Evolved changes in DNA methylation in response to the sustained loss of parental care in the burying beetle

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Abstract

Levels of parental care critically influence the development environment with the capacity to impact the growth, survival, physiology, and behaviour of offspring. Plastic changes in DNA methylation have been hypothesised to modulate gene expression responses to parental environments. Moreover, these effects can be inherited and so may affect the process of adaptive evolution. In this study, using experimental evolution, we investigated how plastic changes in DNA methylation induced by the loss of parental care have evolved in a biparental insect (*Nicrophorus vespilloides*) using experimental evolution. We show that removal of care in a single generation is associated with changes in gene expression in stress-related pathways in 1st instar larvae. However, in larvae that have adapted to the loss of parental care after being deprived of care for 30 generations, gene expression is shifted from stress-related gene expression towards growth and brain development pathways. We found that changes in gene body methylation arose both as a direct response to the loss of parental care and stochastically as populations diverged. Overall, our results suggest that a complex interplay between transcription and DNA methylation shapes the molecular adaptation to environmental change.

Introduction

There has been increasing interest in the idea that epigenetic mechanisms could participate in evolutionary change. Epigenetic mechanisms are the biochemical marks (*e.g.*, DNA methylation, histone modifications) that ultimately determine how accessible DNA is to factors within the cell which, in turn, can regulate gene expression (Holliday, 2006; Jaenisch & Bird, 2003). These processes have been shown to be sensitive to the environment and represent a mechanism through which plasticity can be achieved during development. A hypothesised role for epigenetics in adaptation and evolution is driven primarily from recent work in plants and animals (*e.g.*, *Arabidopsis*, *C. elegans*, mouse) demonstrating the capacity for transgenerational epigenetic inheritance *via* epigenetic modifications. These studies have shown that chronic challenges with stress, dietary changes or chemicals/drugs induce heritable changes in epigenetic marks in the germline that are transmitted to subsequent generations (Curley et al., 2011; Miska & Ferguson-Smith, 2016; Sarkies, 2020). The capacity for such inheritance and the degree to which it persists is highly dependent on the organism and the nature of the epigenetic change in question. Nevertheless, these observations raise the possibility that epigenetic mechanisms could buffer and/or modulate the pace of evolutionary change under changing environments. Indeed, theoretical models have supported this idea (Badyaev & Uller, 2009; Bonduriansky & Day, 2018; Miska & Ferguson-Smith, 2016).

Although these ideas have generated much enthusiasm (and controversy), there is nevertheless limited empirical evidence to support the idea that epigenetic marks are induced by environments and persist in a way that drives long-term adaptive evolutionary change. There have been many observations of population-level differences in epigenetic marks between locally adapted wild populations in a range of diverse taxa (Dixon et al., 2018; Dubin et al., 2015; Heckwolf et al., 2020; Liew et al., 2020; Skinner et al., 2014) which are often correlated with local abiotic environmental conditions (*e.g.*, salinity, temperature, latitude). The accumulation of methylation changes is suggestive of a potential role in adaptation to changing environments. However, there are a number of pathways through which epigenetic marks could participate in adaptative processes and contribute to the emergence of population-level changes. For example, epigenetic marks could be a by-product of transcriptional states or genetic sequence change (Dixon et al., 2018; Dubin et al., 2015; Hu et al., 2021). In this scenario epigenetic modifications are effectors and/or markers rather than drivers of the adaptive change. Taken together, epigenetic modifications could result from a complex interplay between plastic, genetic and stochastic processes acting in the short- and/or long-term (Sarkies, 2020). A critical first step towards understanding the sources of epigenetic variation is to characterise the extent to which epigenetic changes respond to an initial change in the environment, and the degree to which

such changes might persist and be correlated with phenotypic changes (*i.e.*, gene expression) as populations adapt to a changing environment.

Here, we used experimental evolution to examine how patterns of gene expression and DNA methylation are induced and evolve in populations of burying beetles as they adapt to the removal of post-hatching parental care (*i.e.,* a change in the social environment). In natural populations of this locally abundant insect, burying beetle parents raise their young on a carrion nest (formed from a small dead animal, such as a mouse or songbird). There is continuous variation in the level of parental care provisioned (Jarrett, Evans, et al., 2018). At one extreme, parents tend to their offspring throughout their development (including feeding with regurgitated pre-digested carrion) whereas at the other, parents abandon their offspring, leaving them to fend for themselves without any post-hatching care (Eggert et al., 1998). Burying beetles are highly responsive to the levels of parental care they receive within a single generation (Kilner et al., 2015). We have exploited this natural variation in care to establish two types of experimentally evolving populations in the laboratory (each replicated twice), which vary only in the family environment that larvae experience during development, and where the same family environment is created for successive generations within populations. In Full Care populations (FC_{POP}), parents remain with their young throughout development; whereas in No Care populations (NC_{POP}), parents are removed just before their offspring hatch (see Schrader et al., 2017; for more details on the experimental setup). Previous work has found that that NC_{POP} populations rapidly adapt to a life without parental care such that by generation 13 the proportion of successful broods matches that of the FC_{POP} populations (Schrader et al., 2015, 2017). This adaptation to the loss of parental care is associated with a number of changes in morphology and behaviour. For example, NC offspring have evolved larger mandibles which likely helps with feeding on the carcass in the absence of parents (Jarrett, Evans, et al., 2018), hatch more synchronously (Jarrett, Rebar, et al., 2018) and cooperate more with each other (Rebar et al., 2020).

DNA-Me is a highly conserved epigenetic mark across plants and animals. This involves the covalent addition of methyl groups to CG dinucleotides (CpG) within DNA. DNA methylation (mCpG) is added by DNA methyltransferases (DNMTs 1-3; *e.g.*, for maintenance during cell division and *de novo*) and, in mammals, can be actively removed by ten-eleven translocation enzymes (TETs) (Law & Jacobsen, 2010). Although parental care (and its loss) has been associated with plasticity in both DNA-Me and gene expression across a number of species (Arsenault et al., 2018; Franklin et al., 2010; Nyman et al., 2020; Weaver et al., 2006), its role in insect DNA-Me and gene expression plasticity is still poorly understood. Burying beetles have a functioning DNA-Me system with levels of methylation that are comparable to other holometabolous insects and possess single copies of DNMT1-3 (Bewick et al., 2016; Cunningham et al., 2015; Lewis et al., 2020). These enzymes are

