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

Eva L. Koch[1] and Frédéric Guillaume[1]

[1] Department of Evolutionary Biology and Environmental Studies, University of Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland

Corresponding author:
Prof. Dr. Frédéric Guillaume
tel: ++41 44 635 6623
frederic.guillaume@ieu.uzh.ch

Abstract

Plasticity and evolution are two processes 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 in reaction norms (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. Reversion of ancestral plasticity occurs more frequently (Ho & Zhang, 2018), indicating that plastic responses are 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 the 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 few 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 during adaptation to new environments with the model organism *Tribolium castaneum* (red flour beetle) using an experimental evolution approach. With RNA-seq, we quantified plastic and evolutionary changes in gene expression and their contribution to the total phenotypic divergence between populations inhabiting different environments. More specifically, we were interested to test whether the same genes exhibited both evolutionary and plastic changes in a new environment and whether evolved changes were in the same direction as their ancestral plasticity. In doing so, we could test how plasticity

affected evolution. We tested whether plasticity prevented evolution, or whether maladaptive plasticity increased the strength of selection and promoted 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

125 lines to prevent loss of genetic diversity by gene drift and inbreeding, which might impede
126 adaptation: We mixed the six replicate lines of each selection regime in equal proportions (20
127 individuals from each replicate line) four times, resulting in four mixed lines with 120 individuals
128 each. In total we had 39 lines: six normal and four mixed lines per selection regime (one line in D
129 became extinct). The transplant experiment to test for adaptation was conducted in generation
130 22.
131
132 *Reciprocal transplant and fitness assay*
133 Before testing for adaptation, all lines stayed for two generations in the same condition to
134 remove potential maternal or epigenetic effects (Supporting information, Figure S1): Beetles of
135 generation 20 from all selection lines were transferred to control conditions, in which they
136 stayed for one week to mate and lay eggs. After removal of the adults, we waited until their
137 offspring had reached the pupal stage and separated males and females. These individuals
138 (generation 21) developed completely in control conditions. When they reached the adult stage,
139 we created 13 full-sib families per selection line by transferring one virgin male with one virgin
140 female of the same selection line in 15mL tubes with 1g of medium. After four days, in which the
141 beetles could mate and lay eggs, 9g of medium was added to provide food for the developing
142 offspring and each mating pair was transferred to a new vial. We repeated this three times,
143 resulting in four vials per mating pair containing medium and eggs. Immediately after removal of
144 the mating pair, vials of each mating pair were randomly assigned to the four different
145 conditions, resulting in full-sib families split across all conditions. These beetles were transferred
146 to the treatments at the egg stage. As soon as offspring in these vials had reached the pupal
147 stage, males and females (four females and four males per family and condition) were separated
148 and transferred to 15 mL tubes with 5 g of medium and remained there until they were used for
149 the fitness assay two weeks later. They developed completely in treatment conditions. We then

150 assessed their performance in each condition by estimating their fitness to test for adaptation. A
151 virgin male and a virgin female of the same selection line from the same condition but from
152 different families were again placed into a 15 mL tube with 1 g medium. After four days, the
153 mating pair was removed. Males and females were transferred to 1 mL Eppendorf tubes (one
154 individual per tube), immediately frozen in liquid nitrogen and stored at -80°C to use them for
155 gene expression measurements. 9 g medium was added to the mating tube. After four weeks (in
156 CT and H) or five weeks (D and HD), all offspring had reached the adult stage and were counted.
157 We used the number of adult offspring as an estimate of the fitness of a mating pair.
158
159 *Statistical analysis*
160 To test whether selection regime significantly influenced number of offspring produced and test
161 whether 20 generations in the treatments resulted in adaptation, we compared offspring
162 numbers of selection lines in their native condition to CT-lines transferred to the same condition.
163 We applied linear mixed models using the R-packages *lme4* (Bates, Mächler, Bolker, & Walker,
164 2015), and *lmertest* (Kuznetsova, Brockhoff, & Christensen, 2017) and *lsmeans* (Lenth, 2016) to
165 obtain *p*-values and confidence intervals. We included line and family as random factors,
166 selection and line type (mixed/normal) and their interaction as fixed effects. To test whether the
167 selection regime influenced how lines responded to the treatments, we used a linear mixed
168 model with offspring number in CT and treatment conditions as response variable, condition,
169 selection regime, line type (normal/mixed) and interactions as fixed effects and line, family and
170 interaction between line and condition as random effects. A significant interaction between
171 condition and selection regime indicates a significant effect of the selection regime (evolution)
172 on the response to the conditions (plastic response).
173
174 *RNA extraction, library preparation and sequencing*

175 208 female beetles (Table 1) p stored at -80°C were homogenized in Tri-Reagent® (Zymo
176 Research, California, USA) using an electric bead mill. RNA was extracted with the RNA Mini Prep
177 kit (Zimo Research, California, USA) following the instructions of the manufacturer. RNA-quality
178 was checked on a TapeStation (Agilent, Waldbronn, Germany) and concentrations were
179 measured with aQubit® Fluorometer (Life Technologies, California, USA). Libraries were created
180 with 500 ng RNA for each individual separately with the LEXOGEN mRNA-Seq Library Kit
181 following the manual (LEXOGEN GmbH, Vienne, Austria). Library quality was checked on a
182 TapeStation (Agilent, Waldbronn, Germany) and concentrations were determined by qPCR.
183 Libraries were diluted to the same molarity and pooled (33-36 libraries per pool). All treatments
184 and selection regimes were randomized during RNA-extraction, library preparation, and
185 sequencing. Single-end sequencing was performed in five runs on the Illumina NextSeq 500
186 (Illumina, Inc, California, USA) using the 75 cycles High Output Kit. After quality control using
187 FastQC (www.bioinformatics.bbsrc.ac.uk/projects/fastqc) reads (adaptors were trimmed and the
188 first 10 bases were hard trimmed, minimum average quality Q10, minimum tail quality 10,
189 minimum read length 20) were mapped against the reference genome
190 (ftp://ftp.ensemblgenomes.org/pub/release30/metazoa/gtf/tribolium_castaneum/Tribolium_ca
191 staneum.Tcas3.30.gtf.gz) with STAR v.2.5 (Dobin et al., 2013). We then used FeatureCounts (Liao,
192 Smyth, & Shi, 2014) to count the number of reads that mapped to each gene in the reference
193 genome. Mapping as well as read counting was performed within the data analysis framework
194 SUSHI (Hatakeyama et al., 2016).
195
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 S1) or between lines of different origins (CT vs. selection)
204 within conditions (evolutionary changes, see Figure S1). 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 1A-C and
207 Supporting information Figure S1). The ancestral plasticity (PC_{CT}) is the differential expression of
208 CT-lines between CT and treatment conditions, while the evolved plasticity (PC_{Sel}) is the same
209 difference measured in selection lines. The evolutionary changes are EC_T when measured as
210 differential expression between CT and selection lines in the treatments and EC_{CT} when
211 measured in CT (Figure 1). Finally, differences between plastic responses of CT- and selection
212 lines (the interaction between condition and selection regime) give the evolutionary change in
213 plasticity. To partition TC into changes explained by ancestral plasticity (PC_{CT}) and evolutionary
214 changes (EC_T, see Figure 1A-C), we calculated the relative contribution of each component to the
215 total. We used the log2-fold change of each transcript to evaluate and compare the magnitude
216 of the plastic and evolutionary changes. When computing log2-fold changes, we accounted for
217 non-independence among individuals from the same line by using the *duplicateCorrelation*
218 function (Smyth et al. 2005) and added sequencing runs as batch effect. A gene is classified as
219 differentially expressed (DE) with a FDR ≤ 5% after adjusting for multiple testing (Benjamini and
220 Hochberg 1995).
221 In a second step, we used the normalized read counts (cpm, TMM-normalized) corrected for
222 batch effects (sequencing runs) using the *removeBatchEffect* function in the *limma* R package
223 (Ritchie et al., 2015). We used these counts to quantify expression levels in in CT and treatments.

