General DNA methylation patterns and environmentally-induced differential methylation in the eastern oyster (*Crassostrea virginica*)

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Abstract

Epigenetic modification, specifically DNA methylation, is one possible mechanism for intergenerational plasticity. Before inheritance of methylation patterns can be characterized, we need a better understanding of how environmental change modifies the parental epigenome. To examine the influence of experimental ocean acidification on eastern oyster (*Crassostrea virginica*) gonad tissue, oysters were cultured in the laboratory under control (491 ± 49 µatm) or high (2550 ± 211 µatm) pCO_2 conditions for four weeks. DNA from reproductive tissue was isolated from five oysters per treatment, then subjected to bisulfite treatment and DNA sequencing. Irrespective of treatment, DNA methylation was primarily found in gene bodies with approximately 22% of the genome predicted to be methylated In response to elevated pCO_2 we found 598 differentially methylated loci primarily overlapping with gene bodies. A majority of differentially methylated loci were in exons (61.5%) with less intron overlap (31.9%). While there was though there was no evidence of a significant tendency for the genes with differentially methylated loci to be associated with distinct biological processes, the concentration of these loci in gene bodies, including genes involved in protein ubiquitination and biomineralization suggests DNA methylation may be important for transcriptional control in response to ocean acidification. Understanding how experimental ocean acidification conditions modify the oyster epigenome, and if these modifications are inherited, allows for a better understanding of how ecosystems will respond to environmental change.

Keywords: eastern oyster, DNA methylation, epigenetics, ocean acidification, protein ubiquitination

Introduction

As increased anthropogenic carbon dioxide is expected to create adverse conditions for calcifying organisms (IPCC 2019), efforts have been made to understand how ocean acidification impacts ecologically and economically important organisms like bivalves (Parker et al., 2013; Ekstrom et al., 2015). Bivalve species are sensitive to reduced aragonite saturation associated with ocean acidification, with larvae being particularly vulnerable (Barton et al., 2012; Waldbusser et al., 2014). Shell structure may be compromised in larvae, juveniles, and adults (Gazeau et al., 2007; Kurihara et al., 2007; Beniash et al., 2010; Ries, 2011). Aside from affecting calcification and shell growth, ocean acidification can impact protein synthesis, energy production, metabolism, antioxidant responses, and reproduction (Tomanek et al., 2011; Timmins-Schiffman et al., 2014; Dineshram et al., 2016; Boulais et al., 2017; Omoregie et al., 2019). Additionally, adult exposure to ocean acidification may impact their larvae (reviewed in (Ross et al., 2016; Byrne et al., 2019). For example, adult Manila clams (*Ruditapes philippinarum*) and mussels (*Musculista senhousia*) reproductively conditioned in high pCO_2 waters yield offspring that exhibit significantly faster development or lower oxidative stress protein activity in those same conditions (Zhao et al., 2018, 2019). In contrast, northern quahog (hard clam; *Mercenaria mercenaria*) and bay scallop (*Argopecten irradians*) larvae may be more vulnerable to ocean acidification and additional stressors when parents are reproductively conditioned in high pCO_2 waters (Griffith and Gobler, 2017). Some species exhibit both positive and negative carryover effects (ex. *Saccostrea glomerata*; (Parker et al., 2012, 2017). Intergenerational effects have also been documented when adult exposure to ocean acidification does not coincide with reproductive maturity (ex. *Crassostrea gigas*; (Venkataraman et al., 2019)). Although intergenerational carryover effects are now at the forefront of ocean acidification research in bivalve species, it is still unclear how these responses manifest.

Epigenetics is the next frontier for understanding how environmental memory may modulate phenotypic plasticity across generations (Eirin-Lopez and Putnam, 2018). Epigenetics refers to changes in gene expression that do not arise from the original DNA sequence, with methylation of cytosine bases being the most studied mechanism (Bird, 2002; Deans and Maggert, 2015). Unlike highly methylated vertebrate genomes, marine invertebrate taxa have spurious methylation throughout their genomes, similar to a mosaic pattern (Suzuki and Bird, 2008). Genes that benefit from stable transcription, such as housekeeping genes, tend to be more methylated, while environmental response genes that are less methylated are prone to more spurious transcription and alternative splicing patterns, thereby possibly increasing phenotypic plasticity (Roberts and Gavery, 2012; Dimond and Roberts, 2016; Gatzmann et al., 2018). Increased levels of DNA methylation can also correlate with increased transcription. Several loci-level resolution studies in *C. gigas* demonstrate a positive association between DNA methylation and gene expression that is consistent across cell types (Roberts and Gavery, 2012; Gavery and Roberts, 2013; Olson and Roberts, 2014). Since DNA methylation could provide a direct link between environmental conditions and phenotypic plasticity via influencing gene activity, elucidating how invertebrate methylomes respond to abiotic factors is crucial for understanding potential acclimatization mechanisms (Bossdorf et al., 2008; Hofmann, 2017).

While bivalve species have been used as model organisms to characterize marine invertebrate methylomes, how ocean acidification affects bivalve DNA methylation is poorly understood. Methylation responses to ocean acidification have been studied, however, in multiple coral species. When placed in low pH conditions (7.6-7.35), *Montipora capitata* did not demonstrate any differences in calcification, metabolic profiles, or DNA methylation in comparison to clonal fragments in ambient pH (7.9-7.65) (Putnam et al., 2016). DNA methylation increased in another coral species, *Pocillopora damicornis*, in addition to reduced in calcification and more differences in metabolic profiles (Putnam et al., 2016). The coral *Stylophora pistillata* also demonstrates increased global methylation as pH decreases ($pH_{treatment}$ = 7.2, 7.4, 7.8; $pH_{control}$ = 8.0), with methylation reducing spurious transcription (Liew et al., 2018b). Combined whole genome bisulfite sequencing and RNA sequencing revealed differential methylation and expression of growth and stress response pathways controlled differences in cell and polyp size between treatments (Liew et al., 2018b). The association between DNA methylation and phenotypic differences in these corals demonstrates that epigenetic regulation of genes is potentially important for acclimatization and adaptation to environmental perturbation. Recent examination of *C. virginica* methylation patterns in response to a natural salinity gradient suggests that differential methylation may modulate environmental response in this species (Johnson and Kelly, 2019). As bivalves may exhibit a similar response to ocean acidification, continued study could elucidate if DNA methylation could be a mechanism for intergenerational effects.

There is evidence that suggests that methylation patterns can be inherited in marine invertebrates. For example, purple sea urchin (*Strongylocentrotus purpuratus*) offspring have methylomes that reflect maternal rearing conditions (Strader et al., 2019). Different parental temperature and salinity regimes influence larval methylomes in *Platygyra daedalea* (Liew et al., 2018a). In the Pacific oyster (*Crasostrea gigas*), parental exposure to pesticides influence DNA methylation in spat, even though the spat were not exposed to these conditions (Rondon et al., 2017). Methylation changes in reproductive tissue are likely the ones that could be inherited, and may play a role in carryover effects. Before determining if DNA methylation is a viable mechanism for altering subsequent generation phenotypes, the epigenome of bivalve reproductive tissue in response to ocean acidification must be characterized.