106 highly conserved across multiple taxa and methylation occurs in actively transcribed genes
107 suggesting important, yet still poorly understood, roles for DNA-Me in the species that carry
108 this epigenetic modification (Feng et al., 2010; Schmitz et al., 2019).
109 In this study, we first characterized the changes in gene expression might underpin
110 adaptation to the NC environment in developing larvae. To distinguish between evolved and
111 environmentally-induced (plastic) changes, we used a common garden approach where
112 offspring were exposed to the reciprocal parental environment (*i.e.,* FC_{POP} had parents
113 removed to create a NC environment or parents remained post-hatching in NC_{POP} to create a
114 FC environment). We then asked if changes in DNA methylation are correlated with adaptive
115 divergence (see Figure 1a for experimental design summary).
116

117 ## Results

118 **Differential gene expression in response to the sustained removal of care**

119 To quantitatively characterise genome-wide variation in larval gene expression
120 between our populations, we conducted a discriminant analysis of principal components
121 (DAPC) using all expressed genes (n=12,772) within samples. The removal of care was
122 significantly associated with a shift in gene expression (environment posterior mean = 4.22,
123 95% CI = [3.35, 5.15], $p_{MCMC} < 0.001$; Figure 1b) and this response was true whether
124 individuals were derived from the FC or NC populations, as there was no effect of population
125 (population posterior mean = 0.27, 95% CI = [-0.63, 1.20], $p_{MCMC} = 0.576$) nor an interaction
126 between population of origin and the current environment (posterior mean = -7.91, 95% CI =
127 [-2.12, 0.40], $p_{MCMC} = 0.210$). Further, each replicate responded to the care treatment in a
128 similar way, since there was no effect of block on gene expression plasticity (posterior mean
129 = 0.03, 95% CI = [-0.6, 0.72], $p_{MCMC} = 0.940$). Taken together, these results suggest that
130 while there were large shifts in gene expression in response to the absence of parental care
131 in the current environment, evolved changes in gene expression are likely to be more subtle
132 and/or involve smaller subsets of genes.
133 To investigate these differences in more detail we performed differential expression
134 analyses using $FC_{POP}FC_{ENV}$ as the reference population to estimate the number of
135 differentially expressed genes (DEGs) across conditions. We found that 657 genes were
136 differentially expressed when parental care was removed for the first time in the FC_{POP}
137 (n=657; all $\log_2$ fold changes > 1; Figure 1c). By contrast, in the NC_{POP}, which had
138 experienced a NC_{ENV} for the previous 29 generations, 385 genes were differentially
139 expressed which is a significant reduction in response ($X^2(3, n=12772) = 21558$, $p<2 \times 10^{-}$
140 ^{16}). Interestingly, only 20 genes were differentially expressed when NC_{POP} parents were
141 allowed to care for their offspring for the first time in 30 generations, suggesting that gene

142 expression was not constrained by their exposure to divergent selection pressures during
143 these generations.
144 There was significant overlap in identity of the genes being differentially expressed in
145 $FC_{POP}NC_{ENV}$ compared to the $NC_{POP}NC_{ENV}$ (log_2(OR)=122.4265, p<2,2 x 10^{-16}), with many
146 more changes being lost (n=354) than being gained (n=82; Figure 1d). To test whether this
147 reflected changes in the overall strength of response by individual genes to a NC
148 environment within each population, we extracted overlapping DEGs (even those of small
149 effect) and correlated the magnitude of response at each individual gene. This is the
150 equivalent of comparing reaction norms on a genome-wide scale (See Figure 1e inset). We
151 predicted that if the magnitude of change in response to a NC environment was similar
152 across populations then we would expect fold-changes to be highly correlated resulting in a
153 slope close to 1. We found that genes that were differentially expressed in response to a
154 NC_{ENV} in both populations were positively correlated, with no evidence that genes switched
155 direction of expression after evolving without parental care (slope = 0.489, t(3103)=91.875,
156 p<2.2 x 10^{-16}; Figure 1e). However, the slope of this correlation was significantly less than a
157 slope of 1 (F(2,3103)=9176.4, p<2.2 x 10^{-16}). Therefore, while there were similar changes
158 elicited by a NC_{ENV} across both populations, the evolved NC populations expressed those
159 genes at lower levels on average than the FC_{POP}.
160 We extracted significant gene ontology (GO) terms from each contrast and collapsed
161 across significant, but highly redundant terms, to look for overarching patterns. This analysis
162 revealed that changes in gene expression primarily occurred in three broad categories of
163 genes: 1) stress and its cellular response, 2) immune function and 3) growth and
164 development (see Figure 1f for examples of GOs falling within these categories). Taken
165 together, these results suggest that whilst the initial response to the removal of care is
166 associated with an upregulated stress response and increased immune defense, individuals
167 selected under a NC regime express fewer genes associated with these two categories.
168 These changes are accompanied by enhanced expression of genes associated with
169 physiological and neurobiological development (Figure 1g). Moreover, further examination of
170 gene expression changes that emerge after 30 generations of exposure to a NC
171 environment reveal an over-representation in the neurotransmission and neuropeptide
172 categories (*e.g.,* neuropeptide Y, orexin, and glutamate). For full list of GO categories see
173 Supplementary Tables.
174
175 **CpG methylation in the burying beetle**
176 We first sought to characterise the methylomes of first instar burying beetle larvae.
177 We found average CpG methylation across the whole genome to be between 0.7-0.8% for
178 all samples. Average levels of methylation were significantly higher in gene bodies (3.64 ±

