Article (Discoveries)

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

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Abstract

Gene expression is known to be highly responsive to the environment and important for adjustment of metabolism but there is also growing evidence that differences in gene regulation contribute to species divergence and differences among locally adapted populations. However, most studies so far investigated populations when divergence already occurred. Not much is known about the evolutionary processes acting on expression levels when populations first encounter environmental change. Understanding the mechanisms is further complicated by the fact that environmental change is often multivariate, meaning that organisms are exposed to multiple stressors simultaneously, with interactive effects, which can constrain adaptation when multiple responses cannot be optimized at the same time. Here we use a novel approach by combining fitness and whole-transcriptome data in a large-scale experiment to investigate responses to drought, heat and their combination in *Tribolium castaneum*. We found that fitness was reduced by both stressors and their combined effect was almost additive. Expression data showed that both stressors were acting independently and did not interfere physiologically. Since we measured expression and fitness within the same individuals, we were able to estimate selection on single gene expression levels. We found that variation in fitness can be attributed to gene expression variation and that dependent on the environment different selection pressures are acting, which were opposite between control and stress conditions. We could further show that plastic responses of expression are largely adaptive, meaning they changed in the direction that should increase fitness.

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, if not doubted, 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 first had mostly non-consequential effects on fitness (Giaever 2002; Benoit et al. 2009). However, more recent evidence in yeast show that variation in expression can significantly affect fitness (Keren 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).

In contrast to expression differentiation caused by genetic divergence (Brem et al. 2002; Fay and Wittkopp 2008; Romero et al. 2012), phenotypic plasticity is also expected to play an important role in expression 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, thus 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 (Sørensen et al. 2001; Dayan et al. 2015; Ghalambor et al. 2015) repeatedly found that plasticity was non-adaptive because opposed in direction to the evolutionary response. It may thus be that non-adaptive plasticity facilitates evolutionary divergence by increasing the strength of selection (Ghalambor et al. (2007, 2015)).

However, these studies and others compared expression responses of non-adapted individuals to adapted populations or selection lines in common garden experiments (Whitehead and Crawford 2006; McCairns and Bernatchez 2009; Morris et al. 2014; Ghalambor et al. 2015; Leder et al. 2015), thereby examining patterns when divergence has already occurred. They provide little information on how evolutionary forces have acted in the past and shaped expression but show only the current state after divergence. Studying organisms that have been exposed to new conditions recently can give us more insight into the initial processes leading to divergence between populations experiencing novel environmental conditions and how changes in transcription may contribute to it. In particular, it is still unknown how short term selection pressures are linked to long term optimum expression levels. The two may differ because organisms may first activate immediate stress responses that are beneficial and thus adaptive when they appear, but will not persist because costly to maintain on the long term resulting in quite different responses (Moya et al. 2012, 2015), especially for negative stress effects like slowing down of cell cycle and protein synthesis, and protein damage (Enzor and Place 2014; Huth and Place 2016). In contrast to a first-step stress response, long term adaptation necessitates to optimally re-allocate energy resources between reproduction and stress protection. Optimal solutions for this trade-off between reproduction and survival might also differ between conditions (Schlichting and Smith 2002) and because environmental changes in nature are often multivariate, responses may be constrained by trade-offs among multiple stressors. 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 new environments (DeBiasse and Kelly 2016; Kelly et al. 2016). Transcriptomics can give us insight into the mechanisms underlying such trade-offs. It can potentially show which physiological processes are activated, thereby giving us information about how resources are used. These trade-offs in stress responses can not only limit plastic responses but may also constrain evolution and future adaptation (Etterson and Shaw 2001; Pörtner et al. 2006; Kelly et al. 2016). Therefore, linking the immediate plastic responses of gene expression with their fitness effects at the onset of adaptive divergence should allow a better understanding of their adaptive value and evolutionary consequences.

103 In this study we asked how *Tribolium castaneum* (the red flour beetle) was affected by heat and
104 drought in single stressor treatments and in a combination treatment. We combined a fitness
105 assay with measuring gene expression using RNA-seq. This allowed us to get more insight into
106 the physiological processes affected by different stressors, to examine whether the
107 transcriptomic responses to heat and drought overlapped and if gene expression was changed in
108 the same direction by both stressors. Based on this, we can make predictions about how
109 stressors will act in combination and test this in the combined stressor treatment. Since we
110 measured expression and fitness in the same individuals in sufficient sample size, we were able
111 to estimate transcriptome-wide selection intensities on single-gene expression levels giving us an
112 unprecedented overview of selection pressures on gene expression in novel environments.
113 Further, we were able to examine whether plastic responses were adaptive or non-adaptive,
114 thus contributing to our understanding of how plasticity will affect future adaptation.
115

116 ## Results

117 We used a *T. castaneum strain* (Cro1) (Milutinović et al. 2013), which was collected from a wild
118 population in 2010 and adapted to standard control conditions (33 °C, 70% relative humidity
119 (r.h.)) since then. We measured the number of adult offspring as well as gene expression
120 changes relative to control conditions when the beetles were exposed to a drought, a heat and a
121 combined heat-drought treatment (conditions: Dry: 33°C, 30% r.h.; Hot: 37 °C, 70 % r.h; Hot-Dry:
122 37°C, 30% r.h.). Individuals were transferred to treatments in the egg stage and stayed there
123 during their whole life time. Fitness and expression were measured in females at the age of
124 eleven weeks. Gene expression was measured by RNA-seq using mRNA of the whole body.
125

126 *Fitness assay*

127 The proportion of reproducing females was significantly different between conditions ($\chi 2$=
128 627.35, df=3, P< 2.2e-16). The highest proportion of non-reproducing females was found in Hot
129 (Fig. 1A, Tab. 1). Offspring number of reproducing females decreased with increasing
130 temperature ($F_{1,5157}$ = 1981.07 , P < 2.2e-16) and decreasing humidity ($F_{1,5184}$ = 262.05, P < 2.2e-
131 16), with a stronger effect of heat (-15.98 ± 0.57 SE) than of drought (-5.12 ± 0.50 SE). The lowest
132 offspring number was found when heat and drought were combined (Fig. 1B). Interaction
133 between temperature and humidity was also significant ($F_{1,5128}$= 8.37 , P = 0.003835) and led to
134 an additional decrease of -2.22 ± 0.77 compared to purely additive effects.