The present study is the first to determine if ocean acidification induces differential methylation in reproductive tissue in the eastern oyster (*Crassostrea virginica*). Adult *C. virginica* were exposed to control or elevated pCO_2 conditions. We hypothesize that ocean acidification will induce differential methylation in *C. virginica* gonad tissue. Genes that contain differentially methylated loci will have biological functions that allow for acclimatization to environmental perturbation. Understanding how experimental ocean acidification conditions modify the oyster epigenome, and if these modifications are inherited, allows for a better understanding of how ecosystems will respond to environmental change.

Methods

Experimental Design

Adult *C. virginica* (9.55 cm ± 0.45) were collected from an intertidal oyster reef in Plum Island Sound, MA (42.681764, -70.813498) in mid-July 2016. The oysters were transported to the Marine Science Center at Northeastern University (Nahant, MA), where they were cleaned and randomly assigned to one of six flow-through tanks (50L) maintained at ambient seawater conditions. Oysters were acclimated for 14 days under control conditions (500 µatm; 14-15ºC) before initiating a 28-day experimental exposure. Half of the tanks remained at control pCO_2 conditions (500 µatm, $\Omega_{calcite} > 1$), while the other half were ramped up to elevated pCO_2 conditions (2500 µatm, $\Omega_{calcite} < 1$) over 24 hours. This elevated treatment is consistent with observations in other estuarine ecosystems that oysters inhabit (Feely et al., 2010). Five oysters were collected from each treatment at the end of the 28 day exposure. They were immediately dissected with gonadal tissue harvested and immediately flash frozen.

Treatment conditions were replicated across three tanks, with oysters distributed evenly among tanks (1-2 oysters per tank). Each tank had an independent flow-regulator that delivered fresh, natural seawater at approximately 150 ml min^{-1}. Carbonate chemistry was maintained independently for each tank by mixtures of compressed CO_2 and compressed air at flow rates proportional to the target pCO_2 conditions. Gas flow rates were maintained with *Aalborg* digital solenoid-valve-controlled mass flow controllers (Model GFC17, precision = 0.1mL/min). Within a treatment, tanks were replenished with fresh seawater and each tank was independently bubbled with its own mixed gas stream, with partial recirculation and filtration with other tanks in the treatment. As a result, the carbonate chemistry (i.e., the independent variable by which the treatments were differentiated) of the replicate tanks were slightly different from each other, which is evidence of their technical independence. Temperature was maintained at 15ºC using Aqua Euro USA model MC-1/4HP chillers coupled with 50-watt electric heaters. Average salinity was determined by the incoming natural seawater and reflected ambient ocean salinity of Massachusetts Bay near the Marine Science Center (Latitude = 42.416100, Longitude = -70.907737). Oysters were fed 2.81 mL/day of a 10% Shellfish Diet 1800 twice daily following Food and Agriculture Organization's best practices for oysters (Helm and Bourne, 2004).

Measurement and control of seawater carbonate chemistry

The carbonate chemistry of tanks was controlled by bubbling mixtures of compressed CO_2 and compressed air at flow rates proportional to the target pCO_2 conditions. The control pCO_2 treatments were maintained by bubbling compressed ambient air only.

Temperature, pH, and salinity of all replicate tanks was measured three times per week for the duration of the experiment. Temperature was measured using a glass thermometer to 0.1ºC accuracy, pH was measured using an *Accumet* solid state pH electrode (precision = 1mV), salinity was measured using a *YSI 3200* conductivity probe (precision = 0.1 ppt). Every two weeks, seawater samples were collected from each replicate tank for analysis of dissolved inorganic carbon (DIC) and total alkalinity (A_T). Samples were collected in 250 mL borosilicate glass bottles sealed with a greased stopper, immediately poisoned with 100 µL saturated $HgCl_2$ solution, and then refrigerated. Samples were analyzed for DIC via coulomtery and Alk_T via

closed-cell potentiometric Gran Titration with a VINDTA 3C (Marianda Corporation). Other carbonate system parameters, including $\Omega_{calcite}$, pH, and pCO_2, were calculated from DIC, A_T, salinity, and temperature using CO2SYS software version 2.1 (Lewis and Wallace, 1998; Van Heuven et al., 2011), using the seawater pH scale (mol/kg-SW) with K1 and K2 values from (Roy, Rabindra N Roy, Lakshimi N Vogel, Kathleen M Porter-Moore, C Pearson, Tara Good, Catherine E Millero, Frank J Campbell, Douglas M, 1993), a KHSO4 value from (Dickson, 1990), and a [B]Tvalue from (Lee, Kitach Tae-Wook, Kim Byrne, Robert H Millero, Frank J Feely, Richard A Liu, Yong-Ming, 2010).

MBD-BS Library Preparation

DNA was isolated from five gonad tissue samples per treatment using the E.Z.N.A. Mollusc Kit (Omega) according to the manufacturer's instructions. Isolated DNA was quantified using a Qubit dsDNA BR Kit (Invitrogen). DNA samples, ranging from 12.8 ng/µL to 157 ng/µL, were placed in 1.5 mL centrifuge tubes and sonicated using a QSONICA CD0004054245 (Newtown, CT) in 30 second interval periods over ten minutes at 4 ºC and 25% intensity. Shearing size (350bp) was verified using a 2200 TapeStation System (Agilent Technologies). Samples were enriched for methylated DNA using MethylMiner kit (Invitrogen). Library preparation and sequencing was performed by ZymoReearch using Pico Methyl-Seq Library Prep Kit (Cat. #D5455) to generate 100bp paired-end reads on the HiSeq1500 sequencer (Illumina, Inc.).

Global Methylation Characterization

Sequences were trimmed with 10 bp removed from both the 5' and 3' ends using TrimGalore! v.0.4.5 (Martin, 2011). Quality of sequences was assessed with FastQC v.0.11.7 (Andrews, 2010). The *C. virginica* genome (NCBI Accession GCA_002022765.4) was prepared using Bowtie 2-2.3.4 (Linux x84_64 version; (Langmead and Salzberg, 2012)) within the `bismark_genome_preparation` function in Bismark v.0.19.0 (Krueger and Andrews, 2011). Trimmed sample sequences were then aligned to the genome using Bismark v.0.19.0 (Krueger and Andrews, 2011) with non-directionality specified alignment score set using `-score_min L,0,-1.2`. Deduplicated alignments files (ie .bam) were sorted and indexed using SAMtools v.1.9 (Li et al., 2009). Methylation calls were extracted from deduplicated files using `bismark_methylation_extractor`.

Various *C. virginica* genome feature tracks were created for downstream analyses using BEDtools v2.26.0 (Quinlan and Hall, 2010). Genes, mRNA, and exons were derived directly from the *C. virginica* genome on NCBI (Gómez-Chiarri et al., 2015). The complement of the exon track was used to identify introns. Putative promoter regions were defined as those 1kb upstream of transcription start sites. Putative transposable elements were identified using RepeatMasker (v4.07) with RepBase-20170127 and RMBlast 2.6.0 (Smit et al., 2013; Bao et al., 2015). All species available in RepBase-20170127 were used to identify transposable elements.

