected their mean expression levels by shifting
315 the intercept of reaction norms (see Figure 7A). Second, we also found a highly positive
316 correlation (Figure 7A) between PC_{CT} and PC_{Sel} (Figure 3), indicating that plasticity was mainly
317 preserved during adaptation to new conditions. A permutation test showed that this correlation
318 was not significantly different from correlations obtained from permuted datasets (Table 2),
319 where samples were randomly assigned to selection regimes, suggesting that the treatments did
320 not have specific effects on plasticity. When we quantified the relative contributions of changes
321 in the mean versus changes in plasticity to EC_T, we found that evolution of the intercept
322 explained more evolutionary divergence than evolution of the slope of reaction norms, especially
323 in Dry (Figure 6).

324 *Evolution of plasticity in DE genes*

325 We found only five genes with significantly evolved plasticity in the DE analysis, after correcting
326 for multiple testing. Although plasticity seemed to be preserved overall, we found evidence that
327 it evolved among DE genes. The DE analysis revealed a reduced number of plastic genes in

328 selection lines compared to plastic genes in CT-lines (Table 2), although the difference was only
329 significant in HD (Table 3). It further showed that ancestral plasticity PC_{CT} was larger than evolved
330 plasticity PC_{Sel}, in D and HD (Table 3), indicating a reduction of the magnitude of plastic
331 responses after adaptation. We found the same outcome when focusing on genes with
332 significant EC_T, in HD (Mann-Whitney U test: U = 2133, *P* = 2.102E-04; median: CT-lines: 0.25, HD-
333 lines: 0.13), but not in D (U = 144, *P* = 0.584; median: CT-lines: 0.10, D-lines: 0.14) or H (U = 386,
334 *P* = 0.158; median: CT-lines: 0.21, H-lines: 0.12) (Figure 8).

335

336 *Effect of plasticity on evolutionary responses*

337 We could not find evidence that plasticity impeded evolution. In the DE analysis genes with a
338 significant ancestral plastic response were overrepresented among the evolved DE genes in H
339 and HD (H: $\chi2$ = 4.51, df = 1, *P* = 3.38E-02; HD: $\chi2$ = 9.71, df = 1, *P* = 1.84E-03), but not in D (*P* =
340 0.113 Fisher's exact test). Furthermore, when we analysed each selection line separately and
341 compared their expression levels to CT-lines, the proportion of genes exhibiting substantial PC_{CT}
342 ($|Lp - Lo| > 0.2\ Lo$) were also overrepresented among evolved genes with EC_T ($|La - Lp| > 0.2\ Lo$)
343 (Table S2.1).

344 Maladaptive plastic changes may be reverted by evolutionary changes when the plastic response
345 overshoots the optimum expression level in a new environment. Among the genes with
346 significant EC_T in the DE analysis, we found that PC_{CT} was generally in opposite direction (Figure
347 8). Enrichment tests revealed that genes with positive EC_T in the DE analysis were
348 overrepresented among genes with negative PC_{CT} in H and HD (Fisher's exact test D: *P* = 0.076; H:
349 *P* = 0.002; HD: *P* = 9.26e-06), and genes with negative EC_T were overrepresented among genes
350 with positive PC_{CT}, in H and in HD as well (Fisher's exact test D: *P* = 0.073; H: *P* = 0.021; HD: *P* =
351 0.049)(Figure 6). In almost all genes that showed significant EC_T and PC_{CT}, the responses were in

352 opposite direction (Dry: 2 out of 2 genes; Hot: 12 out of 13; Hot-Dry: 33 out of 34). We also
353 found a negative correlation between PC_{CT} and EC_T in H and HD, but not in D (Supporting
354 information, Figure S1.2).

355 When we considered mean expression levels per line with (PC_{CT} > 20% *Lo* and EC_T > 20% *Lo*) we
356 obtained similar results as in the DE analysis. Reinforcements were present but less frequent
357 than reversions in all treatments (proportions of plastic responses with reversion/reinforcement:
358 Dry: 48.5 ± 1.0% / 34.3 ± 0.8 %, P < 2.2e-16 (binomial test); Hot: 44.6 ± 0.9% / 28.8 ± 1.2%, P <
359 2.2e-16; Hot-Dry: P < 2.2e-16; Figure 10A). Different cutoff values did not influence these
360 outcomes (Figure S1.3). Parametric bootstrapping as an additional test (Ho & Zhang, 2019) ,
361 confirmed our results (Supporting information, Figure S1.3). We also found that a substantial
362 proportion of plastic genes (PC_{CT} > 20% *Lo*) had *La* closer to *Lo* than to *Lp* (Dry: 45.4 ± 1.1%; Hot:
363 41.2 ± 1.2%; Hot-Dry: 40.7 ± 2.5 %)(Figure 10B). EC_T (EC_{CT} > 20% *Lo*) genes showed a higher
364 proportion of reversions (Dry: 34.1 ± 0.8%; Hot: 40.0 ± 0.9%; Hot-Dry: 43.0 ± 2.1 %) compared to
365 reinforcements (Dry: 24.1 ± 0.6%; Hot: 25.8 ± 1.1%; Hot-Dry: 26.8 ± 1.6%). 41.8 ± 0.7% (Dry), 34.2
366 ± 0.8% (Hot), 30.2 ± 1.0 % (Hot-Dry) of EC_T genes did not show PC_{CT}.

367 In case of genetic assimilation, i.e. loss of plasticity in selection line and continuous expression of
368 the induced phenotype, evolutionary differences would not be visible in treatment condition.
369 We calculated the mean expression levels of selection lines in Control and defined genes as
370 showing assimilation if they met three criteria: no PC_{Sel} (absolute difference between selection
371 lines in CT and in treatment < 20% *Lo*), PC_{CT} > 20% *Lo*, and *Lp* ~*La* (absolute difference < 20% *La*).
372 The proportion of PC_{CT} genes showing genetic assimilation was small in all conditions (Dry: 13.5
373 %, Hot: 11.5 %, Hot-Dry: 14.7%; Supporting information Table S2.3).

