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 developmental 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 (1,2). 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 (3–6). 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 (4,7,8).

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 (9–13) 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 (9,10,14). 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 (5). 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

69 to the removal of post-hatching parental care (*i.e.,* a change in the social environment). In
70 natural populations of this locally abundant insect, burying beetle parents raise their young
71 on a carrion nest (formed from a small dead animal, such as a mouse or songbird). There is
72 continuous variation in the level of parental care provisioned (15). At one extreme, parents
73 tend to their offspring throughout their development (including feeding with regurgitated pre-
74 digested carrion) whereas at the other, parents abandon their offspring, leaving them to fend
75 for themselves without any post-hatching care (16). Burying beetles are highly responsive to
76 the levels of parental care they receive within a single generation (17). We have exploited
77 this natural variation in care to establish two types of experimentally evolving populations in
78 the laboratory (each replicated twice), which vary only in the family environment that larvae
79 experience during development, and where the same family environment is created for
80 successive generations within populations. In Full Care populations (FC_{POP}), parents remain
81 with their young throughout development; whereas in No Care populations (NC_{POP}), parents
82 are removed just before their offspring hatch (see Schrader et al., 2017; for more details on
83 the experimental setup). Previous work has found that that NC_{POP} populations rapidly adapt
84 to a life without parental care such that by generation 13 the proportion of successful broods
85 matches that of the FC_{POP} populations (18,19). This adaptation to the loss of parental care is
86 associated with a number of changes in morphology and behaviour. For example, NC
87 offspring have evolved larger mandibles which likely helps with feeding on the carcass in the
88 absence of parents (15), hatch more synchronously (20) and cooperate more with each
89 other (21).
90 DNA-Me is a highly conserved epigenetic mark across plants and animals. This
91 involves the covalent addition of methyl groups to CG dinucleotides (CpG) within DNA. DNA
92 methylation (mCpG) is added by DNA methyltransferases (DNMTs 1-3; *e.g.*, for
93 maintenance during cell division and *de novo*) and, in mammals, can be actively removed by
94 ten-eleven translocation enzymes (TETs) (22). Although parental care (and its loss) has
95 been associated with plasticity in both DNA-Me and gene expression across a number of
96 species (23–26), its role in insect DNA-Me and gene expression plasticity is still poorly
97 understood. Burying beetles have a functioning DNA-Me system with levels of methylation
98 that are comparable to other holometabolous insects and possess single copies of DNMT1-3
99 (27–29). These enzymes are highly conserved across multiple taxa and methylation occurs
100 in actively transcribed genes suggesting important, yet still poorly understood, roles for DNA-
101 Me in the species that carry this epigenetic modification (30,31).
102 In this study, we first characterized the changes in gene expression might underpin
103 adaptation to the NC environment in developing larvae. To distinguish between evolved and
104 environmentally-induced (plastic) changes, we used a common garden approach where
105 offspring were exposed to the reciprocal parental environment (*i.e.,* FC_{POP} had parents

removed to create a NC environment or parents remained post-hatching in NC_{POP} to create a FC environment). We then asked if changes in DNA methylation are correlated with adaptive divergence (see Figure 1a for experimental design summary).

Results

Differential gene expression in response to the sustained removal of care

To quantitatively characterise genome-wide variation in larval gene expression between our populations, we conducted a discriminant analysis of principal components (DAPC) using all expressed genes (n=12,772) within samples. The removal of care was significantly associated with a shift in gene expression (environment posterior mean = 4.22, 95% CI = [3.35, 5.15], $p_{MCMC} < 0.001$; Figure 1b) and this response was true whether individuals were derived from the FC or NC populations, as there was no effect of population (population posterior mean = 0.27, 95% CI = [-0.63, 1.20], $p_{MCMC} = 0.576$) nor an interaction between population of origin and the current environment (posterior mean = -7.91, 95% CI = [-2.12, 0.40], $p_{MCMC} = 0.210$). Further, each replicate responded to the care treatment in a similar way, since there was no effect of block on gene expression plasticity (posterior mean = 0.03, 95% CI = [-0.6, 0.72], $p_{MCMC} = 0.940$). Taken together, these results suggest that while there were large shifts in gene expression in response to the absence of parental care in the current environment, evolved changes in gene expression are likely to be more subtle and/or involve smaller subsets of genes.

To investigate these differences in more detail we performed differential expression analyses using $FC_{POP}FC_{ENV}$ as the reference population to estimate the number of differentially expressed genes (DEGs) across conditions. We found that 657 genes were differentially expressed when parental care was removed for the first time in the FC_{POP} (n=657; all $\log_2$ fold changes > 1; Figure 1c). By contrast, in the NC_{POP}, which had experienced a NC_{ENV} for the previous 29 generations, 385 genes were differentially expressed which is a significant reduction in response ($X^2(3, n=12772) = 21558$, $p<2 \times 10^{-16}$). Interestingly, only 20 genes were differentially expressed when NC_{POP} parents were allowed to care for their offspring for the first time in 30 generations, suggesting that gene expression was not constrained by their exposure to divergent selection pressures during these generations.