Overall *C. virginica* gonad methylation patterns were characterized using information from all samples. Individual CpG dinucleotides with at least 5x coverage in each sample were classified

as methylated (≥ 50% methylation), sparsely methylated (10-50% methylation), or unmethylated (< 10% methylation). The locations of all methylated CpGs were characterized in relation to exons, introns, transposable elements, and putative promoter regions. We tested the null hypothesis that there was no association between the genomic location of CpG loci and methylation status (all CpGs versus methylated CpGs) with a chi-squared contingency test (`chisq.test` in R Version 3.5.0).

Differential Methylation Analysis

Differential methylation analysis for individual loci was performed using `methylKit` v.1.7.9 in R (Akalin et al., 2012) using deduplicated, sorted bam files as input. Only loci with at least 5x coverage in each sample were considered for analysis. Methylation differences between treatments were obtained for all loci in the CpG background using `calculateDiffMeth`, a logistic regression built into `methylKit`. The logistic regression models the log odds ratio based on the proportion of methylation at each locus:

$$log(\frac{\pi_i}{1 - \pi_i}) = \beta_0 + \beta_1 Treatment_i$$

A differentially methylated locus was defined as individual CpG dinucleotide with at least a 50% methylation change between treatment and control groups, and a q-value < 0.01 based on correction for false discovery rate with the SLIM method (Wang et al., 2011). Hypermethylated DML were defined as those with significantly higher percent methylation in oysters exposed to high pCO_2, and hypomethylated DML with significantly lower percent methylation in the high pCO_2 treatment. A Principal Components Analysis (PCA) was used to compare oyster sample methylation profiles between treatments for all CpG loci and for differentially methylated loci (DML). The location of DML were characterized in relation to exons, introns, transposable elements, and putative promoter regions using BEDtools `intersect` v2.26.0. The position of DML within genes was characterized by scaling the position of the DML to a hypothetical gene ranging from 0 to 100 bp. Loci that did not overlap with the aforementioned genomic features were also identified. A chi-squared contingency test was used to test the null hypothesis of no association between genomic location and methylation status between MBD-enriched CpGs and DML.

Gene-specific methylation profiles were analyzed using a pipeline modified from (Liew et al., 2018b) to determine if how whole gene methylation patterns might be influenced by ocean acidification.For each sample, loci with 5x coverage were annotated using `intersectBED`. Annotations included gene start and end positions, along with start and end positions for the longest constituent mRNA. Median percent methylation was then calculated for each gene in a sample. Any gene that was not present in all ten samples was excluded from downstream analysis. A non-parametric Kruskal-Wallis test was used to compare median percent methylation of a gene between pCO_2 treatments. Differentially methylated genes (DMG) were defined as those with corrected *P*-values < 0.05.

Enrichment Analysis

Functional enrichment analyses were used to determine if any biological processes were overrepresented in genes based on individual CpG methylation levels. Enrichment was conducted with GO-MWU, a rank-based gene enrichment method initially developed for analyzing transcriptomics data (Wright et al., 2015). Instead of only using genes with DML, GO-MWU identifies GO categories that are overrepresented by genes with any hyper- or hypomethylated CpGs, allowing for more data to contribute to any trends. GO-MWU scripts and a gene ontology database were downloaded from the GO-MWU Github repository (https://github.com/z0on/GO_MWU).

A gene list, containing Genbank IDs and all associated gene ontology terms, and a table of significance measures, with Genbank IDs and signed log *P*-values for all CpGs determined by `methylKit` during the identification of DML, were used as GO-MWU analysis inputs. For the table of significance measures, *P*-values were determined by filtering methylation differences between treatments for the CpG background such that only the CpG locus with the smallest *P*-value within each gene was used in downstream enrichment. Adjusted *P*-values from `methylKit` were converted to signed log *P*-values, such that loci with positive methylation differences had positive log *P*-values, and loci with negative methylation differences had negative log *P*-values. To match the Genbank IDs to CpG loci within mRNAs and create the gene list, overlaps between the *C. virginica* mRNA track from NCBI and the CpG background used in `methylKit` were obtained using BEDtools `intersect` v2.26.0. The mRNAs were then annotated with Uniprot Accession codes using a BLASTx search (v.2.2.29; (Gish and States, 1993; UniProt Consortium, 2019)(Gish and States, 1993). The Uniprot Swiss-Prot Database (downloaded from SwissProt 2018-06-15) was used to obtain protein information and Uniprot Accession codes (Gish and States, 1993; UniProt Consortium, 2019). Genbank IDs provided by NCBI were used to match CpG background-mRNA overlaps with the annotated mRNA track. Gene ontology terms were paired to Uniprot Accession codes using the Uniprot Swiss-Prot Database (UniProt Consortium, 2019).

Once analysis inputs were created, gene ontology terms for each gene were matched with parental terms using default GO-MWU settings. Parental ontology categories with the exact same gene list were combined. Groups were further combined if they shared at least 75% of the same genes. After clustering was complete, a Mann-Whitney U test identified gene ontology categories that were significantly enriched by corresponding hyper- or hypo-methylated loci in genes using the default 10% FDR. Genes with DML were mapped to gene ontology subsets (GO Slim terms) for biological processes to further categorize gene functions.

Results

Water Chemistry

Oysters experienced different water chemistry conditions between control and elevated pCO_2 treatments (Table 1). All oysters were subjected to acclimation pCO_2 conditions (pCO_2 = 521 ± 32 ppm, $\Omega_{calcite}$ = 2.82 ± 0.13) were maintained for 14 days. Oysters in control pCO_2 conditions (pCO_2 = 492 ± 50 µatm; $\Omega_{calcite}$ = 3.01 ± 0.25) experienced low pCO_2 and higher $\Omega_{calcite}$ than those in elevated pCO_2 conditions (pCO_2 = 2550 ± 211 µatm; $\Omega_{calicite}$ = 0.72 ± 0.06) (Table 1).

Experimental Stage	T (ºC)	S (PSU)	DIC	Alk_T	pH_{sw}	pCO_2 (µatm)	$\Omega_{calcite}$
Acclimation	14.6 ± 0.4	31.4 ± 0.1	1978 ± 7	2127 ± 6	7.94 ± 0.00	521 ± 32	2.82 ± 0.13
Control pCO_2 Conditions	14.5 ± 0.4	31.6 ± 0.3	1960 ± 32	2140 ± 15	7.95 ± 0.01	492 ± 50	3.01 ± 0.25
Elevated pCO_2 Conditions	14.5 ± 0.3	31.54 ± 0.32	2173 ± 37	2132 ± 42	7.29 ± 0.01	2550 ± 211	0.72 ± 0.06

Table 1. Summary of water chemistry during the 14-day acclimation period and 28-day experimental exposure. Values indicate mean and standard error for temperature (T), salinity (S), dissolved inorganic carbon (DIC), total alkalinity (A_T), calculated pH on seawater scale, calculated pCO_2, and calculated calcite saturation ($\Omega_{calcite}$).