224 This allowed us to conduct an analysis for each selection lines separately as was described by Ho
225 & Zhang (2018) (details see below).
226
227
228 *Evolution of reaction norms*
229 Reaching a new phenotypic optimum can be achieved by changing the mean (intercept of the
230 reaction norm) or by changing the plasticity of a trait (slope of reaction norm) (Figure 1D,E). We
231 were interested in assessing which of these patterns was more prevalent in gene expression
232 evolution. To test for an evolutionary change in plasticity (slope), we first evaluated the similarity
233 in plasticity between CT- and selection lines by calculating the correlation between PC_{CT} and PC_{Sel}
234 (Suppurting information Figure S2A) among all genes, independent of the significance. Second,
235 to test for an evolutionary change in mean expression (intercept), we calculated the correlation
236 between the evolved differences in CT (EC_{CT}) and in treatment conditions (EC_T). A significant
237 correlation indicates that the overall mean has changed (Figure S2A). Further, for all genes with
238 significant EC_T, we quantified how much of this difference could be explained by a shift in the
239 mean or by a change in plasticity. A shift in the mean corresponds to EC_{CT}, whereas the
240 remaining difference (EC_T-EC_{CT}) gives the evolutionary changes in plasticity (see also Stoks et al.
241 2016). Relative contributions of each component were obtained by dividing them by the total
242 EC_T.
243
244 To test for significant effects of the selection regime on differential expression (i.e., on number
245 of DE genes, log2-fold change, and correlations), we used a permutation test. We randomly
246 assigned samples and their transcriptomes to either CT selection or treatment (D, H, HD)
247 selection (number of samples for each selection was not changed) and repeated the DE analysis.
248 We kept the original assignment to lines and conditions and repeated the DE analysis for each

249 permuted data set. Observed values (e.g. number of DE genes, correlations) were considered
250 significant if higher than the most extreme 5% of the distribution calculated from permutations.
251
252
253 *Comparing plastic and evolutionary responses*
254 To infer the relationship between ancestral plasticity and evolution, we compared the direction
255 of PC_{CT} to the direction of EC_T. Evolution may *reinforce* the plastic response when PC_{CT} is in the
256 same direction as EC_T (Figure 1B). If EC_T is in opposite direction, it *reverses* PC_{CT} (Figure 1A). To
257 test which of these patterns was more prevalent, we followed Ho and Zhang 2018 (Ho & Zhang,
258 2018). Expression levels (cpm) of CT-Lines in CT conditions represented the original stage (*Lo*),
259 CT-Lines in treatment the plastic stage (*Lp*), and selection lines in their respective condition the
260 adaptive stage (*La*) (Figure 2). For subsequent analysis we used genes with appreciable PC_{CT} ($|Lp$
261 $- Lo| > 0.2\ Lo$) and EC_T ($|La - Lp| > 0.2\ Lo$) (Ho & Zhang, 2018) and calculated the proportion of
262 plastic genes with reinforced ($TC > PC_{CT}$) or reversed ($TC < PC_{CT}$) changes.
263 An alternative classification of plastic responses is to assess whether PC_{CT} brings expression
264 levels closer to the new optimum (*La).* In this case, the total change TC would be larger than EC_T
265 (Figure 2C). Such a pattern can occur even if PC_{CT} and EC_T are in opposite directions and classified
266 as reversion (Figure 2C), but may indicate that ancestral plasticity was beneficial. A PC_{CT} that
267 moves *Lp* further away from *La* is strongly indicative of maladaptive plasticity.
268 To confirm that our results were not sensitive to the applied cutoff (20% *Lo*), we repeated the
269 analysis with a cutoff of 50% *Lo* and without any cutoff (Supporting information, Figure S3.1 A-
270 H). Recently, it was pointed out (Mallard et al. 2018; Ho and Zhang 2019) that an excess of
271 reversions relative to reinforcements is expected to be observed due to a statistical artefact that
272 cannot be completely removed by permutation tests. We therefore applied an additional

273 parametric bootstrapping test as proposed by Ho and Zhang (Ho and Zhang 2019), to compare
274 proportion of reversions and reinforcements (Supporting information, Figure S3.1 J-L).
275 To better understand the relationship between the within-line proportions of reversed genes
276 and proportions of genes with *La* closer to *Lo* with adaptation, we calculated the Spearman
277 correlation between the proportions of reversed expression changes (or *La* closer to *Lo*
278 respectively) and mean offspring number in seven selection lines in HD. We focused on HD
279 because it was the most extreme environment with the strongest decline in offspring number.
280 To test for significance, we used permutations: Mean offspring numbers were randomly assigned
281 to lines and correlation was calculated again. Proportion of permutations with a correlation
282 coefficient exceeding the observed value gave the respective *P*-value.
283

284 **Results**

285 *Fitness assay showed evolutionary adaptation*

286 We found that selection lines had a higher offspring number in their native condition compared
287 to CT-lines (Dry: $F_{1,14}$ = 9.20, p = 0.009; Hot: $F_{1,16}$ = 4.78, p = 0.044; Hot-Dry: $F_{1,16}$ = 23.51, p =
288 1.786E-04), confirming that adaptation had occurred (Figure 2). In contrast to treatment
289 conditions, there was no difference in offspring number between CT- and selection lines under
290 CT conditions (CT-lines: 64.89 [61.49, 68.29]; D-lines: 65.76 [61.26, 70.25], H-lines: 67.41 [63.76,
291 71.06], HD-lines: 65.56 [62.17, 68.94]) (Figure 2). Using three additional mixed models, we
292 compared how lines from different selection regimes responded to treatments. We found
293 significant negative effects for all stress treatments (D: $F_{1,23}$ = 45.37, p = 6.85E-07; H: $F_{1,28}$ =
294 507.68, p < 2.200E-16; HD: $F_{1,28}$ = 553.06, p < 2.200E-16) (Figure 2). Interaction between selection
295 and treatments, i.e. whether the response to the treatment was different depending on
296 selection regime, was significant for HD-lines ($F_{1,28}$ = 9.39, p= 4.754E-03) and for D ($F_{1,23}$ = 4.32, p
297 = 0.049), but not for H ($F_{1,28}$ = 0.57, p = 0.455). Interaction between selection regime, treatment
298 and line type (normal/mixed) was not significant in any treatment indicating that mixing lines did
299 not have an effect on adaptation. ANOVA tables as well as results of the linear mixed models are
300 in supporting information S4.