There was significant overlap in identity of the genes being differentially expressed in $FC_{POP}NC_{ENV}$ compared to the $NC_{POP}NC_{ENV}$ ($\log_2(OR)=122.4265$, $p<2{,}2 \times 10^{-16}$), with many more changes being lost (n=354) than being gained (n=82; Figure 1d). To test whether this reflected changes in the overall strength of response by individual genes to a NC environment within each population, we extracted overlapping DEGs (even those of small effect) and correlated the magnitude of response at each individual gene. This is the

143 equivalent of comparing reaction norms on a genome-wide scale (See Figure 1e inset). We
144 predicted that if the magnitude of change in response to a NC environment was similar
145 across populations then we would expect fold-changes to be highly correlated resulting in a
146 slope close to 1. We found that genes that were differentially expressed in response to a
147 NC_{ENV} in both populations were positively correlated, with no evidence that genes switched
148 direction of expression after evolving without parental care (slope = 0.489, $t(3103)=91.875$,
149 $p<2.2 \times 10^{-16}$; Figure 1e). However, the slope of this correlation was significantly less than a
150 slope of 1 ($F(2,3103)=9176.4$, $p<2.2 \times 10^{-16}$). Therefore, while there were similar changes
151 elicited by a NC_{ENV} across both populations, the evolved NC populations expressed those
152 genes at lower levels on average than the FC_{POP}.
153 We extracted significant gene ontology (GO) terms from each contrast and collapsed
154 across significant, but highly redundant terms, to look for overarching patterns. This analysis
155 revealed that changes in gene expression primarily occurred in three broad categories of
156 genes: 1) stress and its cellular response, 2) immune function and 3) growth and
157 development (see Figure 1f for examples of GOs falling within these categories). Taken
158 together, these results suggest that whilst the initial response to the removal of care is
159 associated with an upregulated stress response and increased immune defense, individuals
160 selected under a NC regime express fewer genes associated with these two categories.
161 These changes are accompanied by enhanced expression of genes associated with
162 physiological and neurobiological development (Figure 1g). Moreover, further examination of
163 gene expression changes that emerge after 30 generations of exposure to a NC
164 environment reveal an over-representation in the neurotransmission and neuropeptide
165 categories (*e.g.,* neuropeptide Y, orexin, and glutamate). For full list of GO categories see
166 Supplementary Tables.
167
168 **CpG methylation in the burying beetle**
169 We first sought to characterise the methylomes of first instar burying beetle larvae.
170 We found average CpG methylation across the whole genome to be between 0.7-0.8% for
171 all samples. Average levels of methylation were significantly higher in gene bodies (3.64 ±
172 8.60) compared to coding sequences (CDS; 2.72 ± 10.8), introns (0.952 ± 4.44), repeat/TEs
173 (0.523 ± 1.96) and upstream regions (5' UTR; 0.659 ± 3.22; all FDR-corrected Wilcoxon *p*
174 values $< 2.2 \times 10^{-16}$; Figure 2a). Moreover, we found that gene body methylation was weakly
175 but positively correlated with gene expression ($t(1,11920)=36.34$, $p<2.2 \times 10^{-16}$, $R^2 = 0.10$).
176 When we classified genes into expression state categories (silent, low, medium or highly
177 expressed; Supp Figure 1a), we found that this correlation was mainly driven by expression
178 states that were classified as medium or low. Medium and high levels of expression did not
179 have significantly different methylation values from each other (Figure 2b, Supp Figure 1b).

180 These findings are consistent with previous reports of methylation residing primarily in gene
181 bodies for this species and the presence of higher methylation levels in gene bodies of
182 genes in plants and animals (28–30).
183 Next, we determined whether the removal of care resulted in methylation changes in
184 specific genomic features. We looked for enrichment of differentially methylated CpGs over
185 the expected distribution of methylated CpGs across genomic features (Figure 2b). A CpG
186 was classified as being methylated if the logit probability of it being methylated was greater
187 than the bisulfite conversion error rate in at least 1 sample (see Methods). The density of
188 methylated CpGs was higher in gene bodies (Figure 2c; $X^2(5, n=7400) = 14786$, $p<2.2\times10^{-16}$)
189 and we found differentially methylated CpGs (in both environmental and evolved contrasts)
190 to be significantly enriched in genes and, specifically, introns (FDR-corrected *p*'s<0.05;
191 Figure 1c) but not in any other annotated features tested. We, therefore, chose to focus on
192 gene body methylation for subsequent differential methylation analyses.
193
194 **Differential gene body methylation in response to the sustained removal of care**
195 We performed differential methylation analysis across gene bodies to compare (1)
196 DNA-Me between populations (evolved changes; sustained exposure to a NC_{ENV} over 30
197 generations) with (2) DNA-Me induced by a single generation of exposure to a NC_{ENV} within
198 the FC_{POP} (proxy for initial response) and (3) DNA-Me within the NC_{POP} when exposed to a
199 FC_{ENV} (population-specific responses to NC_{ENV}). As has been previously reported, not all
200 genes in the burying beetle are methylated (28,29). Therefore, we clustered average
201 weighted methylation values (calculated across the entire gene body), identified two distinct
202 distributions of methylation states within genes (see Methods) and focussed our analyses on
203 those that were classified as methylated (Supp Figure 2a, n = 4431; 39% of genes for which
204 methylation was present for 85% of samples). These genes were also more highly
205 expressed (W = 9161933, $p<2.2\times10^{-16}$, Supp Figure 2b) confirming that this procedure
206 extracted functionally-relevant methylated genes. Consistent with previous findings, genes
207 orthologous to housekeeping genes in *Drosophila melanogaster*, displayed higher
208 methylation levels than those that were not housekeeping gene orthologs ($p<2.2\times10^{-16}$,
209 Wilcoxon Test; Supp Figure 3).
210 We first compared the initial environmental response to a NC environment (within the
211 FC_{POP}) to the response 30 generations later (evolved differences). Removal of care in
212 response to one generation of a NC_{ENV} resulted in 1486 differentially methylated genes,
213 whereas we found 1530 differences when comparing the evolved response to the sustained
214 loss of care (all genes FDR-corrected $p<0.01$). We also assessed the overall false discovery
215 rate empirically by randomly shuffling samples between conditions 1000 times and
216 extracting the number of differentially methylated genes (DMGs) after FDR correction at