MBD-BS-Seq

DNA sequencing yielded 280 million DNA sequence reads (NCBI Sequence Read Archive: BioProject accession number PRJNA513384). Of 276 million trimmed paired-end reads, 136 million (49.4%) were mapped to the *C. virginica* genome, providing an average of 13.6 million reads per sample. Sequencing efforts provided data for 4,304,257 CpG loci (30.7% of 14,458,703 total CpGs in the *C. virginica* genome) with at least 5x coverage across all samples combined. As expected, the location of CpGs with 5x coverage in the genome differed from the distribution of all CpG motifs (Contingency test; χ_2 = 906,940, df = 4, *P*-value < 2.2e-16). Of all loci with 5x coverage, 3,255,049 CpGs (75.6%) were found in genic regions in 33,126 out of 38,929 annotated genes in the genome.

The general methylation landscape was defined using all loci with a minimum 5x coverage in each sample. The majority, 3,181,904 (73.9% of MBD-Enriched loci) loci were methylated, with 481,788 (11.2%) sparsely methylated loci and 640,565 (14.9%) unmethylated loci (Figure 1). The *C. virginica* genome was determined to be 22% methylated. Loci methylation was characterized in relation to exons, introns, transposable elements, and putative promoter regions (Figure 2). Methylated CpGs were found primarily in genic regions, with 2,521,653 loci (79.2%) in 25,496 genes. The null hypothesis — CpG methylation status was independent of

genomic location — was rejected, as the proportion of methylated CpG loci was different than expected in exons, introns, transposable elements, putative promoters, and intergenic regions (Contingency test; χ^2 = 872,070, df = 4, *P*-value < 2.2e-16; Figure 2). There was a larger proportion of methylated loci found in exons compared to all CpGs in the genome (Figure 2). Methylated loci were also found in introns (with 1,448,786 loci (47.3% of methylated loci) versus 1,013,691 CpGs (31.9%) in exons), although this was not higher than expected based on the distribution of all CpGs. Transposable elements contained 755,222 methylated CpGs (23.7%). Putative promoter regions overlapped with 106,111 loci (3.3%). There were 372,047 methylated loci (11.7%) that did not overlap with either exons, introns, transposable elements, or promoter regions.

Differential Methylation Analysis

A total of 598 CpG loci were differentially methylated between oysters exposed to control or high pCO_2, with 51.8% hypermethylated and 48.2% hypomethylated between treatments (Figure 3; full list available in Github repository). When considering a PCA using methylation status of all CpG loci with 5x coverage across all samples, the first two principal components explained 29.8% of sample variation (Figure 4A). However, the first two principal components in a PCA with only differentially methylated loci (DML) explained 57.1% of the variation among treatments (Figure 4B). These DML were distributed throughout the *C. virginica* genome (Figure 5). The highest number of DML were in the fifth chromosome, which also has the largest number of genes but was not the largest chromosome (Figure 5A).

Examination of DML within genes revealed that some genes contained multiple DML (Figure 5B-F). Of the 481 genes with DML, the majority only contained one DML (Figure 5B-C). There were 48 genes with 2 DML (Figure 5D), 16 genes with 3 DML (Figure 5E), 6 genes with 4 DML (Figure 5F) and 1 gene with 5 DML. When multiple DML were found within a gene, they could be methylated in either the same or opposite directions (Figure 5C-F).

Within the genome, DML were mostly present in genic regions, with 560 DML in 481 genes (368 DML in exons and 192 in introns). In addition, 42 DML were found in putative promoter regions, 57 in transposable elements, and 21 located outside of defined regions. The distribution of DML in *C. virginica* gonad tissue was higher in exons than expected for MBD-enriched CpG loci with minimum 5x coverage across all samples (Contingency test; χ^2 = 314.18, df = 4, *P*-value < 2.2e-16; Figure 6). Of the 598 DML, 310 were hypermethylated and 288 were hypomethylated in the high pCO_2 treatment. The number of hyper- and hypomethylated DML was almost evenly split within each genomic feature, with the exception of putative promoter regions that had 44 hypermethylated DML versus 23 hypomethylated DML. Within a gene, DML did not appear to be concentrated in one particular region. The distribution of hyper- and hypomethylated DML along a gene do not differ from each other (Figure 7). The DMG analysis did not identify any differentially methylated genes.

The DML were found in genes responsible for various biological processes. However, no gene ontology categories were significantly represented (Figure 8). The majority of genes with DML were involved in protein ubiquitination processes. These genes were not consistently hyper- or

hypomethylated. Certain biomineralization genes did contain DML. The gene coding for calmodulin-regulated spectrin-associated protein contained three hypomethylated and one hypermethylated DML. Genes coding for EF-hand protein with calcium-binding domain, calmodulin-binding transcription activator, and calmodulin-lysine N-methyltransferase contained one or two hypermethylated DML.

Discussion

The present study is one of the first general descriptions of DNA methylation in *C. virginica*, and is one of only a few that have examined epigenetic responses to ocean acidification in the gonad tissue of a mollusk species. Five hundred ninety-eight differentially methylated loci (DML) were identified in response to the elevated pCO_2 treatments, most of which were in exons. Not only was DNA methylation of *C. virginica* altered in response to ocean acidification, but changes in gonad methylation indicates potential for these methylation patterns to be inherited by offspring.

Understanding how environmental stressors influence the epigenome is crucial when considering potential acclimatization mechanisms in marine invertebrates. Our finding that high pCO_2 impacts *C. virginica* DNA methylation adds to a growing body of work about ocean acidification's impact on marine invertebrate methylomes. The coral species *P. damicornis* demonstrated an overall increase in DNA methylation when exposed to low pH conditions (7.3 - 7.6), potentially influencing biomineralization (Putnam et al., 2016). Another coral species, *S. pistillata*, also demonstrated an increase in genome-wide DNA methylation when exposed to low pH conditions. Changes in the methylome also modified gene expression and altered pathways involved in cell cycle regulation (Liew et al., 2018b). The present study on an oyster, however, did not observe the overall genome-wide increase in methylation that was reported for corals. Instead, we found subtle, but significant, increases or decreases in percent methylation at several hundred individual CpGs distributed across the genome. As *C. virginica* and coral species are adapted to different environments and ecological niches, It is possible that species-specific differences in methylation responses contribute to the observed methylation pattern.