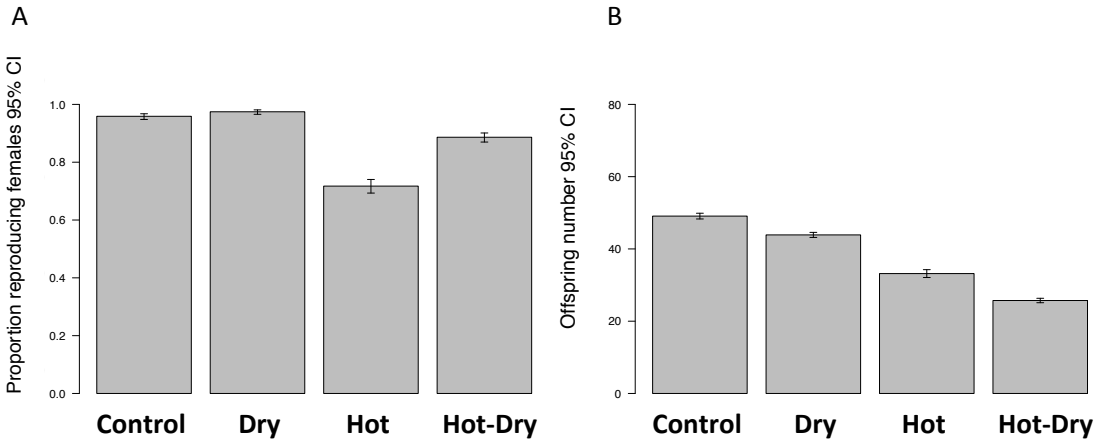


Figure 1 Effect of temperature and humidity on reproduction. Proportion of reproducing females in four different conditions: Control (33°C, 70 % r. h.), Dry (33°C, 30 % r. h.), Hot (37°C, 70% r. h.), Hot-Dry (37°C, 30% r. h.) (A). Offspring number of reproducing females. Females could lay eggs for one week and adult offspring was counted five weeks later (B).

Table 1: Results of the fitness assay. Number of adult offspring that females produced within one week of egg-laying in different conditions (Control: 33°C, 70% relative humidity (r.h.).; Dry: 33°C, 30% r.h.; Hot: 37°C, 70% r.h.; Hot-Dry: 37°C, 70% r.h.). For calculating offspring number per female only reproducing females were used

Condition	N	females with offspring	proportion of reproducing females (± SE)	offspring per reproducing female (±SE)
Control	1575	1514	97.62 ± 0.50 %	48.99 ± 0.44
Dry	1642	1603	98.66 ± 0.25 %	43.87 ± 0.43
Hot	1401	1005	76.38 ± 2.70 %	33.01 ± 0.52
Hot-Dry	1567	1396	92.66 ± 1.01 %	25.68 ± 0.45

Gene expression response to heat is stronger than to drought, with little functional overlap

The number of differentially expressed (DE) genes relative to Control was lowest in Dry and largest in Hot (Fig. 2, Tab. 2). Drought induced up-regulation of 52 and down-regulation of 48 genes. In contrast, the response to heat was much more pronounced, with a significantly higher number of DE genes than in Dry (up: 1594, down: 1255; permutation test $P < 0.004$). The overlap between heat and drought responses included only 26 genes (Fig. 2). To investigate whether the DE genes were involved in specific biological processes, we performed pathway, protein

154 domains, and Gene Ontology (GO) enrichment tests. Because of a low number of DE genes in
155 Dry, only few enrichments could be detected (Supplementary Material S1, S2). Genes up-
156 regulated by drought showed the strongest enrichment in active ion transmembrane transporter
157 activity (GO:0022853), down-regulated genes in hydrolase activity, hydrolyzing O-glycosyl
158 compounds (GO:0004553) and protein family Thaumatin (IPR001938). Analysis of the *Tribolium*
159 genome had revealed a high number of genes thought to be involved in endocrine regulation of
160 diuresis, including several that encode putative neuroendocrine peptides like antidiuretic factors
161 (Aikins et al. 2008; Hauser et al. 2008; Li et al. 2008; Park et al. 2008). Nevertheless, none of
162 these genes was found to respond to the Dry treatment.
163
164 Genes up-regulated in Hot were enriched in many metabolic processes e.g. carbohydrate
165 metabolic process (GO:0005975), Citrate cycle (KEGG 00020), Pyruvate metabolism (KEGG
166 00620) (Supplementary Material S1, S2). The most strongly enriched category was chitin
167 metabolic process (GO:0006030). A protein domain analysis also showed significant enrichment
168 of heat shock proteins (IPR031107, IPR018181, IPR008978) (Supplementary Material S2). Down-
169 regulated genes were enriched in pathways for DNA replication (KEGG 03030), nucleotide
170 excision repair (KEGG 03420) and Ubiquitin mediated proteolysis (KEGG 04120).

