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

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Abstract

The degradation of epigenetic control with age is associated with progressive diseases of ageing, including cancers, immunodeficiency and diabetes. Reduced caloric intake slows the 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 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 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 difference in overall methylation levels between normal and calorie restricted replicates. However, 453 regions were differentially methylated (DMRs) between the normally fed and calorie restricted (CR) replicates. Of these 61% were hypomethylated in the CR group, and 39% were hypermethylated in the CR group. Gene Ontogeny (GO) term enrichment of 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 were kinase and phosphorylation activity, which have a well-known functional link to cancers.

Introduction

Epigenetic modifications play a key role in maintaining gene expression and organismal development. This is particularly evident when epigenetic control degrades, resulting in progressive diseases in humans, including cancers, immunodeficiency and diabetes [1]. The 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): 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 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 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 diabetes [9–11], as well as neurodegenerative diseases [12,13].

DNA Methylation, a reversible covalent modification that regulates gene expression, is the best-studied epigenetic mechanism. DNA Methylation of cytosines occurs when DNA methyltransferase enzymes (DNMTs) transfer a methyl group onto cytosine [14] to create a 5-methylcytosine. Most commonly at a cytosine immediately followed by guanine (CpG site). There are three DNMT enzymes: DNMT3 establishes methylation *de novo*, DNMT1 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 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 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% respectively [19].

CpG methylation can increase or decrease gene expression dependent on the location of 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 enriched for permissive chromatin modification, which condenses the structure of chromatin and further prevents transcription. In contrast, methylation of gene bodies leads 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 [20–25], where it may enhance transcription or mediate alternative splicing [25–27]. Interestingly, in silkworms there is no correlation between methylation in promoters and gene expression [28], suggesting invertebrates and vertebrates differ in their usage of CpG methylation.

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. 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 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 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 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 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 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].

Results

Methylated sites prediction

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 20-32% or reads not aligning to the reference genome (read filtering and coverages per replicate Table 1, Bismark report outputs Supplementary File 1). Of the aligned reads, 29-38% 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 resulted in replicate average read coverages of 8-12-fold (read filtering and coverages per replicate Table 1, Bismark report outputs Supplementary File 1). Global CpG methylation 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 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 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 replicates by methylation status in methylKit [42] demonstrated that mother has a stronger effect on global methylation status than nutritional treatment (Figure 1).

Differential methylation in bsseq

Bsseq [43] testing of differential methylation revealed 453 differentially methylated regions (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 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

per cluster with an average of six. CR hypomethylated DMRs overlapped 357 gene predictions in the *Daphnia magna* geneset, while hypermethylated DMRs overlapped 244. 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 (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. magna* genome annotation version 2.4.

GO term enrichment in methylated genes and DMRs

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 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) 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 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-redundant protein databases for each gene associated with an enriched GO terms is reported in Supplementary File 3.

Discussion

Global methylation and differentially methylated regions

The global CpG methylome of ~0.7%, consistent across replicates, is possibly higher than 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 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, 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 [20,47]. Although this requires confirmation by gene expression data, the expectation is that hypermethylated genes are upregulated in expression and hypomethylated are downregulated.

In what follows, we discuss the genes that are associated with the functional enrichment of 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 or cancers.

Hypermethylation under CR

Perhaps the most prominent difference between CR and control *Daphnia* is the hypermethylation of protein phosphorylation and tyrosine kinase activity (Table 3, BP, 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 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 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, 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 sensing [52,53]. CAM-KK upregulation due to hypermethylation under CR thus might

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

The next major hypermethylated GO term, the molecular function term for ATP binding (GO:0005524), is associated with a gene group that includes kinases, ligases, and eleven uniquely occurring genes (Supplementary File 3). Three of these (ABCD 1, GMP synthase 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 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 gene in humans causes adrenomyeloneuropathy, characterised by an accumulation of unbranched saturated fatty acids [56]. In ABCD1 knockout mice cholesterol levels are 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 [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 lower cognitive performance [61]. The RNA helicase DDX39A has no connection to CR, but its overexpression is associated with poor cancer prognosis [62–66].

Also among the eleven uniquely occurring genes associated with the GO term for ATP binding are several genes associated with DNA stability (Supplementary File 3). SMC2, for example, is a component of the condensing complex which organises and condenses 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].

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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 1 alpha subcomplex subunit. Their methylation may relate to more

efficient respiration because of CR to extract maximum energy from food. Interestingly, in 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 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 results in the expected increase in gene expression. The RNA methylation group for hypermethylated genes (GO:0031167) contains two methyltransferase protein 20s (not DNA methyltransferases) which do not have a clear link to CR in the literature.

Hypomethylation under CR

Phospholipid/glycerolipid metabolism is reduced under CR (Table 3, GO:0006644 and GO:0046486), and both processes are associated with the same genes. These genes are GPI inositol-deacylase, cardiolipin synthase, phosphatidylinositol-glycan biosynthesis class W 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. 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 phospholipids was observed in humans undergoing acute CR [79], and more generally reduces the rick of atherosclerosis and heart disease [80,81]. This is potentially a further 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 overlapping P-P-bond-hydrolysis-driven transporters (GO:0015405) include plasma membrane calcium-transporting ATPase, downregulation of which would increase 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

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.

Conclusion

We have shown that caloric restriction effects the methylation status of a subset of genes, 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. 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 the effect of caloric restriction on DNA methylation status, there are alternative potential epigenetic responses to CR, including small RNAs (sRNAs) and histone modifications. 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, 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 [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 (overall calories, proteins or fatty acids) and examine the joint effects of a range of epigenetic mechanisms.