301

302 *Evolution of gene expression*

303 We found many more genes with expression divergence relative to Control (TC) than genes with
304 evolved changes in the treatments (EC_T) or in control conditions (EC_{CT}) (Table 2). Increased
305 temperature caused expression divergence in a larger number of genes than reduced humidity
306 (Table 2). Most DE genes with significant TC also had significant ancestral plasticity PC_{CT} (Dry:
307 33.9%; Hot: 72.9%, Hot-Dry: 82.2%), but only a minority showed significant evolutionary change

308 EC_T (Dry: 4.9%; Hot: 1.1%; Hot-Dry: 1.0%, see also Supporting information Figure S3.2). About
309 half of the DE genes with EC_T in Hot and Hot-Dry then did not diverge significantly from their
310 ancestral expression levels in Control (TC ~ 0). Overall, TC had a larger log2-fold change
311 contribution from plastic changes (PC_{CT}) than evolved changes in Hot and Hot-Dry (median
312 proportions PC_{CT}/TC; Hot: 71.47%; Hot-Dry: 86.30%), but not in Dry (42.90%). Many genes had
313 PC_{CT} > TC, especially in HD (Figure 3A). In these cases, EC_T was negative and reduced TC.

314 *Evolution of reaction norms*

315 We were then interested to examine whether EC_T were due to a change in mean expression
316 (shift in the intercept of reaction norms) or to a change in plasticity (different slopes of reaction
317 norms, see Figure 3). First, we found a significant correlation between EC_{CT} and EC_T, in all three
318 treatments (permutation tests: $P < 0.0001$) (Table 3, Supporting information Figure S2). This
319 indicates that for a majority of genes, evolution in the past 20 generations affected their mean
320 expression levels by shifting the intercept of reaction norms (see Figure 1D). Second, we also
321 found a highly positive correlation between PC_{CT} and PC_{Sel} (Figure Supporting information S2A),
322 indicating that plasticity was mainly preserved during adaptation to new conditions. A
323 permutation test showed that this correlation was not significantly different from correlations
324 obtained from permuted datasets (Table 3), where samples were randomly assigned to selection
325 regimes, suggesting that the treatments did not have specific effects on plasticity. When we
326 quantified the relative contributions of changes in the mean versus changes in plasticity to EC_T,
327 we found that evolution of the intercept explained more evolutionary divergence than evolution
328 of the slope of reaction norms, especially in Dry (Figure 3B).

329 *Evolution of plasticity in DE genes*

330 We found only five genes with significantly evolved plasticity in the DE analysis, after correcting
331 for multiple testing. Although plasticity seemed to be preserved overall, we found evidence that

332 it evolved among DE genes. The DE analysis revealed a reduced number of plastic genes in
333 selection lines compared to plastic genes in CT-lines (Table 2), although the difference was only
334 significant in HD (Table 3). It further showed that ancestral plasticity PC_{CT} was larger than evolved
335 plasticity PC_{Sel}, in D and HD (Table 3), indicating a reduction of the magnitude of plastic
336 responses after adaptation. We found the same outcome when focusing on genes with
337 significant EC_T, in HD (Mann-Whitney U test: U = 2133, *P* = 2.102E-04; median: CT-lines: 0.25, HD-
338 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,
339 *P* = 0.158; median: CT-lines: 0.21, H-lines: 0.12) (Figure 4).

340

341 *Effect of plasticity on evolutionary responses*

342 We could not find evidence that plasticity in general impeded evolution. In the DE analysis genes
343 with a significant ancestral plastic response were overrepresented among the evolved DE genes
344 in H 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*
345 = 0.113 Fisher's exact test). Furthermore, when we analysed each selection line separately and
346 compared their expression levels to CT-lines, the proportion of genes exhibiting substantial PC_{CT}
347 ($|Lp - Lo| > 0.2\ Lo$) were also overrepresented among evolved genes with EC_T ($|La - Lp| > 0.2\ Lo$)
348 (Table S5.1).

349 Maladaptive plastic changes may be reverted by evolutionary changes when the plastic response
350 overshoots the optimum expression level in a new environment. Among the genes with
351 significant EC_T in the DE analysis, we found that PC_{CT} was generally in opposite direction (Figure
352 4). Enrichment tests revealed that genes with positive EC_T in the DE analysis were
353 overrepresented among genes with negative PC_{CT} in H and HD (Fisher's exact test D: *P* = 0.076; H:
354 *P* = 0.002; HD: *P* = 9.26e-06), and genes with negative EC_T were overrepresented among genes
355 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* =

356 0.049)(Figure 4). In almost all genes that showed significant EC_T and PC_{CT}, the responses were in
357 opposite direction (Dry: 2 out of 2 genes; Hot: 12 out of 13; Hot-Dry: 33 out of 34). We also
358 found negative correlations between PC_{CT} and EC_T in all treatments, although significant only in H
359 and HD, but not in D (Supporting information, Figure S3.3).

360 When we considered mean expression levels per line with PC_{CT} > 20% *Lo* and EC_T > 20% *Lo*, we
361 obtained similar results as in the DE analysis. Reinforcements were present but less frequent
362 than reversions in all treatments (proportions of plastic responses with reversion/reinforcement:
363 Dry: 48.5 ± 1.0% / 34.3 ± 0.8 %, P < 1.151e-07 (binomial test); Hot: 44.6 ± 0.9% / 28.8 ± 1.2%, P =
364 4.369E-13; Hot-Dry: 43.6 / 27.1 P < 2.2e-16; Figure 5A). The reversed genes represented 34.1 ±
365 0.8% (Dry), 40.0 ± 0.9% (Hot), and 42.96 ± 2.1% (Hot-Dry) of the evolved genes. In contrast, 24.1
366 ± 0.6% (Dry), 25.8 ± 1.1% (Hot), 26.80 ± 1.6% (Hot-Dry) of evolved genes showed reinforcement.

367 Different cut-off values did not influence these outcomes (Figure S3.1). Parametric bootstrapping
368 as an additional test (Ho & Zhang, 2019), confirmed our results (Supporting information, Figure
369 S3.1). We also found that a large proportion of ancestrally plastic genes had *La* closer to *Lo* than
370 to *Lp* (Dry: 45.4 ± 1.1%; Hot: 41.2 ± 1.2%; Hot-Dry: 40.7 ± 2.5 %)(Figure 5B). Finally, substantial
371 proportions of genes with EC_T did not show PC_{CT} (Dry: 41.8 ± 0.7%, Hot: 34.6 ± 0.8%, Hot-Dry:
372 30.3 ± 1.0 %).