217 each shuffle. The number of DMGs detected in each comparison far exceeded the number
218 that would be expected at random (p_{FDR} = 0 for both comparisons; Figure 3a). Moreover, the
219 response to the removal of care was highly similar within each population (overlap of 741
220 DMGs; $\log_2$(OR)=1.8, $p<2.2\times10^{-16}$), though a high proportion of these changes remained
221 specific to populations (Supp Figure 4). Taken together, this suggests that epigenetic
222 variation can arise from environmental, population genetic background as well as an
223 interaction between the two.
224
225 **Differential gene body methylation and transcriptional change**
226 Of the DMGs that were altered in response to a single generation of exposure to NC,
227 51% were significantly associated with differences in gene expression (all DEGs FDR-
228 corrected $p<0.01$; $\log_2$(OR)=1.21, $p=0.003$). However, no significant association with gene
229 expression was identified for responses measured after 30 generations of exposure to NC
230 ($\log_2$(OR)=1.16, p=0.27). GO term enrichment analysis revealed that genes differentially
231 methylated after exposure to one generation of NC were enriched in stress and growth
232 reduction pathways (Figure 3d). Differentially methylated genes in the evolved response to a
233 NC_{ENV} were enriched in biological processes related to growth and brain development
234 (Figure 3e; see Supplementary Tables for a full list of GO terms). Furthermore, genes that
235 were differentially methylated and differentially expressed in response to an environmental
236 shift were significantly enriched (FDR-corrected $p<0.05$, Fisher's Test; Figure 3f) for
237 housekeeping genes compared to methylated genes. Interestingly, this enrichment was lost
238 in genes that were differentially expressed in comparison between the two evolved
239 populations (Figure 3f).
240 Given the high degree of overlap in DMGs induced by the environment compared to
241 the evolved response to the NC_{ENV} 48%; Figure 3b), we hypothesised that the latter set of
242 changes might reflect environmentally-induced changes that are no longer coupled with
243 expression (i.e., methylation reflects ancestral rather than current transcriptional state). We,
244 therefore, tested the overlapping DMGs against changes in gene expression induced by a
245 single generation of exposure to NC. We found that overlapping DMGs are associated with
246 the initial environmental response ($\log_2$(OR)=1.17, $p<0.05$) but not current changes in gene
247 expression ($\log_2$(OR)=1.04, p=n.s).
248 To further investigate the idea that differential methylation can occur in evolved
249 responses without associated differential gene expression, we highlighted differentially
250 methylated genes on density plots describing the relationship between expression (FKPM)
251 and average methylation for all genes. We identified a cluster of genes that was persistently
252 differentially methylated but had lost differential expression after 30 generations of sustained
253 exposure to NC (Figure 3c). Differential methylation in this cluster (outlined in Figure 3c) is

254 associated with differential gene expression in populations exposed to NC for one
255 generation (log_2(OR)=1.91, p = 0.01) but not in the evolved response (log_2(OR)=0,
256 p=4.279e-05). Also differentially methylated at this cluster are genes from individuals in the
257 NC_{POP} that were exposed back to the FC_{ENV} for a single generation suggesting they are
258 environmentally labile (*i.e.*, not inherited) (Supp Figure 5). We performed a GO analysis on
259 this cluster and found these genes are almost exclusively enriched in ribosome biogenesis,
260 protein translation and transport processes (see Figure 3f and Supplementary Tables).
261 We then asked if there was a possibility that environmental responses could become
262 fixed evolved differences. In other words, DMGs that were initially responsive to the
263 environment but no longer environmentally sensitive. We reasoned that such changes would
264 not be reversed when NC_{POP} were provided with a $FC_{ENV.}$ There were 273 DMGs (18% of all
265 evolved DMGs) that were differentially methylated in the evolved but not reverted by
266 exposure of the NC_{POP} to a FC_{ENV}. While these genes were enriched in defense, cellular and
267 RNA metabolic processes these were not significantly associated with gene expression
268 changes in larvae (log_2(OR)=0.97, p=ns). This raises the possibility that some heritable
269 methylation changes could become fixed during adaptive divergence, though the functional
270 consequences of such changes remain unclear.
271
272 **Discussion**
273 A role for epigenetic processes in mediating some of the molecular events underlying
274 adaptive divergence is the subject of intense interest and debate (5,8,32,33). Debate
275 persists because empirical studies testing the relationship between early environmentally-
276 induced epigenetic responses and the subsequent adaptive evolution of such modifications
277 to 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
288 environmental, genetic and stochastic processes contribute to the accumulation of DNA
289 methylation differences during adaptive divergence.

