

Genome-wide Methylation Patterns Under Caloric Restriction in *Daphnia*

2 *magna*.

4 Jack Hearn^{*1}, Marianne Pearson¹, Mark Blaxter¹, Philip Wilson², and Tom J. Little¹

6 ¹University of Edinburgh, Institute of Evolutionary Biology, School of Biological Sciences,
Edinburgh, United Kingdom

8 ²Heriot-Watt University, School of Energy, Geoscience, Infrastructure & Environment,
Edinburgh, United Kingdom

10 *corresponding author: Jack.Hearn@ed.ac.uk

12 **Email addresses:**

JH: Jack.Hearn@ed.ac.uk

14 MP: s1674868@sms.ed.ac.uk

16 MB: Mark.Blaxter@ed.ac.uk

PW: pw23@hw.ac.uk

18 TJL: Tom.Little@ed.ac.uk

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28 **Abstract**

30 The degradation of epigenetic control with age is associated with progressive diseases of
ageing, including cancers, immunodeficiency and diabetes. Reduced caloric intake slows the
32 effects of aging and age-related diseases, a process likely to be mediated by the impact of
caloric restriction on epigenetic factors such as DNA methylation. We used whole genome
34 bisulphite sequencing to study how DNA methylation patterns change with diet in a small
invertebrate, the crustacean *Daphnia magna*. *Daphnia* show the classic response of longer life
36 under CR, and they reproduce clonally, which permits the study of epigenetic changes in the
absence of genetic variation. Global CpG methylation was 0.7-0.9%, and there was no
38 difference in overall methylation levels between normal and calorie restricted replicates.
However, 453 regions were differentially methylated (DMRs) between the normally fed and
40 calorie restricted (CR) replicates. Of these 61% were hypomethylated in the CR group, and
39% were hypermethylated in the CR group. Gene Ontology (GO) term enrichment of
42 hyper and hypo-methylated genes showed significant over- and under-representation in
three molecular function terms and four biological process GO terms. Notable among these
44 were kinase and phosphorylation activity, which have a well-known functional link to
cancers.

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50 **Introduction**

Epigenetic modifications play a key role in maintaining gene expression and organismal
52 development. This is particularly evident when epigenetic control degrades, resulting in
progressive diseases in humans, including cancers, immunodeficiency and diabetes [1]. The
54 degradation of epigenetic control with age is proposed to occur in a drift-like process. One
mechanism that may rescue age-related epigenetic dysregulation is caloric restriction (CR):
56 reduced caloric intake without malnutrition or loss of nutrients. CR slows the effects of
aging and postpones the development of age-related diseases [2–5]. In rhesus monkeys and
58 mice, CR of 30% and 40% respectively appears to reduce epigenetic drift in methylation and
increases lifespan, which in rodents can be an extension of up to 50% [6]. Similar results
60 have been seen in yeast, spiders, worms, fish and non-human primates [4,7,8]. CR may also
delay a spectrum of diseases such as cancer, kidney disease, autoimmune disease and
62 diabetes [9–11], as well as neurodegenerative diseases [12,13].

DNA Methylation, a reversible covalent modification that regulates gene expression, is the
64 best-studied epigenetic mechanism. DNA Methylation of cytosines occurs when DNA
methyltransferase enzymes (DNMTs) transfer a methyl group onto cytosine [14] to create a
66 5-methylcytosine. Most commonly at a cytosine immediately followed by guanine (CpG
site). There are three DNMT enzymes: DNMT3 establishes methylation *de novo*, DNMT1
68 maintains methylation, and DNMT2, which has no known role in DNA methylation. A
reduction in expression levels of DNMT enzymes is associated with ageing, leading to a
70 global loss of genomic methylation [15]. In mammals around 70% of CpGs are methylated
[16], however in invertebrates the rate in species sampled to date is lower, from 0% in flies
72 to 15% in the oyster *Crassostrea gigas* [17,18]. The model crustaceans *Daphnia magna* and
Daphnia pulex (Arthropoda: Crustacea) have genomic CpG methylation of 0.52% and 0.7%
74 respectively [19].