The *C. virginica* methylation landscape suggests a role for methylation in gene activity. Only 22% of the *C. virginica* genome is methylated, which is consistent with previous studies of marine invertebrate genomes (Roberts and Gavery, 2012; Dimond and Roberts, 2016; Hofmann, 2017). Methylated loci are concentrated in introns for *C. virginica*, followed by exons and transposable elements. This location of methylated CpGs in gene bodies is consistent to what has been reported across similar taxa (Roberts and Gavery, 2012; Eirin-Lopez and Putnam, 2018). The concentration of methylated CpGs in gene bodies corresponds with proposed functionality in influencing gene activity (Roberts and Gavery, 2012; Dixon et al., 2014; Liew et al., 2018b). Our study also found methylation in transposable elements, putative promoters and intragenic regions. In plants, transposable element methylation has been shown to modulate the effect of transposable element insertion in genic regions (Hosaka and Kakutani,

2018). It is possible that methylation of transposable elements in *C. virginica* could also limit the effect of transposable elements.

While both *C. virginica* and the closely-related Pacific oyster (*C. gigas*) have relatively sparse transposable element and putative promoter methylation, there are differences between the methylomes that could be indicative of species variation or genome assembly. Previously MBD-BS-Seq of ctenidia tissue and whole genome bisulfite sequencing of sperm revealed that 15% of the Pacific oyster genome is methylated, and that this methylation is consistent between tissue types (Gavery and Roberts, 2013; Olson and Roberts, 2014). Our study of gonad tissue found that 22% of the *C. virginica* genome is methylated. This difference can be attributed to the distribution of CpG dinucleotides in both species. There are about 10 million CpG dinucleotides in the described *C. gigas* genome, but over 14 million in the *C. virginica* genome. Additionally, more CpG dinucleotides are located in genic regions in *C. virginica*. Gavery and Roberts (2013) report 38% of total CpGs in *C. gigas* are found in exons and introns, with 47% of CpGs in intergenic regions. The present study with *C. virginica* found 55% of total CpGs in exons and introns, with 45% intergenic CpGs. However, there were more methylated loci in transposable elements for *C. virginica*. Examination of methylation in conserved areas of both genomes may elucidate if these differences are biologically meaningful.

The concentration of DML in gene bodies suggests a role for DNA methylation in gene expression and regulation. A majority of genes with DML were involved in protein ubiquitination. Protein ubiquitination is a post-translational protein modification that is involved in protein synthesis and degradation (Peng et al., 2003; Komander, 2009). Previous studies in which oysters were exposed to experimental ocean acidification conditions have demonstrated changes in this pathway. For example, shotgun proteomic characterization of posterior gill lamellae from adult *C. gigas* exposed to high pCO_2 revealed increased abundance of proteins involved in ubiquitination and decreased protein degradation (Timmins-Schiffman et al., 2014). Elevated pCO_2 levels were also found to upregulate malate dehydrogenase in adult *C. virginica* mantle tissue (Tomanek et al., 2011). Several genes involved in protein ubiquitination, including those for malate dehydrogenase, ubiquitin-protein ligase, RNA polymerase-associated protein, and DNA damage-binding protein, were significantly hypermethylated in gonad tissue exposed to elevated pCO_2. Hypermethylation of these genes may decrease transcriptional opportunities, thus indicating a critical role in the response to ocean acidification. Given that the patterns observed in our study were equivocal, additional research is needed to elucidate the role that methylation plays in protein ubiquitination processes for oysters exposed to environmental stressors.

Four genes involved in biomineralization contained DML, suggesting these genes can be epigenetically regulated. Changes to calcium-binding gene expression have been previously documented in *C. virginica* (Richards et al., 2018). Many studies examining ocean acidification-induced carryover effects in bivalves note changes to calcification processes. For example, the Sydney rock oyster (*Saccostrea glomerata*) larvae exhibit faster shell growth in high pCO_2 conditions when parents mature in those same conditions (Parker et al., 2012, 2015). In contrast, larvae from other species found in the North Atlantic such as northern quahog (hard

clam; *M. mercenaria*) and bay scallops (*A. irradians*) developed slower when parents were reproductively conditioned in low pH conditions (Griffith and Gobler, 2017). There is some evidence to suggest that *C. virginica* larvae may be more resilient to high pCO_2 conditions than *M. mercenaria* or *A. irradians (Gobler and Talmage, 2014)*. Differential methylation of biomineralization genes in *C. virginica* reproductive tissue could be a mechanism to explain when parental experience impacts larval calcification if in fact these DMLs are inherited.

Conclusion

Our study found that *C. virginica* demonstrates a significant epigenetic response to elevated pCO_2 exposure, with 598 DML identified. The concentration of these DML in gene bodies suggests that methylation may be important for transcriptional control in response to environmental stressors. As ocean acidification induced differential methylation in *C. virginica* gonad tissue, there is a potential for intergenerational epigenetic inheritance gene activity control include processes such as biomineralization. As carryover effects can persist even when stressors are long-removed (Venkataraman et al., 2019), understanding the mechanisms involved in intergenerational acclimatization is crucial. Future work should focus on methylation patterns in adult *C. virginica* reproductive tissue, fully-formed gametes, and larvae exposed to various pCO_2 conditions to determine to what degree a difference in methylation influences gene activity and how this might influence phenotypic plasticity.

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Data Accessibility

Associated information for all analyses and supplemental material can be found in the Github repository: https://github.com/epigeneticstoocean/paper-gonad-meth

Figures

Figure 1. Frequency distribution of methylation ratios for CpG loci in *C. virginica* gonad tissue DNA subjected to MBD enrichment. A total of 4,304,257 CpGs with at least 5x coverage summed across all ten samples were characterized. Loci were considered methylated if they were at least 50% methylated, sparsely methylated loci were 10-50% methylated, and unmethylated loci were 0-10% methylated.

Figure 2. Proportion of CpG loci within with genomic features. All CpGs are every dinucleotide in the *C. virginica* genome. Methylated CpGs refers to loci are defined as being at least 50% methylated determined as part of this study.

Figure 3. Heatmap of DML in *C. virginica* reproductive tissue. Samples in control pCO_2 conditions are represented by grey, and samples in elevated pCO_2 conditions are represented by a black bar. Loci with higher percent methylation are represented by darker colors. A logistic regression identified 598 DML, defined as individual CpG dinucleotide with at least a 50% methylation change between treatment and control groups, and a q-value < 0.01 based on correction for false discovery rate with the SLIM method. The density of DML at each percent methylation value is represented in the heatmap legend.

Figure 4. Principal Components Analysis of a) all CpG loci with 5x coverage across samples and b) DML. Methylation status of individual CpG loci explained 29.2% of variation between samples when considering all CpG loci. Methylation status of DML explained 57.1% of sample variation.

Figure 5. Distribution of DML among chromosomes and genes. (a) Number of DML (bars) and number of genes (line) in each *C. virginica* chromosome. (b) Counts of DML in genes containing DML. Most genes that contained DML only had 1 DML. Breakdown of hyper- and hypomethylation patterns in genes with one (c), two (d), three (e), and four (f) DML. The number of hyper- or hypomethylated DML in a gene are indicated below the bars. (c) From left to right, bars represent the number of genes with only hypermethylated DML and only hypomethylated DML; (d) the number of genes with 2 hypermethylated DML, 1 hyper- and 1 hypomethylated DML, and 2 hypomethylated DML; (e) the number of genes with 3 hypermethylated DML, 2 hyper- and 1 hypomethylated DML, 1 hyper- and 2 hypomethylated DML, and 3 hypomethylated DML; (f) the number of genes with 4 hypermethylated DML, 3 hyper- and 1 hypomethylated DML, 2 hyper- and 2 hypomethylated DML, 1 hyper- and 3 hypomethylated DML, and 4 hypomethylated DML. There was one gene with 5 DML (4 hypermethylated and 1 hypomethylated).