290

291 **Evolved changes in gene expression**

292 Levels of parental care can shape the development of a number of physiological and
293 behavioural traits that persist throughout the lifespan, including key fitness-related traits such
294 as lifespan, fecundity and survival (17,26,34,35). Conversely, the removal of parental care
295 has been shown to be associated with disruptions to behavioural development *via* changes
296 in gene expression (24,26). Although much of the molecular level data comes from rodent
297 model species, there is evidence for similar changes across primates, fishes and even a
298 subsocial bee suggesting that there might be suites of conserved molecular mediators
299 (23,25,36). Therefore, it is not surprising that the experience of a NC_{ENV} in burying beetles is
300 the major source of variation in larval gene expression particularly in stress response
301 pathways that disrupt growth and development. Adaptation to the loss of parental care in this
302 species appears to be associated with the evolution of a stress-tolerant phenotype because
303 we observe (1) reduced responsiveness of genes in the NC_{POP} to the NC_{ENV} (in terms of
304 levels and number) and (2) the appearance of a small number of changes that may
305 compensate for stress-related gene expression. This is consistent with the idea that
306 adaptation to stressful environments and/or challenges involves fine tuning the balance
307 between stress- and growth-related gene expression (López-Maury et al., 2008).

308 We further inspected the genes that were differentially expressed in the NC_{POP} but
309 not FC_{POP} in response to the NC_{ENV} (*i.e.*, new DEGs). One striking change was the high
310 expression of a cytochrome P450 gene which appears to be a homolog to the Drosophila
311 *Cyp6a20* gene, the deletion of which has been associated with higher levels of aggression
312 and reduced sociality (37). Also, of particular interest was the differential expression of a
313 number of odorant binding proteins. These proteins are odour sensing proteins that have
314 been shown to be involved in a number of behavioural phenotypes. One such highly
315 expressed gene was a homolog to Drosophila *Obp69a* which is involved in social
316 responsiveness to social experience as well as starvation (38,39). Changes in the
317 expression of both sets of genes was particularly interesting given that previous studies
318 have shown that adaptation to the loss of parental care is associated with a greater level of
319 coordination among larvae (18,20) and a shift from sibling conflict to cooperation (21).

320

321 **Sources of variation in DNA methylation**

322 In rodent model systems, changes in gene expression in developing offspring in
323 response to the loss of parental care have been shown to be, at least in part, mediated by
324 changes in DNA methylation, most notably at promoter regions (24,36,40). Here we show
325 that gene body methylation of developing larvae, though less widespread than vertebrate
326 methylation, is similarly sensitive to the loss of parental care in a subsocial insect and that

327 some of these changes are associated with differential gene expression. We further show
328 patterns of DNA methylation diverge as populations adapt to the recurrent loss of parental
329 care over multiple generations. Some of this change is simply induced afresh, each time the
330 population is exposed to an NC environment. We know this because allowing parents to
331 care for NC_{POP} offspring reversed some of these population-specific differences in DNA
332 methylation. Nevertheless, some of the changes in DNA methylation that were induced
333 originally by the environment did appear to become fixed over time in the NC_{POP}. However,
334 while a significant portion of DNA methylation resembles the initial response to the loss of
335 care, many of the changes in DNA methylation that we observed were not explained solely
336 by the removal of care. Some epigenetic changes are short-lived whereas others appear to
337 persist suggesting that there might be functional differences in the timing and duration of
338 such changes throughout the course of adaptation. These findings do not rule out the
339 possibility of adaptive transgenerational inheritance of environmentally-induced methylation
340 but they do suggest that population differences in epigenetic variation could emerge through
341 multiple parallel processes.
342 Population differences in DNA-Me could be due, at least partially, to genetic variation
343 that emerges through drift and/or selection. For example, in sticklebacks, some but not all
344 population-level variation in DNA-Me is correlated with plasticity-induced by differences in
345 water salinity (11). Some of these methylation changes have been linked to *cis* or *trans*-
346 acting loci which show evidence of local adaptation (14). Similarly, population level changes
347 in gene body methylation that appear in corals inhabiting different quality environments are
348 associated with higher genetic differentiation at those loci (9). Another explanation is that
349 population differences in DNA methylation could have arisen randomly (*i.e.*, epimutations)
350 and then been selected by the environment. This could be made more possible in insects by
351 the reported high heritability of arthropod gene body methylation (12,41). Indeed, theoretical
352 models have shown that stochastic but heritable epigenetic variation can be adaptive under
353 changing environments (42). In plants, there is evidence for both stochastic, genetic and
354 environmental sources of DNA methylation in explaining local adaptation (10,43,44). This
355 further highlights the diverse mechanisms through which these changes in DNA methylation
356 could emerge. Though we were able to distinguish between environmentally-induced and
357 evolved changes in DNA methylation, this lays the groundwork for distinguishing between
358 other (*e.g.*, genetic, epimutational) sources of variation and its functional role.
359 Of particular note is that while a single exposure to the loss of care leads to an
360 association between DNA methylation and gene expression, no such relationship is
361 apparent after populations had diverged and the NC_{POP} had become adapted to a life without
362 parental care. Indeed, over this timeframe, some methylation changes become uncoupled
363 from differential gene expression, which suggests that adaptation to a NC_{ENV} might involve