CpG methylation can increase or decrease gene expression dependent on the location of
76 the methylation. In promoter regions, which can be rich in CpGs and are known as CpG
islands, it represses expression of the gene. Further to this, many CpG islands are also
78 enriched for permissive chromatin modification, which condenses the structure of
chromatin and further prevents transcription. In contrast, methylation of gene bodies leads
80 to an increase in expression of the effected gene. Invertebrates have few CpG Islands, and
methylation predominantly occurs in gene bodies, and is enriched in exonic sequence [20–
82 25], where it may enhance transcription or mediate alternative splicing [25–27].
Interestingly, in silkworms there is no correlation between methylation in promoters and
84 gene expression [28], suggesting invertebrates and vertebrates differ in their usage of CpG
methylation.

86 The relationship between diet and CpG methylation, and subsequent impact on ageing and
health, is well established. Indeed, DNA methylation may be a predictor of biological age.
88 CR in mice caused a two-year difference in biological (0.8) versus chronological (2.8) age,
while in rhesus monkeys CR resulted in a biological age of 20 years for monkeys of
90 chronologically aged 27 years [29]. Specific examples of a diet by methylation interaction
include the expression of DNMTs which have elevated expression in response to CR in
92 cancer cells, which counteracts the global hypomethylation [30] observed during ageing. CR
also causes a reduction in lipid metabolism gene expression by DNA methylation of gene
94 bodies in mouse livers [31]. As a result, older mice undergoing CR were protected from
fatty degeneration, visceral fat accumulation, and hepatic insulin resistance compared to
96 controls. In rats and monkeys short-term CR in older individuals ameliorates the effects of
ageing with respect to disease markers, oxidative stress and damage, and increases the
98 expression of longevity related genes [32,33]. The reverse is seen in obesity-like phenotypes
in rodent models. For example, in Agouti mice, the agouti viable yellow metastable epiallele

100 (A^{vy}) interacts with an upstream retrotransposon intracisternal A particle (IAP) [34].
Unmethylated IAP results in yellow mice and negative health effects associated with obesity,
102 whereas methylation at IAP results in brown healthy mice [35]. Waterland et al (2003)[36]
demonstrated the that supplementing mothers with folic acid, vitamin B12, choline and
104 betaine shifted the offspring of obesity phenotype mice to smaller, brown mice indicative of
increased methylation at IAP.

106 Our work aims to determine if an experimentally controlled nutritional environment directs
changes in methylation status in a small invertebrate, the crustacean *Daphnia magna*. We do
108 this by whole genome bisulphite sequencing of CR and normally-fed (NF) replicates,
identifying and characterizing regions of differential methylation. *Daphnia* show the classic
110 response of longer life under CR and strong maternal effects; the offspring of calorie-
restricted mothers being larger and more resistant to pathogens than their counterparts
112 from better fed mothers. Provisioning of offspring, e.g. with carbohydrates, protein or fats,
is one explanation for these maternal-effect phenotypes, and epigenetic processes, such as
114 methylation, are also potentially key regulators in these plastic responses to fluctuating
environments.

116 *Daphnia* have many attributes that make them favourable for epigenetic study. First, they
reproduce clonally, which permits the study of epigenetic changes in the absence of genetic
118 variation. This also allows powerful study of genetic variation: clonal replicates are
equivalent to identical twin studies, but with an experimentally chosen number of -uplets.

120 CpG-based methylation occurs in *Daphnia*, its genome encodes all three DNMT enzymes
orthologous to mammalian DNMT enzymes, and the global methylation pattern changes in
122 response to environmental factors [19,37–39].

