Combining transcriptomic and fitness data reveals additive and mostly adaptive plastic responses of gene expression to multiple stress in *Tribolium castaneum*

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Abstract

Gene expression is known to be highly responsive to the environment and to vary between species or populations under divergent selection. Yet, its contribution to the process of adaption is still controversial despite growing evidence that differences in gene regulation contribute to adaptive divergence. While most studies so far investigated evolved plasticity in already diverged populations, phenotypic selection acting on gene expression at the onset of adaptation to an environmental change has not been characterized. Here, we combined fitness and whole-transcriptome data in a large-scale experiment with *Tribolium castaneum* to investigate gene expression and fitness responses to drought, heat and their combination. Fitness was reduced by both stressors and their combined effects were nearly additive. Accordingly, expression data showed that both stressors were acting independently and did not interfere physiologically. With expression and fitness within the same individuals, we estimated selection on single gene expression levels. We found that variation in fitness can be attributed to gene expression variation. Selection intensities on expression levels differed between conditions and were opposite between control and stress conditions, showing evidence of evolutionary trade-offs. Plastic expression changes were mostly adaptive when affected by heat stress, and partially non-adaptive when affected by drought.

Keywords: gene expression, plasticity, multiple stressors, selection, *Tribolium castaneum,* fitness

Introduction

One of the major goals of evolutionary biology is to understand the genetic basis of phenotypic variation and how it is shaped by natural selection. The mapping of genetic to phenotypic variation depends on many cellular processes, of which RNA transcript abundance, or gene expression, has been shown to play a central role (Abzhanov et al. 2004; Prud ’homme et al. 2007; Carroll 2008; Fay and Wittkopp 2008; Romero et al. 2012). For variation in expression to be relevant for evolution it needs a heritable genetic basis and a link with fitness variation. While the heritability of expression variation has been established in many cases (Cui et al. 2006; Ayroles et al. 2009; Skelly et al. 2009; Long et al. 2012; Morris et al. 2014; McCairns et al. 2016), we still lack direct estimates of the strength of selection acting on transcript level abundance. The link between expression levels and fitness variation is not obvious, since mRNA abundance must be translated into protein abundance, enzyme activity and ultimately phenotypic variation (Feder and Walser 2005; Evans 2015). So far, the evidence for a link between fitness and gene expression variation is mixed and mostly indirect. For instance, in yeast, the knocking-out of

many genes had inconsequential effects on fitness (Giaever et al. 2002), whereas more recent evidence showed that variation in expression can significantly affect fitness (Keren et al. 2016; Sato et al. 2016). Unfortunately, data in more complex organisms are still scarce, especially on a transcriptome-wide scale. Indirect evidence supporting the importance of gene expression in evolution comes from studies showing differences in expression levels between adaptively divergent populations in yeast (Townsend et al. 2003), humans (Fraser 2013), *Drosophila* (Hutter et al. 2008) or fish (Whitehead and Crawford 2006; McCairns and Bernatchez 2009; Morris et al. 2014; Dayan et al. 2015; Ghalambor et al. 2015; Leder et al. 2015). In such cases, further support can be brought when evolved differences in regulatory DNA sequences are found (Zheng et al. 2011; Sato et al. 2016). Additionally, experimental evolution approaches were successful to detect altered expression levels within a few generations in multiple organisms that adapted to different environmental conditions (Riehle et al. 2003; Telonis-Scott et al. 2009; Yampolsky et al. 2012; Ghalambor et al. 2015; Huang and Agrawal 2016).

Beside genetic divergence causing expression differentiation (Brem et al. 2002; Fay and Wittkopp 2008; Romero et al. 2012), phenotypic plasticity can also play an important role in differentiation, especially at the onset of adaptation to novel environments (Yeh and Price 2004; Lande 2009; Chevin et al. 2010; Ehrenreich and Pfennig 2016). Expression is a highly plastic trait and is involved in the immediate response of organisms to changes in their environment (Gasch et al. 2000; Chen et al. 2003; Gibson 2008; López-Maury et al. 2008; Des Marais et al. 2013). The role of plasticity in evolution is, however, contentious. It is often argued that if plasticity is adaptive, it should impede evolution since it can hide genetic variance on which selection would act, weakening selection (Price et al. 2003). Yet, plasticity is also key for population persistence in a changing environment because it can keep populations at higher sizes, or buffer novel variants against purifying selection (Chevin et al. 2010; Fitzpatrick 2012), thus facilitating long-term adaptation (Yeh and Price 2004; Lande 2009; Ehrenreich and Pfennig 2016). Studies comparing plastic and evolved responses of gene expression in natural populations repeatedly found that plasticity was non-adaptive because opposed in direction to the evolutionary response (Sørensen et al. 2001; Dayan et al. 2015; Ghalambor et al. 2015). It may thus be that non-adaptive plasticity facilitates evolutionary divergence by increasing the strength of selection (Ghalambor et al. 2007, 2015).

Unfortunately, we currently lack estimates of fitness effects of expression changes during early stages of adaptation, on which depends the adaptive value of those changes. Current studies have inferred the adaptive value of a plastic response of expression by comparing the direction

of change of differentially expressed genes between evolved and non-evolved populations or experimental lines in common gardens. An early-stage expression change in the same direction as an evolved change (e.g., when comparing ancestral to evolved lines) would be interpreted as adaptive, without knowledge of the immediate fitness effects of the early-stage expression change (Whitehead and Crawford 2006; McCairns and Bernatchez 2009; Morris et al. 2014; Ghalambor et al. 2015; Leder et al. 2015). However, the two may differ because plasticity can evolve during adaptation (e.g., due to canalization, Heckel et al. 2016), or because beneficial immediate stress responses can be costly to maintain (Moya et al. 2012, 2015), and thus reverted during long-term adaptation (Fong et al. 2005; Rodŕiguez-Verdugo et al. 2016; Ho and Zhang 2018). Evolutionary changes in plasticity make post-adaptation inferences of the adaptive value of early-stage expression changes uncertain. In contrast, measuring fitness directly in organisms that have been exposed to a recent change in conditions can give us deeper insight into the initial processes leading to adaptive divergence between populations. Knowing the strength of selection acting on early-stage plastic transcriptional responses can tell us more about the immediate adaptive value of plasticity in gene expression and the targets of selection during the early stage of adaptation. Ultimately, it can also tell us how short-term selection pressures are linked to long-term optimum expression levels.

The difference between short and long-term gene expression changes will depend on trade-offs between the benefits of immediate stress responses and their long-term costs. Adaptation necessitates optimal re-allocation of energy resources between maintenance and reproduction. However, optimal solutions for this trade-off may differ between environmental stressors (Schlichting and Smith 2002), which further complicates the study of plastic responses in gene expression and their fitness effects in variable environments. A beneficial response elicited by one environmental factor may be overridden by a negative effect in presence of a second factor and generate a pattern of non-adaptive plasticity. The joint effects of stress factors may not be simply deduced from single responses and may result in complex interactions when in combination (Crain et al. 2008; Holmstrup et al. 2010; Byrne and Przeslawski 2013; Gobler et al. 2014; Gunderson et al. 2016). It is thus crucial to understand the trade-offs faced by organisms when adapting to changed environments (DeBiasse and Kelly 2016; Kelly et al. 2016). Transcriptomics can give us insights into the mechanisms underlying trade-offs between 1) responses to different stressors, and 2) energy allocation trade-off between reproduction and maintenance within conditions. It can potentially show which physiological processes are activated, thereby giving us information about how resources are used. Trade-offs in stress responses can not only limit plastic responses but may also constrain evolution and future