171
172 **Table 2:** Number of differently expressed genes (FDR< 5 %) when comparing different
173 conditions. Positive: higher expression in second condition. Negative: lower expression in second
174 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

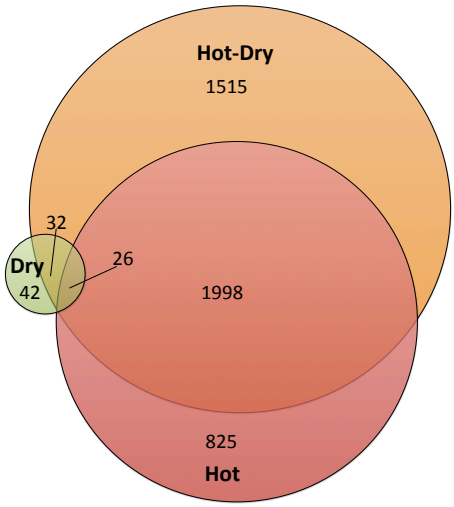


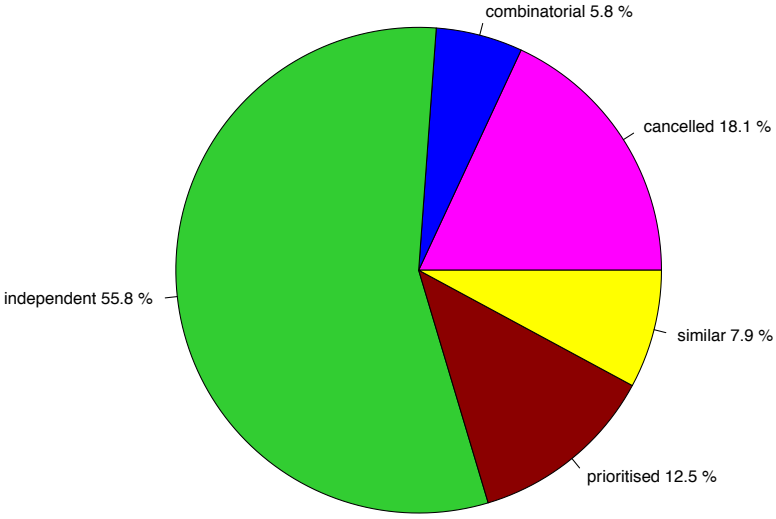
Figure 2 Venn-Diagram showing the number of differentially expressed genes in three treatments relative to control conditions. Overlapping regions represent genes that were found in more than one treatment and changed expression levels in the same direction. Sizes of circles as well as of overlapping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

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

When both stressors were combined in Hot-Dry, we found 3571 DE genes (up: 1866, down: 1705). Among them, 1515 (42.4%) were not found in single stressor treatments. However, only 69 of those genes (up: 30, down: 39) were found significantly DE between Hot-Dry and Dry, or Hot-Dry and Hot. This indicates that in most cases the combined stress did not induce expression changes in a different set of genes but modified their expression levels over and above their responses to single stressors. Compared to Hot, the Hot-Dry response had a significantly higher magnitude of expression change (permutation test: $P < 0.0001$), a higher number of down-regulated genes ($P = 0.007$), but a similar number of up-regulated and total number of DE genes ($P = 0.29$ and, $P = 0.054$, respectively). The functional response to Hot-Dry resembles the response to Hot, but more enriched GO categories and pathways could be found (see Supplementary Material S1, S2).

To further examine how single stress responses are modified during combination, we classified the top 1000 most responding transcripts (based on p-values) of each treatment into different response categories following Rasmussen et al. (2013). Only 8% of the genes showed a *similar* response mode, with same response to Dry, Hot and Hot-Dry (Fig. 3). Most responding genes (56 %) were classified as *independent*, with a response that is not altered in presence of a second

199 stressor. Most of those genes showed the same response in Hot and Hot-Dry (44.5 %) but no
200 response in Dry, in agreement with our DE analysis. Few genes (6 %) had a *combinatorial*
201 response mode in which they did not respond to heat and drought alone, but to their
202 combination. These represent cases in which presence of an additional stressor magnifies the
203 effect of another. Especially interesting are cases with opposite responses to both stressors but
204 with one response *prioritized* when combined, which can help to identify trade-offs. We found
205 13 % of DE genes falling into that category, most of them showing prioritization of the Hot
206 response in Hot-Dry (Supplementary Material S4), also in agreement with our DE analysis. But
207 more common than *prioritized* is the *cancelled* mode with 18 % of the genes, which show
208 opposite responses to both stressors or respond to one stressor only but return to control levels
209 in combination, showing no response to stress combination.



210

211 **Figure 3:** Response modes of the 1000 most significantly responding transcripts in the stress
212 treatments (Dry, Hot, Hot-Dry. *Combinatorial*: Similar levels in the two individual stresses but a
213 different response to combined stresses; *cancelled*: transcript response to either or both
214 individual stresses returned to control levels; *prioritized*: opposing responses to the individual
215 stresses and one stress response prioritized in response to combined stresses; *independent*:
216 response to only one single stress and a similar response to combined stresses; *similar*: similar
217 responses to both individual stresses and to combined stresses. Subcategories of different
218 response modes giving more details about the most prevalent patterns in supplementary
219 material (S4).