373 In case of genetic assimilation, i.e. loss of plasticity in selection line and continuous expression of
374 the induced phenotype, evolutionary differences would not be visible in treatment condition.
375 We calculated the mean expression levels of selection lines in Control and defined genes as
376 showing assimilation if they met three criteria: no PC_{Sel} (absolute difference between selection
377 lines in CT and in treatment < 20% *Lo*), PC_{CT} > 20% *Lo*, and *Lp* ~*La* (absolute difference < 20% *La*).
378 The proportion of PC_{CT} genes showing genetic assimilation was small in all conditions (Dry: 6.8 %,
379 Hot: 10.3 %, Hot-Dry: 10.3 %; Supporting information Table S5.2).

380

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

382 To gain a better understanding of the adaptive value of the changes of expression levels in the
383 evolved lines, we tested for an association between within-line proportion of reversed or
384 reinforced plastic responses and the average fitness of the lines in the HD treatment. We found
385 that lines with a higher proportion of reversions had a higher average offspring number
386 (correlation: 0.82, P = 0.012, Figure 6A) and we found a negative but non-significant correlation
387 between fitness and reinforcements (correlation: -0.43, P = 0.85). When we tested for an
388 association between fitness and proportion of ancestrally plastic genes with *La* closer to *Lo*, we
389 also found a positive correlation (correlation: 0.86, P = 0.006, Figure 6B). Overall, better adapted
390 lines (higher fitness in HD) showed a higher proportion of reversed ancestral plasticity and these
391 plastic genes were more similar to the original expression levels of CT-lines in CT. Performing the
392 analysis with gene expression data in H and D provided similar correlations, although not
393 significant because of lower sample sizes (Supporting information, Table S5.3).

394

395 **Discussion**

396 We studied plastic and evolved responses in gene expression of *T. castaneum* in response to
397 three new environmental conditions (Dry, Hot, Hot-Dry). After 20 generations of experimental
398 evolution, we were able to detect adaptation and found significant evolutionary changes in
399 expression levels. Comparing evolutionary changes with ancestral plastic responses showed that
400 a reversion of plasticity was most frequent (> 95 % genes with significant plastic changes in DE
401 analysis; > 40 % of genes with substantial plastic changes, i.e. changes higher than 20% of
402 ancestral levels). The number of genes where ancestral plasticity was reinforced by evolution
403 was significantly smaller (DE analysis: <5%, 27-34 % of genes with substantial plastic changes)
404 and a smaller proportion of genes (7-10 % of substantially plastic genes) showed expression

405 patterns consistent with genetic assimilation. A high proportion of the originally plastic genes
406 evolved to expression levels that were closer to control levels than to ancestrally plastic levels.
407 Although the proportion of non-reversed plastic genes was still high, positive associations
408 between fitness and proportion of reversions, and compensated plasticity (expression levels
409 closer to control levels) respectively, suggest that ancestral plasticity was maladaptive for a
410 majority of responding genes. Although plasticity showed a high degree of preservation in terms
411 of number of responding genes and direction of the response, we found evidence that selection
412 lines evolved a reduced plasticity and thus partly compensated the maladaptive ancestral
413 response. We were further able to show a positive association between the proportion of
414 reversed plastic responses and adaptation (mean fitness per line) in the most stressful treatment
415 Hot-Dry.
416
417 *Maladaptive plasticity*
418 Different patterns describing the relationship between plastic and evolved changes in gene
419 expression have been documented. It was suggested that plasticity might help populations to
420 persist after environmental change, or to colonize new habitats by bringing phenotypes closer to
421 the new optimum. Studies found support for this hypothesis by showing that plastic responses of
422 non-adapted individuals diminished differences to native populations (Lohman, Stutz, & Bolnick,
423 2017; Mäkinen, Papakostas, Vøllestad, Leder, & Primmer, 2016). Adaptive plasticity can also be
424 indicated when plastic and evolutionary responses are in the same direction (Li, Li, Song, Wang,
425 & Zhang, 2017), or when plasticity is higher in adapted populations (Hasan et al., 2017; McCairns
426 & Bernatchez, 2009), suggesting that most plastic individuals were favored by selection.
427 However, there are also examples for the reversed pattern suggesting that plasticity was
428 maladaptive. In wild populations of *Fundulus heteroclitus*, evolved changes to different
429 temperatures were opposite to plastic responses of the ancestral population (Dayan et al.,

430 2015). *Rhagoletis* flies shifting to a new host fruits showed evolutionary responses opposite to
431 plasticity of non-adapted species (Ragland et al., 2015). Experimental evolution studies found
432 countergradient evolution in *Drosophila* adapting to different diets (Huang & Agrawal, 2016;
433 Yampolsky et al., 2012) and in guppies adapting to low predation environments (Ghalambor et
434 al., 2015). A comparative study (Ho & Zhang, 2018) analyzing data of multiple experimental
435 evolution suggested that reversions of gene expression changes might be a general pattern
436 during adaptation.

437 Our study fits with these previous observations. We found a higher proportion of reversions than
438 reinforcements in all conditions indicating mostly maladaptive plasticity. An alternative
439 explanation for the prevalence of reversions without maladaptive plastic responses would be
440 that CT-lines exhibited a response in the right direction, but overshot an optimum expression
441 level (Figure 1C). Fine-tuning during long-term adaptation could then lead to a partial reversion
442 of the plastic response. We took this possibility into account by not only focusing on a reversion
443 of plasticity, but also testing whether plastic changes brought expression levels closer to the
444 adapted stage *La* than to original stage *Lo.* If, in contrast, we see that *La* is closer to the original
445 level (CT lines in CT conditions), it indicates that plastic responses were maladaptive since they
446 moved expression levels further away from the new optimum and became compensated during
447 evolution (see Figure 1C). We found that adapted lines showed a high proportion of expression
448 levels closer to their ancestral level *Lo* than to *Lp*. We could further show that this proportion is
449 positively associated with higher fitness per selection line. We also found strong positive
450 associations of within-line proportions of reversions with mean reproductive output, indicating a
451 possible fitness advantage to reversions.