374

375 *Proportions of reversed plastic responses and association with fitness*

376 To gain a better understanding of the adaptive value of the changes of expression levels in the
377 evolved lines, we tested for an association between within-line proportion of reversed or
378 reinforced plastic responses and the average fitness of the lines in the HD treatment. We found
379 that lines with a higher proportion of reversions had a higher average offspring number
380 (correlation: 0.82, P = 0.012, Figure 11) and we found a negative but non-significant correlation
381 between fitness and reinforcements (correlation: -0.43, P = 0.85). When we tested for an
382 association between fitness and proportion of genes with *La* closer to *Lo*, we also found a
383 positive correlation (correlation: 0.86, P = 0.006). Overall, better adapted lines (higher fitness in
384 HD) showed a higher proportion of reversed ancestral plasticity and were more similar to the
385 original expression levels of CT-lines in CT. Performing the analysis with gene expression data in
386 H and D provided similar correlations, although not significant because of lower sample sizes
387 (Supporting information, Table S2.2).
388
389 **Discussion**
390 We studied plastic and evolved responses in gene expression of *T. castaneum* in response to
391 three new environmental conditions (Dry, Hot, Hot-Dry). After 20 generation of experimental
392 evolution, we were able to detect adaptation and found significant evolutionary changes in
393 expression levels. Comparing these changes with ancestral plastic responses to the conditions
394 showed that a reversion of plasticity was the more frequent (ca. 60 % of all genes; genes with
395 significant plastic changes in DE analysis: >95%). Number of genes where ancestral plasticity was
396 reinforced by evolution was smaller (ca. 40%; DE analysis: <5%) and only a small proportion of
397 genes (8-10% of plastic genes) showed expression patterns consistent with genetic assimilation.
398 Of the genes with evolutionary differences in treatment conditions, a high proportion (ca. 40%)
399 showed expression levels closer to the ancestral than to the plastic stage suggesting that
400 plasticity was maladaptive. Although plasticity showed a high degree of preservation in terms of

401 responding genes and direction of response, we found evidence that selection lines evolved a
402 reduced plasticity and thus partly compensated the maladaptive ancestral response. We were
403 further able to show an association between the proportion of reversed plastic responses and
404 adaptation (mean fitness per line) in the most stressful treatment Hot-Dry.
405
406 *Maladaptive plasticity*
407 Different patterns describing the relationship between plastic and evolved changes in gene
408 expression have been documented. It was suggested that plasticity might help populations to
409 persist after environmental change, or to colonize new habitats by bringing phenotypes closer to
410 the new optimum. Studies found support for this hypothesis by showing that plastic responses of
411 non-adapted individuals diminished differences to native populations (Lohman, Stutz, & Bolnick,
412 2017; Mäkinen, Papakostas, Vøllestad, Leder, & Primmer, 2016). Adaptive plasticity can also be
413 indicated when plastic and evolutionary responses are in the same direction (Li, Li, Song, Wang,
414 & Zhang, 2017), or when plasticity is higher in adapted populations (Hasan et al., 2017; McCairns
415 & Bernatchez, 2009), suggesting that most plastic individuals were favored by selection.
416 However, there are also examples for the reversed pattern suggesting that plasticity was
417 maladaptive, at least on the long-term. In wild populations of *Fundulus heteroclitus* evolved
418 changes to different temperatures were opposite to plastic responses of the ancestral
419 population (Dayan et al., 2015). *Rhagoletis* flies shifting to a new host fruits showed evolutionary
420 responses opposite to plasticity of non-adapted species (Ragland et al., 2015). Experimental
421 evolution studies found countergradient evolution in *Drosophila* adapting to different diets
422 (Huang & Agrawal, 2016; Yampolsky et al., 2012). A comparative study (Ho & Zhang, 2018)
423 analyzing data of multiple experimental evolutions suggested that reversions of gene expression
424 changes might be a general pattern during adaptation.

425 Our study fits to these previous observations. We found a higher proportion of reversions in all
426 conditions indicating mostly maladaptive plasticity. An alternative explanation for the prevalence
427 of reversions without plastic responses being necessarily maladaptive would be that CT-lines
428 exhibited a response in the right direction, but overshot an optimum expression level (Figure 2C).
429 Fine-tuning during long-term adaptation could then lead to a partial reversion of the plastic
430 response. We took this possibility into account by not only focusing on a reversion of plasticity,
431 but also testing whether *La* was closer to *Lp* or to *Lo* (see Figure 2C). We found that adapted lines
432 showed a high proportion of expression levels that were closer to their ancestral level *Lo*. This
433 confirms that many plastic responses moved phenotypes further away from the new optimum
434 and evolution compensated for these detrimental changes. This pattern might even become
435 more pronounced after some additional generations, given that our selection lines still show
436 incomplete adaptations, i.e. strong reduction in offspring number compared to control levels. In
437 addition we were also able to show a direct association between proportion of reversions and
438 adaptation.
439
440 *Evolution of reaction norms*
441 The ancestral maladaptive plasticity can be compensated by shifts in the intercept or changes in
442 the slope of reaction norms. Both are not mutually exclusive and can occur together in the same
443 trait. We were aiming to quantify their relative importance for evolutionary responses in
444 transcriptomes.
445
446 Gene expression studies so far provided mixed results regarding the evolution of plasticity.
447 *Drosophila* populations adapted to different temperatures showed local adaptation, but there
448 was no evidence for evolution of thermal reaction norms of different transcripts and changes
449 affected mainly expression mean (Clemson, Sgrò, & Telonis-Scott, 2016). Experimental evolution

450 studying *Drosophila* under variable diets found no significant changes in plasticity (Yampolsky et
451 al., 2012) or less than expected (Huang & Agrawal, 2016). In contrast, other studies found
452 differences in temperature responses between tropical and temperate *Drosophila* populations
453 (Levine, Eckert, & Begun, 2011; von Heckel et al., 2016). Other examples for differences in genes
454 expression plasticity between adapted and non-adapted populations include temperature
455 (Morris et al., 2014) and salinity (Gibbons, Metzger, Healy, & Schulte, 2017; McCairns &
456 Bernatchez, 2009) responses of marine and freshwater sticklebacks, temperature response of
457 killifish populations from different latitudes, as well as plastic responses to toxic hydrogen
458 sulphide (H_2S) of fish population from H_2S rich springs versus non-toxic springs (Passow et al.,
459 2017). There was no consistent pattern regarding the direction in which plasticity evolves: In
460 some cases adapted population showed an increase in plasticity (Morris et al., 2014), in other
461 cases plasticity was reduced (Huang & Agrawal, 2016; Ragland et al., 2015; von Heckel et al.,
462 2016) or reduction and enhancement of plasticity were equally frequent (Gibbons et al., 2017;
463 Yampolsky et al., 2014). Overall, there is evidence in multiple species that expression plasticity of
464 some genes can evolve. However, even in some of these studies reporting evolved plasticity
465 (Dayan et al., 2015; Gibbons et al., 2017; Morris et al., 2014) the number of transcripts with
466 significant changes in the mean was much higher and large parts of the plastic responses showed
467 a high degree of preservation.
468
469 In accordance with these previous findings we found that changes in the mean contributed more
470 to the observed differences than changes in plasticity. A possible reason might be that genetic
471 variation in mean expression was higher than genetic variation in plasticity. In addition, we did
472 not select for changes in plasticity directly since the conditions in the treatments were constant.
473 Selection was therefore on expression levels in the treatment and only indirectly on plasticity.
474 Plasticity could evolve if mean expression levels were genetically correlated with plasticity.