364 buffering against or overriding environmentally-induced changes in DNA methylation *via*
365 other mechanisms. Our analysis of GO terms suggests that differentially methylated genes
366 between the environmental and evolved conditions broadly reflect the same functional
367 categories involved in divergent gene expression patterns. So while the initial response to
368 the removal of care involves differential methylation in stress-related pathways, evolved
369 differential methylation is enriched in processes associated with growth and brain
370 development though this was not always associated with gene expression at the
371 developmental point at which we measured it. DNA methylation in insects, including the
372 burying beetle, appears to be stable across the lifespan (41,45). This raises the possibility
373 that DNA methylation may be a product of ancestral transcriptional states. An alternative
374 explanation is that these marks may prime transcriptional responses for activation at
375 different developmental timepoints. What is clear is that while DNA methylation may not
376 always reflect current transcriptional states, it accumulates as populations adapt and diverge
377 and appears to be a marker for past environmental experience.
378
379 **Conclusions**
380 There is accumulating evidence that populations accrue epigenetic variation that is
381 correlated with local abiotic conditions, as they become divergently and locally adapted.
382 These findings, coupled with work showing the transgenerational inheritance of
383 environmentally-induced modifications (primarily in isogenic lab animals and plants) has
384 provoked speculation that epigenetic modifications could play a key causal role in
385 adaptation. An implicit, yet mostly untested, hypothesis here is that epigenetic modifications
386 are inherited, and sustain adaptive divergence through a form of “plasticity-led” evolution.
387 Here, we have taken an experimental approach to test this hypothesis. By manipulating
388 environmental conditions (*i.e.*, the loss of parental care) we have found that a significant
389 amount of variation in DNA methylation can be induced and this is associated with
390 transcriptional activity. However, after several generations of exposure to the same
391 environmental cues, we find that population-level variation and transcription is not solely
392 explained by the environmental differences. These indicate that additional transcriptional
393 and stochastic processes contribute to the accumulation of differential DNA methylation as
394 populations adapt and diverge. In short, although it is still possible that epigenetic variation
395 contributes to adaptation, existing formulations of how this might work in practice are too
396 simplistic to account for the experimental results we obtained.
397

398 **Methods**

399 **Breeding design & Experimental Evolution**

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

418

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

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

426

427 **RNA Sequencing & Read Mapping**

428 Reads were trimmed using TrimGalore (0.5.0; https://github.com/FelixKrueger/TrimGalore)
429 to remove adaptor sequences, perform quality trimming and discard low-quality reads.
430 Reads were mapped and quantified using a custom pipeline using HiSat2 (2.1.0) and
431 Stringtie (2.0.3; Pertea et al., 2016). Transcript abundance estimation was based on
432 counting reads aligned to the *N. vespilloides* reference transcriptome (NCBI Refseq
433 Assembly: GCF_001412225.1; Cunningham et al., 2015). Lowly expressed genes (those

with less than 15 counts in more than 90% of samples) were filtered from raw counts table leaving a total of 12,772 expressed genes in the dataset to be analysed.

DAPC using adegnet and MCMCglmm

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

Differential expression analysis

Differential expression was analysed using DESeq2 using custom scripts in R. Log2-fold change estimates for each differentially expressed gene (DEG) were shrunk to generate more conservative estimates of effect size using the ashr shrinkage estimator (50). Moreover, to focus on genes that are differentially expressed at higher thresholds we considered a gene to be significant differentially expressed only if it had increased by a 2-fold change (*i.e.,* Log_2FoldChange >= 1). We included the separate blocks as a covariate in all the analyses to increase power but also account for minor fluctuations in gene expression across the blocks of replicate populations.

Functional Annotation

Functional enrichment analyses were conducted using the topGO R package (version 2.38.1; Alexa & Rahnenfuhrer, 2016) to identify over-representation of particular functional groups within the DEGs in response to the removal of care as well as the evolved response to the removal of care, based on GO classifications using Fisher's exact test. GO terms were annotated to the *N. vespilloides* genome using the BLAST2GO (version 5.1.1) workflow to assign homologs to the *Drosophila* non-redundant protein databases (51). To increase functional predictions of *N. vespilloides* genes these annotations were supplemented with GO term assignments based on ortholog searches within Arthropods using eggNOG-mapper (52) and OrthoDB (v10.1; Kriventseva et al., 2019).

471

472 **Bisulfite Sequencing & Read Mapping**

473 For each family, DNA from heads of first instar larvae were pooled and extracted using the
474 Qiagen DNEasy Blood & Tissues kit (Qiagen). Total DNA quality was checked using the
475 BioAnalyzer System (Agilent), and yield was quantified using a Qubit DNA HS Assay Kit
476 (Thermo Fisher). DNA methylation libraries were constructed and sequenced (150bp paired-
477 end) at a depth of 30x by Novogene (Hong Kong). The resulting sample group sizes for
478 gene expression analysis were: 3-4 libraries for each group (pooled from the same families
479 used for RNA-sequencing): $FC_{POP}FC_{ENV}$, $FC_{POP}NC_{ENV}$, $NC_{POP}NC_{ENV}$, $NC_{POP}FC_{ENV}$ (n=3-
480 4/group; total of 14 libraries). Following initial QC (using FastQC v0.11.9), reads were
481 trimmed using TrimGalore (0.6.4) to remove adaptor sequences and poor-quality reads.
482 Bisulfite conversion efficiency across all samples was estimated by Novogene and was
483 between 99.14-99.50% for all samples. Bisulfite-converted reads obtained from each library
484 were mapped to the *N. Vespilloides* genome using Bismark (v0.22.3; Krueger & Andrews,
485 2011) and were quantified following deduplication of reads. Mapping rates for samples to the
486 *N. vespilloides* reference genome were 65.45% ± 5.48, which after deduplication gave an
487 average coverage of 17.78x ± 1.79 (mean ± SD).