124 **Results**

Methylated sites prediction

126 Trimmed bisulphite-converted reads aligned to the genome using Bismark [40] exhibited
lower mapping efficiencies than standard short-read alignments typical of WGBS [41], with
128 20-32% of reads not aligning to the reference genome (read filtering and coverages per
replicate Table I, Bismark report outputs Supplementary File I). Of the aligned reads, 29-
130 38% of reads were discarded as PCR duplicates, and 6-10% of the remainder contained
predicted CHH or CHG methylated sites which were also removed from analyses. This
132 resulted in replicate average read coverages of 8-12-fold (read filtering and coverages per
replicate Table I, Bismark report outputs Supplementary File I). Global CpG methylation
134 was 0.7-0.9% in all samples, and no difference in methylation levels is observed between
normal and calorie restricted replicates. Removal of polymorphic CpG sites, where a
136 polymorphic C/T can be miscalled as methylated C, using variants predicted from the
bisulphite-unconverted data had little effect on the total number of sites (table 2). After
138 filtering 99.2% of sites were retained per replicate on average per replicate, with average
total sites across replicates going from 6.9 million to 6.85 million. Hierarchical clustering of
140 replicates by methylation status in methylKit [42] demonstrated that mother has a stronger
effect on global methylation status than nutritional treatment (Figure 1).

142 *Differential methylation in bsseq*

Bsseq [43] testing of differential methylation revealed 453 differentially methylated regions
144 (DMRs) using a t-statistic cutoff of -4.6, 4.6. Of these 278 (61%) were hypomethylated in the
CR group versus normal food, and 175 were hypermethylated (39%) in the CR group versus
146 normal food. DMRs were 164 base-pairs (bp) and 127 bp long for CR hypo- and CR hyper-
methylated regions and ranged from 10-602 bp in length. There are from three to 20 CpGs

148 per cluster with an average of six. CR hypomethylated DMRs overlapped 357 gene
predictions in the *Daphnia magna* geneset, while hypermethylated DMRs overlapped 244.
150 The majority of these also overlapped exonic sequences: 99% (353/357) for CR
hypomethylated DMRs and 80% (194/244) for CR hypermethylated DMRs. Only 63 DMRs
152 (14%) do not overlap a predicted gene body at all. The increase in number of DMR
containing genes versus total DMRs reflects overlapping/redundant predictions in the *D.*
154 *magna* genome annotation version 2.4.

GO term enrichment in methylated genes and DMRs

156 GO term enrichment was explored using the “weight01” and “classic” algorithms in topGO
[44] and molecular function (MF) and biological process (BP) terms are reported. The
158 enrichment analysis (DMRs) using the weight01 algorithm showed significant over and under
representation in three molecular function (MF) GO terms and four biological process (BP)
160 terms. The more permissive classic algorithm revealed significant enrichment in twenty-five
MF GO terms and twenty BP. These results have been condensed into their most-specific
162 terms and direction of methylation in Table 3 (expanded version, Supplementary File 2).
Top-scoring DIAMOND [45] alignment by bit-score against the uniref90 [46] and non-
164 redundant protein databases for each gene associated with an enriched GO terms is
reported in Supplementary File 3.

166

Discussion

Global methylation and differentially methylated regions

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The global CpG methylome of ~0.7%, consistent across replicates, is possibly higher than
172 for the previously sequenced *D. magna* CpG methylome of 0.5% [19], though this earlier

study was performed on a different strain and used different data filtering methods [19]. In
174 line with previously observed methylation patterns in arthropods [20,23,24,47], the majority
(86%) of DMRs are found in gene bodies. Furthermore, most are present in exonic regions,
176 although more so for hypo- (99%) than hypermethylated (80%) regions. This suggests DNA
methylation is regulating expression of targeted genes in *Daphnia* as for other invertebrates
178 [20,47]. Although this requires confirmation by gene expression data, the expectation is that
hypermethylated genes are upregulated in expression and hypomethylated are
180 downregulated.

In what follows, we discuss the genes that are associated with the functional enrichment of
182 differentially methylated regions (GO terms, Table 3), giving particular attention to genes
whose expression is known to respond to CR, or are linked to progressive disease of ageing
184 or cancers.