179 8.60) compared to coding sequences (CDS; 2.72 ± 10.8), introns (0.952 ± 4.44), repeat/TEs
180 (0.523 ± 1.96) and upstream regions (5' UTR; 0.659 ± 3.22; all FDR-corrected Wilcoxon *p*
181 values < 2.2×10^{-16}; Figure 2a). Moreover, we found that gene body methylation was weakly
182 but positively correlated with gene expression ($t(1,11920)=36.34$, $p<2.2 \times 10^{-16}$, $R^2 = 0.10$).
183 When we classified genes into expression state categories (silent, low, medium or highly
184 expressed; Supp Figure 1a), we found that this correlation was mainly driven by expression
185 states that were classified as medium or low. Medium and high levels of expression did not
186 have significantly different methylation values from each other (Figure 2b, Supp Figure 1b).
187 These findings are consistent with previous reports of methylation residing primarily in gene
188 bodies for this species and the presence of higher methylation levels in active genes in
189 plants and animals (Cunningham et al., 2015; Feng et al., 2010; Lewis et al., 2020).
190 Next, we determined whether the removal of care resulted in methylation changes in
191 specific genomic features. We looked for enrichment of differentially methylated CpGs over
192 the expected distribution of methylated CpGs across genomic features (Figure 2b). A CpG
193 was classified as being methylated if the logit probability of it being methylated was greater
194 than the bisulfite conversion error rate in at least 1 sample (see Methods). The density of
195 methylated CpGs was higher in gene bodies (Figure 2c; $X^2(5, n=7400) = 14786$, $p<2.2 \times 10^{-16}$)
196 and we found differentially methylated CpGs (in both environmental and evolved contrasts)
197 to be significantly enriched in genes and, specifically, introns (FDR-corrected *p*'s<0.05;
198 Figure 1c) but not in any other annotated features tested. We, therefore, chose to focus on
199 gene body methylation for subsequent differential methylation analyses.
200
201 **Differential gene body methylation in response to the sustained removal of care**
202 We performed differential methylation analysis across gene bodies to compare (1)
203 DNA-Me between populations (evolved changes; sustained exposure to a NC_{ENV} over 30
204 generations) with (2) DNA-Me induced by a single generation of exposure to a NC_{ENV} within
205 the FC_{POP} (proxy for initial response) and (3) DNA-Me within the NC_{POP} when exposed to a
206 FC_{ENV} (population-specific responses to NC_{ENV}). As has been previously reported, not all
207 genes in the burying beetle are methylated (Cunningham et al., 2015; Lewis et al., 2020).
208 Therefore, we clustered average weighted methylation values (calculated across the entire
209 gene body), identified two distinct distributions of methylation states within genes (see
210 Methods) and focussed our analyses on those that were classified as methylated (Supp
211 Figure 2a, n = 4431; 39% of genes for which methylation was present for 85% of samples).
212 These genes were also more highly expressed (W = 9161933, $p<2.2 \times 10^{-16}$, Supp Figure 2b)
213 confirming that this procedure extracted functionally-relevant methylated genes.
214 We first compared the initial environmental response to a NC environment (within the
215 FC_{POP}) to the response 30 generations later (evolved differences). Removal of care in

216 response to one generation of a NC_{ENV} resulted in 1486 differentially methylated genes,
217 whereas we found 1530 differences when comparing the evolved response to the sustained
218 loss of care (all genes FDR-corrected $p<0.01$). We also assessed the overall false discovery
219 rate empirically by randomly shuffling samples between conditions 1000 times and
220 extracting the number of differentially methylated genes (DMGs) after FDR correction at
221 each shuffle. The number of DMGs detected in each comparison far exceeded the number
222 that would be expected at random ($p_{FDR} = 0$ for both comparisons; Figure 3a). Moreover, the
223 response to the removal of care was highly similar within each population (overlap of 741
224 DMGs; $\log_2(OR)=1.8$, $p<2.2\times10^{-16}$), though a high proportion of these changes remained
225 specific to populations (Supp Figure 3). Taken together, this suggests that epigenetic
226 variation can arise from environmental, population genetic background as well as an
227 interaction between the two.
228
229 **Differential gene body methylation and transcriptional change**
230 Of the DMGs that were altered in response to a single generation of exposure to NC,
231 51% were significantly associated with differences in gene expression (all DEGs FDR-
232 corrected $p<0.01$; $\log_2(OR)=1.21$, $p=0.003$). However, no significant association with gene
233 expression was identified for responses measured after 30 generations of exposure to NC
234 ($\log_2(OR)=1.16$, p=0.27). GO term enrichment analysis revealed that genes differentially
235 methylated after exposure to one generation of NC were enriched in stress and growth
236 reduction pathways (Figure 3d). Differentially methylated genes in the evolved response to a
237 NC_{ENV} were enriched in biological processes related to growth and brain development
238 (Figure 3e; see Supplementary Tables for a full list of GO terms).
239 Given the high degree of overlap in DMGs induced by the environment compared to
240 the evolved response to the NC_{ENV} 48%; Figure 3b), we hypothesised that the latter set of
241 changes might reflect environmentally-induced changes that are no longer coupled with
242 expression (i.e., methylation reflects ancestral rather than current transcriptional state). We,
243 therefore, tested the overlapping DMGs against changes in gene expression induced by a
244 single generation of exposure to NC. We found that overlapping DMGs are associated with
245 the initial environmental response ($\log_2(OR)=1.17$, $p<0.05$) but not current changes in gene
246 expression ($\log_2(OR)=1.04$, p=n.s).
247 To further investigate the idea that differential methylation can occur in evolved
248 responses without associated differential gene expression, we highlighted differentially
249 methylated genes on density plots describing the relationship between expression (FKPM)
250 and average methylation for all genes. We identified a cluster of genes that was persistently
251 differentially methylated but had lost differential expression after 30 generations of sustained
252 exposure to NC (Figure 3c). Differential methylation in this cluster (outlined in Figure 3c) is

253 associated with differential gene expression in populations exposed to NC for one
254 generation (log_2(OR)=1.91, p = 0.01) but not in the evolved response (log_2(OR)=0,
255 p=4.279e-05). Also differentially methylated at this cluster are genes from individuals in the
256 NC_{POP} that were exposed back to the FC_{ENV} for a single generation suggesting they are
257 environmentally labile (*i.e.*, not inherited) (Supp Figure 4). We performed a GO analysis on
258 this cluster and found these genes are almost exclusively enriched in ribosome biogenesis,
259 protein translation and transport processes (see Figure 3f and Supplementary Tables).
260 We then asked if there was a possibility that environmental responses could become
261 fixed evolved differences. In other words, DMGs that were initially responsive to the
262 environment but no longer environmentally sensitive. We reasoned that such changes would
263 not be reversed when NC_{POP} were provided with a $FC_{ENV.}$ There were 273 DMGs (18% of all
264 evolved DMGs) that were differentially methylated in the evolved but not reverted by
265 exposure of the NC_{POP} to a FC_{ENV}. While these genes were enriched in defense, cellular and
266 RNA metabolic processes these were not significantly associated with gene expression
267 changes in larvae (log_2(OR)=0.97, p=ns). This raises the possibility that some heritable
268 methylation changes could become fixed during adaptive divergence, though the functional
269 consequences of such changes remain unclear.
270