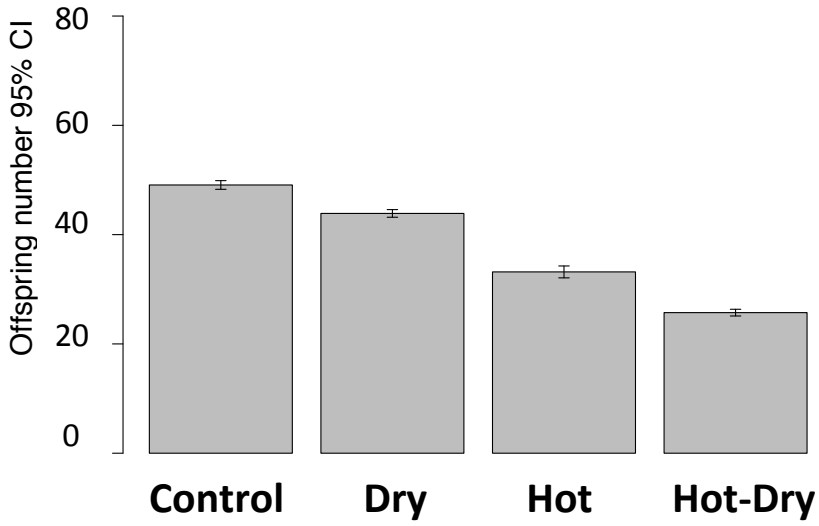
114 adaptation (Etterson and Shaw 2001; Pörtner et al. 2006; Kelly et al. 2016). Therefore, linking the
115 immediate plastic responses of gene expression with their fitness effects at the onset of adaptive
116 divergence should allow a better understanding of their adaptive value and evolutionary
117 consequences.
118
119 In this study we asked how *Tribolium castaneum* (the red flour beetle) was affected by heat and
120 drought in single stressor treatments and in a combination treatment. We combined a fitness
121 assay with measuring gene expression using RNA-seq. This allowed us to get more insight into
122 the physiological processes affected by different stressors, to examine whether the
123 transcriptomic responses to heat and drought overlapped and if gene expression was changed in
124 the same direction by both stressors. Based on this, we can make predictions about how
125 stressors will act in combination and test this in the combined stressor treatment. We further
126 examined the potential resource allocation trade-off between reproduction and stress response
127 by focusing on specific genes known to be involved in reproduction. We tested how these genes
128 were affected by the different treatments, and whether this corresponded with the observed
129 fitness consequences. Since we measured expression and fitness in the same individuals in
130 sufficient sample size, we were also able to estimate transcriptome-wide selection intensities on
131 gene expression levels giving us an unprecedented view of selection pressures on gene
132 expression in different environments. Further, we were able to examine whether immediate
133 plastic responses were adaptive or non-adaptive, thus contributing to our understanding of how
134 plasticity may affect future adaptation.
135

136 ## Results

137 We used a *T. castaneum strain* (Cro1) (Milutinović et al. 2013), which was collected from a wild
138 population in 2010 and adapted to standard control conditions (33 °C, 70% relative humidity
139 (r.h.)) since then. We measured the number of adult offspring as well as gene expression
140 changes relative to control conditions when the beetles were exposed to a drought, a heat and a
141 combined heat-drought treatment (conditions: Dry: 33°C, 30% r.h.; Hot: 37 °C, 70 % r.h; Hot-Dry:
142 37°C, 30% r.h.). Individuals were transferred to treatments in the egg stage and stayed there
143 during their whole life time. Fitness and expression were measured in females at the age of
144 eleven weeks. Gene expression was measured by RNA-seq using mRNA of the whole body.
145

146 *Fitness assay*

147 Offspring number of reproducing females decreased with increasing temperature ($F_{1,5157}$ =
148 1981.07 , P < 2.2e-16) and decreasing humidity ($F_{1,5184}$ = 262.05, P < 2.2e-16), with a stronger
149 effect of heat (-15.98 ± 0.57 SE) than of drought (-5.12 ± 0.50 SE). The lowest offspring number
150 was found when heat and drought were combined (Fig. 1B). Interaction between temperature
151 and humidity was also significant ($F_{1,5128}$= 8.37 , P = 0.003835) and led to an additional decrease
152 of -2.22 ± 0.77 compared to purely additive effects. The proportion of reproducing females was
153 significantly different between conditions (χ2= 627.35, df=3, P< 2.2e-16). The highest
154 proportion of non-reproducing females was found in Hot (Supplementary Material S1).



155
156
157 **Figure 1** Offspring number of reproducing females in four different conditions: Control (33°C, 70
158 % r. h.), Dry (33°C, 30 % r. h.), Hot (37°C, 70% r. h.), Hot-Dry (37°C, 30% r. h.) Females could lay
159 eggs for one week and adult offspring was counted five weeks later.
160
161 **Table 1:** Results of the fitness assay. Number of adult offspring that females produced within one
162 week of egg-laying in different conditions (Control: 33°C, 70% relative humidity (r.h.).; Dry: 33°C,
163 30% r.h.; Hot: 37°C, 70% r.h.; Hot-Dry: 37°C, 70% r.h.). For calculating offspring number per
164 female only reproducing females were used

Condition	N	Females with offspring	Offspring per reproducing female (±SE)
Control	1575	1514	48.99 ± 0.44
Dry	1642	1603	43.87 ± 0.43
Hot	1401	1005	33.01 ± 0.52
Hot-Dry	1567	1396	25.68 ± 0.45

165

166 *Gene expression response to heat is stronger than to drought*

167 The number of differentially expressed (DE) genes relative to Control was lowest in Dry and
168 largest in Hot (Fig. 2, Tab. 2). Drought induced up-regulation of 52 and down-regulation of 48
169 genes. In contrast, the response to heat showed a significantly higher number of DE genes than
170 in Dry (up: 1594, down: 1255; permutation test $P < 0.004$). Overlap between heat and drought
171 responses was significantly higher than expected by chance ($\chi 2 = 17.75$, d.f.= 1, p-value = 2.516e-
172 05) and included 26 genes (Fig. 2) with responses in the same direction (up: 25, down: 1) and 17
173 genes with responses in opposite direction. To investigate whether the DE genes were involved
174 in specific biological processes, we performed pathway, protein domains, and Gene Ontology
175 (GO) enrichment tests. Because of a low number of DE genes in Dry, only few enrichments could
176 be detected in Dry (Supplementary Material S2), with up-regulated genes enriched in active ion
177 transmembrane transporter activity (GO:0022853), and down-regulated genes enriched in
178 hydrolase activity, hydrolyzing O-glycosyl compounds (GO:0004553), and protein family
179 Thaumatin (IPR001938). Analysis of the *Tribolium* genome had revealed a high number of genes
180 thought to be involved in endocrine regulation of diuresis, including several that encode putative
181 neuroendocrine peptides like antidiuretic factors (Aikins et al. 2008; Hauser et al. 2008; Li et al.
182 2008; Park et al. 2008). Nevertheless, none of these genes was found to respond to the Dry
183 treatment.

184

185 Genes up-regulated in Hot were enriched in many metabolic processes, e.g. carbohydrate
186 metabolic process (GO:0005975), Citrate cycle (KEGG 00020), and Pyruvate metabolism (KEGG
187 00620) (Supplementary Material S1). The most strongly enriched category was chitin metabolic
188 process (GO:0006030). A protein domain analysis also showed significant enrichment of heat
189 shock proteins (IPR031107, IPR018181, IPR008978) (Supplementary Material S2). Down-
190 regulated genes were enriched in pathways for DNA replication (KEGG 03030), nucleotide
191 excision repair (KEGG 03420) and Ubiquitin mediated proteolysis (KEGG 04120) (Supplementary
192 Material S2).