452 There was still a large proportion of plastic genes that did not show reversion. They are either
453 close to the levels of adapted lines, or showed reinforcement. The first case might indicate that
454 plasticity prevented evolution by matching the new optimum. Reinforced plastic changes could

455 be examples of adaptive plasticity. However, correlation between proportion of reinforcements
456 and fitness was not significant and negative. The observed positive correlation between
457 proportion of reversed plastic genes and mean fitness per line in HD rather suggests that
458 reversions were favoured during adaptations. Reversions might become more pronounced after
459 more generations, once evolution had sufficient time to further reverse maladaptive ancestral
460 plastic responses. Our selection lines still show a strong reduction in offspring number compared
461 to control levels suggesting further potential to adapt.
462
463 *Evolution of reaction norms*
464 The ancestral maladaptive plasticity can be compensated by shifts in the intercept or changes in
465 the slope of reaction norms. Both are not mutually exclusive and can occur together in the same
466 trait. We were aiming to quantify their relative importance for evolutionary responses in
467 transcriptomes.
468
469 Gene expression studies so far provided mixed results regarding the evolution of plasticity.
470 *Drosophila* populations adapted to different temperatures showed local adaptation, but there
471 was no evidence for evolution of thermal reaction norms of different transcripts and changes
472 affected mainly expression mean (Clemson, Sgrò, & Telonis-Scott, 2016). Experimental evolution
473 studying *Drosophila* under variable diets found no significant changes in plasticity (Yampolsky et
474 al., 2012) or less than expected (Huang & Agrawal, 2016). In contrast, other studies found
475 differences in temperature responses between tropical and temperate *Drosophila* populations
476 (Levine, Eckert, & Begun, 2011; von Heckel et al., 2016). Other examples for differences in genes
477 expression plasticity between adapted and non-adapted populations include temperature
478 (Morris et al., 2014) and salinity (Gibbons, Metzger, Healy, & Schulte, 2017; McCairns &
479 Bernatchez, 2009) responses of marine and freshwater sticklebacks, temperature response of

480 killifish populations from different latitudes, as well as plastic responses to toxic hydrogen
481 sulphide (H_2S) of fish population from H_2S rich springs versus non-toxic springs (Passow et al.,
482 2017). There was no consistent pattern regarding the direction in which plasticity evolves: In
483 some cases adapted population showed an increase in plasticity (Morris et al., 2014), in other
484 cases plasticity was reduced (Huang & Agrawal, 2016; Ragland et al., 2015; von Heckel et al.,
485 2016) or reduction and enhancement of plasticity were equally frequent (Gibbons et al., 2017;
486 Yampolsky et al., 2014). Overall, there is evidence in multiple species that expression plasticity of
487 some genes can evolve. However, even in some of these studies reporting evolved plasticity
488 (Dayan et al., 2015; Gibbons et al., 2017; Morris et al., 2014) the number of transcripts with
489 significant changes in the mean was much higher than transcripts with changed plasticity and
490 large parts of the plastic responses showed a high degree of preservation.
491
492 In accordance with these previous findings we found that changes in the mean contributed more
493 to the observed expression differences in the treatments than changes in plasticity. A possible
494 reason might be that genetic variation in mean expression was higher than genetic variation in
495 plasticity. In addition, we did not select for changes in plasticity directly since the conditions in
496 the treatments were constant. Selection was therefore on expression levels in the treatment and
497 only indirectly on plasticity. Plasticity could evolve if mean expression levels were genetically
498 correlated with plasticity. Although we did not directly test for such correlations, we found
499 evidence for evolution of plasticity of the DE genes, that is of the genes with significant changes
500 in expression level between CT and treatment conditions. Although plastic responses showed a
501 high degree of preservation in terms of affected genes and direction, we found evidence for
502 evolutionary changes in the magnitude of plastic responses, i.e. the slope of the reaction norm.
503
504

505 *Why can plasticity be maladaptive?*

506 New stressors might disturb homeostasis resulting in inappropriate responses and long-term
507 adaptation therefore restores ancestral phenotypes by genetic changes, referred to as genetic
508 compensation (Grether, 2005) or counter-gradient variation (Conover et al., 2009). However, in
509 our study we applied relatively mild stressor treatments, i.e. individuals were able to survive and
510 reproduce. Drought and heat are also stressors, which *T. castaneum* had experienced in the past
511 (Sokoloff, 1972), so there had probably been selection on plastic responses to be beneficial.
512 However, plastic responses might be optimized for a short-term exposure: Allocation of
513 resources from reproduction to protection might increase survival probability and allow
514 individuals to continue reproduction as soon as the stress has disappeared, but this response
515 becomes maladaptive during continuous exposure and should therefore be under negative
516 selection. Expression of stress related genes is in general accompanied by a down-regulation of
517 genes involved in growth and reproduction due to an allocation of resources (Schwenke, Lazzaro,
518 & Wolfner, 2016; Sokolova, 2013). A well-studied example are heat shock proteins (hsp). Hsp are
519 well known for their protective function and to be crucial for survival (Feder & Hoffman, 1999),
520 but it was also shown that their expression comes at a cost (Feder et al., 1998; Sørensen,
521 Kristensen, & Loeschcke, 2003). Accordingly, it was often found that hsp expression in
522 populations adapted to warmer climates is lower compared to non-adapted populations
523 (Fangue, Hofmeister, & Schulte, 2006; Narum & Campbell, 2015; J G Sørensen, Dahlgaard, &
524 Loeschcke, 2001). In general, other protection mechanisms independent of ancestral plasticity
525 may arise during long-term adaptation (e.g. enzymes, which are more stable at high
526 temperature) and make the costly stress response expendable.

527

528 An alternative explanation for the reduced plasticity in adapted lines is that the signal
529 responsible for eliciting the plastic responses is based on any kind of damage (e.g. deformations

530 in macromolecules, membrane lipids, proteins, and DNA) caused by heat or stress in general
531 (Kültz, 2005). Higher resistance in adapted lines might shift the inducing thresholds, i.e. the
532 temperature when damages occur and stress response is induced (Sikkink, Reynolds, Ituarte,
533 Cresko, & Phillips, 2014) above the levels we applied in the treatments.
534
535 Interestingly, we found no differences in fitness between lines from different selection regimes
536 under CT conditions (Figure 2). We could detect some genes with different expression levels in
537 the treatments and found in general a correlated change in expression levels in control
538 conditions, but this did not affect offspring number. It indicates a lack of fitness trade-offs,
539 where alleles providing a fitness advantage in one environment (treatment) are detrimental in
540 another (CT). Together with the observation that selection lines evolved to bring expression
541 closer to ancestral CT-levels, it suggests that for many genes the optimal expression level is not
542 different between conditions. They might be involved in processes important for maintenance
543 and reproduction. Under stress, limited resources have to be invested into protection, that are
544 then not available for reproduction (Sokolova, 2013). Long-term adaptation should then work to
545 restore control levels that are likely to be optimized for highest reproductive output and to
546 reduce costly stress responses resulting in improved canalization of traits associated with fitness
547 (Stearns & Kawecki, 2006). Canalization, i.e. robustness against environmental variation, was
548 found previously in gene expression adaptation (Levine et al., 2011; Shaw et al., 2014; von
549 Heckel et al., 2016). Genetic differences between control and selection lines that are responsible
550 for adaptation to the treatments did not have an effect in control conditions and thus represent
551 cryptic genetic variation (Gibson & Dworkin, 2004). They might either concern genes that are not
552 expressed in control conditions, or represent changes neutral under control conditions.
553
554

555 *Potential caveats*

556 The number of genes with significant plastic changes in the DE analysis was much higher
557 compared to genes showing evolutionary changes. One possible explanation would be that
558 adaptive plasticity prevented evolution. If the plastic responses matched the optimum, no
559 genetic changes in the selection lines are expected to occur. However, when we analysed each
560 line separately and considered a gene as evolved if the mean difference between *La* and *Lp* was
561 more than 20% of the *Lo*, we found approximately the same number of genes with EC_T and PC_{CT}
562 (Supporting information Table S5.1).