271 **Discussion**

272 A role for epigenetic processes in mediating some of the molecular events underlying
273 adaptive divergence is the subject of intense interest and debate (Bonduriansky & Day,
274 2018; Charlesworth et al., 2017; Laland et al., 2014; Sarkies, 2020). Debate persists
275 because empirical studies testing the relationship between early environmentally-induced
276 epigenetic responses and the subsequent adaptive evolution of such modifications to
277 changing environments have been rare. How and when such changes are adaptive would
278 depend on the persistence, heritability and function of epigenetic modifications during
279 adaptation. Here, using experimental evolution in a wild-caught population of a subsocial
280 insect, the burying beetle, we describe how gene expression and genome-wide patterns of
281 DNA methylation diverge as beetles adapt to the experimentally imposed loss of parental
282 care in the lab. We show that (1) DNA methylation and gene expression is highly sensitive to
283 the loss of parental care, in both the initial and the evolved response (30 generations later),
284 (2) The relationship between gene expression and DNA methylation at methylated genes
285 shifts as populations diverge and (3) There is more variation in DNA methylation between
286 populations (evolved change) and in population-specific responses to the environment than
287 is reflected in gene expression profiles. Taken together, these data suggest that

environmental, genetic and stochastic processes contribute to the accumulation of DNA methylation differences during adaptive divergence.

Evolved changes in gene expression

Levels of parental care can shape the development of a number of physiological and behavioural traits that persist throughout the lifespan, including key fitness-related traits such as lifespan, fecundity and survival (Gustafsson & Sutherland, 1988; Kilner et al., 2015; Steiger, 2013). Conversely, the removal of parental care has been shown to be associated with disruptions to behavioural development *via* changes in gene expression (Franklin et al., 2010; Weaver et al., 2006). Although much of the molecular level data comes from rodent model species, there is evidence for similar changes across primates, fishes and even a subsocial bee suggesting that there might be suites of conserved molecular mediators (Arsenault et al., 2018; Massart et al., 2014; Nyman et al., 2020). Therefore, it is not surprising that the experience of a NC_{ENV} in burying beetles is the major source of variation in larval gene expression particularly in stress response pathways that disrupt growth and development. Adaptation to the loss of parental care in this species appears to be associated with the evolution of a stress-tolerant phenotype because we observe (1) reduced responsiveness of genes in the NC_{POP} to the NC_{ENV} (in terms of levels and number) and (2) the appearance of a small number of changes that may compensate for stress-related gene expression. This is consistent with the idea that adaptation to stressful environments and/or challenges involves fine tuning the balance between stress- and growth-related gene expression (López-Maury et al., 2008).

We further inspected the genes that were differentially expressed in the NC_{POP} but not FC_{POP} in response to the NC_{ENV} (*i.e.*, new DEGs). One striking change was the high expression of a cytochrome P450 gene which appears to be a homolog to the Drosophila *Cyp6a20* gene, the deletion of which has been associated with higher levels of aggression and reduced sociality (Wang et al., 2008). Also, of particular interest was the differential expression of a number of odorant binding proteins. These proteins are odour sensing proteins that have been shown to be involved in a number of behavioural phenotypes. One such highly expressed gene was a homolog to Drosophila *Obp69a* which is involved in social responsiveness to social experience as well as starvation (Bentzur et al., 2018; Farhadian et al., 2012). Changes in the expression of both sets of genes was particularly interesting given that previous studies have shown that adaptation to the loss of parental care is associated with a greater level of coordination among larvae (Jarrett, Rebar, et al., 2018; Schrader et al., 2017) and a shift from sibling conflict to cooperation (Rebar et al., 2020).

325 **Sources of variation in DNA methylation**

326 In rodent model systems, changes in gene expression in developing offspring in
327 response to the loss of parental care have been shown to be, at least in part, mediated by
328 changes in DNA methylation, most notably at promoter regions (Franklin et al., 2010;
329 Massart et al., 2014; Suderman et al., 2012). Here we show that gene body methylation of
330 developing larvae, though less widespread than vertebrate methylation, is similarly sensitive
331 to the loss of parental care in a subsocial insect and that some of these changes are
332 associated with differential gene expression. We further show patterns of DNA methylation
333 diverge as populations adapt to the recurrent loss of parental care over multiple generations.
334 Some of this change is simply induced afresh, each time the population is exposed to an NC
335 environment. We know this because allowing parents to care for NC_{POP} offspring reversed
336 some of these population-specific differences in DNA methylation. Nevertheless, some of
337 the changes in DNA methylation that were induced originally by the environment did appear
338 to become fixed over time in the NC_{POP}. However, while a significant portion of DNA
339 methylation resembles the initial response to the loss of care, many of the changes in DNA
340 methylation that we observed were not explained solely by the removal of care. Some
341 epigenetic changes are short-lived whereas others appear to persist suggesting that there
342 might be functional differences in the timing and duration of such changes throughout the
343 course of adaptation. These findings do not rule out the possibility of adaptive
344 transgenerational inheritance of environmentally-induced methylation but they do suggest
345 that population differences in epigenetic variation could emerge through multiple parallel
346 processes.

347 Population differences in DNA-Me could be due, at least partially, to genetic variation
348 that emerges through drift and/or selection. For example, in sticklebacks, some but not all
349 population-level variation in DNA-Me is correlated with plasticity-induced by differences in
350 water salinity (Heckwolf et al., 2020). Some of these methylation changes have been linked
351 to *cis* or *trans*-acting loci which show evidence of local adaptation (Hu et al., 2021). Similarly,
352 population level changes in gene body methylation that appear in corals inhabiting different
353 quality environments are associated with higher genetic differentiation at those loci (Dixon et
354 al., 2018). Another explanation is that population differences in DNA methylation could have
355 arisen randomly (*i.e.*, epimutations) and then been selected by the environment. This could
356 be made more possible in insects by the reported high heritability of arthropod gene body
357 methylation (Liew et al., 2020; Yagound et al., 2020). Indeed, theoretical models have
358 shown that stochastic but heritable epigenetic variation can be adaptive under changing
359 environments (Feinberg & Irizarry, 2010). In plants, there is evidence for both stochastic,
360 genetic and environmental sources of DNA methylation in explaining local adaptation
361 (Becker et al., 2011; Dubin et al., 2015; Kawakatsu et al., 2016). This further highlights the

