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1 # Environmentally-induced DNA methylation is inherited across generations in an
2 aquatic keystone species (*Daphnia magna*)

3

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26 **Key words:**

27 epigenetics, extra-genetic inheritance, transgenerational plasticity, maternal effects, whole-

28 genome bisulfite sequencing, stress response, crustacean;

29

30 **Short title:**

31 Transgenerational inheritance of naturally-induced epigenetic marks

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32 **Abstract**

33 Environmental stress can result in epigenetic modifications that are passed down several
34 generations. Such epigenetic inheritance can have significant impact on eco-evolutionary
35 dynamics, but the phenomenon remains controversial in ecological model systems. Here, we
36 used whole-genome bisulfite sequencing on individual water fleas (*Daphnia magna*) to assess
37 whether environmentally-induced DNA methylation can persist for up to four generations.
38 Genetically identical females were exposed to a control treatment, one of three natural
39 stressors (high temperature, zinc, microcystin), or the methylation-inhibitor 5-azacytidine.
40 After exposure, lines were propagated clonally for four generations under control conditions.
41 We identified between 70 and 225 differentially methylated CpG positions (DMPs) between
42 controls and F1 individuals whose mothers (and therefore they themselves as germ cells)
43 were exposed to one of the three natural stressors. Between 46% and 58% of these
44 environmentally-induced DMPs persisted until generation F4 without attenuation in their
45 magnitude of differential methylation. DMPs were enriched in exons and largely stressor-
46 specific, suggesting a possible role in environment-dependent gene regulation. In contrast,
47 treatment with the compound 5-azacytidine demonstrated that pervasive hypo-methylation
48 upon exposure is reset almost completely after a single generation. These results suggest that
49 environmentally-induced DNA methylation is non-random and stably inherited across
50 generations in *Daphnia*, making epigenetic inheritance a putative factor in the eco-
51 evolutionary dynamics of fresh-water communities.

52

53 **Author summary**

54 Water fleas are important keystone species mediating eco-evolutionary dynamics in lakes and
55 ponds. It is currently an open question in how far epigenetic inheritance contributes to the
56 ability of *Daphnia* populations to adapt to environmental stress. Using a range of naturally

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57 occurring stressors and a multi-generational design, we show that environmentally-induced
58 DNA methylation variants are stably inherited for at least four generations in *Daphnia*
59 *magna*. The induced variation in DNA methylation are stressor-specific and almost
60 exclusively found in exons, bearing the signatures of functional adaptations. Our findings
61 imply that ecological adaptations of *Daphnia* to seasonal fluctuations can be underpinned by
62 epigenetic inheritance of DNA methylation without changes in gene frequencies.

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63 **Introduction**

64 Environmental stress can cause systemic changes in development and physiology. Such
65 changes have been shown to occasionally span several generations [e.g., 1, 2, 3]. Many of the
66 responses involve changes in the molecular machinery that is associated with DNA and
67 contributes to gene regulation. DNA methylation is one of the most well studied epigenetic
68 mechanisms in this context, but its involvement in transgenerational effects remains
69 controversial in animals [4-7]. In mammals, inheritance of environmentally induced DNA
70 methylation is limited by the fact that epigenetic marks are typically reset during
71 reproduction [8-10], and this may explain why transgenerational persistence of
72 environmentally induced DNA methylation appears rather uncommon [5]. In invertebrates,
73 the transgenerational persistence of stochastic or environmentally induced DNA methylation
74 variation is poorly studied. This is partly because DNA methylation is of limited significance
75 in traditional model systems [e.g., Drosophila; 11]. However, recent studies suggest that
76 environmentally induced variation in DNA methylation can be passed on to subsequent
77 generations in insects, and perhaps other invertebrates as well [12-14].

78 Water fleas of the genus *Daphnia* are common in lakes and ponds, where they play
79 central roles in the functioning of ecological interactions, food webs, and nutrient cycling
80 [15]. How *Daphnia* respond to environmental change can have strong impact on community
81 and ecosystem dynamics, making *Daphnia* a model system to understand the interactions
82 between phenotypic plasticity, adaptive evolution, and ecology on contemporary time scales
83 [16]. Such eco-evolutionary dynamics may be fundamentally altered if environmentally
84 induced responses are inherited, for example, via epigenetic mechanisms [17]. However, the
85 extent and specificity of transgenerational persistence of environmentally induced epigenetic
86 variation remains poorly understood, not only in *Daphnia* but in ecological model systems in
87 general [18, 19].

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88 In addition to its role as a keystone species, there are several others reasons why
89 *Daphnia* is particularly useful to study epigenetic inheritance. Individuals frequently
90 reproduce clonally, which makes it possible to study epigenetic inheritance without the
91 confounding effects of genetic variation [20, 21]. Furthermore, *Daphnia* inhabit waters with
92 seasonal environmental variation, spanning periods of multiple asexual generations, a
93 situation that should favour incomplete epigenetic resetting [22-24]. Thus, *Daphnia* may be
94 particularly likely to have evolved mechanisms that enable context- and gene-specific
95 inheritance of gene regulation. Since *Daphnia* are carrying their offspring in an actively
96 ventilated brood pouch, maternal exposure to environmental stressors can have effects on
97 future generations by directly affecting embryos or germ cells, similar to the situation in
98 mammals. Such effects on offspring phenotype and fitness are commonly observed and
99 occasionally carry over to more than two generations (e.g., UV [25], microcystin [26],
100 temperature [27], and predator cues [28]). A candidate epigenetic mechanism underlying
101 transgenerational plasticity in *Daphnia* is DNA methylation. Despite that DNA methylation
102 in water fleas is occurring at low levels [~0.6%; 29, 30], it is typically enriched in exons and
103 is positively correlated with levels of gene expression [29]. There is also some evidence that
104 environmentally induced variation in DNA methylation can be passed on to subsequent
105 generations [31-33]. However, a rigorous assessment of genome-wide patterns of inheritance
106 on the individual level has not been performed to date.
107 In this study, we chose four stressors that have been shown to affect global DNA
108 methylation. Three are stressors that *Daphnia* encounter in the wild (*Microcystis aeruginosa*,
109 a cyanobacteria producing the toxin microcystin, zinc, and elevated temperature) and one is a
110 toxin not naturally encountered by *Daphnia* (5-azacytidine, a compound that inhibits the
111 function of the DNA methyltransferase DNMT1 and thereby causes hypo-methylation [34]).
112 Using a multi-generational experimental design (Figure 1), we explored (1) the immediate