563 In the DE analysis in *limma* we did not analyse each line separately but treated them as biological
564 replicates. Since lines were split across conditions comparisons between conditions, i.e. plastic
565 changes, can be made within lines. They should thus be more precise and statistical power
566 should be higher than comparisons between selection regimes, i.e. evolved changes, that have
567 to be made between lines. Differences between lines from the same selection regime lower the
568 ability to obtain significant evolutionary changes. These differences can arise from genetic drift.
569 Since our population size was relatively small (120 individuals per line) this might have been an
570 important factor. Another explanation is that lines from the same selection regime differed how
571 exactly they improved their fitness in the respective treatment. Since fitness is a highly polygenic
572 trait, the genes contributing to a fitness increase may not be the same in different lines (see
573 Barghi et al., 2019). For the most extreme treatment HD, where we sequenced seven lines, we
574 further found considerable differences in fitness between the lines, suggesting that not all of
575 them were at the same stage of adaptation. It is therefore not surprising that expression levels
576 did not evolve in the same way among lines.

577 The DE analysis in *limma* requires that a gene shows similar changes in all replicate lines, and is
578 therefore more conservative. If the main interest of a study is to identify promising candidate
579 genes for future more detailed analyses it is the appropriate approach to keep FDR as low as

580 possible. In contrast, if the focus is more on general patterns, a less stringent analysis using mean
581 expression levels can give us a more complete picture. Since genetic drift is random it cannot
582 explain the observed excess of reversions over reinforcements.
583
584 Although we demonstrated that gene expression changed during evolution, it is not clear
585 whether these changes are the cause of an increased fitness in these conditions or whether they
586 are rather the consequence of adaptation and being less stressed. One disadvantage in studying
587 whole transcriptomes is that not all responding genes might be of functional importance but are
588 correlated to other adaptive changes. High intercorrelations within the transcriptome (Ayroles et
589 al., 2009; McGraw et al., 2011) might lead to correlated responses in many other genes.
590 Furthermore, observed evolutionary changes might be caused by indirect selection and other
591 mechanisms, e.g. changes in protein structure of enzymes, were responsible for adaptation of
592 selection lines. Future studies that manipulate expression and test for correlated changes in
593 offspring are needed to confirm adaptive value of expression changes.
594
595 **Conclusions**
596 We found that genes with the strongest plastic responses showed evolutionary changes in
597 opposite direction suggesting that ancestral plasticity was maladaptive for long-term adaptation.
598 In the most stressful treatment, selection lines with higher fitness show a higher proportion of
599 reversions and a higher proportion of originally plastic genes that are closer to ancestral
600 expression levels. Differences between adapted lines and CT-lines in the treatment were mainly
601 due to a change in mean expression (i.e. shift in the intercept of reaction norms), while plasticity
602 was preserved in terms of affected genes and direction of change. However, we found that a
603 part of the differences in the treatments can be explained by a reduction in the magnitude of
604 plasticity in adapted lines. Our results add to growing evidence that plasticity and evolution are

605 often in opposite direction and maladaptive plastic responses might increase strength of
606 selection. In contrast to previous studies, including fitness data allowed us to give evidence for
607 adaptation and in the most stressful condition we were able to show an association between
608 reversion of plasticity and adaptation. Similar results in all three stress treatments indicated that
609 these findings may represent a general pattern of gene expression adaptation.
610

611 **Data Accessibility**

612 RNA-seq reads will be available at GEO.
613 Fitness data will be available at Dryad.
614

615 **Author Contribution**

616 FG and ELK designed experiment. ELK conducted experiment, laboratory work and analysed the
617 data. FG and ELK wrote the manuscript.
618

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625 data.
626

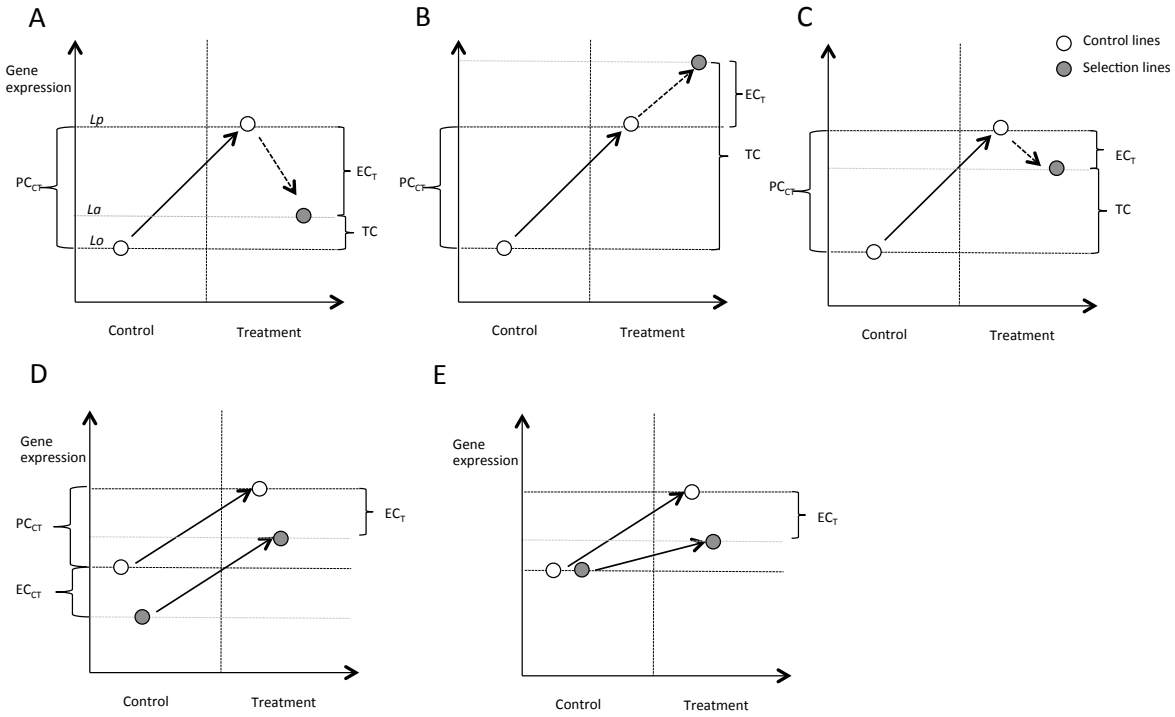

Figure 1: Possible relations between plastic change PC and evolutionary change EC. Gene expression levels of control lines (white) in the ancestral control condition represent the original stage Lo, expression in the treatment the plastic stage Lp. Expression levels of selection lines (grey) in the treatment give the adapted stage La Arrows indicate the direction of PC (dashed line) and EC (solid line). 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)**. During adaptation, lines could have reached the optimum by either changing mean expression, i.e. shift in the intercept of the reaction norm **(D)** or by changing their plasticity, i.e. the slope of the reaction norm **(E)**. 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 **(D)**. 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 **(E)**.