475 Although we did not directly test for such correlations, we found evidence for evolution of
476 plasticity in DE genes that is in genes with largest changes in expression level between CT and
477 treatment conditions. Although plastic responses showed a high degree of preservation, we
478 found evidence for evolutionary changes in the magnitude of plastic responses.
479
480 *Why can plasticity be maladaptive?*
481 New stressors might disturb homeostasis resulting in inappropriate responses and long-term
482 adaptation therefore restores ancestral phenotypes by genetic changes, referred to as genetic
483 compensation (Grether, 2005) or counter-gradient variation (Conover et al., 2009). However, in
484 our study we applied relatively mild stressor treatments, i.e. individuals were able to survive and
485 reproduce. Drought and heat are also stressors, which *T. castaneum* had experienced in the past
486 (Sokoloff, 1972), so there had probably been selection on plastic responses to be beneficial.
487 However, plastic responses might be optimized for a short-term exposure: Allocation of
488 resources from reproduction to protection might increase survival probability and allow
489 individuals to continue reproduction as soon as the stress has disappeared, but this response
490 becomes maladaptive during continuous exposure and should therefore be under negative
491 selection. Expression of stress related genes is in general accompanied by a down-regulation of
492 genes involved in growth and reproduction due to an allocation of resources (Schwenke, Lazzaro,
493 & Wolfner, 2016; Sokolova, 2013). A well-studied example are heat shock proteins (hsp). Hsp are
494 well known for their protective function and to be crucial for survival (Feder & Hoffman, 1999),
495 but it was also shown that their expression came at a cost (Feder et al., 1998; Sørensen,
496 Kristensen, & Loeschcke, 2003). Accordingly, it was often found that hsp expression in
497 populations adapted to warmer climates is lower compared to non-adapted populations
498 (Fangue, Hofmeister, & Schulte, 2006; Narum & Campbell, 2015; J G Sørensen, Dahlgaard, &
499 Loeschcke, 2001). In general, other protection mechanisms independent of ancestral plasticity

500 may arise during long-term adaptation (e.g. enzymes, which are more stable at high
501 temperature) and make the costly stress response expendable.
502
503 An alternative explanation for the reduced plasticity in adapted lines is that the signal
504 responsible for eliciting the plastic responses is based on any kind of damage caused by heat or
505 stress in general (Kültz, 2005). Higher resistance in adapted lines might shift the inducing
506 thresholds (Sikkink, Reynolds, Ituarte, Cresko, & Phillips, 2014) above the levels we applied in the
507 treatments.
508
509 Interestingly, we found no differences in fitness between lines from different selection regimes
510 under CT conditions (Figure 4). We could detect some genes with different expression levels in
511 the treatments and found in general a correlated EC_{CT}, but this did not affect offspring number. It
512 indicates a lack of fitness trade-offs, where alleles providing a fitness advantage in one
513 environment are detrimental in another. Together with the observation that selection lines
514 evolve to bring expression closer to ancestral CT-levels, it suggests that for most genes the
515 optimal expression level is not different between conditions.
516
517 *Potential caveats*
518 The number of genes with significant PC_{CT} in the DE analysis was much higher compared to genes
519 showing EC_T. One possible explanation would be that adaptive plasticity prevented evolution. If
520 the plastic responses matched the optimum, no genetic changes in the selection lines are
521 expected to occur. However, when we analysed each line separately and considered a gene as
522 evolved if the mean difference between *La* and *Lp* was more than 20% of the *Lo*, we found
523 approximately the same number of genes with EC_T and PC_{CT} (Supporting information Table S2.2).

524 In the DE analysis in *limma* we did not analyse each line separately but treated them as biological
525 replicates. Comparisons between conditions, i.e. plastic changes, can be made within lines. They
526 should thus be more precise and statistical power should be higher than comparisons between
527 selection regimes, i.e. evolved changes, that have to be made between lines. Differences
528 between lines from the same selection regime lower the ability to obtain significant evolutionary
529 changes. These differences can arise from genetic drift. Since our population size was relatively
530 small (120 individuals per line) this might have been an important factor. Another explanation is
531 that lines from the same selection regime differed how exactly they improved their fitness in the
532 respective treatment. Since fitness is a highly polygenic trait, the responsible genes contributing
533 to an increase may not be the same. For the most extreme treatment HD, where we sequenced
534 seven lines, we further found considerable differences in fitness between the lines, suggesting
535 that not all of them were at the same stage of adaptation. It is therefore not surprising that
536 expression levels did not evolve in the same way.
537 We used two different approaches, a classical DE analysis treating lines as replicates and
538 analysing lines separately using mean expression level of samples. The DE analysis resulted in a
539 much smaller number of genes with evolved changes. Since it requires that a gene shows similar
540 changes in all replicate lines, it is more conservative. If the main interest of a study is to identify
541 promising candidate genes for future more detailed analyses it is the appropriate approach to
542 keep FDR as low as possible. In contrast, if the focus is more on general patterns, a less stringent
543 analysis using mean expression levels can give us a more complete picture. The number of
544 evolved genes was much higher using this approach. Many of the observed differences between
545 selection lines and Control-lines might be caused by drift and are therefore not significant in the
546 DE analysis. However, since genetic drift is random it cannot explain the observed excess of
547 reversions over reinforcements.
548