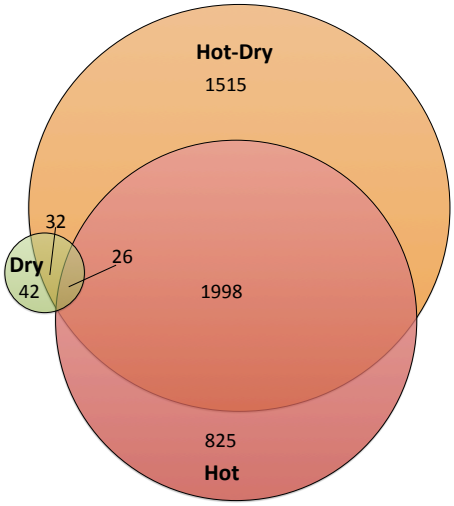
193

194 The significant overlap between heat and drought response suggests that these genes are
195 involved in a general stress response. However, no significant functional enrichment could be
196 detected.

197

198 **Table 2:** Number of differently expressed genes (FDR < 5%) when comparing different
199 conditions. Positive: higher expression in second condition. Negative: lower expression in second
200 condition. Differential expression analysis was conducted in edgeR (Robinson et al. 2010).

	positive	negative	total
Dry vs Control	52	48	100
Hot vs Control	1594	1255	2849
Hot-Dry vs Control	1866	1705	3571
Hot vs Dry	1553	1290	2843
Hot-Dry vs Dry	1928	1813	3741
Hot-Dry vs Hot	101	164	265



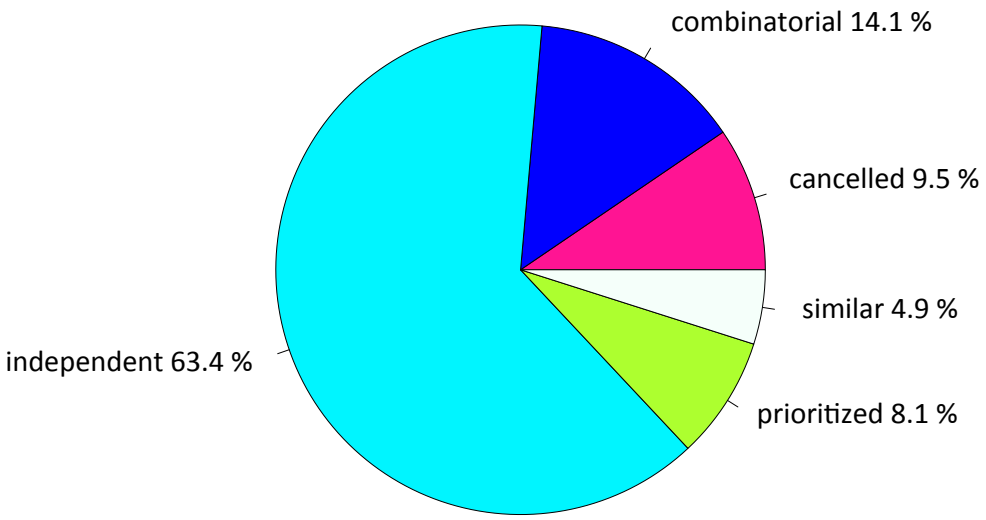
201

202 **Figure 2** Venn-Diagram showing the number of differentially expressed genes in three
203 treatments relative to control conditions. Overlapping regions represent genes that were found
204 in more than one treatment and changed expression levels in the same direction. Sizes of circles
205 as well as of overlapping regions are proportional to number of genes.

206 *Response to stressor combination is dominated by heat response, single stress responses are*
207 *mainly not modified in combination*

208 When both stressors were combined in Hot-Dry, we found 3571 DE genes (up: 1866, down:
209 1705). Among them, 1515 (42.4%) were not found in single stressor treatments. However, only
210 69 of those genes (up: 30, down: 39) were found significantly DE between Hot-Dry and Dry, or
211 Hot-Dry and Hot. This indicates that in most cases the combined stress did not induce expression
212 changes in a different set of genes, but modified their expression levels over and above their
213 responses to single stressors. Compared to Hot, the Hot-Dry response had a significantly higher
214 magnitude of expression change (permutation test: $P < 0.0001$), a higher number of down-

215 regulated genes (P = 0.007), but a similar number of up-regulated and total number of DE genes
216 (P = 0.29 and, P = 0.054, respectively). The functional response to Hot-Dry resembles the
217 response to Hot, but more enriched GO categories and pathways could be found (see
218 Supplementary Material S2).
219
220 To further examine how single stress responses are modified during combination, we classified
221 the DE genes of all treatments into different response categories following Rasmussen et al.
222 (2013) (see Methods and Fig.3). Only 5% of the genes showed a *similar* response mode, with
223 same response to Dry, Hot and Hot-Dry (Fig. 3). Most responding genes (63 %) were classified as
224 *independent*, with a response that is not altered in presence of a second stressor. Most of those
225 genes showed the same response in Hot and Hot-Dry (60.1 % of all genes, Supplementary
226 Material S4), but no response in Dry, in agreement with our DE analysis. 14% had a
227 *combinatorial* response mode: They did not respond to heat and drought alone, but to their
228 combination. These represent cases, in which presence of an additional stressor magnifies the
229 effect of another. Especially interesting are genes with opposite responses to both stressors, but
230 with one response *prioritized* when stressors occur simultaneously. These genes can be
231 indicative of physiological trade-offs that constrain responses to stress combination. We found 8
232 % of DE genes falling into that category. Most of them showed prioritization of the Hot response
233 in Hot-Dry (7.5 %, Supplementary Material S4). This is in agreement with our DE analysis, which
234 showed a high similarity between responses to Hot and Hot-Dry. 9.5 % of expression responses
235 were classified as *cancelled,* i.e. response disappears when another stressor is added. Most of
236 these genes (6.7 %) showed a significant response in Hot, but not to Dry and returned to control
237 levels in Hot-Dry.



238

239 **Figure 3:** Response modes of genes, which showed significant responses to at least one of the
240 treatments (Dry, Hot, Hot-Dry). *Combinatorial*: Similar levels in the two individual stresses but a
241 different response to combined stresses; *cancelled*: transcript responses to either or both
242 individual stresses returned to control levels; *prioritized*: opposing responses to the individual
243 stresses and one stress response prioritized in response to combined stresses; *independent*:
244 response to only one single stress and a similar response to combined stresses; *similar*: similar
245 responses to both individual stresses and to combined stresses. Subcategories of different
246 response modes, with more details about the most prevalent patterns, are given in
247 supplementary material (S4).