362 diverse mechanisms through which these changes in DNA methylation could emerge.
363 Though we were able to distinguish between environmentally-induced and evolved changes
364 in DNA methylation, this lays the groundwork for distinguishing between other (*e.g.*, genetic,
365 epimutational) sources of variation and its functional role.
366 Of particular note is that while a single exposure to the loss of care leads to an
367 association between DNA methylation and gene expression, no such relationship is
368 apparent after populations had diverged and the NC_{POP} had become adapted to a life without
369 parental care. Indeed, over this timeframe, some methylation changes become uncoupled
370 from differential gene expression, which suggests that adaptation to a NC_{ENV} might involve
371 buffering against or overriding environmentally-induced changes in DNA methylation *via*
372 other mechanisms. Our analysis of GO terms suggests that differentially methylated genes
373 between the environmental and evolved conditions broadly reflect the same functional
374 categories involved in divergent gene expression patterns. So while the initial response to
375 the removal of care involves differential methylation in stress-related pathways, evolved
376 differential methylation is enriched in processes associated with growth and brain
377 development though this was not always associated with gene expression at the
378 developmental point at which we measured it. DNA methylation in insects, including the
379 burying beetle, appears to be stable across the lifespan (Cunningham et al., 2019; Yagound
380 et al., 2020). This raises the possibility that DNA methylation may be a product of ancestral
381 transcriptional states. An alternative explanation is that these marks may prime
382 transcriptional responses for activation at different developmental timepoints. What is clear
383 is that while DNA methylation may not always reflect current transcriptional states, it
384 accumulates as populations adapt and diverge and appears to be a marker for past
385 environmental experience.
386
387 **Conclusions**
388 There is accumulating evidence that populations accrue epigenetic variation that is
389 correlated with local abiotic conditions, as they become divergently and locally adapted.
390 These findings, coupled with work showing the transgenerational inheritance of
391 environmentally-induced modifications (primarily in isogenic lab animals and plants) has
392 provoked speculation that epigenetic modifications could play a key causal role in
393 adaptation. An implicit, yet mostly untested, hypothesis here is that epigenetic modifications
394 are inherited, and sustain adaptive divergence through a form of “plasticity-led” evolution.
395 Here, we have taken an experimental approach to test this hypothesis. By manipulating
396 environmental conditions (*i.e.*, the loss of parental care) we have found that a significant
397 amount of variation in DNA methylation can be induced and this is associated with
398 transcriptional activity. However, after several generations of exposure to the same

399 environmental cues, we find that population-level variation and transcription is not solely
400 explained by the environmental differences. These indicate that additional transcriptional
401 and stochastic processes contribute to the accumulation of differential DNA methylation as
402 populations adapt and diverge. In short, although it is still possible that epigenetic variation
403 contributes to adaptation, existing formulations of how this might work in practice are too
404 simplistic to account for the experimental results we obtained.
405

406 ## Methods

407 **Breeding design & Experimental Evolution**

408 We analysed experimental populations of *Nicrophorus vespilloides* that had been evolving
409 under different regimes of parental care, and which were founded from a genetically diverse
410 founding population generated by interbreeding beetles from multiple wild populations
411 across Cambridgeshire. These populations are described in detail in Schrader et al., 2017.
412 and comprise a total of 4 populations, two blocks (Block 1 and Block 2; separated by 1
413 week) containing two populations evolving with (FC_{POP}) or without parental care (NCpop).
414 On the 29th generation, seventeen days after their emergence as adults, when individuals
415 were sexually mature, we paired 15 males and females within each population (N=30 pairs
416 in total). Each pair was placed in a separate breeding box. Each pair was placed in a
417 separate breeding box with moist soil and a thawed carcass (10-12g). We then placed each
418 breeding box in a cupboard, and allowed parents to prepare the carcass and for the female
419 to lay the clutch of eggs. After 53h, populations were split such that both parents were either
420 removed (in keeping with the procedure experienced by the NC_{POP}) or left in the breeding
421 box. This produced offspring from both populations in their evolved condition ($FC_{POP}FC_{ENV}$ &
422 $NC_{POP}NC_{ENV}$) as well as their reciprocal environments ($FC_{POP}NC_{ENV}$, $NC_{POP}FC_{ENV}$). At 80h
423 post-pairing (approx. 10h post-hatching), first-instar larvae were collected from surviving
424 families. Only families that with brood sizes between 20-30 larvae were used for subsequent
425 RNA-sequencing analyses.
426

427 **Larval tissue dissection, RNA extraction & Sequencing**

428 For each family, RNA from four heads of first instar larvae were pooled and extracted using
429 Trizol (Invitrogen). Total RNA quality was checked using the BioAnalyzer System (Agilent),
430 and yield was quantified using a Qubit RNA Assay Kit (Thermo Fisher). PolyA-selected RNA
431 libraries were constructed and sequenced (150bp paired-end) at a depth of 30x by
432 Novogene (Hong Kong). The resulting sample group sizes for gene expression analysis
433 were: 5-6 libraries for each group within each block (total of 46 libraries).
434

435 **RNA Sequencing & Read Mapping**

436 Reads were trimmed using TrimGalore (0.5.0; https://github.com/FelixKrueger/TrimGalore)
437 to remove adaptor sequences, perform quality trimming and discard low-quality reads.
438 Reads were mapped and quantified using a custom pipeline using HiSat2 (2.1.0) and
439 Stringtie (2.0.3; Pertea et al., 2016). Transcript abundance estimation was based on
440 counting reads aligned to the *N. vespilloides* reference transcriptome (NCBI Refseq
441 Assembly: GCF_001412225.1; Cunningham et al., 2015). Lowly expressed genes (those
442 with less than 15 counts in more than 90% of samples) were filtered from raw counts table
443 leaving a total of 12,772 expressed genes in the dataset to be analysed.
444

445 **DAPC using adegnet and MCMCglmm**

446 To compare overall patterns of gene expression plasticity, we performed a discriminant
447 analysis of principal components (DAPC), which linearizes principal components within a
448 dataset into a single discriminant function, enabling the comparison of overall levels of
449 variation between groups. Gene counts were normalized and log-transformed using a
450 regularized log transform in DESeq2 (version 1.26.0; Love et al., 2014) and a discriminant
451 function was built by defining each populations response to their reciprocal environments
452 using adegenet (version 2.1.3; Jombart, 2008). To compare differences in gene expression
453 plasticity between populations we used the MCMCglmm package (version 2.29; Hadfield,
454 2010) to model DAPC score as a function of population background and current
455 environment (and an interaction between the two) using a default prior that assumes a
456 normal posterior distribution with large variances for the fixed effects and a weakly
457 informative (flat) prior.
458