549 Although we demonstrated that gene expression changed during evolution, it is not clear
550 whether these changes are the cause for an increased fitness in these conditions or whether
551 they are rather the consequence of adaptation and being less stressed. One disadvantage in
552 studying whole transcriptomes is that not all responding genes might be of functional
553 importance but are correlated to other adaptive changes. Reversion of plasticity during
554 adaptation can implicate that responding genes were not the direct target of selection and other
555 mechanisms, e.g. changes in protein structure of enzymes, were responsible for adaptation of
556 selection lines. Future studies that manipulate expression and test for correlated changes in
557 offspring are needed to confirm adaptive value of expression changes.
558
559 **Conclusions**
560 Evolution compensated ancestral plasticity and reduced total phenotypic divergence between
561 control and treatment conditions suggesting that ancestral plasticity was maladaptive for long-
562 term adaptation. In the most stressful treatment, selection lines with higher fitness show a
563 higher proportion of reversions and a higher similarity with ancestral expression. Differences
564 between adapted lines and CT-lines in the treatment were mainly due to a change in mean
565 expression (i.e. shift in the intercept of reaction norms), while plasticity was preserved in terms
566 of affected genes and direction of change. However, we found that a part of the differences in
567 the treatments can be explained by a reduction in the magnitude of plasticity in adapted lines.
568 Plastic genes were clearly overrepresented among evolved genes, thus showing that plasticity
569 did not impede evolution. In contrast, our results suggested that maladaptive plasticity increased
570 selection and promoted evolution. Similar results in all three stress treatments indicated that
571 these findings may represent a general pattern of gene expression adaptation.
572
573

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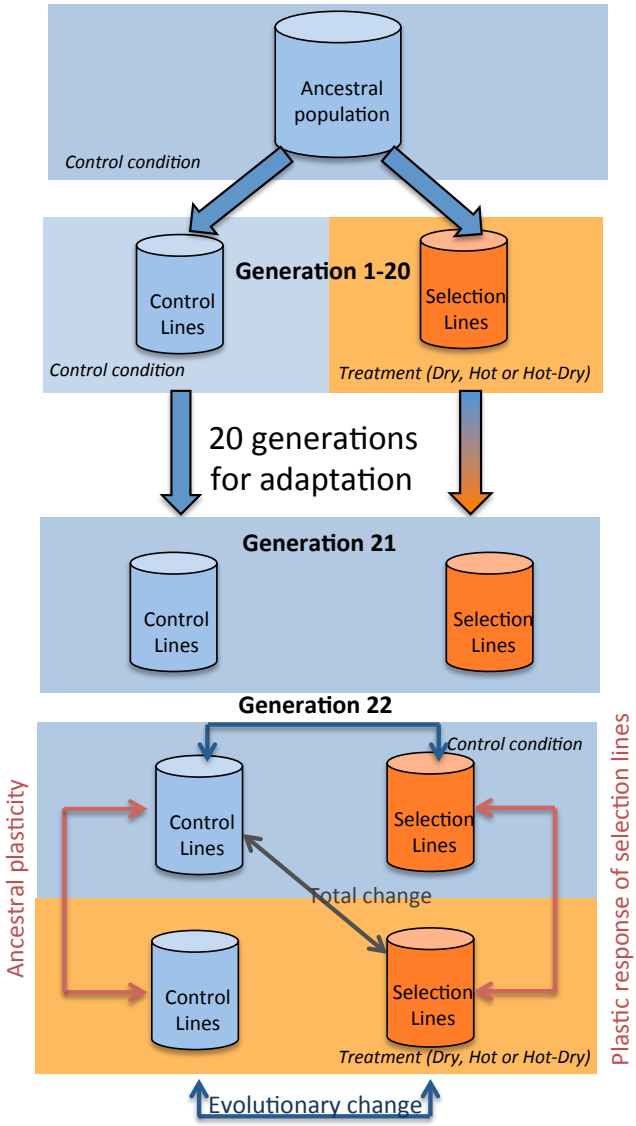

581

582 **Figure 1:** Schematic representation of the experimental evolution design. Lines were derived from a single outbred
583 *Tribolium castaneum* population (collected in 2013, strain Cro1) that had lived under control conditions for ca. 30
584 generations. For each selection regime we used six replicate lines. In generation 15, four additional lines were created

by mixing the six lines of each selection regime in equal proportions, resulting in a total of 10 lines per selection regime. Lines stayed in control or treatment conditions for 20 generations. After one generation in control condition to remove potential maternal and epigenetic effects, individuals from all lines were transferred to control and treatment conditions in the egg stage. Fitness and gene expression were measured in adult females. To get plastic responses in gene expression, expression levels in treatment conditions were compared to levels under control conditions of the same line. Evolved response is the difference within treatment conditions between control and selection lines. Control conditions: 33°C, 70% relative humidity r.h.; Treatments: Dry: 33°C, 30% r.h.; Hot 37°C, 70% r.h.; Hot-Dry: 37°C, 30% r.h.

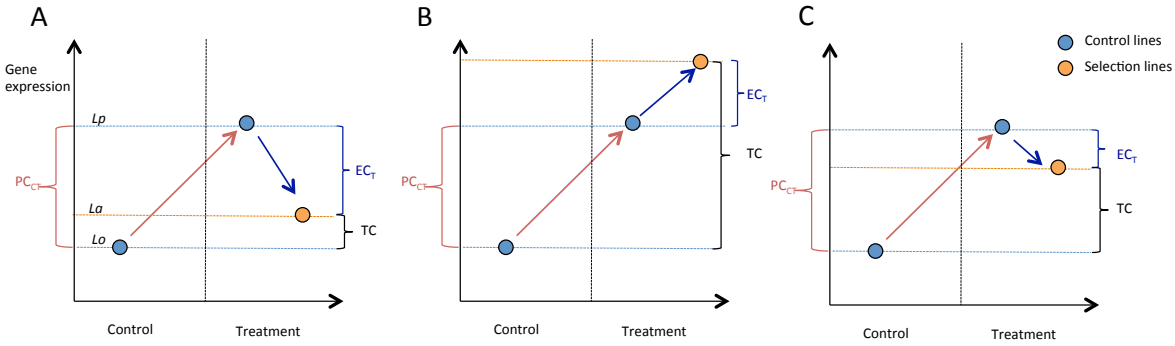

Figure 2: Possible relations between plastic change PC and evolutionary change EC. Gene expression levels of control lines (blue) in the ancestral control condition represent the original stage Lo, expression in the treatment the plastic stage Lp. Expression levels of selection lines (orange) in the treatment give the adapted stage La Arrows indicate the direction of PC (red) and EC (blue). PC can be in opposite direction to EC (reversion **(A))** or it can be in the same direction (reinforcement **(B))**. However, even if PC and EC are opposite to each other, PC can bring expression levels closer to levels of the adapted lines. In this case, the total change TC, (difference between expression levels of control lines in control conditions and selection lines in treatment) is larger than EC **(C)**.