Hypermethylation under CR

186 Perhaps the most prominent difference between CR and control *Daphnia* is the
hypermethylation of protein phosphorylation and tyrosine kinase activity (Table 3, BP,
188 GO:0006468; MF, GO:0004713). These GO terms are associated with genes that include
four calcium/calmodulin-dependent protein kinase kinases (CAM-KK). CAM-KKs are
190 involved in regulating cell apoptosis and promote cell survival by activating protein kinase B
(Akt) [48], which also has roles in cell-cycle progression. In humans, aberrant expression of
192 CAM-KK is a known factor in several cancers, and is considered a therapeutic target for
prostate and stomach cancers [49,50]. CAM-KK responses to CR are less well understood,
194 but can protect against atherosclerosis by activation of AMP-activated protein kinase and
sirtuin-1 [51]. Sirtuin proteins are involved in the response to CR and general nutrient
196 sensing [52,53]. CAM-KK upregulation due to hypermethylation under CR thus might

activate sirtuin-based responses and subsequent whole organism phenotypes. Further
198 investigation of this might included characterising differences in downstream expression of
AMPK, sirtuins, mTORC1 [54] and sirtuin triggered pathways.

200

The next major hypermethylated GO term, the molecular function term for ATP binding
202 (GO:0005524), is associated with a gene group that includes kinases, ligases, and eleven
uniquely occurring genes (Supplementary File 3). Three of these (ABCD1, GMP synthase
204 and The RNA helicase DDX39A), have established links to diet or ageing. ATP-binding
cassette sub-family D member 1 (ABCD1), is involved in the catabolism of long chain fatty
206 acids [55]. This suggests upregulation of energy production in our CR lines (though we also
find evidence of down-regulation of lipid metabolism, discussed below). Mutations in this
208 gene in humans causes adrenomyeloneuropathy, characterised by an accumulation of
unbranched saturated fatty acids [56]. In ABCD1 knockout mice cholesterol levels are
210 higher than in wildtypes, and are unaffected by cholesterol feeding [57]. Most pertinent to
CR, however, is the defective antioxidant response correlated with ABCD1 dysfunction
212 [58], because reducing oxidative stress is a proposed mechanism by which CR increases
longevity [6,59,60]. GMP synthase expression gradually decreases with age resulting in
214 lower cognitive performance [61]. The RNA helicase DDX39A has no connection to CR,
but its overexpression is associated with poor cancer prognosis [62–66].

216

Also among the eleven uniquely occurring genes associated with the GO term for ATP
218 binding are several genes associated with DNA stability (Supplementary File 3). SMC2, for
example, is a component of the condensin complex which organises and condenses
220 chromosomes during mitosis and meiosis [67,68]. SMC2 also acts to repair double-stranded
breaks in DNA and maintains ribosomal DNA stability in yeast, dysregulation of which is

222 linked to cancer in humans [67,69]. Although no link has been established between CR and
SMC2, CR enhances genomic stability through several pathways including double-strand
224 repair [70]. The condensin complex is a possible mechanism by which this enhancement
occurs. The presence of DNA polymerase θ , and the MCM2 component of the MCM2-7
226 complex, which is essential in initiating DNA replication by unwinding double-stranded
DNA, is further evidence of a genomic stability maintenance response of CR. A reduction in
228 expression of the MCM2-7 complex leads to aneuploidy and in mice reduced life-spans due
to cancer [71,72]. DNA polymerase θ also acts to repair double-stranded breaks in DNA,
230 and higher expression of this gene is associated with better cancer treatment outcomes
[73].