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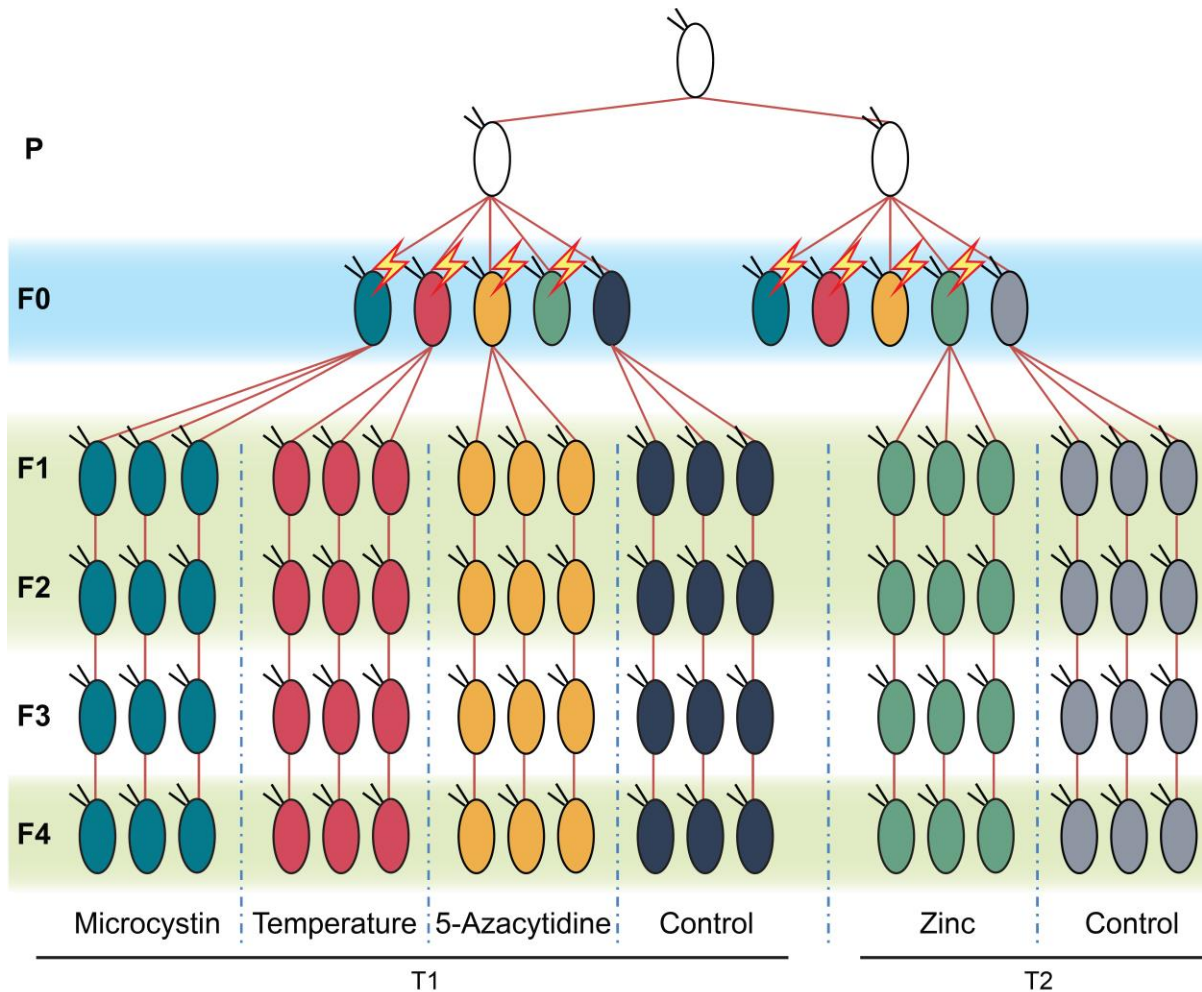
113 impact of the stressors on genome-wide DNA methylation levels in F1 individuals, which
114 were germ cells during maternal exposure, by identifying differentially methylated cytosine
115 positions (DMPs), (2) whether these DMPs are specific for each stressor, (3) whether DMPs
116 persist across four generations and (4) the putative biological function of these DMPs.



117

118 **Figure 1.** Schematic representation of the experimental design. Clonal siblings (generation P) were
119 divided into two lines (experiment T1 and T2) and their offspring (F0) were exposed to environmental
120 stressors (microcystin, high temperature, 5-azacytidine, or zinc) or kept under control conditions (one
121 per line). This experiment was replicated twice (T1 and T2) and run in parallel to account for potential
122 incompleteness due to the extinction of maternal lines. The most complete experiment for each
123 stressor was subjected to further analyses (as indicated, mortality in the zinc-exposed line resulted in
124 incomplete data and the analyses for zinc therefore make use of data from the second experiment).
125 Individual *Daphnia* of the generation F0 were exposed to environmental stressors from birth to first

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126 reproduction (more detail in Table S1). Since the maternal treatment stopped before the egg cells were
127 released into the brood pouch, the F1 generation was exposed as germ cells to the stressors but not as
128 embryos. Five offspring per exposed (or control) mother were selected and allowed to propagate until
129 generation F4 under control conditions. Red lines represent propagation of second brood offspring.
130 Individual *Daphnia* of each treatment group and generation F1, F2 and F4 were subjected to whole-
131 genome bisulfite sequencing (indicated by light green boxes). Each experimental unit included in the
132 final analysis consisted of three individuals, except for T1-5-azacytidine-F1 and T1-Control-F4,
133 which consisted of two replicate individuals, and T2-control-F1 and T1-control-F2, which consisted
134 of four replicate individuals. Tests of transgenerational persistence of environmentally induced DNA
135 methylation were analysed separately for T1 and T2. For more details, see Methods and Tables S1
136 and S2.
137
138 **Results**

139 **Environmental stressors negatively affect the reproductive output of exposed *Daphnia***

140 Fitness assays on the reproductive output of individuals showed a direct effect on the
141 stressor-exposed F0 generation relative to control samples for all stressors except elevated
142 temperature (Figure 2). These negative fitness effects were most pronounced for *Daphnia*
143 exposed to toxic cyanobacteria (microcystin treatment). However, this treatment line, as well
144 as the zinc treatment line, regained reproductive fitness already in the F1 generation. The
145 negative fitness effect of the 5-azacytidine treatment persisted until generation F3. Elevated
146 temperature negatively affected reproductive fitness in generations F1 and F3 (Figure 2).
147 Thus, the treatments significantly affected reproductive output of exposed individuals and
148 caused maternal and transgenerational effects on fitness that demonstrate the physiological
149 relevance of the stressors.