220

221

222 *Immune response: Interaction between drought and heat*

223 We noticed that among the genes up-regulated in Hot many are involved in immune response
224 (Oppert et al. 2012; Behrens et al. 2014). These transcripts encode putative chitin-interacting
225 proteins (binding, deacetylase), allergens, thaumatins, glucosidases, and serine proteases,
226 whereas these genes are down-regulated at low humidity. For instance, Thaumatin protein
227 family, which is involved in antimicrobial response (Altincicek et al. 2008) showed the strongest
228 enrichment in down-regulated genes (Supplementary Material S2) and a pathogenesis related
229 protein and several antimicrobial peptides are among those genes that showed the strongest
230 response in Hot. We applied a gene set enrichment analysis to test for an overrepresentation of
231 immune genes in responses to the different treatments. To avoid including immune genes also
232 involved in heat stress response (Behrens et al. 2014), we used a test set of 15 antimicrobial
233 peptides, which should be specific to a pathogen response, based on Zou et al. 2007, Oppert et
234 al. 2012, Altincicek et al. 2013 (Supplementary Material S3A). We found that antimicrobial
235 peptides were significantly up-regulated in Hot (p-value = 0.001) and down-regulated in Dry (p-
236 value = 0.001) (Tab. 3). When we compared Hot-Dry with Hot, these genes became down-
237 regulated (p-value = 0.002), indicating that it is mainly the combination of high temperature with
238 relatively high humidity (i.e. 70% r.h.) that activates this response, pointing to a possible higher
239 parasite load under these conditions.

240

241 **Table 3** Results of enrichment analysis of antimicrobial peptides. Gene set enrichment test was
242 conducted in edgeR (Robinson et al. 2010). Prop.Down and Prop.Up give the proportion of
243 genes that are down- and up-regulated. The direction of change is determined from the
244 significance of changes in each direction, and is shown in the Direction column. The p-value
245 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

246

247

248 *Treatment effects on reproduction related processes*

249 To get further insights into the molecular processes that link the observed decline in offspring
250 number with transcriptomic data, we focused on the juvenile hormone (JH), 20-hydroxecdysone
251 (20 E), and insulin/insulin-like peptides (IIS) target of rapamycin (TOR) signaling pathways (IIS-

252 TOR), which are important mediators in the trade-off controlling stress response and
253 reproduction (Schwenke et al. 2016), along with vitellogenin, which is the main nutrient source
254 of eggs, and vitellogenin receptors. We found that heat and heat-drought stress, led to a
255 significant down-regulation of all pathways and repression of vitellogenin as well as vitellogenin
256 receptors (Supplementary Material S3B). Drought did not show any significant effect. A gene set
257 test based on a selection of key reproduction genes within these pathways (Supplementary
258 Material S3C) confirmed that the reproduction genes were mainly down-regulated in Hot and
259 Hot-Dry (Tab. 4).
260

261 **Table 4:** Results of gene set enrichment analysis of genes involved in reproduction. Gene set
262 enrichment test was conducted in edgeR (Robinson et al. 2010). Prop.Down and Prop.Up give
263 the proportion of genes that are down- and up-regulated. The direction of change is determined
264 from the significance of changes in each direction, and is shown in the Direction column. The *P*-
265 value provides evidence for whether the majority of genes in the set are DE in the specified
266 direction. The genes were selected based on (Parthasarathy, Sheng, et al. 2010; Parthasarathy,
267 Sun, et al. 2010; Xu et al. 2010; Parthasarathy and Palli 2011).

Contrast	PropDown	PropUp	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

268

269 *Selection on expression levels is environment specific*

270 Since we measured offspring number and transcription within the same individuals, we could
271 estimate selection intensity on single gene expression levels in each condition separately by
272 performing a linear regression of relative fitness on standardized expression levels (z-score). In
273 control conditions, expression levels of 2158 genes showed a negative and 21 a positive and
274 significant correlation with fitness. The two genes under strongest positive selection coded for
275 vitellogenin. In Dry, Hot and Hot-Dry we could not detect any significant selection after
276 correcting p-values for multiple comparisons. To gain a better overview of the variation in
277 selection pressures among treatments and to avoid stringent significance thresholds on single-
278 gene fitness-expression correlations, we then compared selection intensities on all genes
279 between conditions. We found significant negative correlations of selection intensities between
280 Control and all stress treatments, and positive correlations among stress treatments (p-values <

2.2e-16) (Fig 4). Control and Dry had the strongest negative correlation (-0.24), while Hot and Hot-Dry had the highest positive correlation (0.34). Furthermore, significantly DE genes responding to Hot and Hot-Dry were overrepresented among those that were negatively selected in control conditions (Hot: $\chi2$= 158.62, df = 1, p-value < 2.2e-16, Hot-Dry: $\chi2$= 177.97, df = 1, p-value < 2.2e-16), with 361 (22.6 %) up-regulated genes in Hot and 417 (22.3 %) in Hot-Dry. The magnitudes of selection intensities were also significantly different between conditions (median (and SD) of absolute values in Control: 0.09 (0.06), Dry: 0.03 (0.03), Hot: 0.09 (0.07), Hot-Dry: 0.05 (0.04); Kruskal-Wallis rank sum test: $\chi2$= 9333.3, df = 3, p-value < 2.2e-16), with the majority of genes negatively selected in Control, and similar proportions of genes under positive and negative selection in stress treatments (Fig. 5). Estimated selection intensities were rather low compared to those that were reported for morphological and life-history traits in natural populations (Kingsolver et al. 2001; Hereford et al. 2004), but within the range that was estimated for selection on expression levels (McCairns and Bernatchez 2009). Although the Dry treatment had the largest negative correlation with Control expression levels and largest number of genes switching sign relative to Control (9591), it had the lowest magnitude of change in selection intensity of those genes (median: 0.14). In contrast, the Hot environment had the strongest magnitude of change in selection intensity compared to Control (median: 0.19, 6644 genes) followed by Hot-Dry (median: 0.16, 7412 genes). Overall, a greater proportion of genes switched from negative to positive selection (Dry: 0.86, Hot: 0.76, Hot-Dry: 0.8).