248

249 *Immune response: Interaction between drought and heat*

250 We noticed that among the genes up-regulated in Hot, many are involved in immune response
251 (Oppert et al. 2012; Behrens et al. 2014). These transcripts encode putative chitin-interacting
252 proteins (binding, deacetylase), allergens, thaumatins, glucosidases, and serine proteases,
253 whereas these genes are down-regulated at low humidity. For instance, Thaumatin protein
254 family, which is involved in antimicrobial response (Altincicek et al. 2008) showed the strongest
255 enrichment in down-regulated genes (Supplementary Material S2) under drought, and a
256 pathogenesis related protein and several antimicrobial peptides are among those genes that
257 showed the strongest up-regulation in Hot, but were significantly down-regulated in Dry . We
258 applied a gene set enrichment analysis to test for an overrepresentation of immune genes in
259 responses to the different treatments. To avoid including immune genes, which could also be
260 involved in heat stress response (Behrens et al. 2014), we used a test set of 15 antimicrobial
261 peptides, which should be specific to a pathogen response, based on Zou et al. 2007, Oppert et

262 al. 2012, Altincicek et al. 2013 (Supplementary Material S3A). We found that antimicrobial
263 peptides were significantly up-regulated in Hot (p-value = 0.001) and down-regulated in Dry (p-
264 value = 0.001) (Tab. 3). When we compared Hot-Dry with Hot, these genes became down-
265 regulated (p-value = 0.002), indicating that it is mainly the combination of high temperature with
266 relatively high humidity (i.e. 70% r.h.) that activates this response, pointing to a possible higher
267 parasite load under these conditions.
268
269 **Table 3** Results of enrichment analysis of antimicrobial peptides. Gene set enrichment test was
270 conducted in edgeR (Robinson et al. 2010). Prop.Down and Prop.Up give the proportion of
271 genes that are down- and up-regulated. The direction of change is determined from the
272 significance of changes in each direction, and is shown in the Direction column. The p-value
273 provides evidence for whether the majority of genes in the set are DE in the specified direction

Contrast	Prop. Down	Prop. Up	Direction	p-value
Dry-Control	0.5333	0	Down	0.001
Hot-Control	0	0.4667	Up	0.001
Hot-Dry–Control	0.1333	0.2667	Up	0.832
Hot-Dry–Hot	0.4	0.0667	Down	0.002

274
275 *Treatment effects on reproduction related processes*
276 To get further insights into the molecular processes that link the observed decline in offspring
277 number with transcriptomic data, we focused on the juvenile hormone (JH), 20-hydroxecdysone
278 (20 E), and insulin/insulin-like peptides (IIS) target of rapamycin (TOR) signaling pathways (IIS-
279 TOR), which are important mediators in the trade-off controlling stress response and
280 reproduction (Schwenke et al. 2016), along with vitellogenin, which is the main nutrient source
281 of eggs, and vitellogenin receptors. We found that heat and heat-drought stress led to a
282 significant down-regulation of all pathways and repression of vitellogenin and vitellogenin
283 receptors (Supplementary Material S3B). Drought did not show any significant effect. A gene set
284 test based on a selection of key reproduction genes within these pathways (Supplementary
285 Material S3C) confirmed that the reproduction genes were mainly down-regulated in Hot and
286 Hot-Dry (Tab. 4).
287

288 **Table 4:** Results of gene set enrichment analysis of genes involved in reproduction. Gene set
289 enrichment test was conducted in edgeR using the *roast* function (Robinson et al. 2010).
290 Prop.Down and Prop.Up give the proportion of genes that are down- and up-regulated. The
291 direction of change is determined from the significance of changes in each direction, and is

292 shown in the Direction column. The *P*-value provides evidence for whether the majority of genes
293 in the set are DE in the specified direction. The genes (N=56) were selected based on
294 (Parthasarathy, Sheng, et al. 2010; Parthasarathy, Sun, et al. 2010; Xu et al. 2010; Parthasarathy
295 and Palli 2011).

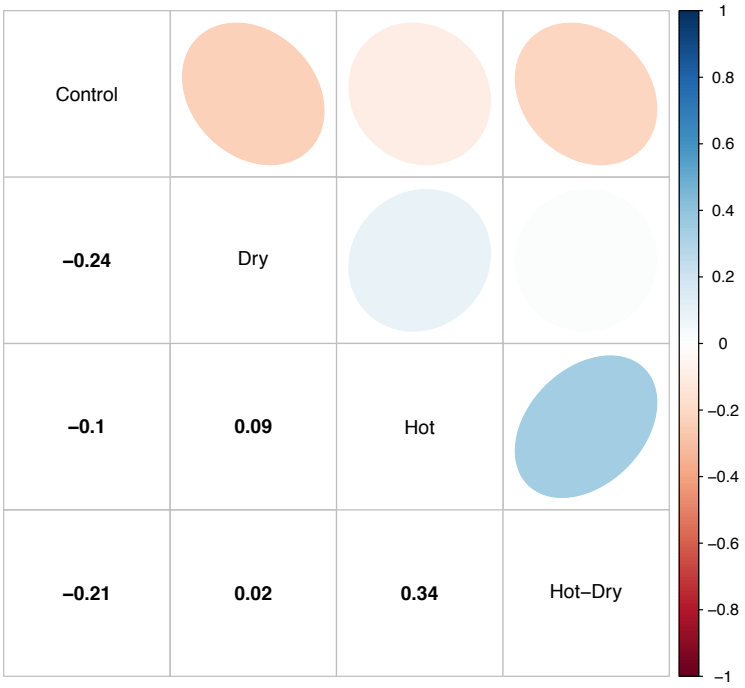
Contrast	Prop. Down	Prop. Up	Direction	p-value
Dry–Control	0.054	0.036	Up	0.912
Hot–Control	0.464	0.071	Down	0.001
Hot-Dry–Control	0.589	0.071	Down	0.001

296

297 *Selection on expression levels is environment specific*

298 Since we measured offspring number and transcription within the same individuals, we could
299 estimate selection intensity on single gene expression levels in each condition separately by
300 performing a linear regression of relative fitness on standardized expression levels (z-score). In
301 control conditions, expression levels of 2179 genes showed a significant correlation with
302 offspring number (negative: 2158, positive: 21). The two genes under strongest positive selection
303 coded for vitellogenin. Another positively selected gene coded for a serine protease (TC000870)
304 and is involved in oocyte development (GO:0048599). In Dry, Hot and Hot-Dry we could not
305 detect any significant selection on single gene expression levels after correcting p-values for
306 multiple comparisons. To gain a better overview of the variation in selection pressures among
307 treatments and to avoid stringent significance thresholds on single-gene fitness-expression
308 correlations, we then compared selection intensities on all genes between conditions. We found
309 significant negative correlations of selection intensities between Control and all stress
310 treatments, and positive correlations among stress treatments (p-values < 2.2e-16) (Fig 4).
311 Control and Dry had the strongest negative correlation (-0.24), while Hot and Hot-Dry had the
312 highest positive correlation (0.34). Furthermore, significantly DE genes responding to Hot and
313 Hot-Dry were overrepresented among those that were negatively selected in control conditions
314 (Hot: $\chi2$= 158.62, df = 1, p-value < 2.2e-16, Hot-Dry: $\chi2$= 177.97, df = 1, p-value < 2.2e-16), with
315 361 (22.6 %) up-regulated genes in Hot and 417 (22.3 %) in Hot-Dry. The magnitudes of selection
316 intensities were also significantly different between conditions (median (and SD) of absolute
317 values in Control: 0.09 (0.06), Dry: 0.03 (0.03), Hot: 0.09 (0.07), Hot-Dry: 0.05 (0.04); Kruskal-
318 Wallis rank sum test: $\chi2$= 9333.3, df = 3, p-value < 2.2e-16), with the majority of genes negatively
319 selected in Control, and similar proportions of genes under positive and negative selection in
320 stress treatments (Fig. 5). Estimated selection intensities were rather low compared to those
321 that were reported for morphological and life-history traits in natural populations (Kingsolver et
322 al. 2001; Hereford et al. 2004), but within the range that was estimated for selection on

323 expression levels (McCairns and Bernatchez 2009). Although the Dry treatment had the largest
324 negative correlation with Control expression levels and largest number of genes switching sign
325 relative to Control (9591), it had the lowest magnitude of change in selection intensity of those
326 genes (median: 0.14). In contrast, the Hot environment had the strongest magnitude of change
327 in selection intensity compared to Control (median: 0.19, 6644 genes) followed by Hot-Dry
328 (median: 0.16, 7412 genes). Overall, a greater proportion of genes switched from negative to
329 positive selection (Dry: 0.86, Hot: 0.76, Hot-Dry: 0.8).