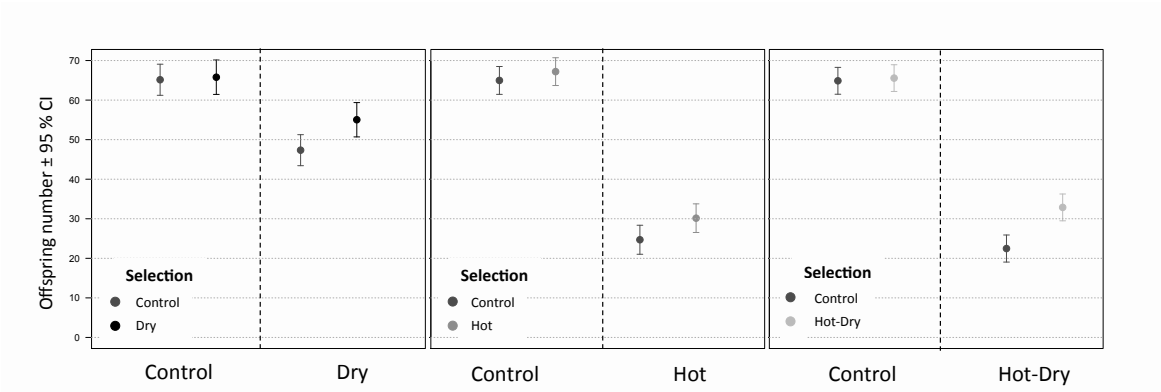

Figure 2: Offspring number of Control and selection lines under different conditions. Selection lines could adapt for 20 generations.

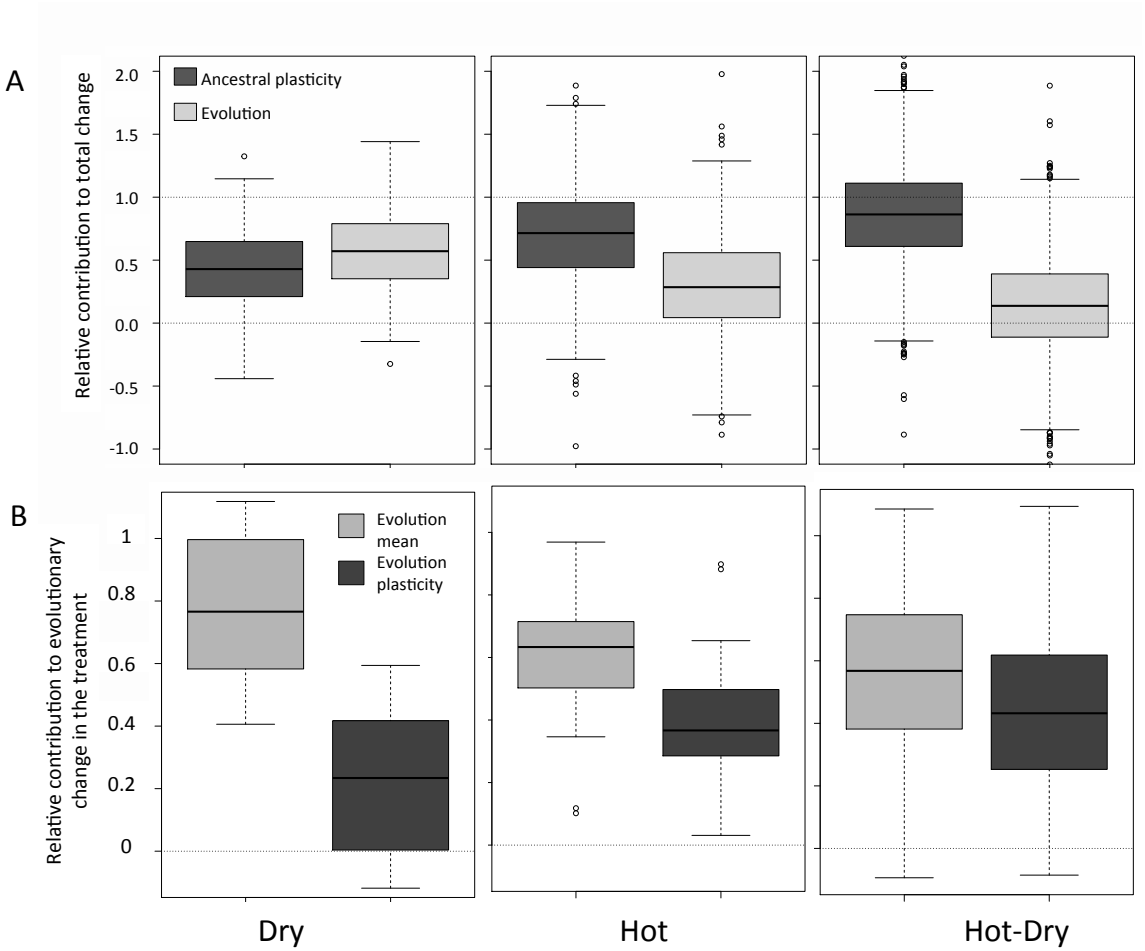

Figure 3: Relative contribution of ancestral plasticity and evolutionary change to total divergence for all genes showing significant total divergence in the DE analysis. Number of genes: Dry: 283, Hot: 1192, Hot-Dry: 2045 **(A)**. Relative contribution of changes in the mean (shift in the intercept 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 **(B)**.