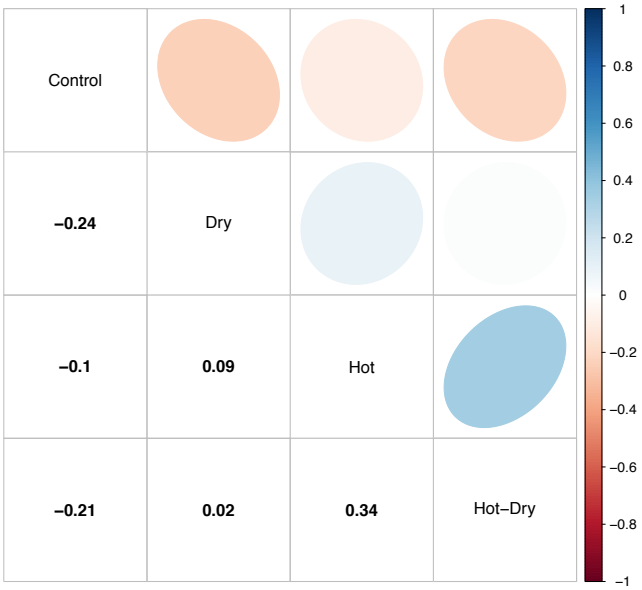


Figure 4: Pairwise correlations of selection intensities on single gene expression levels in different conditions. Blue indicates a positive and red a negative correlation. Values are given in the lower triangle. Confidence intervals for correlations: Control-Dry: -0.25 ,-0.22; Control-Hot: -

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-Dry: 0.33, 0.36.

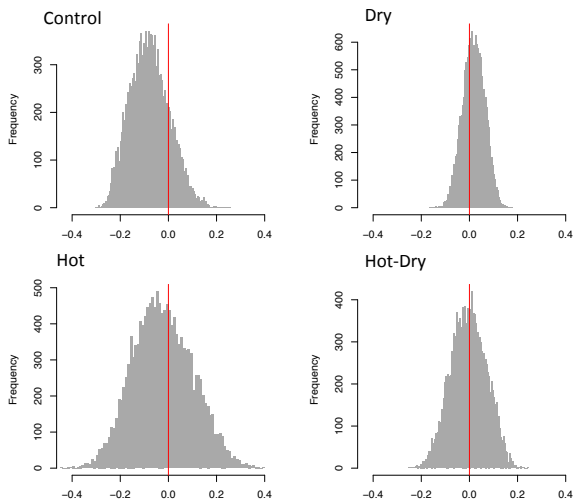


Figure 5: Distribution of selection intensities on gene expression levels under different conditions. Linear regression of relative fitness (number of adult offspring) on normalized RNA-seq read counts (z-score) was used to estimate selection intensity (slope of selection gradient). 0 is marked by the vertical line.

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 with up- and down-regulated genes significantly more positively and negatively selected than non-responding genes, respectively (permutation tests: $P < 0.0001$) (Fig. 6C). 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. 6A). In Hot, the response is partly adaptive since down-regulated genes were significantly more negatively selected, while up-regulated genes were not under significantly different selection (Fig. 6B).

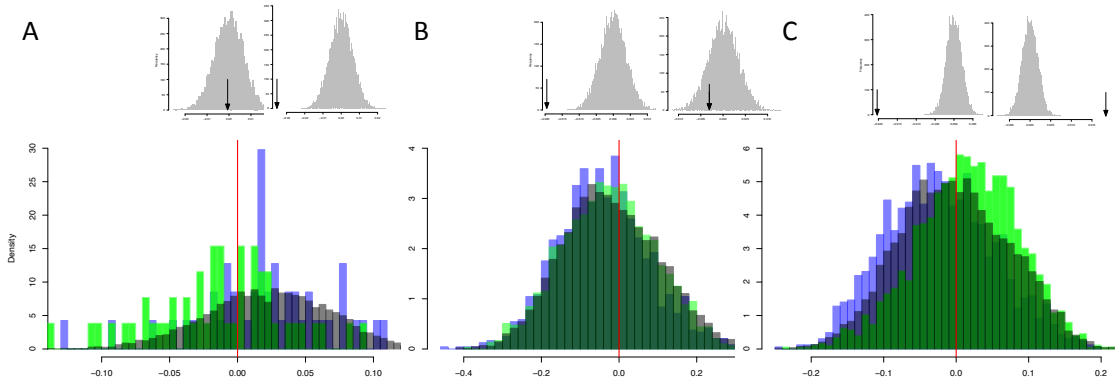


Figure 6: Distribution of selection intensities on gene expression levels under different conditions: Dry (A), Hot (B), Hot-Dry (C). Linear regression of relative fitness (number of adult offspring) on normalized RNA-seq read counts (z-score) was used to estimate selection intensity (slope of selection gradient). Genes that became significantly up-regulated (FDR< 5%) in the respective condition relative to control are represented in green, significantly down-regulated genes in blue and genes that did not show a significant response in grey. Insets show the distribution of differences in the mean selection intensity between down-regulated and not responding genes (left) and up-regulated and not responding genes (right) from 10,000 permutations. Arrows indicate the observed difference.

Discussion

Physiological trade-offs caused by single stressors and their combination

We found that the effects of heat and drought on expression differed in the processes affected and the magnitude of their response. Heat stress triggered a response in a larger number of genes and affected a larger number of biological processes than drought, with little overlap between the two responses. These results are in agreement with many studies showing that heat is a major driver of expression change, especially in ectotherms (Neven 2000; Nguyen et al. 2009; Levine et al. 2011; Chen and Stillman 2012; Morris et al. 2014). The heat-induced expression changes were characteristic of stress responses: Up-regulation of heat shock proteins and metabolic pathways, whereas genes involved in cell growth and cell cycle, and reproduction were repressed (Gasch et al. 2000; Kültz 2005; Kassahn et al. 2009; Enders et al. 2015). This pattern of change in gene expression together with the overall reduction of the reproductive output of the females assayed suggests a shift of resource utilization from growth to stress protection, indicative of a physiological trade-off between stress response and reproduction