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331 **Figure 4:** Pairwise correlations of selection intensities on single gene expression levels in
332 different conditions. Blue indicates a positive and red a negative correlation. Values are given in
333 the lower triangle. Confidence intervals for correlations: Control-Dry: -0.25 ,-0.22; Control-Hot: -
334 0.11, -0.08; Control–Hot-Dry: -0.23, -0.20; Dry-Hot: 0.08, 0.11; Dry–Hot-Dry: 0.00, 0.03; Hot–Hot-
335 Dry: 0.33, 0.36.

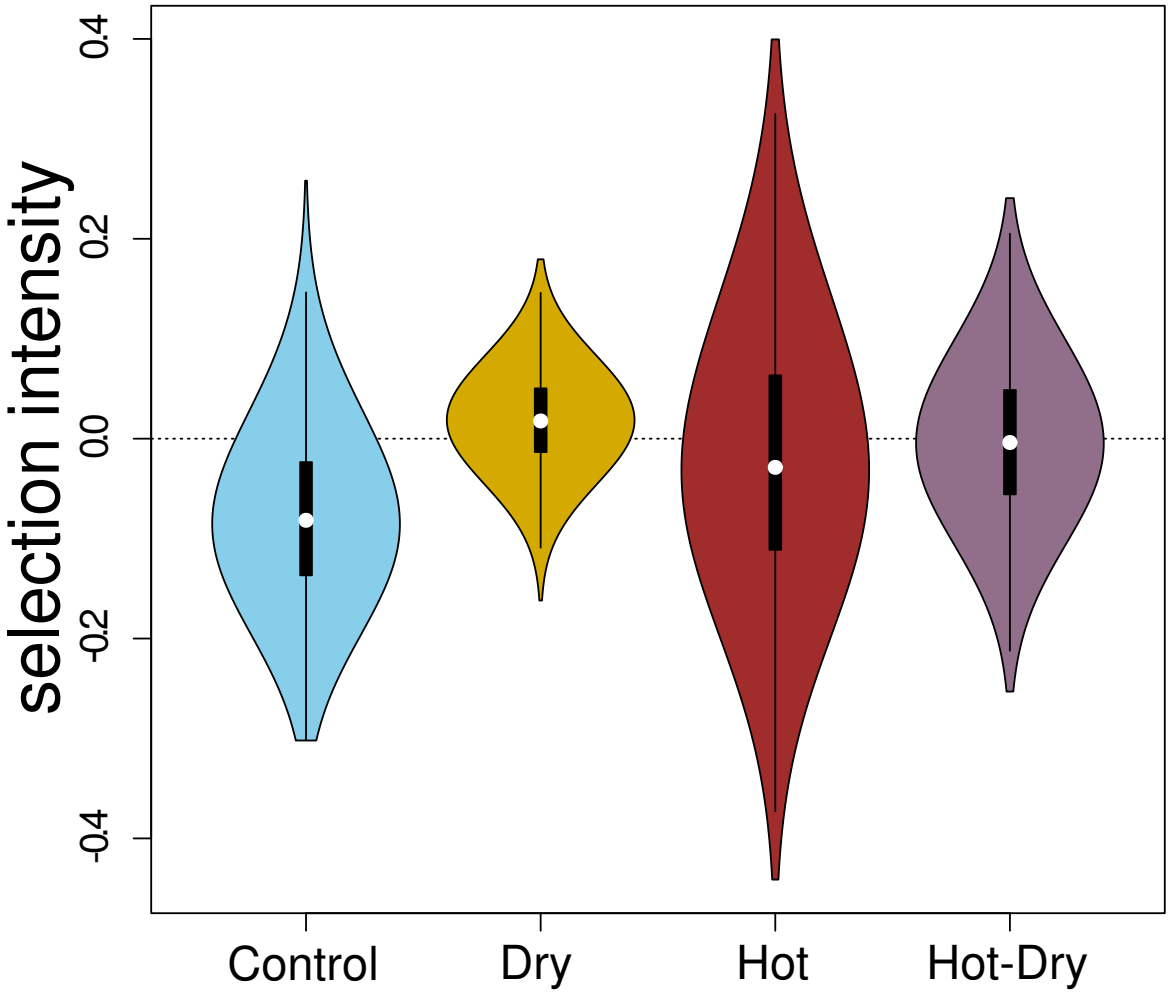
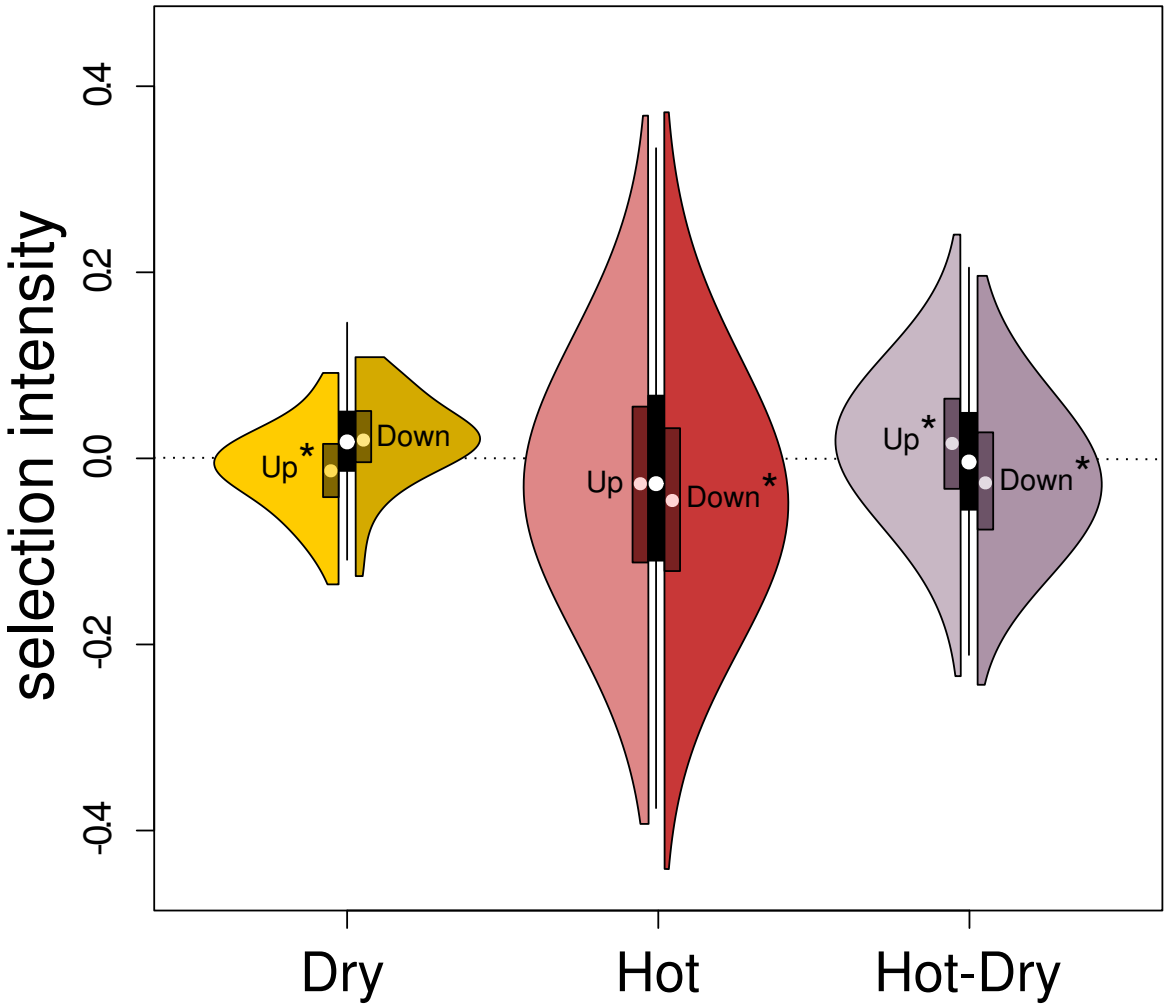


Figure 5: Distribution of selection intensities on gene expression levels under Control and treatment conditions. Each violin plot contains a boxplot of the data. White dots are medians and black rectangles represent inter-quartiles. The selection intensities were obtained as linear regression coefficients of relative fitness (number of adult offspring) on normalized RNA-seq read counts (z-score).