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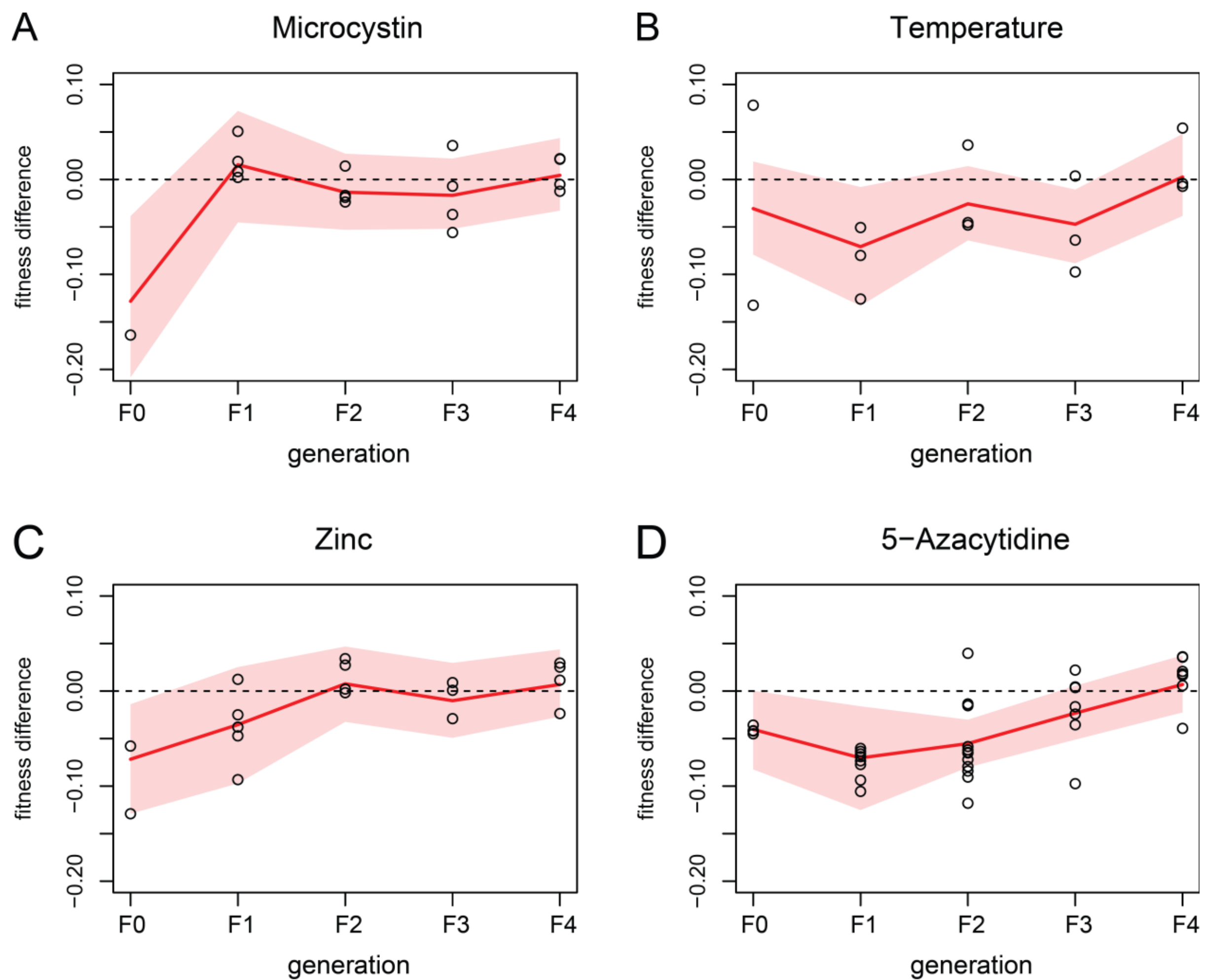

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151 **Figure 2. Fitness effects of the environmental stressors.** (A-D) Each plot shows the fitness
152 difference of exposed *Daphnia* per generation relative to the control conditions (marked as dashed
153 line). Fitness estimates refer to lifetime reproductive output derived from the age at first and second
154 reproduction and the size of the first and second brood (see Methods). Red lines mark the means and
155 shaded areas the 95% credible intervals. Observed values (statistically corrected for clone line effects)
156 are plotted as black circles. Note that fitness data was recorded for all individuals included in this
157 study, including those not selected for bisulfite sequencing. For (D) 5-azacytidine the fitness effects
158 were sustained until generation F2, whereas for (A) microcystin and (C) zinc the effects disappeared
159 after generation F1. For (B) high temperature, fitness effects lasted for several generations (until
160 generation F3), though fitness was significantly different from the control samples only for generation
161 F1 and F3.

162

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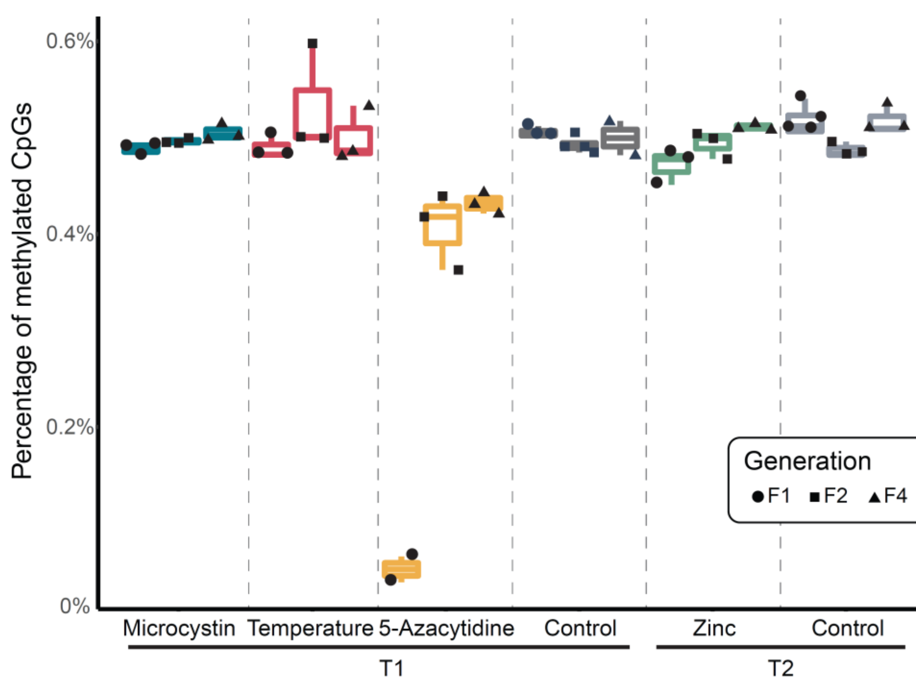
163 **Genome-wide methylation levels are consistently low and reduced by 5-azacytidine**

164 Consistent with previous studies in *D. magna* [0.74%; 29] [0.52%; 30], we found an overall
165 low proportion of CpG sites in a methylated state (Figure 3). In control samples, 0.50% (SD:
166 0.02%) of all CpG sites were methylated, and similar proportions were found in *Daphnia* that
167 were exposed to one of the natural stressors (high temperature, zinc or microcystin) as germ
168 cells (F1), and in their non-exposed descendants (F2 and F4). As expected, the stressor 5-
169 azacytidine, which inhibits DNA methylation, caused a tenfold decrease in methylation levels
170 of CpG sites in the generation F1 (0.05%; SD: 0.02%, *P*-value <0.01) relative to control F1.
171 Subsequent generations (F2 and F4) of 5-azacytidine exposed *Daphnia* showed CpG
172 methylation levels that approached the levels of the control samples, but remained at a
173 consistently lower level (Figure 3).



174

175 **Figure 3.** Overall levels of CpG methylation across samples. Plots show the percentages of CpGs that
176 are methylated relative to the total number of CpGs in the genome for each treatment group. Boxes
177 are coloured according to treatment group. The lower, median and upper hinges correspond to the
178 first, second and third quartiles respectively. Whiskers indicate the range that lies within 1.5 times of
179 the interquartile ranges. Black symbols indicate the individual data points according to generation.

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180

181 **Germ-cell exposure to environmental stressors leads to differential methylation of CpG**
182 **sites**

183 We detected an effect on genome-wide patterns of methylation in individual F1 *Daphnia* that
184 were exposed to natural stressors as germ cells. We identified 70 DMPs in the F1 generation
185 exposed to thermal stress (relative to the nine control samples in the F1, F2 and F4
186 generations), 76 DMPs in *Daphnia* exposed to zinc, and 225 DMPs in *Daphnia* exposed to
187 microcystin (at 5% FDR; Tables S3-S5).

188 Consistent with the strong signal of demethylation through 5-azacytidine, we found
189 2,231 DMPs in F1 *Daphnia* of this treatment line. While pairs of treatments shared a low
190 number of DMPs (Table S6), we found no DMPs in F1 that were shared by all four stressor
191 groups, and also no DMPs shared between the three natural stressors (thermal stress, zinc and
192 microcystin). Thus, the induced methylation changes were largely stressor-specific.