459 **Differential expression analysis**

460 Differential expression was analysed using DESeq2 using custom scripts in R. Log2-fold
461 change estimates for each differentially expressed gene (DEG) were shrunk to generate
462 more conservative estimates of effect size using the ashr shrinkage estimator (Stephens,
463 2016). Moreover, to focus on genes that are differentially expressed at higher thresholds we
464 considered a gene to be significant differentially expressed only if it had increased by a 2-
465 fold change (*i.e.,* Log_2FoldChange >= 1). We included the separate blocks as a covariate in
466 all the analyses to increase power but also account for minor fluctuations in gene expression
467 across the blocks of replicate populations.
468

469 **Functional Annotation**

470 Functional enrichment analyses were conducted using the topGO R package (version
471 2.38.1; Alexa & Rahnenfuhrer, 2016) to identify over-representation of particular functional

472 groups within the DEGs in response to the removal of care as well as the evolved response
473 to the removal of care, based on GO classifications using Fisher's exact test. GO terms were
474 annotated to the *N. vespilloides* genome using the BLAST2GO (version 5.1.1) workflow to
475 assign homologs to the *Drosophila* non-redundant protein databases (Sun et al., 2020). To
476 increase functional predictions of *N. vespilloides* genes these annotations were
477 supplemented with GO term assignments based on ortholog searches within Arthropods
478 using eggNOG-mapper (Huerta-Cepas et al., 2017) and OrthoDB (v10.1; Kriventseva et al.,
479 2019).
480
481 **Bisulfite Sequencing & Read Mapping**
482 For each family, DNA from heads of first instar larvae were pooled and extracted using the
483 Qiagen DNEasy Blood & Tissues kit (Qiagen). Total DNA quality was checked using the
484 BioAnalyzer System (Agilent), and yield was quantified using a Qubit DNA HS Assay Kit
485 (Thermo Fisher). DNA methylation libraries were constructed and sequenced (150bp paired-
486 end) at a depth of 30x by Novogene (Hong Kong). The resulting sample group sizes for
487 gene expression analysis were: 3-4 libraries for each group (pooled from the same families
488 used for RNA-sequencing): $FC_{POP}FC_{ENV}$, $FC_{POP}NC_{ENV}$, $NC_{POP}NC_{ENV}$, $NC_{POP}FC_{ENV}$ (n=3-
489 4/group; total of 14 libraries). Following initial QC (using FastQC v0.11.9), reads were
490 trimmed using TrimGalore (0.6.4) to remove adaptor sequences and poor-quality reads.
491 Bisulfite conversion efficiency across all samples was estimated by Novogene and was
492 between 99.14-99.50% for all samples. Bisulfite-converted reads obtained from each library
493 were mapped to the *N. Vespilloides* genome using Bismark (v0.22.3; Krueger & Andrews,
494 2011) and were quantified following deduplication of reads. Mapping rates for samples to the
495 *N. vespilloides* reference genome were 65.45% ± 5.48, which after deduplication gave an
496 average coverage of 17.78x ± 1.79 (mean ± SD).
497
498 **Differential Methylation Analysis**
499 For differential methylation analysis of individual CpGs we first tested each site in every
500 sample to determine sites that were significantly methylated using a binomial test. We then
501 tested for differential methylation across sites using a weighted binomial glm (*p*-values were
502 adjusted using Benjamini-Hochperg corrections for multiple testing). We then mapped CpGs
503 to their feature using our custom genome annotation (see below for details) and bedtools
504 (Quinlan & Hall, 2010). For analyses of gene body methylation, we collapsed across all CpG
505 sites within each genic region (using bedtools) to compute the average weighted methylation
506 value for each gene for each sample. Genes were clustered into methylated and
507 unmethylated clusters using mixture models using the mixtools R package. To determine
508 which genes were differentially methylated we used a weighted binomial GLM (base R stats

509 package) and adjusted for multiple testing using the Benjamini-Hochberg procedure. Genes
510 were considered differentially methylated if they had an adjusted $p<0.01$. Fishers' exact tests
511 for enrichment were run using the base R stats packages.
512
513 **Genome Annotation**
514 To annotate exons in each genome we used existing annotations, excluding genes that were
515 split across multiple contigs. To annotate regions which may contain promoters or
516 enhancers (5' UTR), we took 1,000 bases upstream of each gene, excluding genes where
517 this exceeded the contig start or end point. We annotated introns based on the position of
518 exons, excluding genes that were split across multiple contigs. To annotate TEs, we used
519 RepeatModeller (v2.0.1) to generate a model of TEs for the *N. vespilloides* genome, and
520 then RepeatMasker (v4.1.0) to annotate TEs based on the model for that genome.
521
522 **Data Access**
523 All raw sequencing data generated have been submitted to the NCBI Gene Expression
524 Omnibus (GEO) under accession number (TBC).