232

The tRNA aminoacylation ligase (GO:0043039) group (Table 3) is associated with four
234 tRNA ligases (for glutamate, proline, histidine, and phenylalanine). There is no research
directly linking these ligase genes to CR, but increased expression via methylation may be a
236 response to low abundance of these amino acids, which indicates that future studies of diet
should vary protein availability. Indeed, it may be that protein restriction is more important
238 than overall calorie restriction for longevity (cites). Additionally, fragments of tRNAs called
5' tRNA halves are a class of signalling molecules that are modulated by CR and ageing in
240 mice [74,75], in which CR 'rescues' older 5' tRNA halves in line with other CR phenotypes.
An as yet undiscovered mechanism of diet and ageing could involve tRNA ligases regulating
242 levels of 5' tRNA halves in response to CR.

244 Respiratory electron transport chain (GO:0022904; Table 3) contains two proteins:
cytochrome b-c1 complex subunit and NADH (Nicotinamide adenine dinucleotide reduced
246 form) dehydrogenase I alpha subcomplex subunit. Their methylation may relate to more

efficient respiration because of CR to extract maximum energy from food. Interestingly, in
248 yeast, CR is associated with increased longevity due to a reduction in NADH levels because
of NADH dehydrogenase activity [76] to create NAD⁺ (oxidised form). NADH is a
250 competitive inhibitor of yeast sirtuin, leading to its activation on decreased NADH levels
[76,77]. This could also be occurring in *Daphnia* under CR if methylation of the NADH gene
252 results in the expected increase in gene expression. The RNA methylation group for
hypermethylated genes (GO:0031167) contains two methyltransferase protein 20s (not
254 DNA methyltransferases) which do not have a clear link to CR in the literature.

256 **Hypomethylation under CR**

Phospholipid/glycerolipid metabolism is reduced under CR (Table 3, GO:0006644 and
258 GO:0046486), and both processes are associated with the same genes. These genes are
GPI inositol-deacylase, cardiolipin synthase, phosphatidylinositol-glycan biosynthesis class W
260 protein, and phosphatidylserine synthase. Assuming that decreased methylation lowers gene
expression, this result is in keeping with previous work on effects of CR on phospholipids.
262 In mice myocardium, phospholipids undergo a reduction in mass and are remodelled when
facing CR [78], which is speculated to maximise energy efficiency. The same drop in
264 phospholipids was observed in humans undergoing acute CR [79], and more generally
reduces the risk of atherosclerosis and heart disease [80,81]. This is potentially a further
266 common mechanism of response to CR in which DNA methylation is an important
component. The molecular function GO term for ATPase activity (GO:0042626) and
268 overlapping P-P-bond-hydrolysis-driven transporters (GO:0015405) include plasma
membrane calcium-transporting ATPase, downregulation of which would increase
270 calcium/calmodulin-dependent protein kinase activity within cells by maintaining calcium
levels. The remaining genes encode ATP binding protein sub-family B proteins, which pump

272 various substrates out of cells and have no clear links to known CR phenotypes. This may
reflect the broad-range of substrates ATP binding protein are able to efflux.

274 **Conclusion**

We have shown that caloric restriction effects the methylation status of a subset of genes,
276 despite the low overall CpG methylation found in *Daphnia*. There is a strong concordance
between these results and CR experiments in humans, mice and yeast among other species.
278 We show that hypermethylated genes and processes are in line with upregulation in
previous CR and hypomethylated genes with downregulation. Although we have focused on
280 the effect of caloric restriction on DNA methylation status, there are alternative potential
epigenetic responses to CR, including small RNAs (sRNAs) and histone modifications.
282 Previously, we established that CR induces differential miRNA expression in *D. magna* under
an equivalent experimental design [82], but other sRNAs, for example piRNAs and tsRNAs,
284 could also have a role in CR-dependent gene regulation [83–85]. Histone modifications in
response to CR or protein restriction (PR) are known from work on humans, rats and mice
286 [86–89] and are proposed to increase longevity [87] by delaying and repressing ageing-
related processes and diseases. Future studies could vary a range of dietary components
288 (overall calories, proteins or fatty acids) and examine the joint effects of a range of
epigenetic mechanisms.