193

194 **A large proportion of environmentally-induced DMPs persist until the F4 generation**

195 After calling DMPs for each stressor and for each generation (at 5% FDR), we intersected
196 DMPs across generations per stressor to assess their overlap and thus the persistence of the
197 stress response. For example, of the 225 DMPs detected in F1 *Daphnia* from the microcystin
198 treatment, 57.8% (130 sites) were also differentially methylated compared to control samples
199 in generation F2 and F4 (Figure 4A; Table S7). For zinc and temperature, 53.9% and 45.7%
200 of the environmentally induced methylation variants in F1 persisted until the F4 generation
201 (Tables S8 and S9). Across the natural stressors (high temperature, zinc or microcystin), the
202 number of DMPs shared among three generations (F1, F2 and F4) was greater than expected
203 by chance (*P*-value derived from 1000 permutations: <0.001 for all three stressors), and also

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204 greater than the number of DMPs unique to F2 or F4 (Figure 4A). These results demonstrate
205 transgenerational stability of stress-induced methylation marks.
206 Since 5-azacytidine induced a strong hypomethylation in the F1 generation exposed
207 as germ cells, followed by a re-methylation in following generations (Figure 3), the patterns
208 of transgenerational inheritance were different from that of the other stressors (Figure 4B).
209 Only 1% (26 sites) of the environmentally induced methylation variants in F1 persisted until
210 the F4 generation, all of which remained hypomethylated (Figure 4A; Table S10). Thus,
211 compared to the other three stressors, the number of DMPs within each generation were
212 consistently higher, but their inter-generational overlap was lower.
213 To robustly verify that the treatment-induced transgenerational DMPs are stably
214 persisting across generations and not due to stochastic events, we used two additional
215 strategies for data analyses. Firstly, we applied permutations by randomly shuffling sample
216 labels to generate a null hypothesis. These permutations demonstrated deflated *P*-values
217 relative to the observed *P*-values (Figures S1 and S2) and produced no transgenerational
218 DMPs except two in 100 permutations in the 5-azacytidine case. Secondly, to mitigate the
219 potential bias stemming from using the same set of control samples (i.e., all nine control
220 samples) in each statistical test, we used an alternative strategy that identified candidate
221 DMPs in the F1 and subsequently tested the significance of those candidates in F2 and F4
222 (for details and results, see Methods section). Both additional strategies broadly confirmed
223 the existence of environmentally induced DMPs that persist for at least four generations.
224 Consistent with the limited overlap of DMPs of F1 *Daphnia* between the four
225 stressors, we also found that transgenerational DMPs are largely stressor-specific. However,
226 pairs of treatments (e.g., temperature and microcystin treatments) shared up to 7 DMPs,
227 which was more than expected by chance (*P*-value derived from 1000 permutations: <0.001;
228 Figure 4C).

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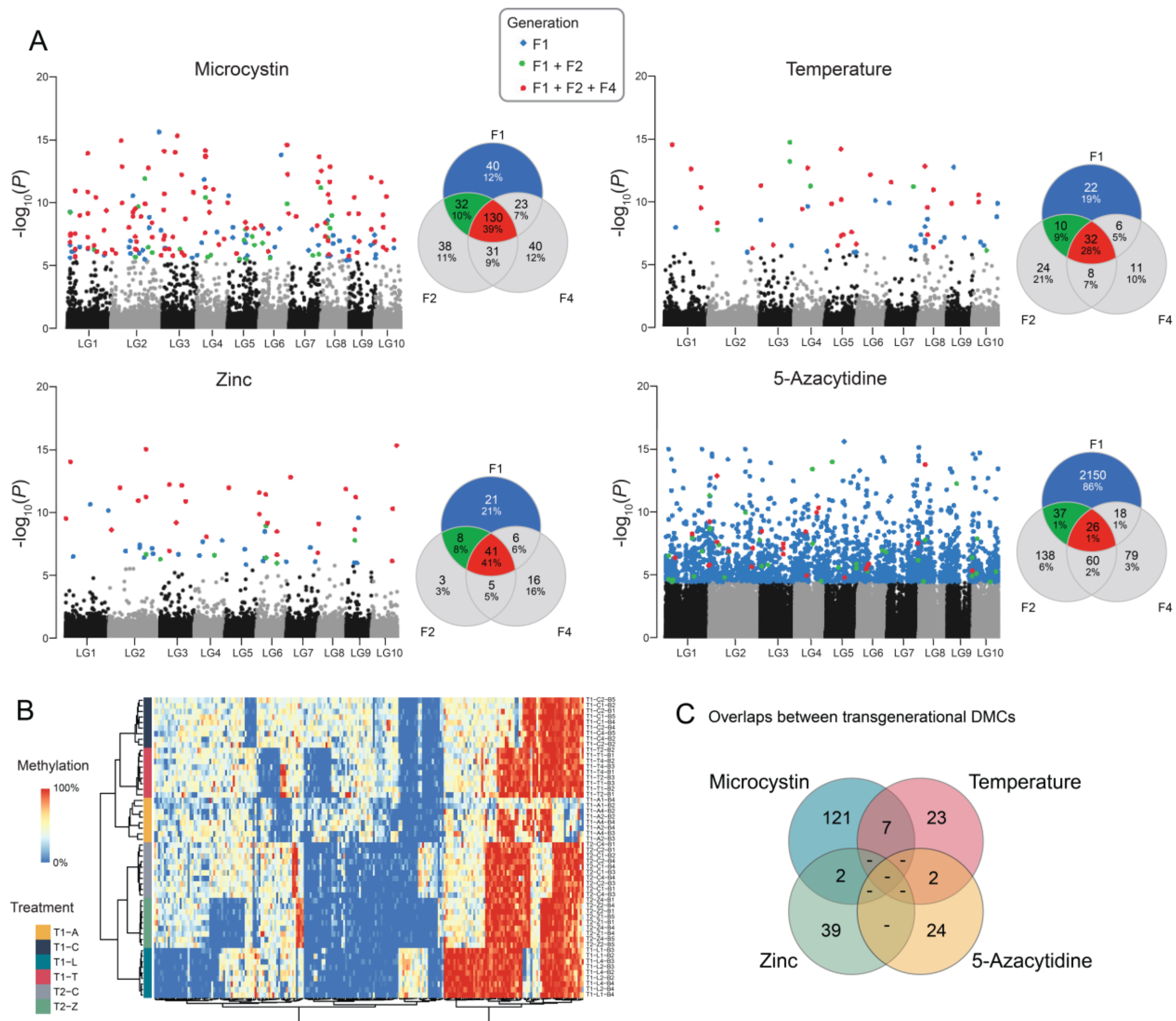

230

231 **Figure 4.** Transgenerational inheritance of DMPs. (A) Manhattan plots showing the genomic
232 positions of DMPs (left) and Venn-diagrams showing the overlap of DMPs across generation F1, F2
233 and F4 (right) for each of the four stressors. DMPs are color-coded according to their
234 transgenerational persistence as indicated. Numbers in the centre of the Venn-diagrams give the
235 number of transgenerational DMPs. Percentages of all DMPs are provided below absolute numbers of
236 DMPs in each group. (B) Heatmap of the methylation level for all 229 transgenerational DMPs across
237 all samples included in this study. Label names consist of experiment (T1 or T2), treatment and
238 generation (A, 5-azacytidine; L, microcystin; T, temperature; Z, zinc; followed by a number
239 indicating the generation), and clonal line number (B1-B5). (C) Venn diagram presenting the overlap

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240 of transgenerational DMPs across the treatments. No DMP was shared across three or all four
241 treatments, but between two and seven are shared by two stressors.
242

243 **Transgenerational DMPs retain a consistent methylation pattern and are almost**
244 **exclusively located in gene bodies**