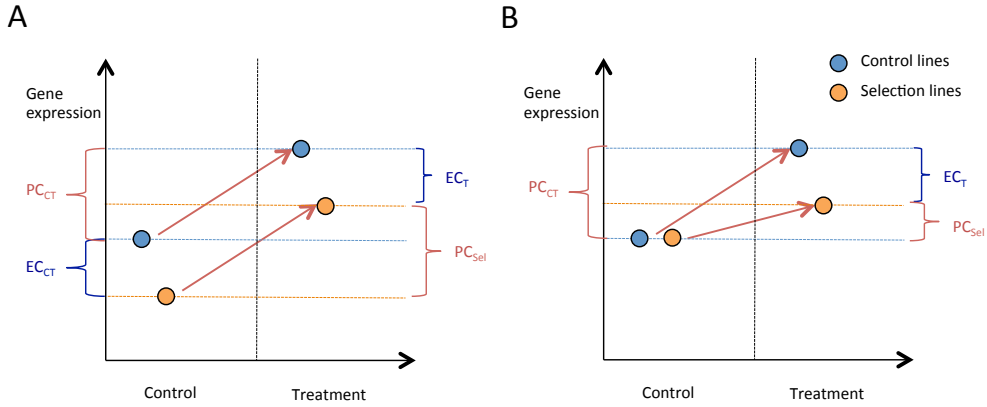

Figure 3: Possible ways how non-adapted lines (in blue) can reach the phenotypic optimum in a new environment. We assume that expression levels of adapted selection lines (in orange) represent the optimum level in the treatment. During adaptation, lines could have reached the optimum by either changing mean expression, i.e. shift in the intercept of the reaction norm **(A)** or by changing their plasticity, i.e. the slope of the reaction norm **(B)**. In case of a change in the mean, plastic changes of Control lines PC_{CT} and selection lines PC_{Sel} as well as observed evolutionary change EC_T in treatment and in Control EC_{CT} would be highly correlated because reaction norms (red arrows) remain parallel **(A)**. If observed EC_T in treatment is due to a change in plasticity only, no correlation between EC_T and EC_{CT} should exist, and the correlation between PC_{Sel} and PC_{CT} should be small **(B)**.

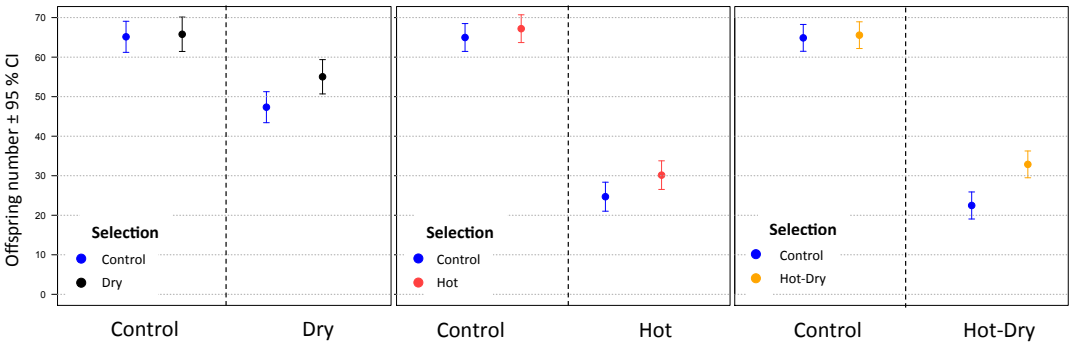

Figure 4: Offspring number of Control and selection lines under different conditions. Selection lines could adapt for 20 generations.

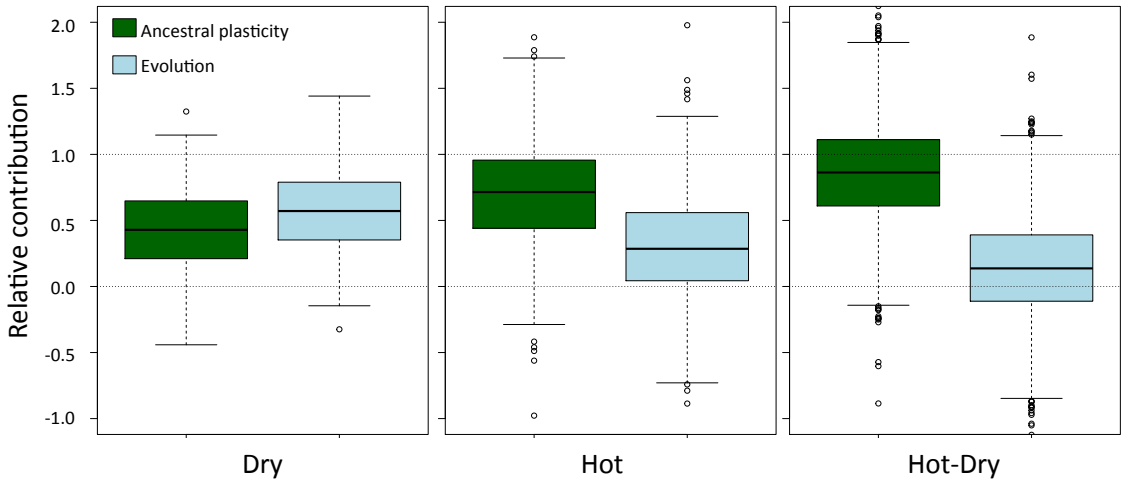

Figure 5: Relative contribution of ancestral plasticity and evolutionary change to total divergence for all genes showing significant total divergence in the DE analysis. Number of genes: Dry: 283, Hot: 1192, Hot-Dry: 2045.