Is response in gene expression adaptive?

To examine whether the plastic responses in gene expression are adaptive, we tested whether significantly up-regulated genes were more positively selected and significantly down-regulated genes more negatively selected than non-responding genes. We found that the response to Hot-Dry was mainly adaptive: Down-regulated genes were under significantly more negative selection and up-regulated genes under more positive selection compared to non-responding genes, respectively (permutation tests: $P < 0.0001$) (Fig. 6). In contrast, some parts of the response in Dry seemed maladaptive: Down-regulated genes were not under significantly different selection, but up-regulated genes were more negatively selected (Fig. 6). In Hot, the

352 response is partly adaptive since down-regulated genes were significantly more negatively
353 selected, while up-regulated genes were not under significantly different selection (Fig. 6).



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355 **Figure 6:** Selection intensities on expression levels of genes that showed significant responses to
356 stress treatments (DE genes). The left half of each violin plot is the distribution of selection
357 intensities of up-regulated genes, relative to Control, while the right half is for down-regulated
358 genes. The median and inter-quartile range are represented as a white dot and a dark rectangle,
359 respectively. The central boxplot represents variation of selection intensities of genes whose
360 expression was not significantly different from expression in Control (at 5% FDR). Significance of
361 the shift in selection intensities of the up- and down-regulated DE genes relative to the non-DE
362 genes is marked with the star symbol (*). Significance was determined with a permutation test
363 with 10,000 permutations (see Methods).

364

365 ## Discussion

366 *Selection on gene expression*

367 By measuring gene expression and one component of fitness within the same individuals, we
368 could assess the strength of selection acting on gene expression variation within each
369 environment. The distribution of selection intensities, measuring the change in fitness relative to
370 a change of the phenotypes under selection (Lande and Arnold 1983; Falconer and MacKay
371 1996), was informative of the type of selection at play in each environment. Under Control
372 conditions, to which the beetles were best adapted, the distribution of selection intensities had
373 the most negative mean and median with a small proportion of transcripts under significant
374 positive selection. This pattern is strongly suggestive of selection against costly gene expression,
375 and thus of some form of purifying selection against non-adaptive variation in expression levels.
376 Gene expression is known to be costly (Castillo-Davis et al. 2002; Wagner 2005; Lang et al. 2009;
377 Frumkin et al. 2017), and selection should therefore favor low expression levels under those mild
378 laboratory conditions. Genes under strongest positive selection were vitellogenin genes playing
379 an important role in reproduction. Vitellogenin genes are also among the most highly expressed
380 genes in the transcriptome. Their strong correlation with offspring number in Control suggests
381 that variation in this component of fitness is mainly driven by differences in egg production
382 among individuals. We could confirm that our measure of fitness is strongly related to fecundity
383 (see Supplementary Material S5), which also confirms that our experimental design was able to
384 capture meaningful correlation of gene expression with reproductive output. Second, shifts in
385 the environment caused an increase of the proportion of beneficial trait variation, with higher
386 median and mean selection intensities in the stress treatments. The changes of the direction of
387 selection, from mostly negative to more positive, affected both the genes that significantly
388 changed their expression plastically (DE genes), and those that did not. The protection provided
389 by stress responses might thus have outweighed the costs of expression seen previously in
390 Control conditions, especially since the direction of selection switched to positive in 80% of the
391 cases. This led to strong negative correlations with selection intensities in Control and suggests
392 that the physiological stress imposed by the stress treatments modified the optimum expression
393 level of a majority of genes in the genome.

394 In Dry, and to some extent in Hot-Dry, the selection intensities were weaker with a distribution
395 more centered on zero. This may be indicative of stabilizing selection on gene expression levels.
396 Yet, beetles in all three stressful conditions incurred a significant decrease in fitness, and not all
397 plastic changes in gene expression were adaptive. That is, not all were in the direction that

398 increased fitness. The immediate stress responses were thus likely still costly because of trade-
399 offs between stress resistance mechanisms and reproduction, or trade-offs in stress responses
400 between stressors.

401 *Physiological trade-off between stress response and reproduction within treatments*

402 The heat-induced expression changes were characteristic of stress responses: Up-regulation of
403 heat shock proteins and metabolic pathways, and repression of genes involved in cell growth,
404 cell cycle, and reproduction (Gasch et al. 2000; Kültz 2005; Kassahn et al. 2009; Enders et al.
405 2015). This pattern of change in gene expression, and the overall reduction of females'
406 reproductive output, suggests a shift of resource utilization from growth to stress protection.
407 This would be indicative of a physiological trade-off between stress response and reproduction
408 (Salmon et al. 2001; Fedorka et al. 2004; Harshman and Zera 2007). We could confirm that this
409 trade-off was at play because the decrease of females' reproductive output was mainly driven by
410 a loss of fecundity (egg number) in the Hot and Hot-Dry treatments and not a decrease of larvae
411 survival (see Supplementary Material S5). This matches well with the down-regulation of
412 vitellogenin and other reproduction-related genes (JH, the IIS-Tor and 20E signaling pathways) in
413 Hot and Hot-Dry. Those genes are indeed expected to play an important role in mediating the
414 trade-offs between stress-responses and cellular growth and maintenance (Flatt et al. 2005;
415 Gruntenko and Rauschenbach 2008; Schwenke et al. 2016). Reduction in fecundity was also
416 likely affected by the expression of heat shock proteins, whose fitness costs are known (Feder
417 and Hoffman 1999; Silbermann and Tatar 2000; Kassahn et al. 2009). Finally, a trade-off between
418 immune response and reproduction may also have affected our results. We detected an up-
419 regulation of immune response genes in the Hot treatment, which might be indicative of a
420 higher pathogen load. Alternatively, it might be an indirect response induced by hot and humid
421 conditions, which has evolved in adaptation to pathogens, and might also occur in absence of
422 parasites. Since we did not measure pathogen load, we were not able to distinguish between
423 both. However, a high proportion of non-reproducing females in Hot (Supplementary Material
424 S1) may suggest infections.

425 *No physiological trade-offs between single stressors*

426 The transcriptomic responses to heat and drought were very contrasted with a small but
427 significant overlap when taken separately. Only a minority of overlapping genes (17 out of 43)
428 showed a trade-off in expression between the two conditions, and no functional enrichment
429 could be detected. Different physiological processes are thus likely affected by the two stresses.
430 This lack of a strong trade-off between the individual stress responses was also evident when

431 investigating the combined transcriptomic response in the Hot-Dry treatment. Most DE genes in
432 Hot-Dry responded to a single stressor independently of the presence of the second stressor (Fig.
433 3). Such an additive combined stress response can be expected when individual stressors require
434 different protection mechanisms and affect different pathways, and are thus likely not
435 interfering with one another (Folt and Chen 1999). Furthermore, as expected from the single
436 stress responses, the combined response was dominated by the heat response. This is in
437 agreement with many studies showing that heat is a major driver of expression change,
438 especially in ectotherms (Neven 2000; Nguyen et al. 2009; Levine et al. 2011; Chen and Stillman
439 2012; Morris et al. 2014). We also detected an additive combined effect of heat and drought on
440 fitness, where the average reproductive output in Hot-Dry was close to the combined reduction
441 of fitness in Hot and Dry. Nevertheless, and thanks to our large sample size, we could still detect
442 a significant interaction between heat and drought on offspring number, although the effect was
443 small (-2.22 ± 0.77). Therefore, the addition of a drought stress to the heat stress apparently
444 emphasized the effect of the heat stress, as seen in the transcriptomic response where the Hot-
445 Dry response mainly differed in magnitude compared to the Hot response.