245 For all transgenerational DMPs of a given stressor, the sign of differential methylation (i.e.,
246 hypo- or hypermethylated) was consistent across the generations. Moreover, when comparing
247 the effect sizes of differential methylation relative to control samples for a given stressors, we
248 found that those observed in the F1 and in the F4 generation are remarkably similar in
249 magnitude, and no sign of attenuation in the F4 generation was observed (Figure 5A).
250 To assign a putative functional role to the identified DMPs, we systematically
251 characterized the genomic positions for two groups of DMPs: those that occurred in the F1
252 generation after developmental exposure (as germ cell) to a stressor (direct DMPs) and a
253 subset of these, namely those that were stably inherited until generation F4 (transgenerational
254 DMPs). We found that both direct and transgenerational DMPs are predominantly found in
255 gene bodies (direct: 96.1%; transgenerational: 98.7%; Tables S3-S10, Figure 5B). Of all
256 DMPs occurring in gene bodies, the majority lies in exons rather than introns (direct: 87.70%;
257 transgenerational: 85.81%; Tables S3-S10, Figure 5B). Roughly half of the DMP-containing
258 gene bodies contained at least two DMPs (direct: 54%; transgenerational: 42%) in close
259 proximity to each other (median distance in bp, direct: 17; transgenerational, 1).
260

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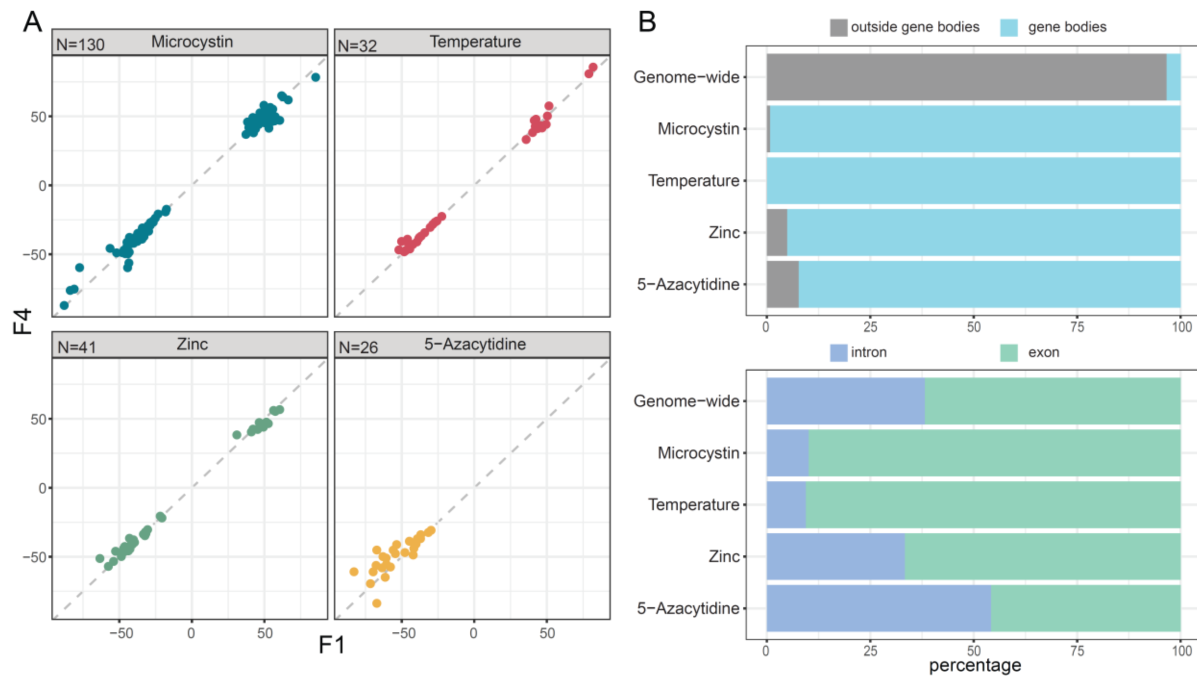

261

262 **Figure 5.** Characterization of transgenerational DMPs. (A) Effect sizes of the strength of differential
263 methylation (methylation difference relative to the nine control samples) for the transgenerational
264 DMPs of the F1 generation plotted against the corresponding effect sizes for the F4 generation. All
265 DMPs lie close to the dashed line indicating equally strong effect sizes in the two generations, which
266 shows that the effect sizes are consistent across generations. Note that all effect sizes for 5-azacytidine
267 are negative, as expected, due to the hypomethylation caused by this compound. (B) Top panel shows
268 the proportion of transgenerational DMPs that lie within gene bodies, and bottom panel further details
269 the distribution of those to exons or introns. The comparison with the distribution of genome-wide
270 CpG sites (N = 10,806,885) shows that the stressor-induced transgenerational DMPs are
271 overrepresented in gene bodies, and tend to lie in exons rather than introns, except for the stressor 5-
272 azacytidine.

273

274 **Transgenerational DMPs are occurring in genes that exhibit stressor-specific functions**

275 Although we did not find any overlap in the exact genes containing transgenerational DMPs
276 induced by different stressors, members of the 60S (large) ribosomal protein family are
277 consistently hypomethylated following exposure to a natural stressor (temperature, zinc or
278 microcystin). A more systematic assessment of functional overlap using GO enrichment

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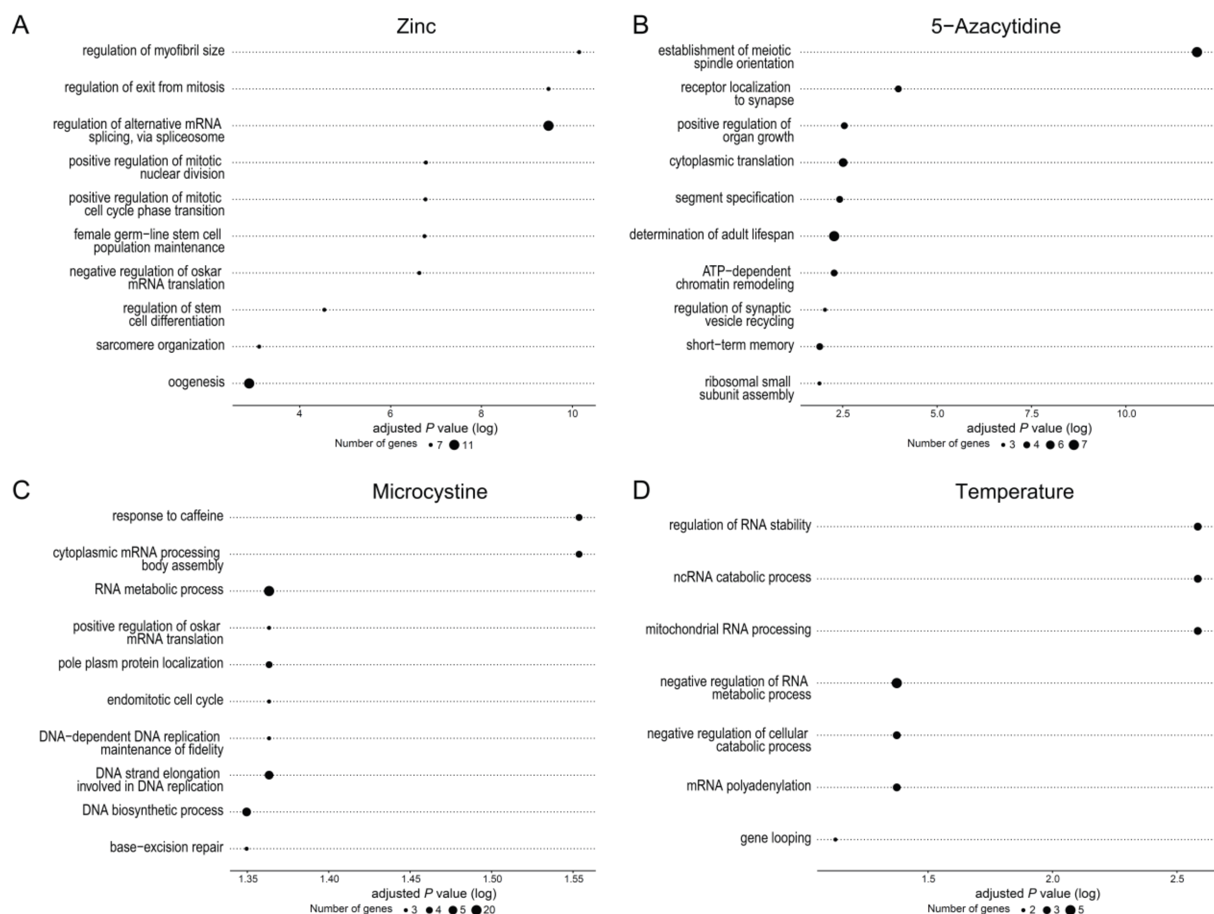
279 analysis showed that transgenerational DMPs were significantly enriched for a number of
280 different functions. We found between 7 and 15 significant GO terms of biological processes
281 and the top ten of these terms are shown in Figure 6. None of the GO terms were shared
282 between two or more stressors (Tables S11 and S12). This shows that DMPs are largely
283 occurring in different sets of genes for each stressor, and that these genes show no functional
284 similarity (i.e., not the same GO terms).