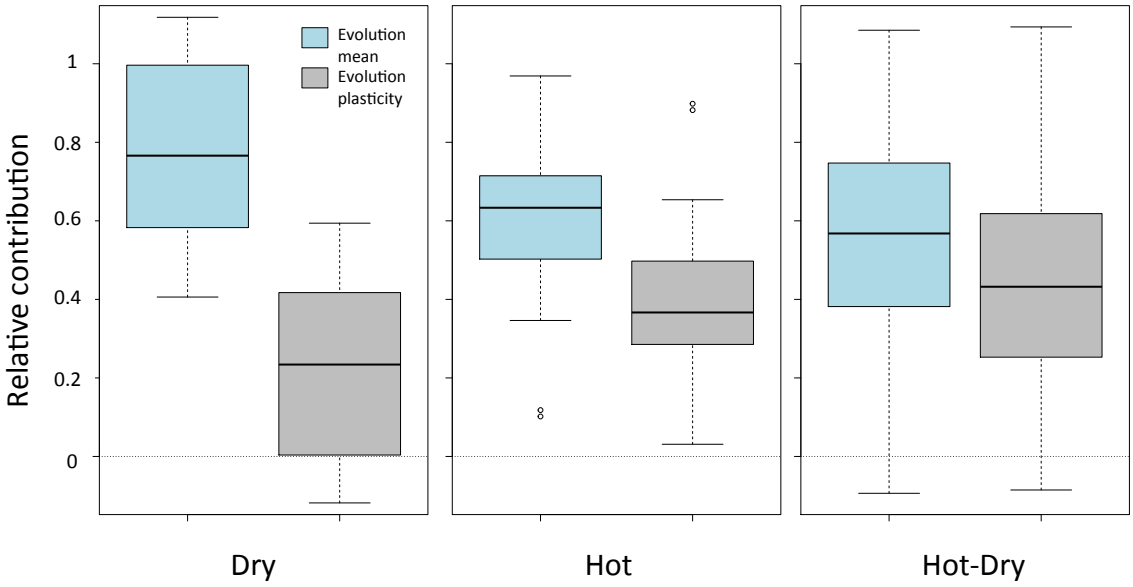

Figure 6: Relative contribution of changes in the mean (shift in the intercept of reaction norm) and changes in plasticity (different slopes of reaction norms) to evolutionary differences between control-lines and selection lines in

the treatment. Only genes with significant differences in the DE analysis were included. Number of genes: Dry: 18, Hot: 25, Hot-Dry: 55.

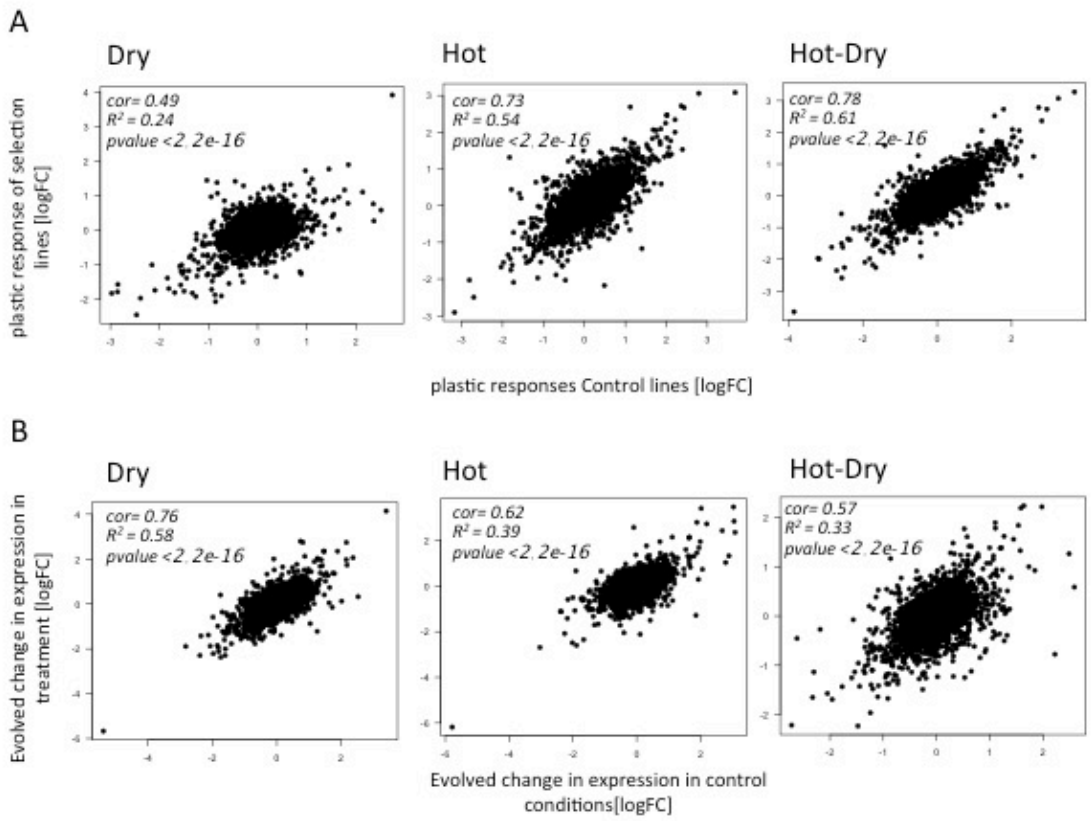

Figure 7: Correlation of plastic responses in gene expression of control lines and selection lines (**A**) and correlation of evolved divergence in gene expression in control and treatment conditions (**B**). Selection lines had lived in the respective treatment for 20 generations. After filtering lowly expressed genes (minimum of 1cpm in at least 2 samples) 11534 genes remained that were used for calculating correlations.

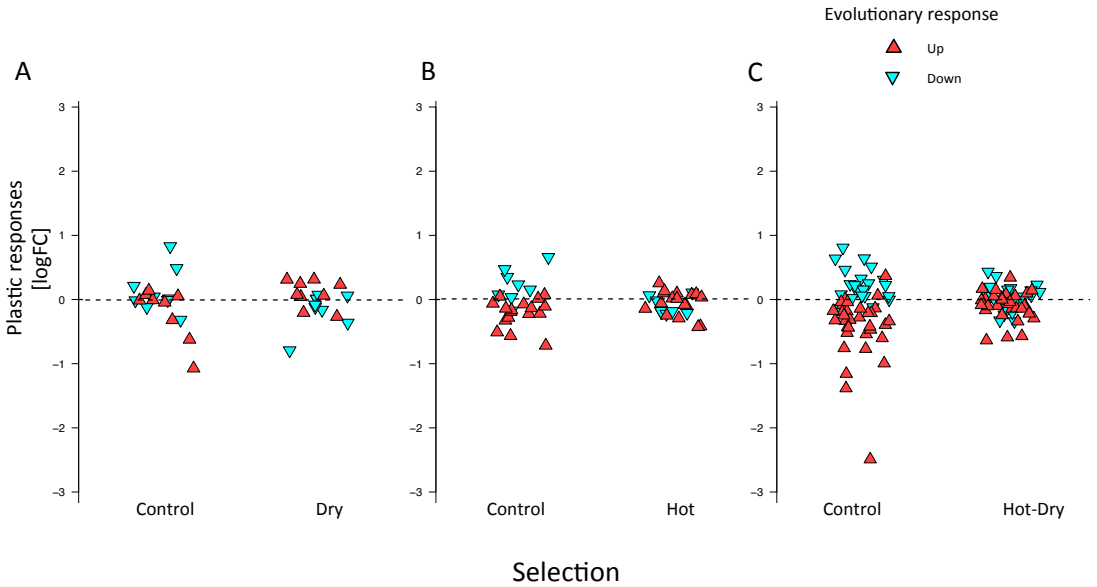

Figure 8: Plastic responses in control-lines and adapted selection-lines of genes showing significant evolutionary changes in expression in the treatments Dry **(A)**, Hot **(B)** and Hot-Dry **(C)**. Plastic responses in adapted selection lines

are weaker (smaller logFC). Furthermore, plastic changes are mainly opposite to evolved changes. Genes that evolved to lower expression in the treatments (represented in blue) show positive plastic changes in control-lines, i.e. they are up-regulated. Genes that evolved to higher expression levels (in red) are down-regulated in the non-adapted Control-Lines.