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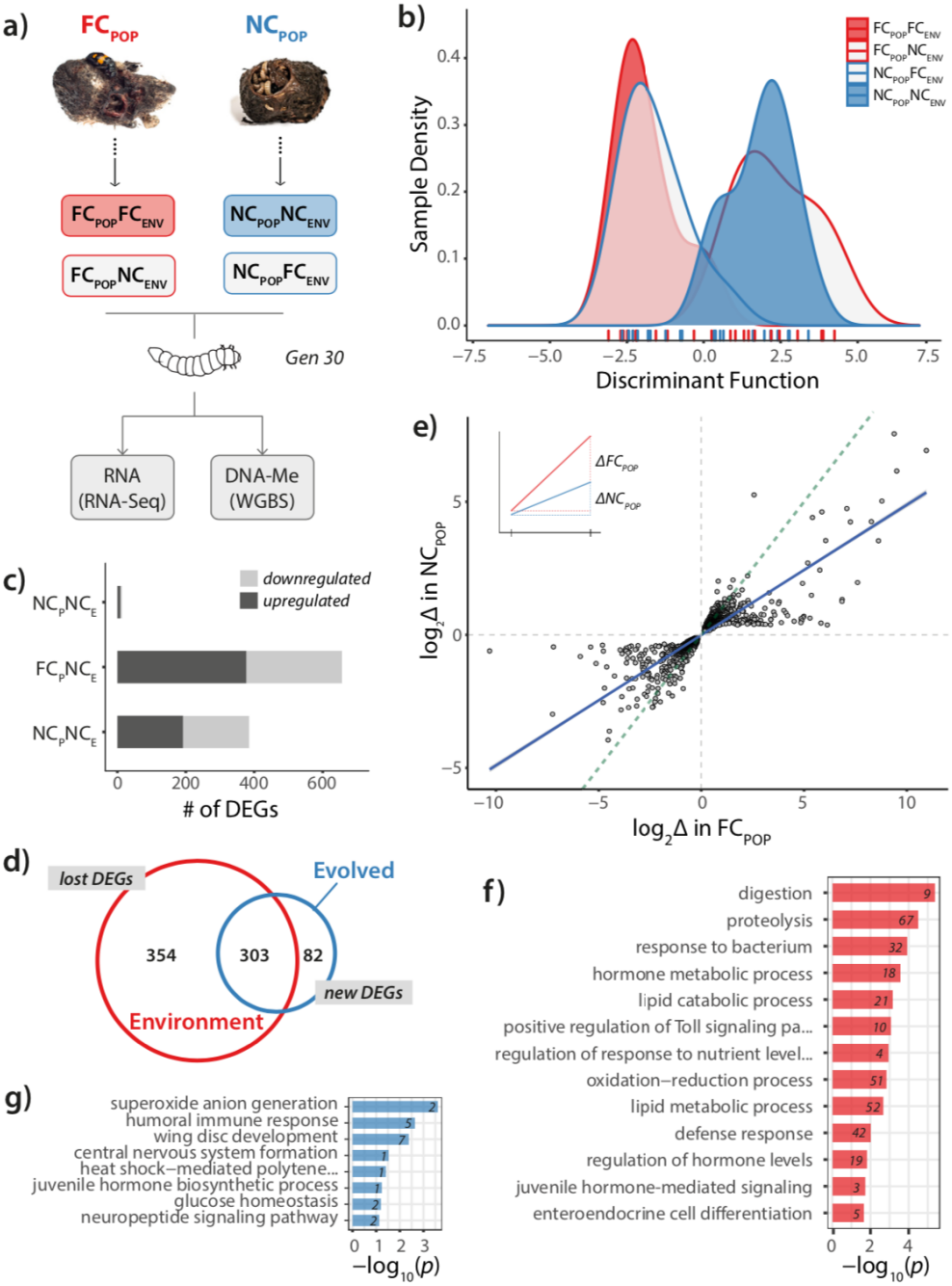
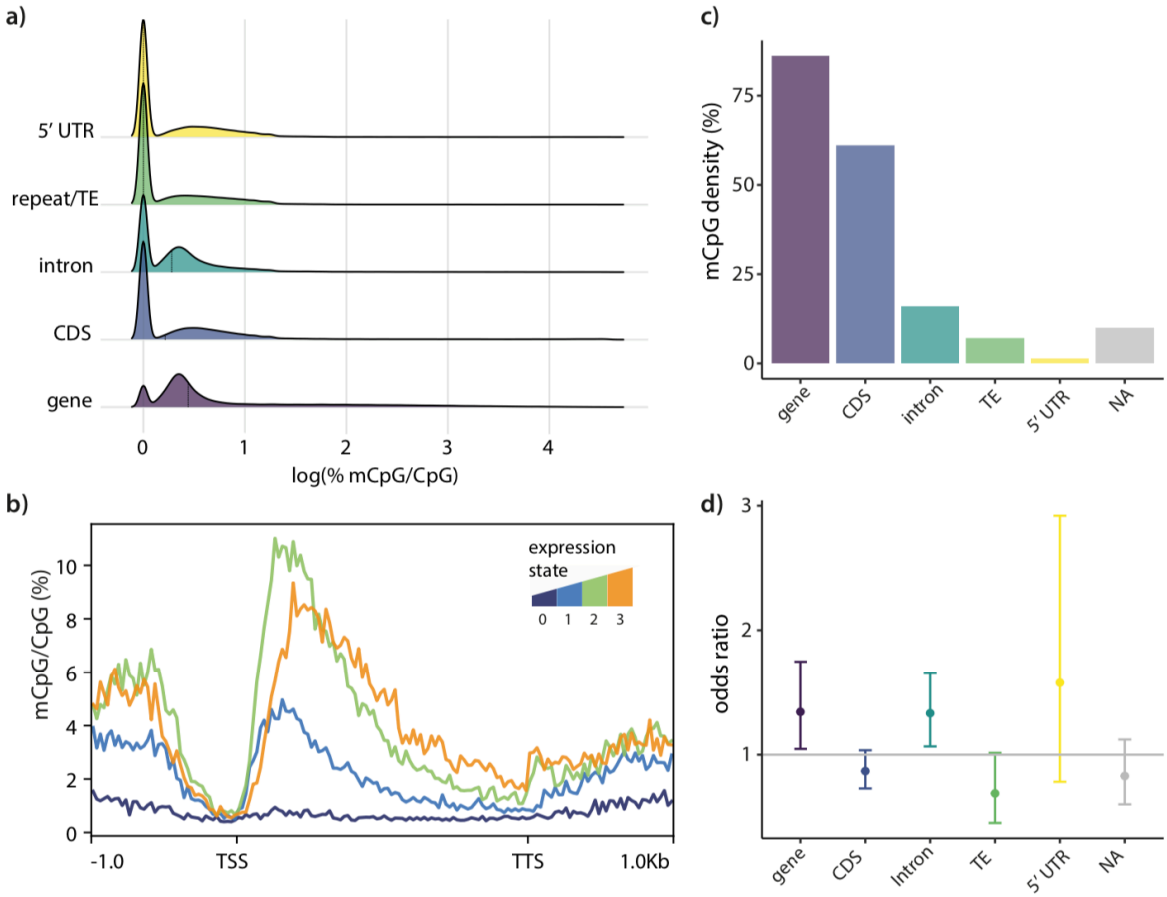
719 **Figure 1**