349 (Salmon et al. 2001; Fedorka et al. 2004; Harshman and Zera 2007). We can further confirm that
350 this physiological trade-off is at play because the decrease of females' reproductive output was
351 mainly driven by a loss of fecundity (egg number) in the Hot and Hot-Dry treatments and not a
352 decrease of larvae survival (see Supplementary Material S5), which matches well with the down-
353 regulation of vitellogenin and the other reproduction-related genes we monitored (JH, the IIS-
354 Tor and 20E signaling pathways). It has been suggested that those genes indeed play an
355 important role in mediating the trade-offs between stress-responses and cellular growth and
356 maintenance (Flatt et al. 2005; Gruntenko and Rauschenbach 2008; Schwenke et al. 2016). The
357 fitness costs associated with expression of heat shock proteins are also known (Feder and
358 Hoffman 1999; Silbermann and Tatar 2000; Kassahn et al. 2009). Finally, a trade-off between
359 immune response and reproduction may also have affected our results. Indeed, we found a
360 larger proportion of non-reproducing females (Fig.1B, Tab. 1) and an up-regulation of immune
361 response genes in the Hot treatment. These two results may point to a higher pathogen load
362 when high temperature (37°C) and relatively high humidity (70% r.h.) are combined, as described
363 before (Sokoloff 1972). However, the similar or even stronger down-regulation of reproduction-
364 related genes in the Hot-Dry treatment in which antimicrobial peptides were not up-regulated,
365 would suggest that the trade-off between reproduction and immune response was not the main
366 cause of reduction in fecundity in Hot, although it may have plaid a role in the number of
367 reproducing females. Overall, our analysis shows that different physiological trade-offs between
368 reproduction and multiple stressors exist in complex environments and elicit gene expression
369 responses that overlap with each other, resulting in a general decrease of population fitness.
370 However, precisely separating the responses of multiple stressors is difficult because of the large
371 pleiotropic effects of the core stress response genes part of the pathways (JH, IIS-TOR, 20E) and
372 stress response mechanisms (e.g. Hsp) mentioned above.
373
374 In contrast to the above energy allocation trade-offs, there was a lack of a strong trade-off
375 between heat and drought responses of gene expression. We investigated this trade-off by
376 comparing the single stressor transcriptomic responses to their combined response in the Hot-
377 Dry treatment. As expected from the small functional and DE genes overlap between the Hot
378 and Dry treatments, the top responding genes in the Hot-Dry treatment showed an independent
379 action mode of the individual stress responses as the most common response mode. When
380 stressors require different protection mechanisms and affect different pathways, it is unlikely
381 that they interfere with each other during combination and the effect of combination should
382 thus be additive (Folt and Chen 1999). Accordingly, we found that the observed reproductive

383 output in the combined Hot-Dry treatment was close to what would be expected for purely
384 additive effects between Hot and Dry. Detection of a significant interaction between heat and
385 drought on offspring number was due to high sample size and accordingly high statistical power
386 and it should be noted that the effect was small (-2.22 ± 0.77). Together with a larger overall
387 expression response, including stronger down-regulation of reproduction-related genes, this
388 points to a larger shift of resource allocation from growth to stress protection in the Hot-Dry
389 compared to the Hot treatment caused by the addition of a milder drought stress to the large
390 heat stress.
391
392 *Evolutionary trade-offs in gene expression across environments*
393 Our experiment revealed the fitness effects of the gene expression changes in the new
394 environments from which evolutionary trade-offs can be uncovered. A shift of the direction of
395 selection on gene expression variation from one environment to the next is symptomatic to an
396 evolutionary trade-off and can lead to phenotypic changes between environments when
397 selection acts on existing genetic variation. We found strong evidence of such switches of the
398 direction of selection between Control and test environments. The distribution of selection
399 intensities in Control showed that most expression levels were under negative selection,
400 probably because gene expression is costly (Castillo-Davis et al. 2002; Wagner 2005; Lang et al.
401 2009; Frumkin et al. 2017), and selection should therefore favor low expression levels under mild
402 laboratory conditions to which the beetles were adapted. The distribution changed when
403 conditions became stressful with a shift towards more transcripts under positive selection (about
404 80% of the shifting genes, see Results) in the three stress treatments. This translated into strong
405 negative correlations of selection intensities between Control and treatments. The protection
406 provided by stress response genes might thus have outweighed the costs of expression. Yet,
407 more importantly, although stress response trades-off negatively with reproduction and growth,
408 leading to an overall decrease of fitness in stress treatments, the gene expression change of the
409 underlying genes is mainly in the direction favored by selection (i.e. negative selection intensities
410 for down-regulated genes and vice-versa for positive selection, see Fig. 6). This means that the
411 physiological trade-off in energy allocation may not constrain the evolutionary response of those
412 genes because their responses were aligned with the direction of selection. There is thus a
413 strong potential for the evolution of gene expression, mostly in the Hot-Dry and to a lesser
414 extent in the Hot environments depending on the amount of genetic variance in expression
415 levels, which we haven't attempted to estimate here.
416