446 *Adaptive value of plastic responses and indications for evolutionary adaptations*

447 Our results suggest that plastic responses in Hot-Dry and down-regulation in Hot were mainly
448 adaptive to short-term exposures. This adaptive plasticity probably depends on whether *T.*
449 *castaneum* encountered similar stress in the past and evolved adaptive plastic responses. This
450 should especially be the case for temperature protection mechanisms, essential to ectotherms.
451 However, immediate stress responses might not remain beneficial during long-term adaptation
452 to constant high temperature because they trade off with reproduction. Therefore, costly plastic
453 responses may become maladaptive over time and be reversed despite the immediate benefits
454 provided by their protective functions. This might be particularly the case for heat shock
455 proteins, which were the most strongly responding genes in this experiment. Their immediate
456 protective functions are well known (King and MacRae 2015), as well as the reproductive costs of
457 their overexpression (Silbermann and Tatar 2000). Although adaptive, some of the plastic gene
458 expression changes that we observed may thus be reversed in future evolved populations
459 because other resistance mechanisms may arise during long-term evolution (e.g. enzymes, which
460 are more stable at higher temperatures), making stress protection expendable. On the other
461 hand, part of the genes whose response became associated with lower reproductive output
462 (down-regulation of vitellogenin and other reproduction related processes), would be good
463 candidates for future adaptive up-regulation. Their immediate down-regulation, imposed by
464 trade-offs with stress responses, may then also be reversed. Overall, we expect long-term

465 evolution to decrease differentiation of gene expression relative to the ancestral condition (here
466 Control) in those genes involved in strong fitness trade-offs, but to increase differentiation in
467 genes whose response will remain adaptive.

468 In the most stressful treatment, Hot-Dry, up-regulated genes under positive selection were
469 enriched in many metabolic processes (e.g. aerobic respiration, citrate metabolic process), while
470 down-regulated genes under negative selection showed enrichment in negative regulation of
471 metabolic processes (GO:0009892). Thus, a part of the adaptive response of gene expression
472 seemed to enhance metabolic activity, potentially improving females' reproductive output.
473 Those changes constitute candidates for future evolutionary divergence relative to the ancestral
474 population. In Hot, only the down-regulation of genes under negative selection reflected
475 adaptive plasticity. Nevertheless, we can expect to see convergence of the evolutionary patterns
476 of long-term adaptation between the Hot and Hot-Dry selection lines because of their similarity
477 in expression responses and correlation in selection intensities. Results in Dry suggest that
478 maladaptive responses were predominant since up-regulated genes were under more negative
479 selection than non-responding genes. However, responses as well as selection intensities in Dry
480 were generally small, which may reflect the fact that *T. castaneum* has special anatomical
481 adaptations to cope with drought (King and Denholm 2014). Modifying gene expression might
482 therefore play only a minor role in adaptation to dry conditions.

483 Taken together, our results show a mix of responses sometimes in the direction of natural
484 selection and sometimes in an opposite direction. But, more often than not we found evidence
485 that a significant majority of plastic responses is immediately adaptive, especially in the most
486 stressful conditions. Only in the Dry treatment have we found evidence of maladaptive plasticity.
487 As suggested by Ghalambor et al. (2015), genes with maladaptive plastic responses might be
488 those that will show the strongest changes during evolution. Future evolutionary changes in the
489 three treatments may follow a direction predicted by the selection gradients we measured,
490 although this also depends on the existence of additive genetic variation in expression and
491 genetic correlation with fitness (Robertson 1966; Price 1970; Rausher 1992; Morrissey et al.
492 2010, 2012; Bonnet et al. 2017). In any case, plastic and non-plastic genes may or may not show
493 evolutionary divergence in expression, depending on underlying trade-offs with reproduction or
494 other traits. While we have estimated the direction and strength of phenotypic selection acting
495 on gene expression, we cannot predict evolutionary changes because we did not attempt to
496 estimate the additive genetic co-variance of the traits under selection (estimated with their G-
497 matrix: Lande 1979; Walsh and Blows 2009). Genetic covariation among phenotypic traits and

498 with fitness are tedious to estimate and necessitate much larger sample sizes than we had here.
499 (but see McGraw et al. 2011, Blows et al. 2015 for recent attempts using transcriptomic data).
500 Genetic covariance in expression can be caused by shared regulatory elements (e.g., same
501 pleiotropic transcription factors) and lead to correlated responses to selection among genes,
502 which can strongly deviate from predictions of single-gene selection responses (Lande 1979;
503 Walsh and Blows 2009). Nevertheless, our measures of phenotypic selection allowed us to test if
504 up-regulated or down-regulated genes were targets of positive or negative selection pressures,
505 respectively. We could thus test if plastic expression changes were responding to environmental
506 changes in a direction favored by selection. Future work based on a deeper sampling and long-
507 term evolution will address whether those selection pressures translated into corresponding
508 evolutionary changes as predicted by genetic covariation of expression with fitness.

509 One drawback of our study is the exclusion of males from the gene expression measurements.
510 We decided to focus on females, because they have to invest more into reproduction compared
511 to males. Energy allocation trade-offs between responses to different stressors, as well as
512 between stress protection and reproduction should be more pronounced here. We could also
513 show that number of adult offspring was mainly dependent on egg number (Supplementary
514 Material S5), suggesting a minor contribution of males to fitness. Correlation of male expression
515 levels with fitness, and hence selection intensity, would probably be lower, because also
516 dependent on the status of mating females.

517 Conclusions

518 Our approach shows how transcriptomics can be used to get information about the relative
519 importance of different stressors, their interaction and the potential constraints acting on plastic
520 and evolutionary responses when several environmental variables change at the same time. We
521 were thus able to evaluate the immediate adaptive value of the plastic changes in gene
522 expression. By adaptive, we here meant immediate fitness advantage or disadvantage associated
523 with changes in gene expression. Therefore, our study strongly contributes to understanding
524 how plasticity affects fitness at the early onset of adaptive divergence and gives indications of
525 potential future changes in gene expression. It shows that some parts of the plastic response are
526 adaptive, whereas others are maladaptive. However, further work is needed to clarify how we
527 can use plastic responses to predict long-term evolutionary outcomes, for instance by using long-
528 term evolution experiments.