488

489 **Differential Methylation Analysis**

490 For differential methylation analysis of individual CpGs we first tested each site in every
491 sample to determine sites that were significantly methylated using a binomial test. We then
492 tested for differential methylation across sites using a weighted binomial glm (*p*-values were
493 adjusted using Benjamini-Hochperg corrections for multiple testing). We then mapped CpGs
494 to their feature using our custom genome annotation (see below for details) and bedtools
495 (55). For analyses of gene body methylation, we collapsed across all CpG sites within each
496 genic region (using bedtools) to compute the average weighted methylation value for each
497 gene for each sample. Genes were clustered into methylated and unmethylated clusters
498 using mixture models using the mixtools R package. To determine which genes were
499 differentially methylated we used a weighted binomial GLM (base R stats package) and
500 adjusted for multiple testing using the Benjamini-Hochberg procedure. Genes were
501 considered differentially methylated if they had an adjusted $p<0.01$. Fishers' exact tests for
502 enrichment were run using the base R stats packages.

503

504 **Genome Annotation**

505 To annotate exons in each genome we used existing annotations, excluding genes that were
506 split across multiple contigs. To annotate regions which may contain promoters or
507 enhancers (5' UTR), we took 1,000 bases upstream of each gene, excluding genes where

508 this exceeded the contig start or end point. We annotated introns based on the position of
509 exons, excluding genes that were split across multiple contigs. To annotate TEs, we used
510 RepeatModeller (v2.0.1) to generate a model of TEs for the *N. vespilloides* genome, and
511 then RepeatMasker (v4.1.0) to annotate TEs based on the model for that genome.
512

513 **Data Access & Code Availability**

514 All raw sequencing data generated have been submitted to the NCBI Gene Expression
515 Omnibus (GEO) under accession number (GSE171776). All code for the analyses contained
516 within this paper can be found at: https://github.com/r-mashoodh/nves_meth_evol.
517
518
519

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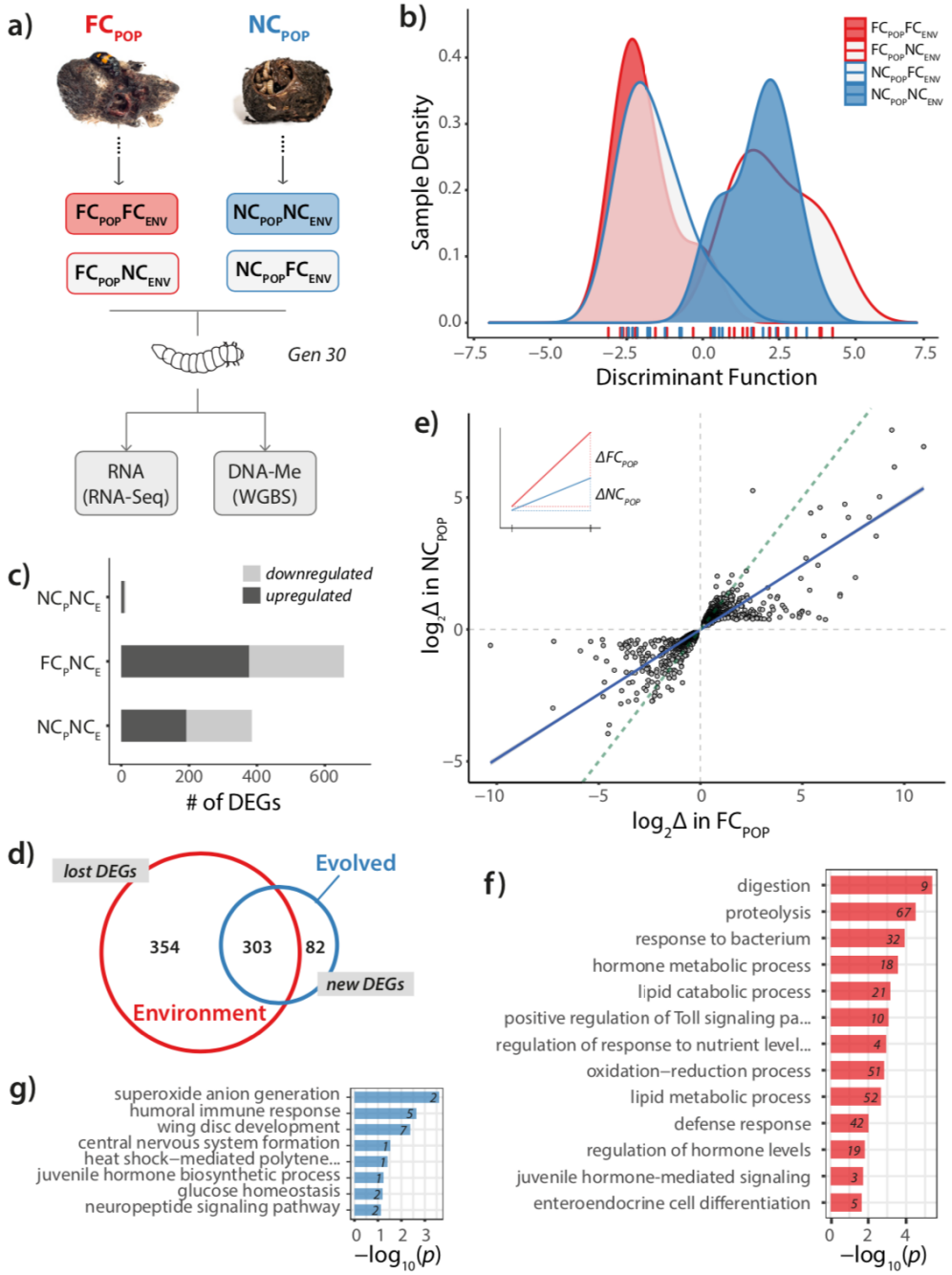
673