720

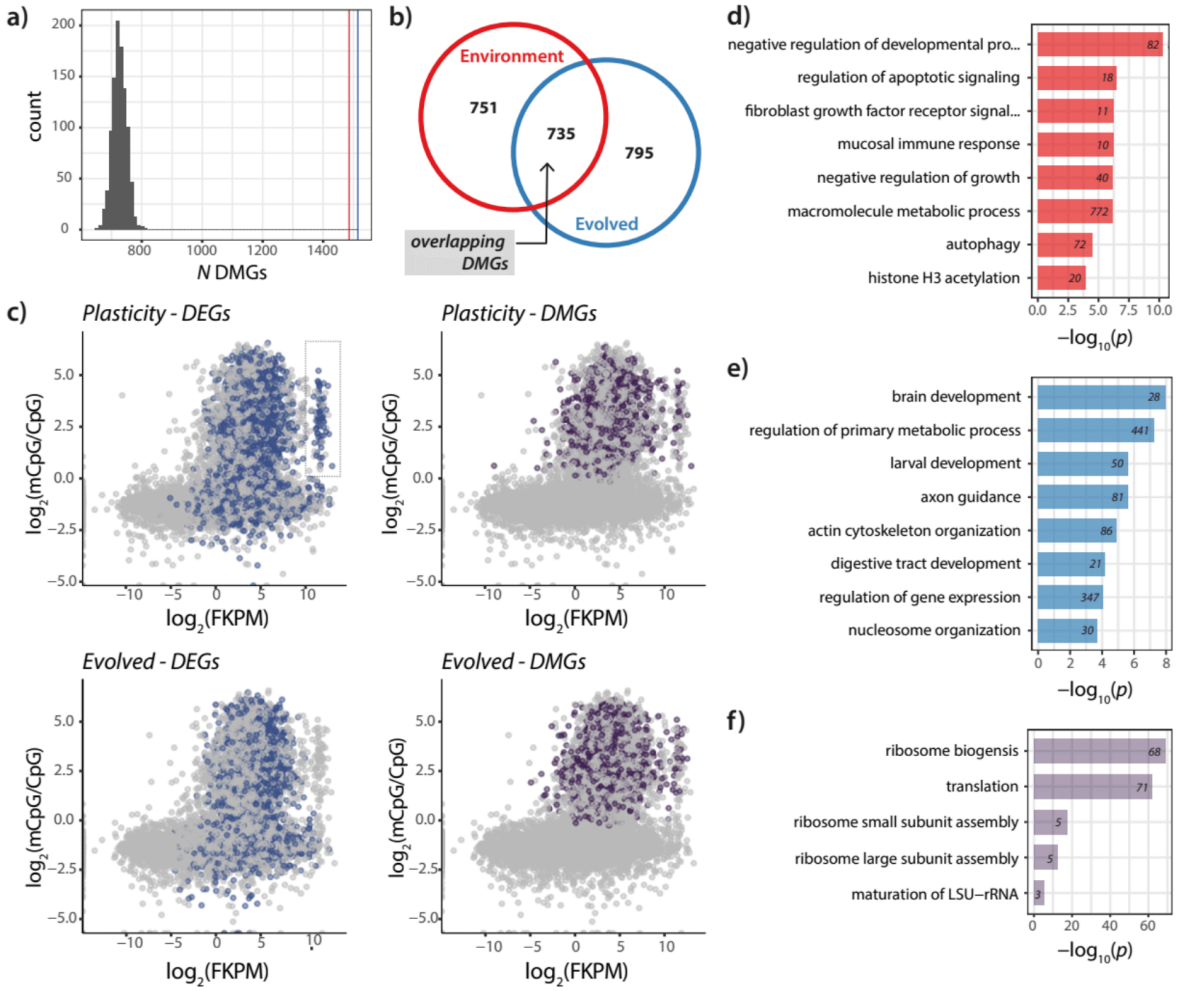
721 **Figure 1. a)** Summary of sampling from experimentally evolving populations and sequencing strategy.
722 Larval heads from Full Care (FC_{POP}) and No Care (NC_{POP}) populations in native ($FC_{POP}FC_{ENV}$;
723 $NC_{POP}NC_{ENV}$) and reciprocal environments ($FC_{POP}NC_{ENV}$; $NC_{POP}FC_{ENV}$) were sampled and used to
724 measure gene expression (RNA-Seq) and DNA methylation (whole-genome bisulfite sequencing;
725 WGBS). **b)** Density plots of discriminant function values for each family indicating that variation in
726 gene expression is primarily due to the current social environment. **c)** Number of differentially
727 expressed genes (DEGs) relative to the $FC_{POP}FC_{ENV}$ group. **d)** Venn diagram indicating degree of
728 overlap between plastic and evolved DEGs. **e)** Scatterplot showing the correlation between log-fold
729 changes ($\log_2\Delta$) in the response to a NC_{ENV} within each population. **f)** Gene ontology (GO; biological
730 processes) enrichment for plastic response to a NC_{ENV}. **g)** GO terms for "new" DEGs following
731 adaptation. Only GO terms with FDR-corrected $p<0.05$ are shown. Numbers indicate number of
732 genes associated with that category.

733 **Figure 2**
734



735
736
737 **Figure 2. a)** Ridge plots showing the distribution of percent methylated CpG levels (mCpG/CpG %) in
738 different genomic features: 1000bp upstream of genes (5' UTR), repeat or transposable elements
739 (TE), introns, coding sequences (CDS) and across the entire gene body (gene). Median mCpG values
740 are indicated by vertical dotted lines. **b)** Average mCpG levels across gene bodies (± 1kb) of genes
741 categories by transcriptional activity: from inactive (0) to genes showing low (1), intermediate (2) and
742 high (3) levels of expression. Plotted regions include 1kb upstream and downstream of transcription
743 start sites (TSS) and transcription termination sites (TTS), respectively for each gene **c)** Bar plot
744 indicating the distribution of methylated CpGs across gene features. **d)** Enrichment scores (odds-
745 ratio; $\log_2$(OR)) for differentially methylated CpGs across different gene features. **Fishers' FDR-*
746 *corrected p<0.05*.

747 **Figure 3**



748

749 **Figure 3. a)** Histogram of number of differentially methylated genes (DMGs) passing FDR-corrected
750 *p*<0.01 when samples are shuffled randomly. Vertical lines indicate number of DMGs found between
751 plastic (red) and evolved contrasts (blue). **b)** Venn diagram indicating degree of overlap between
752 plasticity and evolved DMGs. **c)** The relationship between gene expression (FKPM) and percent CpG
753 methylation (mCpG/CpG) for all genes analysed for plasticity (top) and evolved (bottom) contrasts.
754 Colours indicated differentially expressed genes (DEGs; blue) and DMGs (purple) for each contrast.
755 Gene ontology (GO; biological processes) enrichment for plastic response DMGs **(e)** Evolved DMGs
756 **(f)** and differentially methylated but not differentially expressed in the Evolved contrast **(g)**. Only GO
757 terms with FDR-corrected *p*<0.05 are shown. Numbers indicate number of genes associated with that
758 category.