529 Material and Methods

530 *Animal rearing and stress treatments*

531 We used the *Tribolium castaneum* Cro1 strain (Milutinović et al. 2013), collected from a wild
532 population in 2010 and adapted to lab standard conditions (33°C, 70% relative humidity) for
533 more than 20 generations. Beetles were kept in 24h dark on organic wheat flour mixed with 10%
534 organic baker's yeast. We sterilized the flour and yeast by heating them for 12h at 80°C before
535 use. We tested the response of fitness and gene expression of the beetles to heat, drought, and
536 a combination of both stressors. The conditions in the treatments were: Hot: 37°C and 70% r. h.,
537 Dry: 33°C and 30% r. h., Hot-Dry: 37°C and 30% r. h. Parents of the experimental beetles were
538 reared and mated in control conditions at the age of four weeks in 15 mL tubes with 1 g of
539 medium. Each virgin male was mated with a virgin female. After four days, in which the beetles
540 could mate and lay eggs, each mating pair was transferred to a new vial. We repeated this three
541 times, resulting in four vials per mating pair containing medium and eggs. Vials of each mating
542 pair were randomly assigned to the four different conditions, resulting in full-sib families split
543 across all conditions. Male and female offspring (four females and four males per family and
544 condition) were separated at the pupal stage and transferred to 10 mL tubes with 1 g of medium
545 and remained there until they were used for the fitness assay eight weeks later. After the fitness
546 assay, males and females were transferred to 1 mL tubes, frozen in liquid nitrogen and stored at
547 -80°C. The fitness assay was started in the morning and stopped in the afternoon one week later
548 by removing the mating pair, which was then immediately frozen. Beetles should not show a
549 diurnal cycle since they were kept in 24h dark.
550
551 *Fitness assay*
552 To test the effects of the different conditions on fitness, we measured reproduction in 6183
553 virgin females (ca. 1500 per condition, Table 1). We mated each virgin female with one unrelated
554 male from the same condition in 15 mL tube with 1 g medium. The male was removed after 24 h.
555 Females were removed from the tubes after one week of egg laying, and 9 g medium was added
556 to provide food for the developing offspring. After five weeks the number of offspring was
557 counted. At this time, all offspring had reached the adult stage. Some females did not produce
558 any offspring, in proportions that differed between conditions. To test whether there was an
559 effect of treatment on the number of reproducing and non-reproducing females, we used a
560 generalized linear mixed model with reproduction success (binomial: offspring/no offspring) as
561 response and condition as fixed effect. Since some of the tested females and males were full-sibs
562 and developed within the same tube, we used male and female families as random factors to
563 account for non-independence due to relatedness and a shared environment during
564 development. To test how offspring number of reproducing females was influenced by

565 conditions we used a linear mixed model with offspring number as response, temperature,
566 humidity and their interaction as fixed effects and female and male family as random factors.
567 Denominator degrees of freedom were estimated using Satterthwaite approximation. Statistical
568 analyses were performed using the Lme4 package (Bates et al. 2015) version 1.1-17 in R (R Core
569 Team 2015).
570
571 *RNA extraction, library preparation and sequencing*
572 183 female beetles with known fitness (ca. 45 per condition), which had been stored at -80°C,
573 were homogenized in Tri-Reagent® (Zymo Research, California, USA) using an electric bead mill.
574 RNA was extracted with the RNA Mini Prep kit (Zimo Research, California, USA) following the
575 instructions of the manufacturer. RNA-quality was checked with the Bioanalyzer 2100 (Agilent,
576 Waldbronn, Germany) and concentrations were measured with aQubit® Fluorometer (Life
577 Technologies, California, USA). Libraries were created with 500 ng RNA for each individual
578 separately with the LEXOGEN mRNA-Seq Library Kit following the manual (LEXOGEN GmbH,
579 Vienne, Austria). Library quality was checked on a TapeStation (Agilent, Waldbronn, Germany)
580 and concentrations were determined by qPCR. Libraries were diluted to the same molarity.
581 Concentrations of dilutions were checked again by qPCR and libraries were pooled (36 libraries
582 per pool). All treatments were randomized during RNA-extraction, library preparation, and
583 sequencing. The single-end sequencing was performed in five runs on the Illumina NextSeq 500
584 (Illumina, Inc, California, USA) using the 75 cycles High Output Kit. Each run resulted in 550-600
585 million reads that passed the internal sequencer filter. After quality control using FastQC
586 (www.bioinformatics.bbsrc.ac.uk/projects/fastqc), reads were mapped against the reference
587 genome(ftp://ftp.ensemblgenomes.org/pub/release30/metazoa/gtf/tribolium_castaneum/Tribo
588 lium_castaneum.Tcas3.30.gtf.gz) with STAR v.2.5 (Dobin et al. 2013) (adaptors were trimmed
589 and the first 10 bases were hard trimmed, minimum average quality Q10, minimum tail quality
590 10, minimum read length 20). We then used FeatureCounts (Liao et al. 2014) to count the
591 number of reads that mapped to each gene in the reference genome. Mapping as well as read
592 counting was performed within the data analysis framework SUSHI (Hatakeyama et al. 2016).
593
594 *Differential expression and enrichment analysis*
595 We conducted a differential expression analysis using the R package edgeR (Robinson et al.
596 2010). We tested for differently expressed genes between the treatments (Dry, Hot, Hot-Dry)
597 relative to the control as well as to each other. A gene is classified as DE with a FDR ≤5% after
598 adjusting for multiple testing (Benjamini and Hochberg 1995). To test whether the number of DE

genes (relative to Control) was significantly different between two environmental conditions a permutation tests was used. For each permutation entire RNA-seq samples of the two groups were randomly assigned to conditions and the edgeR analysis repeated. Significance was assessed by number of times the observed DE number was higher than the DE number obtained by permutations. To test whether the magnitude of change in expression levels relative to control was significantly different between Hot and Hot-Dry, we performed a permutation test. Absolute log2-fold changes of each transcript were randomly assigned to the two groups and differences in the mean were calculated. We then compared the distribution of differences obtained by permutations to the observed difference between mean absolute log2-fold changes in expression. Gene set enrichment analyses for immune response genes and reproduction related genes were conducted in edgeR using the *roast* function (Wu et al. 2010). The significance cutoff for genes contributing to the proportion of down-regulated genes is $z < -\sqrt{2}$ and $z > \sqrt{2}$ for proportion of up-regulated genes (Robinson et al. 2010). A GO enrichment analysis of DE genes was performed with gProfiler Version: r1622_e84_eg31 (Reimand et al. 2011) and pathway and protein domain enrichment analysis with STRING v.10.0 (Szklarczyk et al. 2015).

Classification of response mode

Following Rasmussen et al. (2013), we created 20 predefined expression profiles each representing a potential response when two single stressors are combined: *Combinatorial*: similar expression levels in single stress treatments but a different level in stress combination, *cancelled*: response to one or both single stressors but expression levels similar to control conditions when both are combined, *prioritized*: opposite responses to single stressors and expression levels in combination similar to one of them, *independent*: response to only one single stressor and the same response in combination, *similar*: same response in each of the two single stressor treatments, and combination. For creating predefined expression profiles we used 0 as control level, 1 and -1 as expression levels for up- and down-regulation, e.g. expression profile for an independent response could be: CT:0, D:0, H:1, HD:1. We then created a dataset consisting of all genes showing a significant response in at least one treatment (4419 genes in total). Correlation between normalized read counts (cpm, TMM normalization) of these genes and each of the predefined expression profiles was tested and genes were assigned to the category with the highest correlation.

633 *Selection*

634 We measured selection intensity on gene expression separately for each treatment using
635 univariate linear regression methods (Lande and Arnold 1983; Brodie III et al. 1995). Fitness
636 (number of adult offspring) was normalized by dividing each individual value by the mean ($w' =$
637 w_i/mean(w)). For each gene, expression levels were first normalized to cpm (counts per million,
638 TMM normalization) using edgeR and then transformed to standardized z-scores by subtracting
639 the mean and dividing by the standard deviation ($z = (x_i$- mean(x))/ SD(x)). Resulting regression
640 coefficients of relative fitness on standardized expression levels give an estimate of the selection
641 intensity. P-values were corrected for multiple comparisons. To test whether up- and down-
642 regulated genes were under significantly different selection compared to genes without a
643 significant response, we used permutation tests. For each permutation (10,000 for each test) we
644 randomly assigned the categories "not DE" and "up" (or "down" respectively) to each estimated
645 selection intensity and calculated the difference in mean selection intensity between both
646 groups. Significance was tested by counting the number of permutations that showed a
647 difference higher or equal to the observed one.

648

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656

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