285



286

287 **Figure 6.** Putative functional role of transgenerational DMPs. (A-D) Top ten significantly enriched
288 GO terms of biological processes for genes containing transgenerationally inherited DMPs across the
289 four stressors. The size of each GO term represents the number of genes that it is represented by, and
290 the position along the x-axis indicates its significance (log-transformed adjusted *P*-value).

291

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292 **Candidate genes for stress responses reported in the literature are not differentially**
293 **methylated**
294 Finally, we cross-referenced genes identified as differentially methylated in our study with
295 genes identified as differentially expressed upon stress exposure reported in the literature. We
296 retrieved six studies that assessed gene expression differences in response to stressor
297 exposure in *Daphnia sp.* We only included experimental designs that are comparable to the
298 treatment conditions applied in our study, and that used genome-wide, unbiased approaches
299 such as microarrays (four studies) or RNA-sequencing (two studies; Tables S13 and S14).
300 Three of these studies used microcystin as stressor [35-37], two used zinc [38, 39], and one
301 used temperature [40]. Between 6% and 15% of the key genes differentially expressed in
302 response to microcystin, zinc, or temperature as stressor are highly functionally similar to
303 genes identified as differentially methylated in our data, though gene identity was not
304 identical (Tables S13 and S14). Most instances of genes identified as differentially expressed
305 as well as differentially methylated concern genes with a well-described, broad functionality
306 such as heat shock proteins or large ribosomal proteins (Tables S13 and S14).
307 The effects of 5-azacytidine on gene expression has not been assessed in a genome-
308 wide, unbiased approach in *Daphnia*, but two studies reported the differential expression of
309 several candidate genes (mostly DNA-methyltransferases, which are known to be the target
310 of this drug) [41, 42]. We found that none of the key candidate genes differentially expressed
311 in response to 5-azacytidine treatment was identified as differentially methylated in our
312 analyses.
313
314 **Discussion**
315 Despite extensive interest in epigenetic inheritance, the extent to which environmentally
316 induced epigenetic marks are heritable in animals remains an open question. Here, we show

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317 that, in *Daphnia*, environmentally induced variation in DNA methylation in germ cells are
318 specific to the stressor, and are stably inherited for at least four generations.
319 Maternal exposure to the natural stressors microcystin, zinc, and high temperature
320 caused stressor-specific DNA methylation changes in their F1 offspring. Since the maternal
321 treatment stopped before the egg cells were released into the brood pouch, the DMPs in F1
322 were likely induced in germ cells and persisted during cell differentiation to be evident in
323 most, or all, cell types (and thus possible to detect by bisulfite sequencing of whole
324 individuals; although differences in the relative number of cell types between treatments may
325 also contribute). As expected, 5-azacytidine led to genome-wide hypomethylation, while the
326 other stressors induced both hypo- and hypermethylation. One of the two genes that were
327 affected in all three of the naturally occurring stressors – microcystin, zinc, and high
328 temperature – was a 60S (large) ribosomal protein, which was consistently hypomethylated.
329 Large ribosomal proteins have been repeatedly reported as being implemented in stress
330 responses of a variety of organisms [43, 44], including salinity stress in *Daphnia* [32].
331 However, in general, the specific DMPs, the genes they reside in, and the putative functions
332 of those genes (i.e., GO terms) were largely specific to each stressor.
333 The majority of environmentally induced DMPs were located in gene bodies, more
334 precisely in exons, which is consistent with how DNA methylation appears to regulate gene
335 expression in invertebrates [45, 46]. Indeed, some genes with DMPs do have putative
336 functions for responding to these stressors, but few of the *a priori* candidate genes or
337 pathways were identified as being differentially methylated. For example, DNA-
338 methyltransferases, which are strongly down-regulated upon exposure to 5-azacytidine [41,
339 42], showed no signs of changes in methylation levels. Similarly, ABC transporter genes and
340 nucleoside transporters, which are strongly expressed in response to microcystin [36] and
341 zinc [38], respectively, were not differentially methylated. This might be explained by the

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342 fact that the differential expression of these candidate genes tends to be restricted to particular
343 tissues (e.g., gut cells), and our whole-body measurements might not have been sensitive
344 enough to pick up these subtle effects on the level of DNA methylation in specific tissues. In
345 contrast, genes that appear to be both differentially expressed and differentially methylated
346 were those with a rather general function, such as heat shock proteins and large ribosomal
347 proteins. Genes encoding these proteins might perhaps show a more consistent expression
348 across a range of cell types. Further analysis of DNA methylation in germ cells and
349 differentiated cell types, and data on the relationship between DNA methylation and gene
350 expression, could substantiate the breadth and stressor-specificity of DMPs in germ cells,
351 their persistence during somatic cell differentiation, and functional relevance.

352 The epigenetic changes induced in offspring of exposed mothers commonly persisted
353 until at least the fourth generation. The exception was the 5-azacytidin treatment, which
354 demonstrates that modification of DNA methylation typically is restored from one generation
355 to the next. Overall, only about 1% of sites that became hypomethylated in offspring of 5-
356 azacytidin-exposed mothers remained hypomethylated in the F4 generation. This epigenetic
357 resetting makes it the more striking that nearly half of the DMPs observed in the offspring of
358 mothers exposed to microcystin, zinc, or high temperature actually persisted until the F4
359 generation. This suggests that naturally occurring stressors modify DNA methylation in a
360 way that reliably allow those modifications to be passed on to subsequent generations in
361 *Daphnia*. These results substantiate and extend previous work on pools of *Daphnia*
362 individuals that indicated that methylation patterns induced upon salinity stress or gamma
363 radiation can be detected until the F3 generation [32].