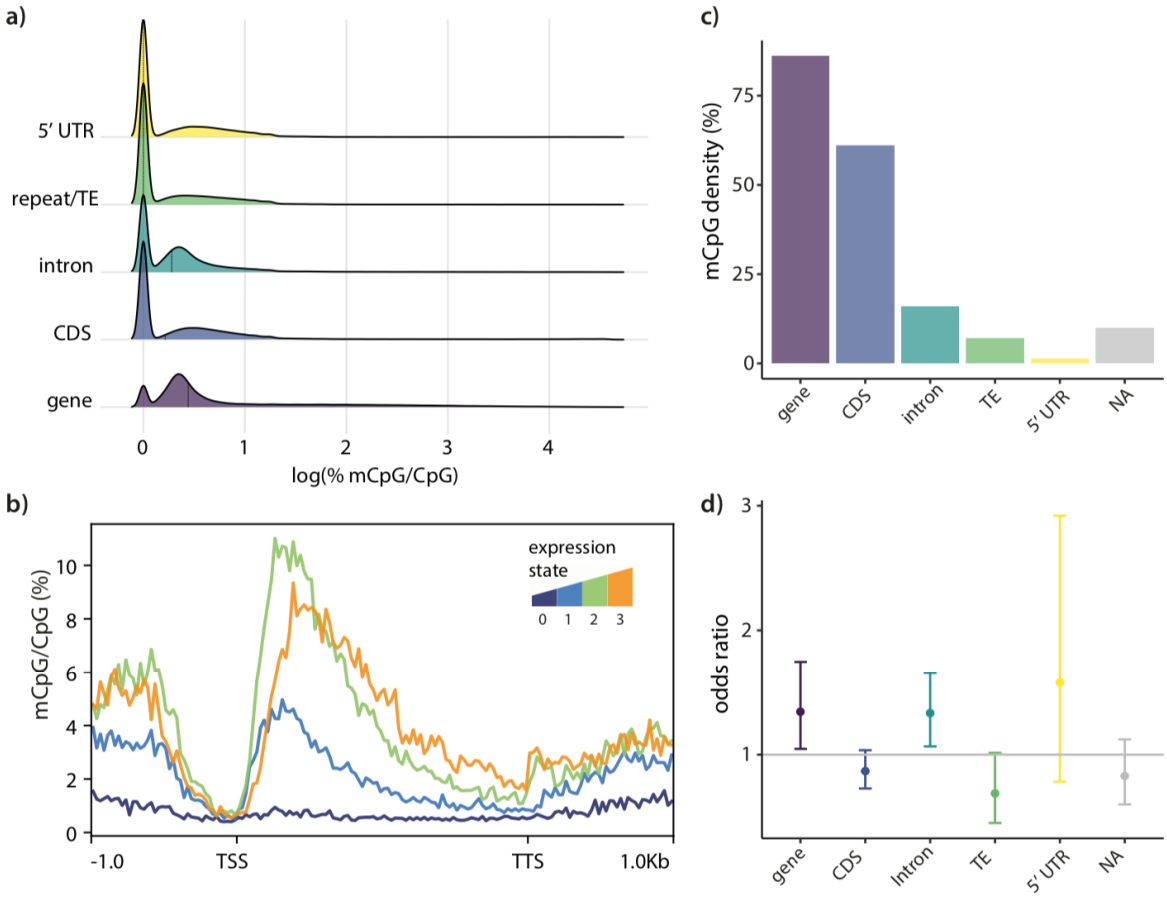
674 **Figure 1**



675

676 **Figure 1. a)** Summary of sampling from experimentally evolving populations and sequencing strategy.
677 Larval heads from Full Care (FC_{POP}) and No Care (NC_{POP}) populations in native ($FC_{POP}FC_{ENV}$;
678 $NC_{POP}NC_{ENV}$) and reciprocal environments ($FC_{POP}NC_{ENV}$; $NC_{POP}FC_{ENV}$) were sampled and used to
679 measure gene expression (RNA-Seq) and DNA methylation (whole-genome bisulfite sequencing;
680 WGBS). **b)** Density plots of discriminant function values for each family indicating that variation in
681 gene expression is primarily due to the current social environment. **c)** Number of differentially
682 expressed genes (DEGs) relative to the $FC_{POP}FC_{ENV}$ group. **d)** Venn diagram indicating degree of
683 overlap between plastic and evolved DEGs. **e)** Scatterplot showing the correlation between log-fold
684 changes ($\log_2\Delta$) in the response to a NC_{ENV} within each population. **f)** Gene ontology (GO; biological
685 processes) enrichment for plastic response to a NC_{ENV}. **g)** GO terms for "new" DEGs following
686 adaptation. Only GO terms with FDR-corrected $p<0.05$ are shown. Numbers indicate number of
687 genes associated with that category.