417 *Plasticity and the evolutionary potential for adaptation*

418 Based on our findings, we could expect divergence in gene expression to evolve during
419 adaptation to stressful conditions compared to the baseline expression in Control, with some
420 convergence of the evolutionary responses in the Hot and Hot-Dry conditions because of the
421 similarity of their expression responses and selection pressures (evident by positive correlation).
422 However, we have here documented the first-step plastic response of the adaptive process to a
423 new stressful environment. Although there is some evidence for a relationship between plastic
424 and evolved responses in gene expression (Riehle et al. 2003; Landry et al. 2007; Swindell et al.
425 2007; Tirosh et al. 2009; Ragland et al. 2015; Mäkinen et al. 2016), the changes observed may
426 not indicate an evolutionary trend. A common observation is that adaptation reverses first-step
427 plastic responses, as shown in experimental evolution studies (Fong et al. 2005; Rodŕiguez-
428 Verdugo et al. 2016; Ho and Zhang 2018). Stress response is costly and long-term adaptation
429 might involve different genes that are not part of the immediate stress response processes. After
430 adaptation has occurred, stress protection mechanisms are not required anymore and
431 expression reverses to pre-stress levels after rebalancing of the energy allocation trade-off in
432 favor of growth and reproduction (Rodŕiguez-Verdugo et al. 2016). Therefore, previous studies
433 comparing evolved with plastic responses inferred that plastic responses were mainly
434 maladaptive because in a direction opposed to the evolved responses (Ghalambor et al. 2015;
435 Huang and Agrawal 2016; Ho and Zhang 2018). Here, we showed that the first-step plastic
436 responses were adaptive and increased fitness, thereby helping populations to persist, at least in
437 Hot-Dry and partially in Hot. Heat and drought are stressors that *T. castaneum* has certainly
438 encountered in the past (Sokoloff 1972) meaning that there has probably been selection on
439 plastic responses to be adaptive. The results in Dry are less clear. Changes in expression were
440 small and included only a few genes for which the response was not in the direction that should
441 increase fitness. This is consistent with previous studies showing that well-adapted populations
442 have weaker responses in expression (Heckel et al. 2016; Levis et al. 2017). Special anatomical
443 adaptations allow *T. castaneum* to cope with dry conditions by reducing water loss dramatically
444 (King and Denholm 2014), and selection on expression levels was weak in Dry, indicating that
445 modifying transcription will play a minor role during potential future adaptation. More generally,
446 adaptation can occur by either changing the mean of the expression levels or by modifying the
447 extent of plasticity to match the optimal expression level in a new environment. It was found
448 that most genes change rather mean expression than plasticity during adaptation (Yampolsky et
449 al. 2012; Morris et al. 2014), but it was also shown that plasticity in expression could evolve
450 during adaptation to novel conditions (Levine et al. 2011; Morris et al. 2014; Huang and Agrawal

2016; Levis et al. 2017; Passow et al. 2017). With our data it is not yet clear, which of these mechanisms will be more prevalent or whether they are of equal importance.

Conclusions

We contend that current approaches of comparing already divergently adapted populations cannot reveal the selection pressures acting at the beginning of divergence when populations are exposed to different environments. They thus cannot say whether the first-step plastic changes were adaptive or non-adaptive in the population where they appeared. In contrast, our experimental approach gives a more accurate account of the selection pressures acting on gene expression variation at the early onset of adaptive divergence by integrating fitness and expression data in a large-scale stress-response experiment. Our approach also shows how transcriptomics can be used to get information about the relative importance of different stressors, their interaction and the potential constraints acting on plastic and evolutionary responses when several environmental variables change at the same time. We were thus able to evaluate the immediate adaptiveness of the plastic changes in gene expression. However, further work is needed to clarify how we can use plastic responses to predict long-term evolutionary outcomes, for instance by using long-term evolution experiments.

Material and Methods

Animal rearing and stress treatments

We used the *Tribolium castanuem* Cro1 strain (Milutinović et al. 2013), collected from a wild population in 2010 and adapted to lab standard conditions (32°C, 70% relative humidity) for more than 20 generations. Beetles were kept in 24h dark on organic wheat flour mixed with 10% organic baker's yeast. We sterilized the flour and yeast by heating them for 12h at 80°C before use. We tested the response of fitness and gene expression of the beetles to heat, drought, and a combination of both stressors. The conditions in the treatments were: Hot: 37°C and 70% r. h., Dry: 33°C and 30% r. h., Hot-Dry: 37°C and 30% r. h. Parents of the studied beetles grew up and mated in control conditions at the age of four weeks in 15 mL tubes with 1 g of medium. Each virgin male was mated with a virgin female. After four days, in which the beetles could mate and lay eggs, each mating pair was transferred to a new vial. We repeated this three times, resulting in four vials per mating pair containing medium and eggs. Vials of each mating pair were randomly assigned to the four different conditions, resulting in full-sib families split across all conditions. Male and female offspring (four females and four males per family and condition)

485 were separated at the pupal stage and transferred to 10 mL tubes with 1 g of medium and
486 remained there until they were used for the fitness assay eight weeks later. After the fitness
487 assay, males and females were transferred to 1 mL tubes, frozen in liquid nitrogen and stored at
488 -80°C.
489
490 *Fitness assay*
491 To test the effects of the different conditions on fitness, we measured reproduction in 6183
492 virgin females (ca. 1500 per condition, Table 1). We mated each virgin female with one unrelated
493 male from the same condition in 15 mL tube with 1 g medium. The male was removed after 24 h.
494 Females were removed from the tubes after one week of egg laying, and 9 g medium was added
495 to provide food for the developing offspring. After five weeks the number of offspring was
496 counted. At this time, all offspring had reached the adult stage. Some females did not produce
497 any offspring, in proportions that differed between conditions. To test whether there was an
498 effect of treatment on the number of reproducing and non-reproducing females, we used a
499 generalized linear mixed model with reproduction success (binomial: offspring/no offspring) as
500 response and condition as fixed effect. Since some of the tested females and males were full-sibs
501 and developed within the same tube, we used male and female families as random factors to
502 account for non-independence due to relatedness and a shared environment during
503 development. To test how offspring number of reproducing females was influenced by
504 conditions we used a linear mixed model with offspring number as response, temperature,
505 humidity and their interaction as fixed effects and female and male family as random factors.
506 Denominator degrees of freedom were estimated using Satterthwaite approximation. Statistical
507 analyses were performed using the Lme4 package (Bates et al. 2015) version 1.1-17 in R (R Core
508 Team 2015).
509
510 *RNA extraction, library preparation and sequencing*
511 183 female beetles with known fitness (ca. 45 per condition), which had been stored at -80°C,
512 were homogenized in Tri-Reagent® (Zymo Research, California, USA) using an electric bead mill.
513 RNA was extracted with the RNA Mini Prep kit (Zimo Research, California, USA) following the
514 instructions of the manufacturer. RNA-quality was checked with the Bioanalyzer 2100 (Agilent,
515 Waldbronn, Germany) and concentrations were measured with aQubit® Fluorometer (Life
516 Technologies, California, USA). Libraries were created with 500 ng RNA for each individual
517 separately with the LEXOGEN mRNA-Seq Library Kit following the manual (LEXOGEN GmbH,
518 Vienne, Austria). Library quality was checked on a TapeStation (Agilent, Waldbronn, Germany)