364 The mechanism by which DNA methylation is inherited remains poorly understood.
365 Both direct copying of methylation states and involvement of small RNA molecules in RNA-
366 directed DNA methylation [47] are potential mechanisms. MicroRNA expression in eggs of

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367 *Daphnia* vary as a result of maternal stress, but there is no evidence that differences in the
368 expression of these RNAs persist for several generations [48]. However, this does not rule out
369 that other forms of small RNAs, such as piRNA or tsRNA, are involved. RNA-mediated
370 mechanisms may make it more likely that environmentally induced variation in DNA
371 methylation will be inherited also during sexual reproduction and through both parents. More
372 generally, establishing the mechanisms of transgenerational persistence of variation in DNA
373 methylation will help to understand the extent to which it represents a flexible mechanism of
374 inheritance that can contribute to ecological and evolutionary dynamics.
375 Despite a high incidence of parthenogenesis, *Daphnia* are famous for their ability to
376 adapt rapidly to environmental stressors, including to all three naturally occurring stressors of
377 this study (e.g., toxic cyanobacteria [49]; metal pollution [50], and high temperature [51]).
378 Interestingly, such adaptations can be rapidly lost if conditions improve [50]. Laboratory
379 studies have also demonstrated strong environmentally induced maternal effects (e.g., toxic
380 cyanobacteria [52]; metal pollution [53]; temperature [54]), sometimes persisting for several
381 generations [27, 55, 56]). The persistence of environmentally induced DNA methylation from
382 one generation to the next that we demonstrate here could partly contribute to such
383 transgenerational effects, and suggests that low genetic diversity may not prevent *Daphnia*
384 populations from responding to selection. Thus, our results suggest that epigenetic
385 inheritance can contribute to the adaptability of *Daphnia,* allowing populations to persist
386 even under rapid and severe environmental change.
387

388 **Materials and Methods**

389 *Daphnia husbandry and experimental design*

390 A stock of *Daphnia magna* was sourced from Lake Bysjön (surface area 10 ha, 55°40'32"N
391 13°32'42"E) in Southern Sweden. Single clonal lines were kept under laboratory conditions

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392 [52] and allowed to reproduce asexually for 12 months before the onset of the experiment.
393 All experiments in this study used a single clone to minimize any genetic effects. Applying
394 the experimental design shown in Figure 1, individual *Daphnia* of the generation F0 were
395 exposed to environmental stressors from birth to first reproduction (more detail in Table S1).
396 Since the maternal treatment stopped before the egg cells were released into the brood pouch,
397 the F1 generation was exposed as germ cells to the stressors but not as embryos [57].
398 Following the first brood, all individuals of the F0 generation were maintained under control
399 conditions. We propagated these lines down to generation F4 by isolating five offspring from
400 the second brood in each generation and keeping them under control conditions. Subsequent
401 generations (F2, F3 and F4) did not encounter the stressors. We collected individuals of
402 generations F1, F2 and F4 for whole-genome bisulfite sequencing directly after they
403 produced their second brood (i.e., as adults). We omitted the F3 generation and used the F4
404 generation instead to gain insights into truly transgenerational effects on DNA methylation.
405 This experiment was performed twice simultaneously (T1 and T2), to account for potential
406 incompleteness due to the extinction of maternal lines, and the most complete experiment for
407 each stressor was subjected to further analyses. The experiment T1 for 5-azacytidine,
408 microcystin and high temperature, and experiment T2 was selected for the zinc treatment.
409 Controls were matched within each of these two experimental groups (i.e., the effects of zinc
410 were evaluated against its corresponding T2 control line, and the effects of the other three
411 treatments against the T1 control line). Previous analyses of DNA methylation in *Daphnia*
412 have relied on pools of individuals [32, e.g., 58], but to avoid confounding effects, we applied
413 a newly developed low input methodology [59] that allowed us to sequence individual
414 *Daphnia*.
415
416 *Reproductive output as a proxy of fitness effects*

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417 To assess how the stressor treatments affect the lifetime reproductive success of exposed
418 individuals and their descendants, we collected the age of first and second reproduction (in
419 days) and the sizes of the first and second brood for all individuals of the selected experiment
420 (T1 or T2; including those individuals not selected for bisulfite sequencing). We estimated
421 fitness by calculating, for each individual, the intrinsic rate of population increase r with a
422 univariate root finding algorithm (*uniroot* in R) using the Euler equation [for details, see 52].
423 To test whether fitness varied by treatment and generation we estimated fitness for each
424 treatment by generation in a nested multilevel model, with generation nested within treatment
425 (in Stan 2.21.0 accessed from R with rstan 2.21.2). We present fitness effects as the
426 difference between the fitness for a particular treatment by generation and the fitness of the
427 control treatment of the same generation (with negative effects indicating a reduction of
428 fitness compared to the control).

429

430 *WGBS, read mapping and extraction of methylation values*

431 DNA was extracted from whole individual *Daphnia* samples using the DNeasy blood and
432 tissue kit (Qiagen™, Valencia, CA, USA) and DNA concentrations were estimated using a
433 Qubit Fluorometer (ThermoFisher Scientific). Three individuals per experimental unit were
434 initially processed, but units containing samples with low DNA concentrations were
435 supplemented with a fourth back-up sample (i.e., for some units, four rather than three
436 samples were processed). Extracted DNA samples were subjected to library preparation using
437 the SPLAT protocol [59] with minor modifications. Adapter oligos were modified at all the
438 5'- and 3'-ends not involved in ligation to reduce adapter dimer formation. The following
439 adapter oligos were used: 5'AmMC6/GACGTGTGCTCTTCCGATCTNNNNNN/3'AmMo,
440 5'Phos/AGATCGGAAGAGCACACGTC/3'AmMo,
441 5'AmMC6/ACACGACGCTCTTCCGATCT, and

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442 5'AmMC6/NNNNNNAGATCGGAAGAGCGTCGTGT/3'AmMo. All oligos were
443 purchased from IDT. Libraries were sequenced on six lanes of an Illumina HiSeqX
444 instrument in randomized order. Sequencing data were processed within the framework of the
445 nf-core methylseq workflow version 1.5 [60] (Figure S3). In summary, raw reads of 64 fastq
446 files were trimmed of adapter sequences using Trim Galore! with default
447 parameters. Trimmed reads were mapped to the *Daphnia magna* reference genome
448 GCA_003990815.1 [genome size: 123 Mb; 61] using Bismark [62] with the paired-end
449 setting and with parameter settings "-q --score-min L,0,-0.2 --ignore-quals --no-mixed --no-
450 discordant --dovetail --maxins 500 --directional". Cytosine methylation from deduplicated
451 sequence data was generated using bismark_methylation_extractor [62] with parameter
452 settings "--ignore_r2 2 --ignore_3prime_r2 2 --no_overlap".

453 Six libraries were excluded from the analyses due to low read mapping rate and
454 cytosine site coverage (<15% mapping rate, <1X mean coverage and <5X median
455 coverage). Furthermore, four libraries were excluded on the basis of being PCA outliers in
456 CpG percent methylation. Of these outliers, three were characterised by high percentage of
457 methylated CpG (>97% percentile of the remaining libraries), which were at levels similar to
458 the six libraries excluded due to low read mapping rates. In total, 54 libraries passed the
459 quality control and proceeded to further analyses. These libraries had a mean coverage of
460 5.3X and a mean mapping rate of 48% (Table S2).