290

Methods

292 *Daphnia* preparation and experiment

294 Six replicates of control (i.e. well-fed) *Daphnia magna* were compared to six replicates of
caloric restricted *Daphnia* to identify differentially methylated regions. We used a single
296 clone (known to us as Clone 32) of *D. magna*, collected from the Kaimes pond near *Leitholm*

in the Scottish Borders [90]. Maternal lines were first acclimatized for three generations.
298 For this, individuals were kept in artificial pond medium at 20°C and on a 12h:12h light:dark
cycle and fed 2.5×10^6 cells of the single-celled green algae *Chlorella vulgaris* daily. Following
300 three generations of acclimatisation (detailed in [82]), 40 offspring from each mother were
isolated and split to form a replicate. Twenty were fed a normal diet of 5×10^6 algal cells/day
302 and the remaining twenty that were fed a calorie restricted diet of 1×10^6 algal cells/day. Each
replicate was split and reared in four sub-replicate jars of five *Daphnia* which were
304 subsequently pooled at DNA extraction. Hence, normal food and calorie restricted
replicates and were paired by mother and each consisted of twenty *Daphnia* total. The
306 experiment was ended after the birth of 2nd clutch (approximately day 12 of the treatment
generation). *Daphnia* were ground by motorized pestle in Digsol and proteinase K and
308 incubated overnight at 37°C and stored at -70°C until DNA extraction.

310 *DNA extraction and sequencing*

DNA was extracted from pooled *Daphnia* per replicate by phenol-chloroform followed by a
312 Riboshredder RNA digestion step and repeat of the phenol-chloroform step. DNA was
eluted into 100 ul of TE buffer and quantified by Qubit fluorimeter. Sample purity was
314 checked by 260:280 ratio on nanodrop, and DNA integrity was examined by running
approximately 35 ng DNA on a 0.8% agarose gel stained with ethidium bromide. Each DNA
316 extraction was split in two for creation of a bisulphite converted library and corresponding
bisulphite unconverted library (all steps the same except bisulphite conversion). Thus, 24
318 libraries were created: 12 bisulphite-converted and 12 corresponding unconverted samples.
This was done to identify per replicate mismatches from the reference and remove false
320 positive methylation calls. All libraries were created by Edinburgh Genomics using the
Zymogen EZ DNA Methylation-Lightning Kit and Methyseq Library prep Illumina TruSeq

322 DNA Methylation Kit and 125 base pair paired-end sequenced on Illumina HiSeq. Raw read
data has been deposited in the European Nucleotide Archive under accession PRJEB24784,
324 (file names and conversion status, Supplementary File 4).

326 *Quality Assessment and Mapping*

Before aligning reads to the *D. magna* reference genome (version 2.4 downloaded from:
328 http://arthropods.eugenescience.org/EvidentialGene/daphnia/daphnia_magna/Genes/earlyaccess/),
the reference was converted to clone 32 as for Hearn et al (2018) [82]. This was to
330 increase mapping efficiency, and accuracy of the analysis, by reducing polymorphism
between the reference (assembled from a different clone) and our data.

332

Reads from both bisulphite converted and unconverted libraries were trimmed of the first
334 and last nine bases of every read using TrimGalore! (version 0.4.1) [91] after initial
inspection of Bismark m-bias plots. TrimGalore! was also used to remove base calls with a
336 Phred score of 20 or lower, adapter sequences, and sequences shorter than 20 bp. FastQC
0.11.4 [92] was used to before and after quality control to inspect the data. Bisulphite calls
338 were made with Bismark 0.16.3 [40]. Bismark alignments were performed with options “–
score_min L,0,-0.6”. PCR duplicates were removed using deduplicate_bismark script.
340 Bismark reports indicated that libraries were not fully bisulphite converted and raw
methylation calls were approximately 3% for CpG, CHH and CHG sites. Previous research
342 has shown that CHH and CHG methylation is negligible in the *D. magna* genome [19]. As a
result, we used the filter_non_conversion script to remove reads that contain either CHH
344 and CHG methylation sites as diagnostic of a non-bisulphite converted read. Finally,
methylated sites were identified using bismark_methylation_extractor and reports created
346 with bismark2report.