Methods

Daphnia preparation and experiment

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 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. For this, individuals were kept in artificial pond medium at 20°C and on a 12h:12h light:dark cycle and fed 2.5 x 10^6 cells of the single-celled green algae *Chlorella vulgaris* daily. Following 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 5x10^6 algal cells/day and the remaining twenty that were fed a calorie restricted diet of 1x10^6 algal cells/day. Each replicate was split and reared in four sub-replicate jars of five *Daphnia* which were 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 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 incubated overnight at 37°C and stored at -70°C until DNA extraction.

DNA extraction and sequencing

DNA was extracted from pooled *Daphnia* per replicate by phenol-chloroform followed by a 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 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 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 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 positive methylation calls. All libraries were created by Edinburgh Genomics using the Zymogen EZ DNA Methylation-Lightning Kit and Methylseq 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.eugenes.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

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: http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/function/cddrps-dapmaevg14.gotab2. Fisher's exact test with an α of 0.01 was used to identify enriched 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 extracted and blast [96] searched against uniref90 and the non-redundant protein databases downloaded January 18th 2018 using DIAMOND [45].

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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.

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

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

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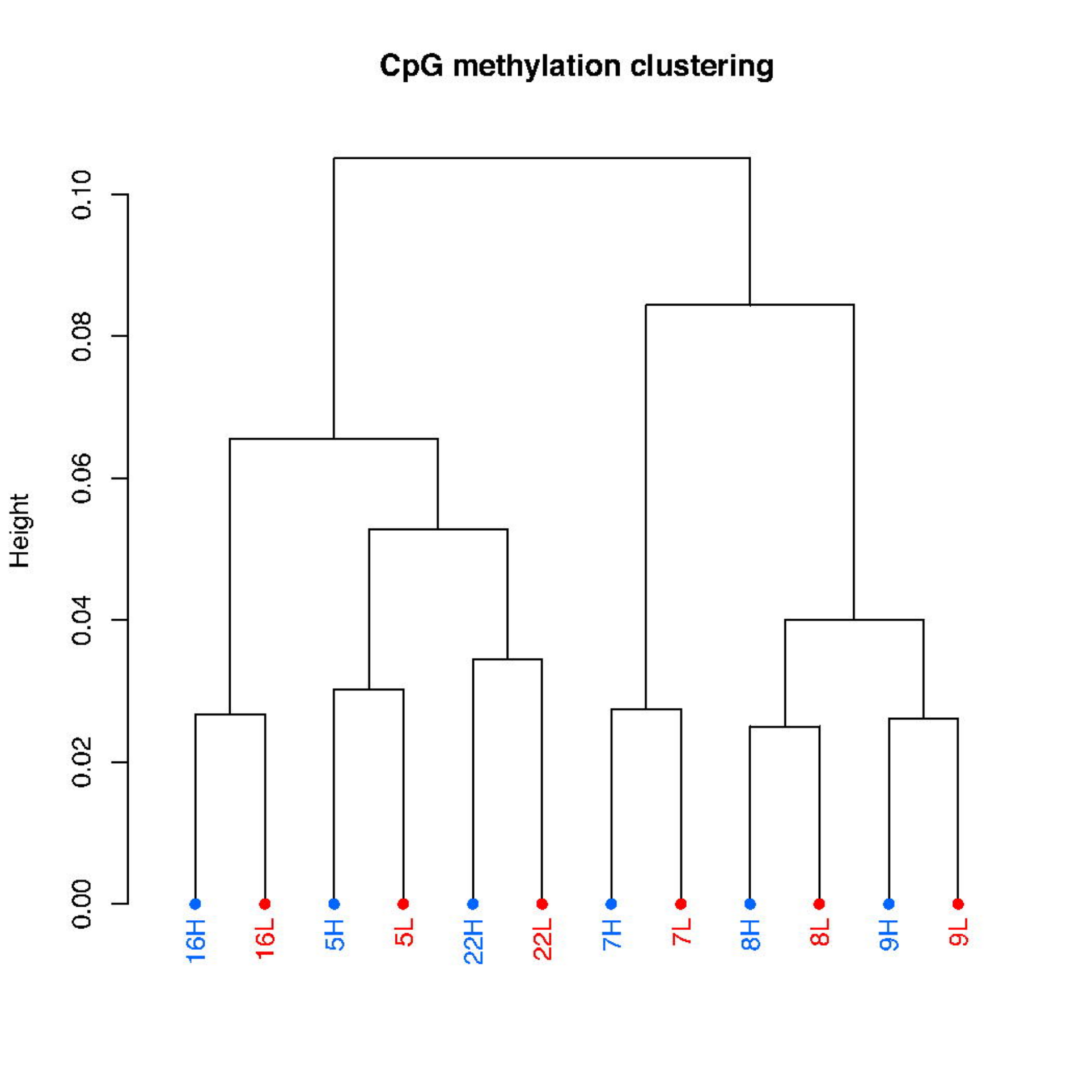
Supplementary File 1. MultiQC report in html format reporting general statistics from the Bismark alignment process for all replicates. Including alignment rates, deduplication effect, overall cytosine methylation and m-bias plot. This plot shows average methylation level per position across reads, demonstrating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

Supplementary File 2. Functional enrichment of DMRs using the Biological Process Gene 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 from the same hierarchy, identified by the classic algorithm.

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 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 column fourteen is the description of the best-matching hit for that gene.

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 that replicate, mother identification and treatment: H is normal food and L is caloric restriction.


CpG methylation clustering
Height
0.00
0.02
0.04
0.06
0.08
0.10
16H
16L
5H
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22L
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