688 **Figure 2**
689



690
691
692 **Figure 2. a)** Ridge plots showing the distribution of percent methylated CpG levels (mCpG/CpG %) in
693 different genomic features: 1000bp upstream of genes (5' UTR), repeat or transposable elements
694 (TE), introns, coding sequences (CDS) and across the entire gene body (gene). Median mCpG values
695 are indicated by vertical dotted lines. **b)** Average mCpG levels across gene bodies (± 1kb) of genes
696 categories by transcriptional activity: from inactive (0) to genes showing low (1), intermediate (2) and
697 high (3) levels of expression. Plotted regions include 1kb upstream and downstream of transcription
698 start sites (TSS) and transcription termination sites (TTS), respectively for each gene **c)** Bar plot
699 indicating the distribution of methylated CpGs across gene features. **d)** Enrichment scores (odds-
700 ratio; $\log_2$(OR)) for differentially methylated CpGs across different gene features. **Fishers' FDR-*
701 *corrected p<0.05*.

Figure 3

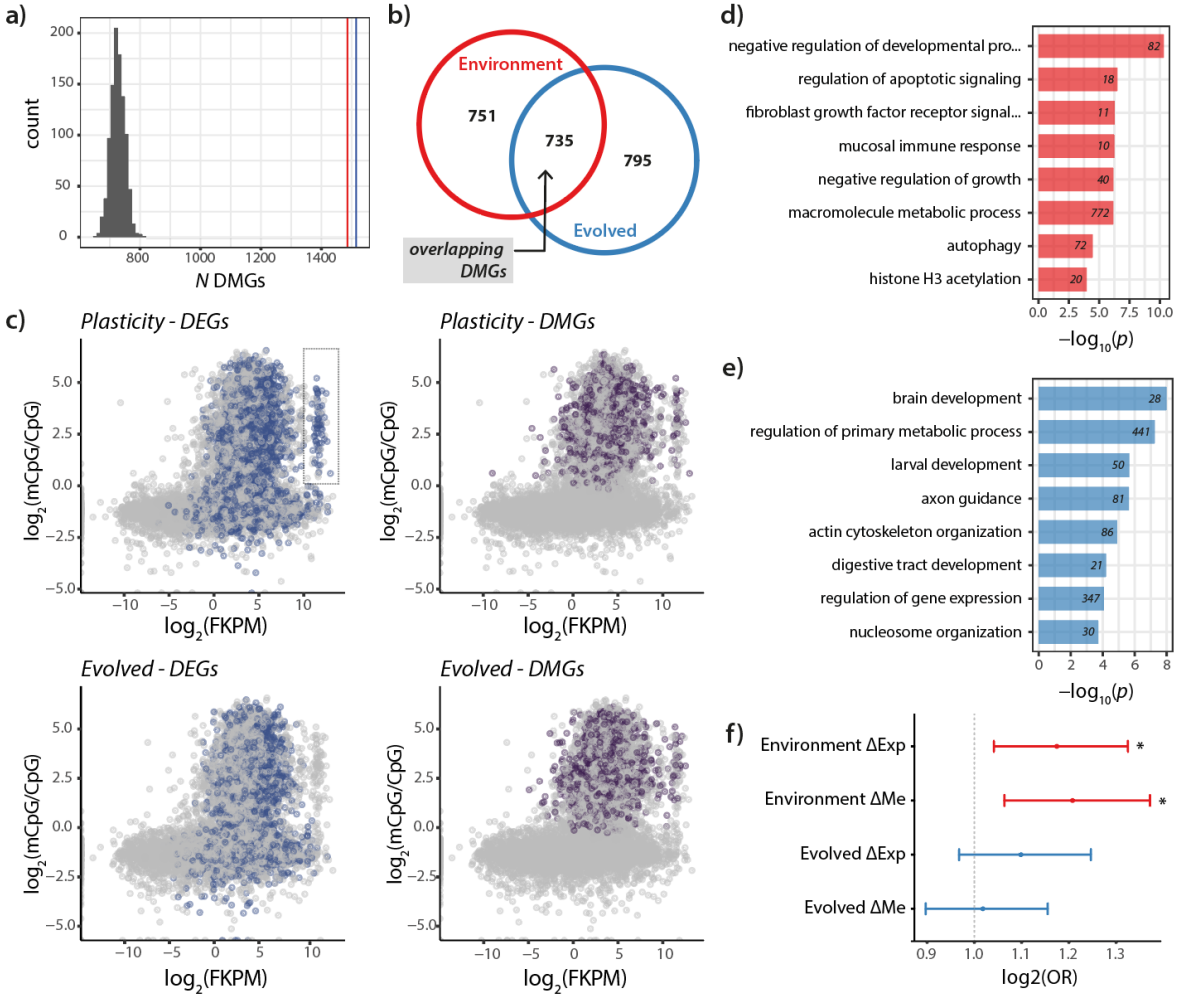


Figure 3. a) Histogram of number of differentially methylated genes (DMGs) passing FDR-corrected *p*<0.01 when samples are shuffled randomly. Vertical lines indicate number of DMGs found between plastic (red) and evolved contrasts (blue). **b)** Venn diagram indicating degree of overlap between plasticity and evolved DMGs. **c)** The relationship between gene expression (FKPM) and percent CpG methylation (mCpG/CpG) for all genes analysed for plasticity (top) and evolved (bottom) contrasts. Colours indicated differentially expressed genes (DEGs; blue) and DMGs (purple) for each contrast. **d)** Gene ontology (GO; biological processes) enrichment for environmental response to the loss of care DMGs and **(e)** Evolved DMGs. Only GO terms with FDR-corrected *p*<0.05 are shown. Numbers indicate number of genes associated with that category. **f)** Enrichment scores for associations between differential expression (ΔExp) and differential methylation (ΔMe) in housekeeping genes (* indicates corrected *p*<0.05).