348 Further variants were predicted per replicate using the unconverted library reads by
following the GATK pipeline [93,94] and converted strain 32 reference sequence. One
350 round of base quality score recalibration was sufficient using variants previously identified
from strain 32. Sites with single nucleotide polymorphisms at methylated positions were
352 removed from the analysis using BEDtools [95].

354 *Differential Methylation Analysis*

All analyses were performed using methylation calls from the bisulphite converted libraries
356 only. Hierarchical sample clustering of genome-wide methylation patterns across replicates
was generated using methylKit [42]. The six-normal food- and six calorie restricted
358 replicates were then compared using bsseq [43] Bioconductor package in R to identify
regions of differential methylation. We ran bsseq with a paired t-test to control for the
360 batch effect of mother on methylation. DMRs were selected using t-statistic cutoff of -4.6
and 4.6, a greater than 0.1 average difference in methylation between groups, and at least
362 three methylated CpGs. Genes overlapping DMRs were identified using the *D. magna*
version 2.4 genome annotation file and BEDTools. This list of overlapping genes was used as
364 a basis for functional enrichment analysis.

366 *Functional Enrichment Analysis*

Enriched GO (gene ontology) terms were identified using topGO [44] (weight01 and classic
368 algorithm) and molecular function (MF) and biological process (BP) GO terms are reported.
We tested if differentially methylated genes were enriched for specific GO terms against a
370 database of GO terms for all the genes in the *Daphnia magna* genome. Differentially

372 methylated regions were also split into hyper and hypo-methylated in calorie restricted
categories to test for direction-specific enrichment. *D. magna* GO terms were downloaded
from:

374 [http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/function/cddrp
s-dapmaevgl4.gotab2](http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/function/cddrp-s-dapmaevgl4.gotab2). Fisher's exact test with an α of 0.01 was used to identify enriched
376 genes, and no p-value correction was applied as per topGO author recommendation. The
group of genes present in each hyper- or hypo- methylated enriched GO terms were
378 extracted and blast [96] searched against uniref90 and the non-redundant protein databases
downloaded January 18th 2018 using DIAMOND [45].

380

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644 Figure 1. Samples cluster by mother and not by treatment in global CpG similarity.
Dendrogram created by ward.D method in methylKit. Number refers to mother from
646 which replicate was derived; H for normal food diet, L for caloric restriction.

Diet	Mother	Code	Converted pairs	Unconverted pairs	Alignments analysed	Deduplicated mapped reads	non CpG filtered reads	Bases remaining	Average coverage	CpG % methylated
H	5	5H	25601600	14305707	18634549	12764776	12026017	2320675218	9.67	0.7
H	7	7H	29785044	132653916	21608960	14878231	13894067	2693793276	11.22	0.8
H	8	8H	26405910	20875768	19223306	12837344	12060578	2312369294	9.63	0.7
H	9	9H	29296515	12172764	21000058	14155807	13242208	2622392293	10.93	0.8
H	16	16H	34120276	15802459	23704762	15726061	14675418	2914262560	12.14	0.8
H	22	22H	34878128	23164024	15908472	15908472	14830368	2919581788	12.16	0.8
L	5	5L	22562863	21579618	14154784	10164439	9447332	1871992335	7.80	0.7
L	7	7L	21248753	22953919	15063166	10412350	9743258	1937024597	8.07	0.7
L	8	8L	31610110	24339207	21765927	14712500	13701673	2657817364	11.07	0.8
L	9	9L	34684920	20342139	23809968	15706084	14477225	2870659128	11.96	0.8
L	16	16L	29775932	19257227	19183633	13586009	12679198	2523165965	10.51	0.8
L	22	22L	32239102	32281921	14083083	14083083	12718680	2438782524	10.16	0.9

Table 1. Read sequenced per replicate for both converted and unconverted libraries. Alignments analysed is number of read-pairs per bisulphite converted library aligned by Bismark; deduplicated is number of read pairs after removal of PCR duplicates; non CpG filtered is read-pairs remaining after removal of CHH and CHG containing reads; bases remaining are number of bases left for CpG methylation prediction in Bismark; average coverage is for filtered bases at a D. magna genome size of 240 megabases; CpG % methylated is the genome-wide percentage of CpG methylation. Diet, H: normal food, L: caloric restriction, code: combination of diet and mother.