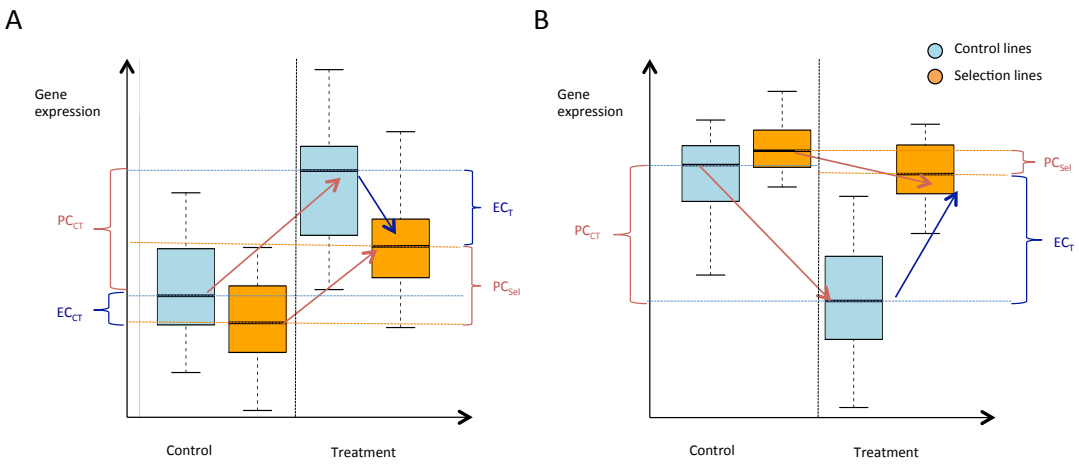

Figure 9: Examples of gene expression levels in control and treatment conditions of control lines (blue) and adapted selection lines (orange). **A:** example of one gene with no change in plasticity and a reversion of the plastic response, which was the commonly observed pattern. Plastic changes PC of control lines (PC_{CT}) and selection lines (PC_{Sel}) are positively correlated and very similar. Evolutionary changes EC in treatment (EC_T) are positively correlated with evolutionary changes in control conditions (EC_{CT}) due to a change in mean expression. In most cases, EC_{CT} is smaller than EC_T . **B:** Example of a gene with significant evolutionary divergence in expression in treatment conditions: If selection lines reduce their plastic response PC_{Sel}, it can increase divergence EC_T in the treatment. For most genes we observed that plastic changes (red arrows) are in the opposite direction to evolved changes (blue arrows).

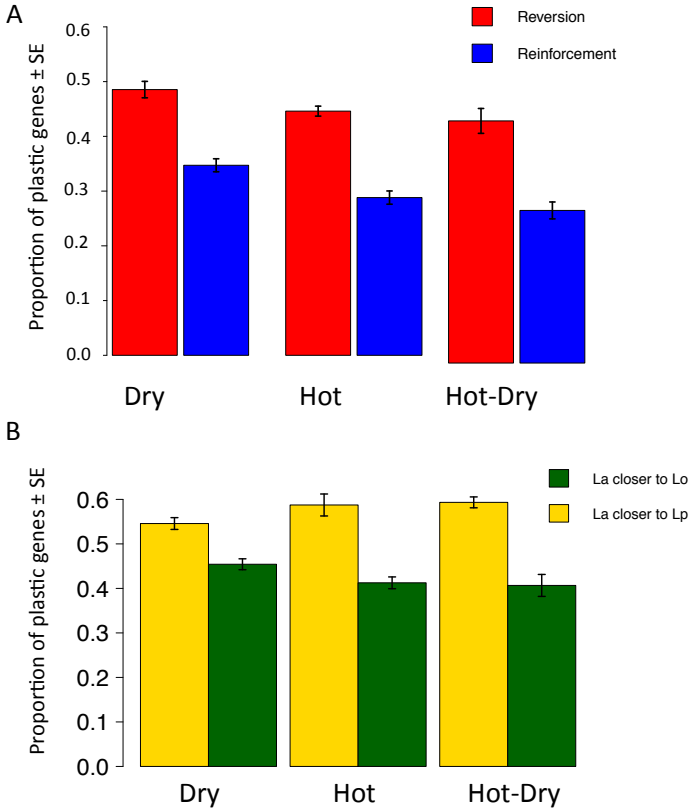

Figure 10: Comparison of plastic and evolutionary changes in gene expression in response to three environmental conditions. **A:** Proportion of genes (average over selection lines) showing a reversion or reinforcement of ancestral plasticity during evolution. **B:** Proportion of genes with expression levels after evolution (adapted stage La) closer to original levels (Lo, i.e. Control-lines in control conditions), or closer to plastic levels (Lp, i.e. expression levels of Control-lines in treatment). Only genes exhibiting substantial plastic changes (|Lp-Lo| > 20% of Lo) as well as evolutionary changes (La-Lp| > 20% of Lo) were used for analysis.