519 and concentrations were determined by qPCR. Libraries were diluted to the same molarity.
520 Concentrations of dilutions were checked again by qPCR and libraries were pooled (36 libraries
521 per pool). All treatments were randomized during RNA-extraction, library preparation, and
522 sequencing. The single-end sequencing was performed in five runs on the Illumina NextSeq 500
523 (Illumina, Inc, California, USA) using the 75 cycles High Output Kit. Each run resulted in 550-600
524 million reads that passed the internal sequencer filter. After quality control using FastQC
525 (www.bioinformatics.bbsrc.ac.uk/projects/fastqc), reads were mapped against the reference
526 genome(ftp://ftp.ensemblgenomes.org/pub/release30/metazoa/gtf/tribolium_castaneum/Tribo
527 lium_castaneum.Tcas3.30.gtf.gz) with STAR v.2.5 (Dobin et al. 2013) (adaptors were trimmed
528 and the first 10 bases were hard trimmed, minimum average quality Q10, minimum tail quality
529 10, minimum read length 20). We then used FeatureCounts (Liao et al. 2014) to count the
530 number of reads that mapped to each gene in the reference genome. Mapping as well as read
531 counting was performed within the data analysis framework SUSHI (Hatakeyama et al. 2016).
532
533 *Differential expression and enrichment analysis*
534 We conducted a differential expression analysis using the R package edgeR (Robinson et al.
535 2010). We tested for differently expressed genes between the treatments (Dry, Hot, Hot-Dry)
536 relative to the control as well as to each other. A gene is classified as DE with a FDR ≤5% after
537 adjusting for multiple testing (Benjamini and Hochberg 1995). To test whether the number of DE
538 genes (relative to Control) was significantly different between two environmental conditions a
539 permutation tests was used. For each permutation entire RNA-seq samples of the two groups
540 were randomly assigned to conditions and the edgeR analysis repeated. Significance was
541 assessed by number of times the observed DE number was higher than the DE number obtained
542 by permutations. To test whether the magnitude of change in expression levels relative to
543 control was significantly different between Hot and Hot-Dry, we performed a permutation test.
544 Absolute log2-fold changes of each transcript were randomly assigned to the two groups and
545 differences in the mean were calculated. We then compared the distribution of differences
546 obtained by permutations to the observed difference between mean absolute log2-fold changes
547 in expression. Gene set enrichment analyses for immune response genes and reproduction
548 related genes were conducted using edgeR. A GO enrichment analysis of DE genes was
549 performed with gProfiler Version: r1622_e84_eg31 (Reimand et al. 2011) and pathway and
550 protein domain enrichment analysis with STRING v.10.0 (Szklarczyk et al. 2015).
551
552 *Classification of response mode*

553 Following Rasmussen et al. (2013), we created 20 predefined expression profiles each
554 representing a potential response when two single stressors are combined: *Combinatorial*:
555 similar expression levels in single stress treatments but a different level in stress combination,
556 *cancelled*: response to one or both single stressors but expression levels similar to control
557 conditions when both are combined, *prioritized*: opposite responses to single stressors and
558 expression levels in combination similar to one of them, *independent*: response to only one
559 single stressor and the same response in combination, *similar*: same response in single stressor
560 treatments and combination. For creating predefined expression profiles we used 0 as control
561 level, 1 and -1 as expression levels for up- and down-regulation. We then tested the correlation
562 of normalized read counts of the top 1000 genes that showed the most significant responses
563 (based on significance of the DE analysis) in Dry, Hot and Hot-Dry conditions with each of these
564 expression profiles and assigned the genes to the category to which they showed the highest
565 correlation (Pearson correlation).
566
567 *Selection*
568 We measured selection intensity on gene expression separately for each treatment using
569 univariate linear regression methods (Lande and Arnold 1983; Brodie III et al. 1995). Fitness
570 (number of adult offspring) was normalized by dividing each individual value by the mean ($w' =$
571 $w_i/\text{mean}(w)$). For each gene, expression levels were first normalized to cpm (counts per million,
572 TMM normalization) using edgeR and then transformed to standardized z-scores by subtracting
573 the mean and dividing by the standard deviation ($z = (x_i - \text{mean}(x))/ SD(x)$). Resulting regression
574 coefficients of relative fitness on standardized expression levels give an estimate of the selection
575 intensity. P-values were corrected for multiple comparisons. To test whether up- and down-
576 regulated genes were under significantly different selection compared to genes without a
577 significant response, we used permutation tests. For each permutation (10,000 for each test) we
578 randomly assigned the categories “not DE” and “up” (or “down” respectively) to each estimated
579 selection intensity and calculated the difference in mean selection intensity between both
580 groups. Significance was tested by counting the number of permutations that showed a
581 difference higher or equal to the observed one.
582
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