648 Table 2. CpG sites remaining in each replicate after filtering of polymorphic sites
demonstrating minimal effect of this filtering. Polymorphic sites were identified from
650 unconverted libraries against a reference genome converted to Clone 32. Diet, H: normal
food, L: caloric restriction, code: sample code.

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Diet	Mother	Code	Sites before SNP filtering	After SNP filtering	% Remaining
H	5	5H	6298891	6247063	99.18
H	7	7H	6983214	6926563	99.19
H	8	8H	6283291	6231498	99.18
H	9	9H	7299503	7240944	99.20
H	16	16H	7325180	7266539	99.20
H	22	22H	7450910	7391057	99.20
L	5	5L	6577176	6523622	99.19
L	7	7L	6456008	6403288	99.18
L	8	8L	6934926	6878855	99.19
L	9	9L	7476524	7416772	99.20
L	16	16L	7335517	7276624	99.20
L	22	22L	6392175	6339761	99.18

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664 Table 3. Functional enrichment of DMRs using the Biological Process and Molecular
Function Gene Ontology (GO). Table lists all significant terms identified with the 'weight01'
666 algorithm, which accounts for GO term hierarchy, plus any that were only identified with
the 'classic' algorithm, which ignores hierarchy.
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Biological process

GO ID	GO term	Ancestors	Algorithm	Direction
GO:0006468	protein phosphorylation	7	weight01	Hyper
GO:0043039	tRNA aminoacylation	2	weight01	Hyper
GO:0031167	rRNA methylation	4	weight01	Hyper
GO:0022904	respiratory electron transport chain	0	classic	Hyper
GO:0006644	phospholipid metabolic process	1	classic	Hypo
GO:0046486	glycerolipid metabolic process	1	classic	Hypo

Molecular function

GO:0004713	protein tyrosine kinase activity	5	weight01	Hyper
GO:0005524	ATP binding	12	weight01	Hyper
GO:0042626	ATPase activity, coupled to transmembran...	1	weight01	Hypo
GO:0015405	P-P-bond-hydrolysis-driven transmembrane...	1	classic	Hypo

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Supplementary File 1. MultiQC report in html format reporting general statistics from the
676 Bismark alignment process for all replicates. Including alignment rates, deduplication effect,
overall cytosine methylation and m-bias plot. This plot shows average methylation level per
678 position across reads, demonstrating minimal bias at 5' and 3' reads after trimming of first
and last nine base pairs of each read.

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Supplementary File 2. Functional enrichment of DMRs using the Biological Process Gene
682 Ontology (GO), Most specific term listed first, and is identified by the weight01 algorithm,
indented beneath are GO term description using topGo to test for enrichment blog post
684 from the same hierarchy, identified by the classic algorithm.

686 Supplementary File 3. DIAMOND aligner results in blast output format six for best match to
uniref 90 and non-redundant protein databases of each *D. magna* gene associated with an
688 enriched GO term under the weight01 algorithm. Column one is GO term, column two is
D. magna gene, columns three-thirteen correspond to standard blast tabular output, and
690 column fourteen is the description of the best-matching hit for that gene.

692 Supplementary File 4. Raw read files in the European Nucleotide Archive for each replicate,
including ENA alias, bisulphite converted or unconverted status, unique identification for
694 that replicate, mother identification and treatment: H is normal food and L is caloric
restriction.

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CpG methylation clustering