461

462 *Differential methylation analysis*

463 The Bioconductor R package methylKit_1.12.0 [63] was used to carry out differential
464 methylation analysis comparing each treatment (case) against untreated (control) groups. For
465 each case and control selection, we only consider CpG sites with a minimum of 5 total read
466 counts in all samples in all F-generations. The read counts of all sites that passed this filter

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467 were normalised by a library specific scaling factor as computed by a median coverage
468 normalisation in methylKit. Furthermore, sites were filtered to consider only variable sites
469 with sample standard deviations in percent methylation values of ≥0.5 (per case-vs-control
470 group; see below). Overall, the initial set of >8 M CpG sites called per sample was reduced to
471 an average of 2.8 M sites (range from 2.3-3.3 M) that were tested for differential methylation
472 analysis.

473 Within the methylKit framework, we used the Wald test for hypothesis testing and
474 beta binomial with overdispersion correction and parameter shrinkage to model the
475 proportion of methylated CpG at a site. We quantitatively confirmed the main results using
476 logistic regression models (Figure S4). The Benjamini-Hochberg method was used for
477 multiple testing correction.

478 The case-vs-control statistical tests to identify differentially methylated CpG sites
479 were carried out independently for each F-generation of case samples. However, to control
480 for any generational epigenetic drift that could add to stochastic noise in the controls, the
481 same set of all control samples across generations was used for each statistical test, i.e., 3
482 cases of F1 vs 9 controls (F1+F2+F4), 3 cases of F4 vs 9 controls (F1+F2+F4). Lastly, the
483 statistical significance of differentially methylated CpG (DMP) sites was adjusted with a 5%
484 FDR in each test.

485

486 *Identification of transgenerational DMPs*

487 We defined transgenerational DMPs as being CpG sites that have acquired a treatment
488 induced methylation state in the F1 generation (i.e., differentially methylated in the three F1
489 samples compared to the nine control samples), and for which methylation states are
490 consistently maintained in the succeeding F2 and F4 generations (i.e., differentially
491 methylated in both F2 vs control and F4 vs control). No minimum methylation difference was

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492 imposed. Furthermore, the statistical significance of DMP overlap across generations was
493 obtained using the permutation function *permTest* in the R package regioneR_1.20.0 [64] and
494 resampling randomly from all tested CpGs.

495 To robustly verify that the treatment-induced transgenerational DMPs are stably
496 inherited across generations and not due to stochastic events, we used two additional
497 strategies for data analyses. First, we carried out a permutation test by randomly assigning
498 sample labels. For each selected treatment and control pair, we permuted their sample labels
499 by shuffling case/control labels (e.g., zinc and control) and generation labels (i.e., F1, F2 and
500 F4). We carried out 100 permutations of sample labels. After permuting sample labels,
501 differential analysis was carried out as described above, i.e. 3-vs-9 per generation, with the
502 same set of 9 "controls" in each generation. The null hypothesis was no association between
503 CpG methylation and sample labels and we expect that randomly shuffling the sample labels
504 would fulfil the null hypothesis. By inspecting quantile-quantile (Q-Q) plots (Figure S1 and
505 S2), we compared the distribution of true labels with that of randomly shuffled labels and
506 assessed if the former was associated with lower *P*-values and a higher number of
507 transgenerational DMPs (i.e., significant DMPs shared across the three generations). Since
508 this strategy does not mitigate the potential bias stemming from using the same set of control
509 samples (i.e., all nine control samples) in each of the statistical tests, we also adopted a
510 second strategy of identifying DMPs to exclude the possibility that this non-independence
511 inflates the number of DMPs. To this end, we selected candidate environmentally induced
512 CpGs by selecting outliers from comparisons of three cases versus three controls in the F1
513 generation using a lenient 20% FDR. We then tested these candidate CpGs and asked which
514 of them also meet the criterion of being differentially methylated at a 5% un-adjusted *P*-value
515 cut-off in the F2 and F4 generations by performing three cases versus three controls tests
516 within these two generations. When comparing this alternative set of DMPs against the set

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517 obtained using the original 3-vs-9 approach, we found that between 77% and 25% of the
518 original approach were also identified by the alternative approach (Table S15), with the
519 alternative approach being generally more stringent (i.e., producing lower numbers of
520 transgenerational DMPs).
521
522 *Annotation of DMPs and gene ontology analyses*
523 We assigned each DMP to a nearest gene or a gene unit (i.e., exon or intron) by cross-
524 referencing its genomic position with the GTF annotation from the reference assembly. This
525 was carried out using BEDOPS closest-feature [65]. To obtain functional annotation such as
526 gene ontology for the *Daphnia* genome, we used eggnog 5.0 [66] (emapper-2.1.2) with
527 default parameters but restricting to the taxon Arthropoda. The enrichment analysis of GO
528 terms was carried out using the R package topGO (version 2.40.0) and Fisher's exact test.
529
530 *Cross-referencing differentially methylated genes with differentially expressed genes*
531 *identified in the literature*
532 To assess if hypo- or hyper-methylated genes in this study are those demonstrated to be
533 differentially expressed upon exposure to a given stressor, we systematically screened the
534 literature for relevant transcriptomic studies. We conducted a literature search using ISI *Web*
535 *of Science* (v.5.30) with search terms specific to each dataset. We used the search terms
536 'Daphnia' and 'transcriptomic*', 'RNAseq', 'gene expression', 'microarray', along with one
537 of the following: '*zinc*', 'microcystin*', 'temperature*' or '*azacytidine*'. We excluded
538 studies that used experimental designs that are too dissimilar from our settings (e.g., in terms
539 of exposure duration). Quantitative comparisons between the set of differentially methylated
540 genes identified in the present study and differentially expressed genes taken from the
541 literature is hampered by a number of facts (e.g., differentially expressed genes are not

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542 reported in a standardized way, overrepresented GO terms are rarely reported, assigning gene
543 orthologs between different *Daphnia* species, or assigning corresponding genes between
544 different genome versions of the same species, is not straightforward). In addition, these
545 studies often report up to 30% of all transcripts as differentially expressed, which precludes
546 quantitative enrichment analyses. We therefore restricted our analysis to cross-referencing the
547 key genes singled out in genome-wide, unbiased approaches against the differentially
548 methylated genes identified in our study. We manually compared gene sets and regarded
549 genes as shared when they are semantically highly similar. For example, we considered 'heat
550 shock protein 70 Bbb' similar to 'heat shock factor protein-like, transcript variant X7'.
551

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556 **Competing interests**
557 The authors declare no competing interests.
558

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572

573 **Data availability**

574 All sequences generated in this study have been deposited in NCBI Sequence Read Archive
575 (SRA) with accession number PRJNA760269. Data for the fitness analyses are deposited in
576 Dryad (*DOI to be generated during submission*).
577

578 **Code availability**

579 Code for the analysis of DNA methylation is available on Bitbucket
580 (https://bitbucket.org/scilifelab-lts/t_uller_1801/). Code for the fitness analyses is deposited
581 in Zenodo (DOI: 10.5281/zenodo.5635792).
582

583 **Author contributions**

584 R.R. and T.U. conceived the study; N.F. and T.U. coordinated the study; R.R., E.W.T, B.T.H.
585 and T.U designed the study; R.R. performed the experiments; A.R. performed optimization of
586 library preparation and generated the sequencing libraries; L.V. analysed the sequence data
587 with input from N.F., R.R., M.R., B.N. and T.U.; R.R. analysed fitness data with input from
588 N.F. and T.U.; N.F. and T.U. wrote the manuscript with input from L.V., R.R., E.W.T and
589 B.T.H. All authors approved the final manuscript.
590

591

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