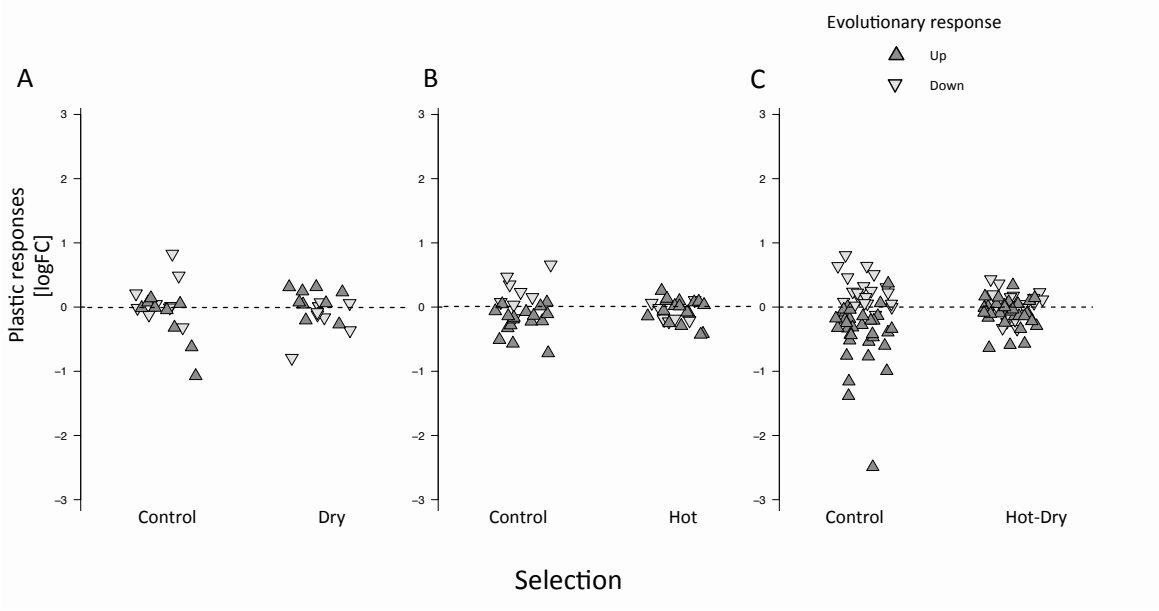

Figure 4: Plastic responses in control-lines and adapted selection-lines of genes showing significant evolutionary changes in expression in the treatments Dry **(A)**, Hot **(B)** and Hot-Dry **(C)**. Plastic responses in adapted selection lines are weaker (smaller logFC). Furthermore, plastic changes are mainly opposite to evolved changes. Genes that evolved to lower expression in the treatments (represented in lightgrey) show positive plastic changes in control-lines, i.e. they are up-regulated. Genes that evolved to higher expression levels (in darkgrey) are down-regulated in the non-adapted Control-Lines.

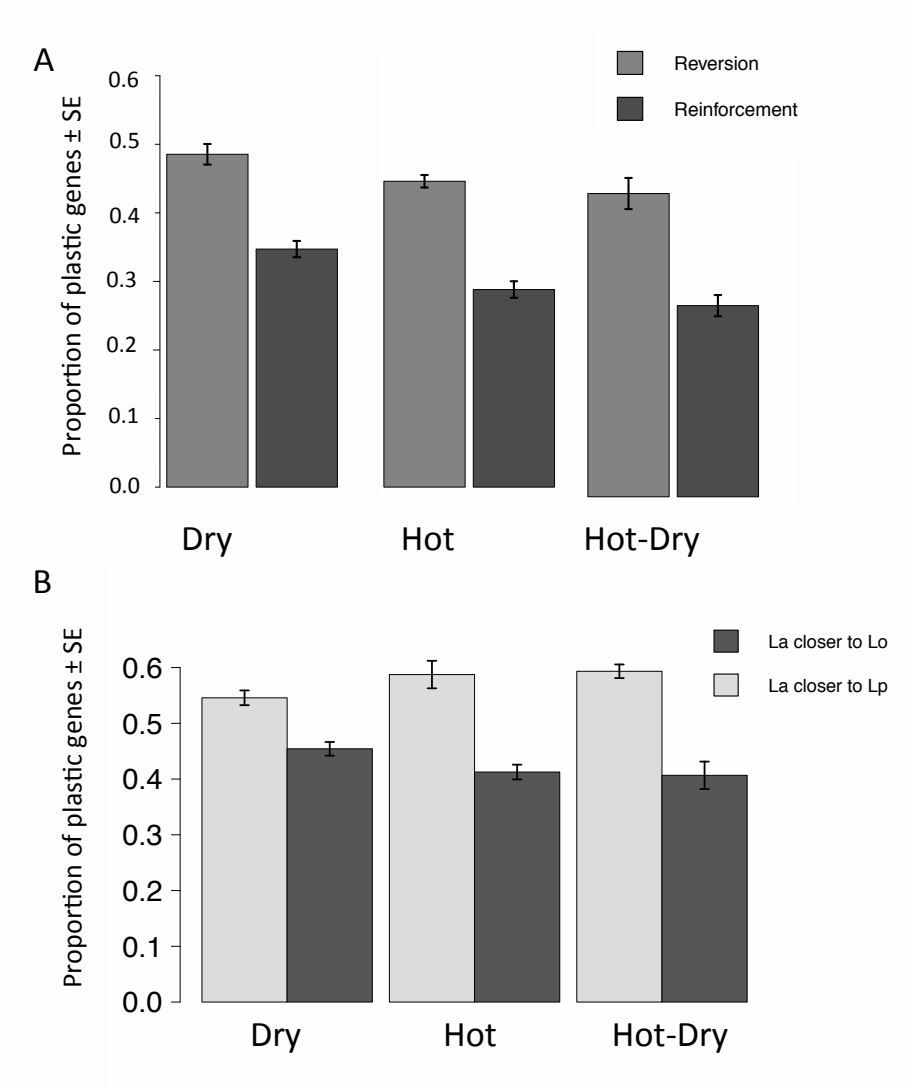

Figure 5: Comparison of plastic and evolutionary changes in gene expression in response to three environmental conditions. **A:** Proportion of genes (average over selection lines) showing a reversion or reinforcement of ancestral plasticity during evolution. **B:** Proportion of genes with expression 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.

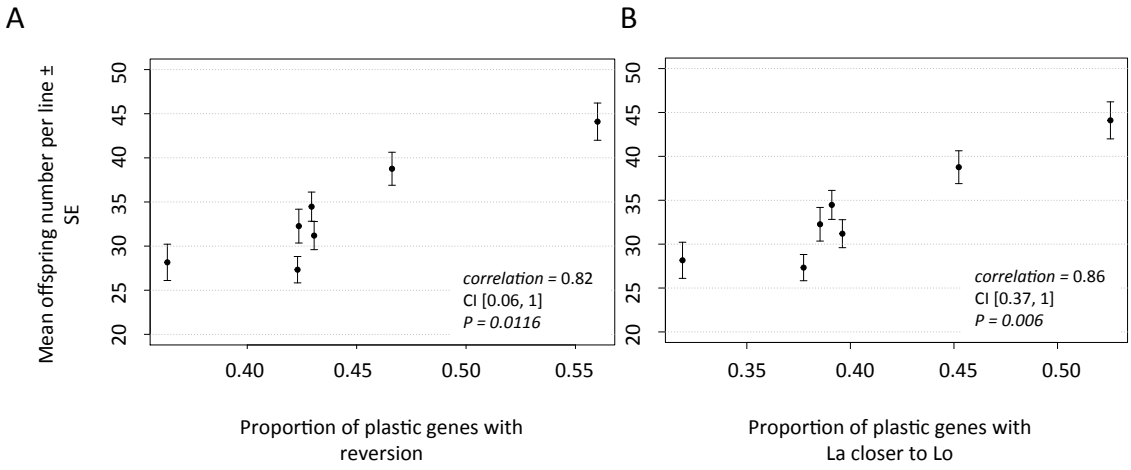

Figure 6: Relationship between plastic and evolved changes in gene expression in response to hot-dry conditions. Expression levels of Control-lines in control conditions represent the ancestral stage Lo, Control-Lines in hot-dry conditions the plastic stage Lp and adapted Hot-Dry-lines in hot-dry conditions the adapted stage La. **A:** Relationship between the proportion of reversed plastic responses and mean fitness (=offspring number) per Hot-Dry line. **B:** Relationship 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: 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 (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**

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

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