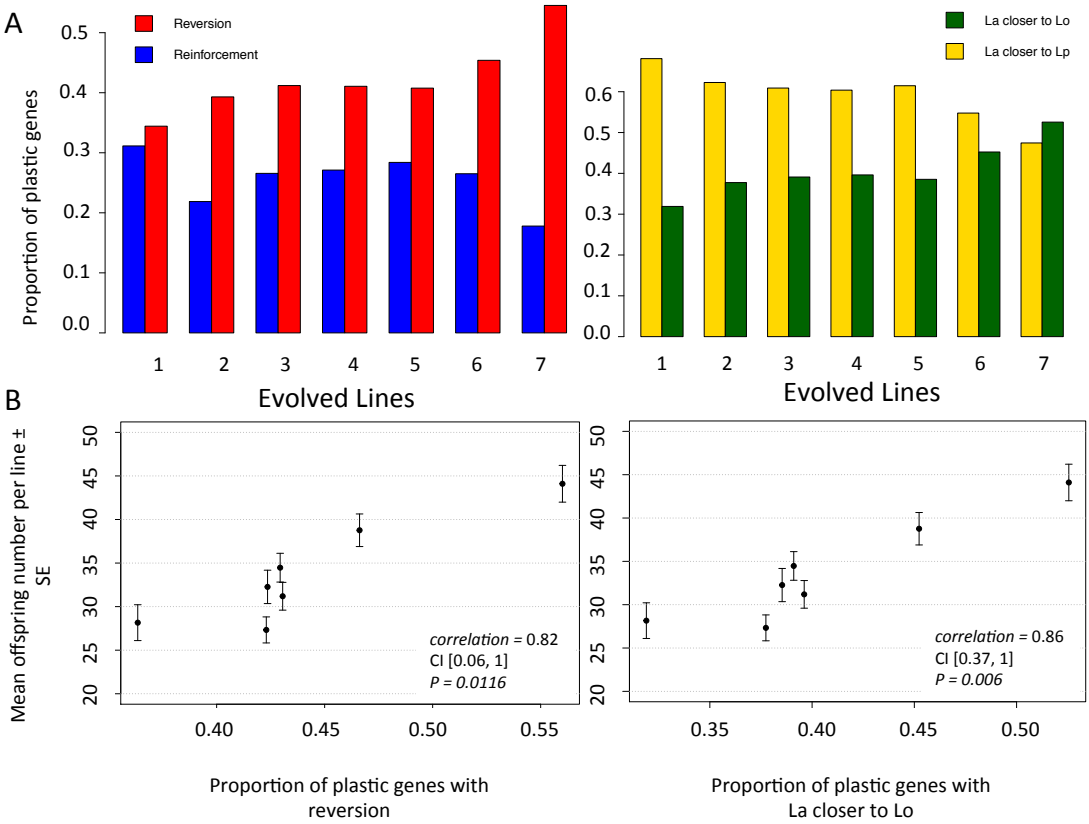

Figure 11: Relationship between plastic and evolved changes in gene expression in response to hot-dry conditions. Expression levels of Control-lines in control conditions represent the ancestral stage Lo, Control-Lines in hot-dry conditions the plastic stage Lp and adapted Hot-Dry-lines in hot-dry conditions the adapted stage La. **A:** Barplots show the proportion of genes in each Hot-Dry line with a reversion (evolved response in opposite direction to plastic response of Control-Lines) or a reinforcement of the plastic response (same direction as plastic response of Control-Lines) in the left graph. The right graph shows the proportion of genes with La closer to Lp (in yellow) and La closer to Lo (in green). **B:** Relationship between the proportion of reversed plastic responses and mean fitness (=offspring number) per Hot-Dry line and between proportion of genes with La closer to Lo and mean fitness. Only genes exhibiting substantial plastic changes (|Lp-Lo| > 20% of Lo) as well as evolutionary changes (La-Lp| > 20% of Lo) were used for analysis. P-values of the spearman correlations were obtained by 10,000 permutations. 95 % Confidence intervals are based on a non-parametric bootstrap test.

Table 1: Number of sequenced replicate lines and individuals per selection and treatment, which were used for this study. Selection lines could adapt to conditions for 20 generations. Control (CT) conditions: 33°C, 70% relative humidity (r.h.); treatments: Dry (D): 33°C, 30% r.h.; Hot (H): 37°C, 70% r.h.; Hot-Dry (HD): 37°C, 30% r.h.

Selection	Conditions				
	Total	CT	D	H	HD
CT					
Number lines	**7**	7	5	5	7
Number Ind.	**89**	25	20	19	26
D					
Number lines	**4**	4	4	–	–
Number Ind.	**32**	16	16	–	–
H					
Number lines	**5**	5	–	5	–
Number Ind.	**40**	20	–	20	–
HD					
Number lines	**7**	7	–	–	7
Number Ind.	**56**	28	–	–	28

Table 2: Significantly differently expressed genes. For evolved differences, control-lines and selection-lines are compared within condition (e.g. Dry-lines vs Control-lines in Dry). Plastic response gives the number of genes that changed expression between control and treatment conditions (e.g. Dry-lines in Dry vs Dry-lines in control). Different plasticity gives the number of genes with significant different plastic responses in lines from different selection regimes. Analysis was conducted using the R package limma (Ritchie et al., 2015).

		Dry	**Hot**	**Hot-Dry**
Evolved difference in Control	down	3	0	6
	up	1	9	4
	total	**4**	**9**	**10**
Evolved difference in treatment	down	9	8	22
	up	9	17	33
	total	**18**	**25**	**55**
Plastic response Control-Lines	down	209	1765	2451
	up	156	1714	2200
	total	**365**	**3479**	**4651**
Plastic response Selection-Lines	down	28	1417	1649
	up	21	1381	1470
	total	**49**	**2798**	**3119**
Total change (Selection-lines in treatment vs Control-lines in Control)	down	154	564	1016
	up	129	628	1029
	total	**283**	**1192**	**2045**
different plasticity	**total**	**0**	**1**	**4**

Table 3: P-values obtained from permutation tests (10,000 permutations). Samples were randomly assigned to either control or treatment selection and differential expression analyses were repeated. Significance was assessed by calculating the proportion of permutations with more extreme values than the observed one. Control (CT) conditions:

682 33°C, 70% relative humidity r.h. Conditions in treatments: Dry (D): 33°C, 30% r.h.; Hot (H): 37°C, 70% r.h.; Hot-Dry
683 (HD): 37°C, 30% r.h.

	Selection		
	D	**H**	**HD**
CT-Lines have more genes with significant plastic responses compared to adapted selection lines	0.2981	0.3037	**0.0325**
Magnitude of plastic response is higher in CT-Lines	**0.0493**	0.1676	**0.0491**
Individuals from different selection regimes show lower similarity in plastic responses †	0.8608	0.3914	0.5217
Number of genes with significant differences in expression levels is higher in treatment than in CT conditions	**0.0095**	**0.0003**	**0.0031**
Differences in expression levels in CT conditions and treatment are correlated	**< 0.0001**	**<0.0001**	**< 0.0001**

684 †: observed correlation of plastic responses of CT- and selection lines would be lower compared to results of permutations

685
686

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688

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