Figure 6. Proportion CpG loci within with exons, introns, transposable elements (TE), and putative promoters for MBD-enriched CpGs and differentially methylated loci (DML),The distribution of DML in *C. virginica* gonad tissue differed from distribution of MBD-enriched loci with 5x coverage across control and treatment samples (Contingency test; χ^2 = 314.18, df = 4, *P*-value < 2.2e-16).

Figure 7. Distribution of hyper- and hypomethylated DML along a hypothetical gene. The scaled position of a DML within a gene was calculated by dividing the base pair position of the DML by gene length. Counts of hypermethylated DML are plotted above the x-axis, and hypomethylated DML counts are below the x-axis.

Figure 8. Biological processes represented by all genes used in enrichment background (% Genes) and those with DML (% Genes with DML). Gene ontology categories with similar functions are represented by the same color. Genes may be involved in multiple biological processes. No gene ontologies were significantly enriched.

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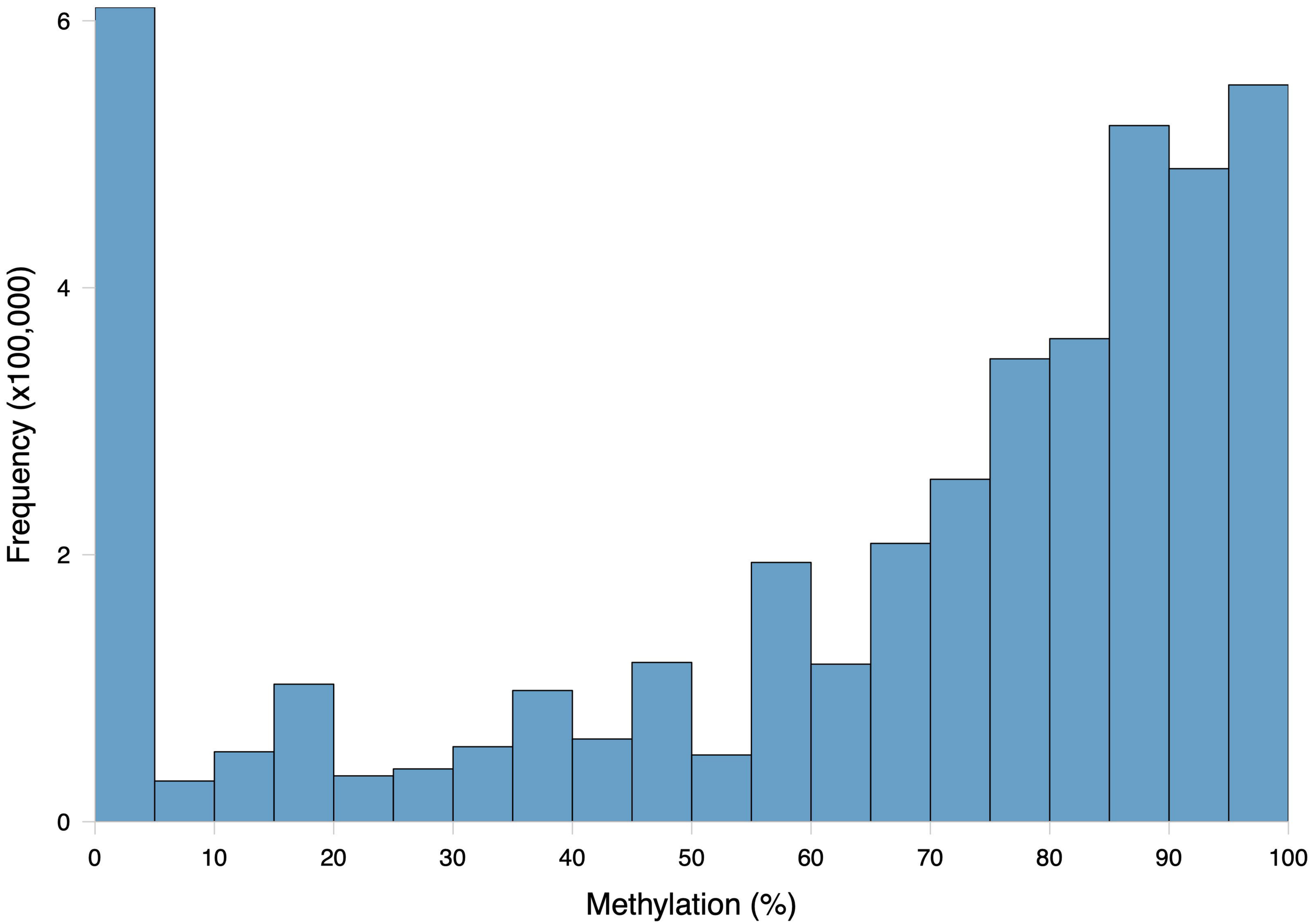
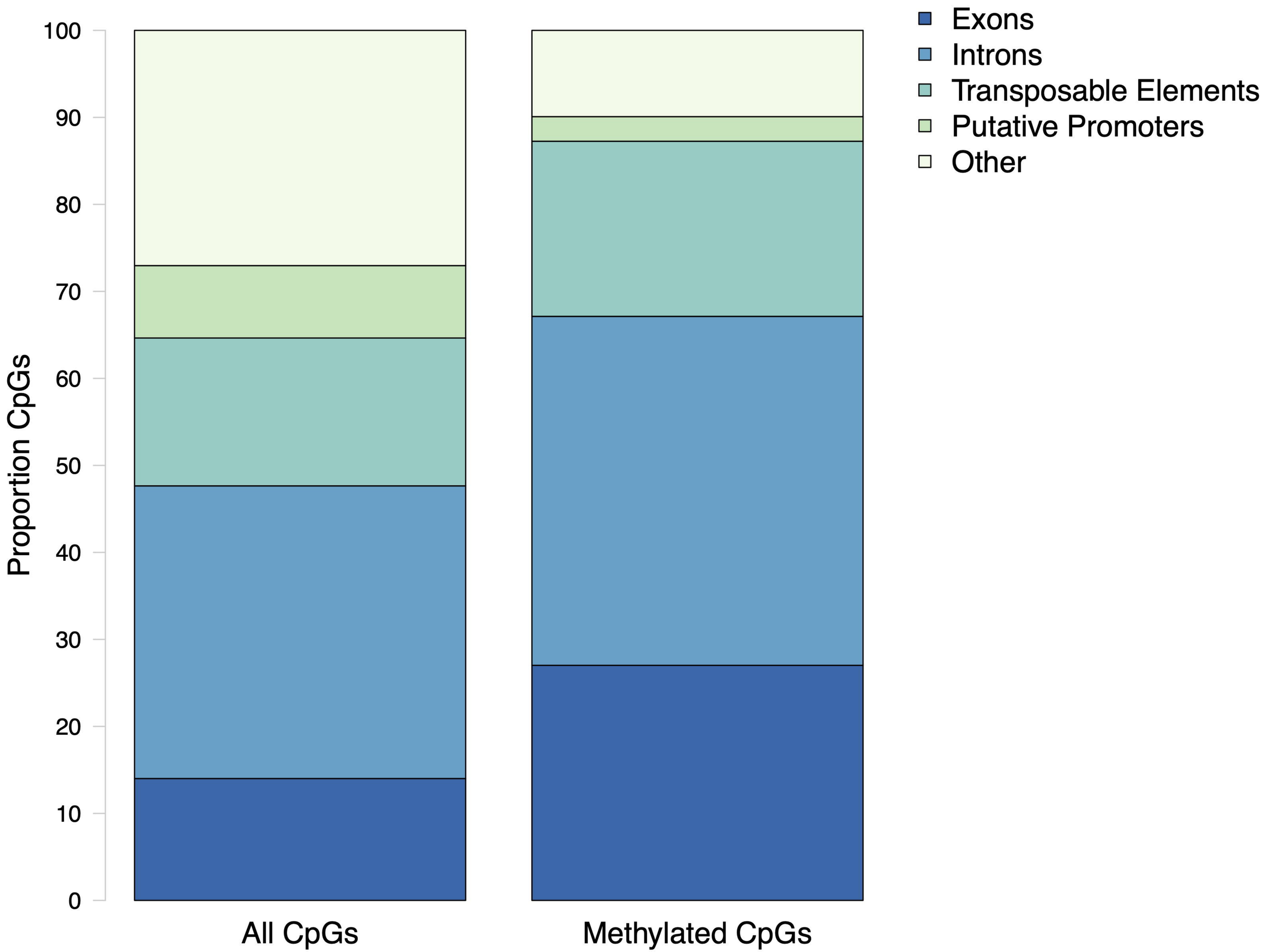
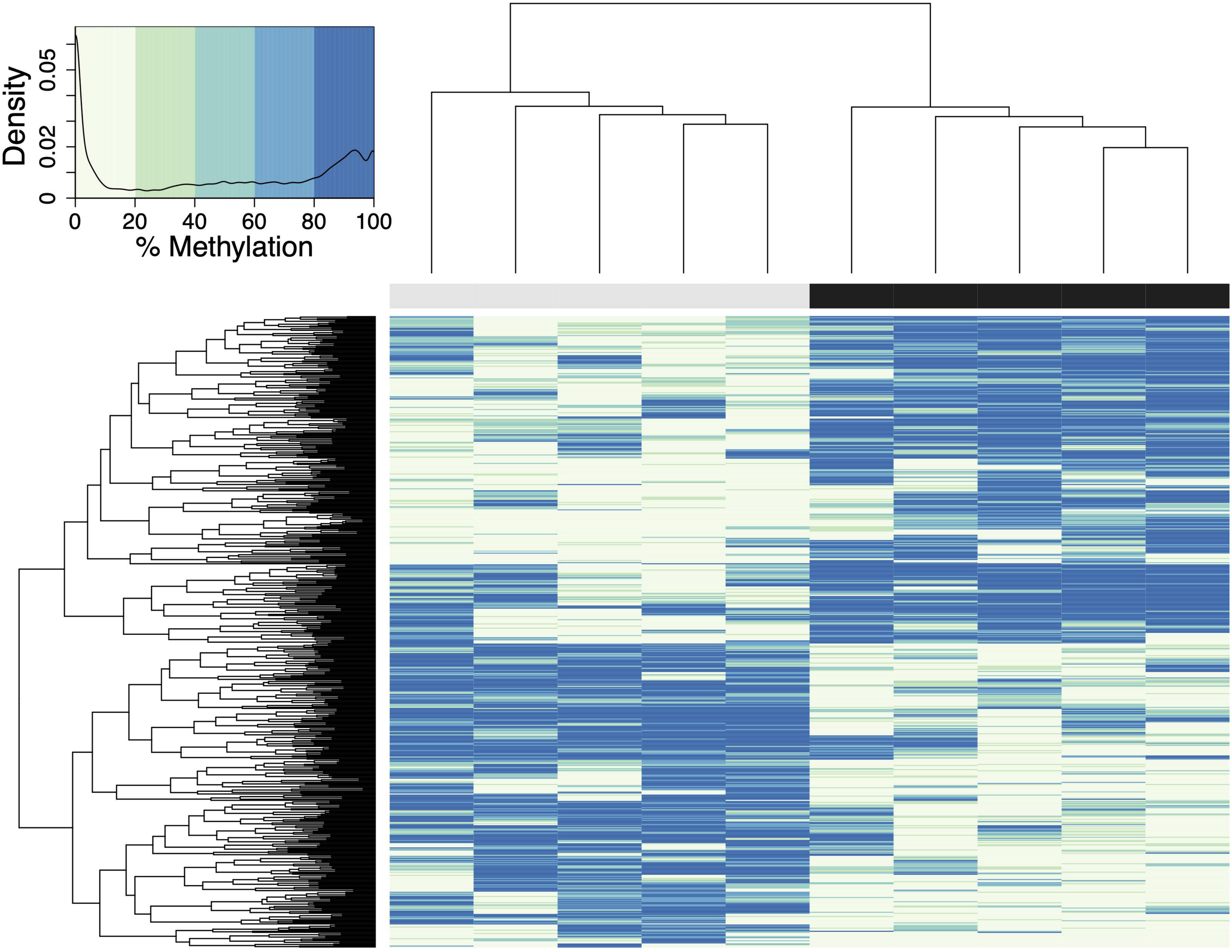
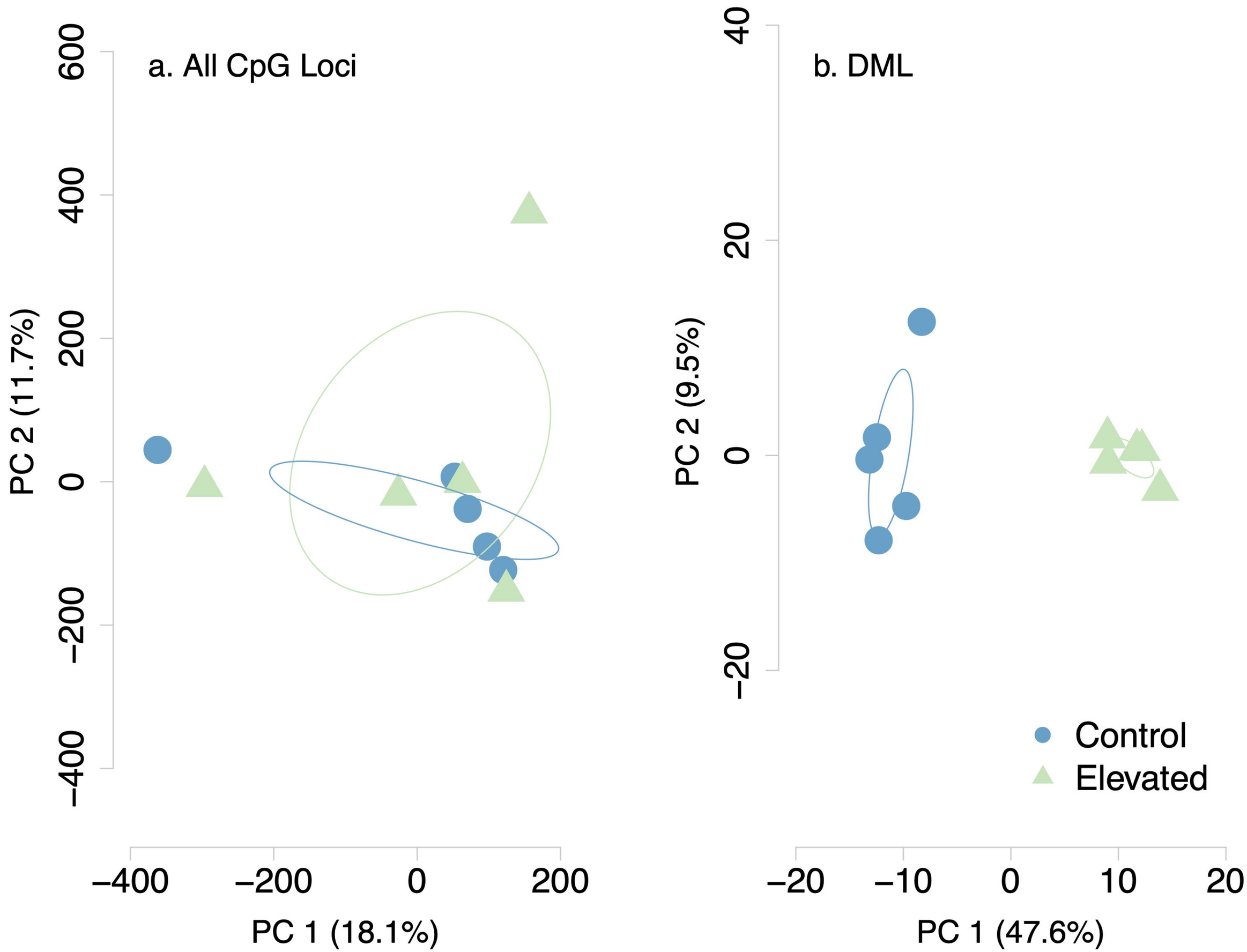
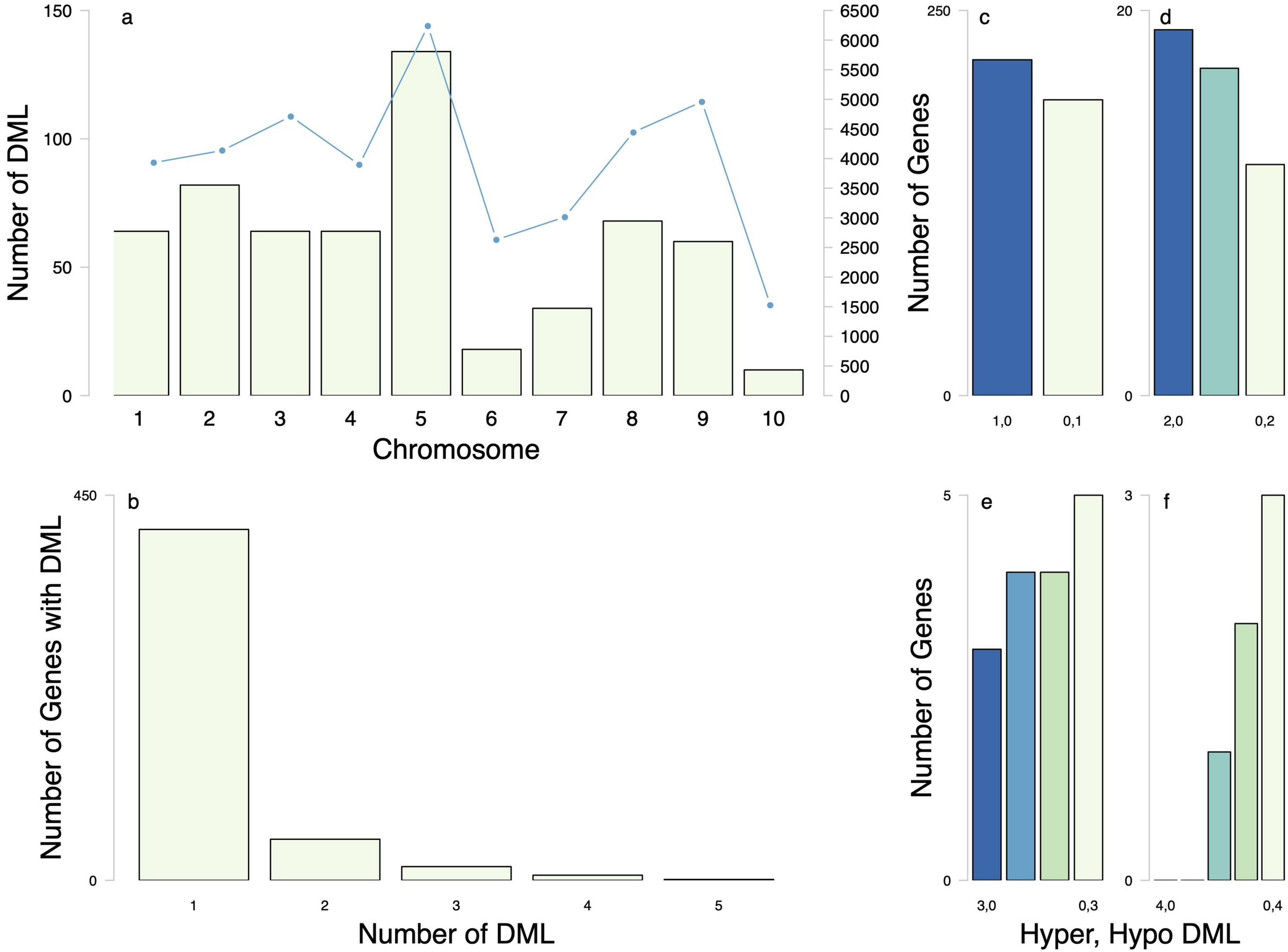

Frequency (x100,000)
6
4
2
0
0
10
20
30
40
50
60
70
80
90
100
Methylation (%)


Exons
Introns
Transposable Elements
Putative Promoters
Other
Proportion CpGs
100
90
80
70
60
50
40
30
20
10
0
All CpGs
Methylated CpGs

Density
0
0.02
0.05
0
20
40
60
80
100
% Methylation




a. All CpG Loci
b. DML
PC 2 (11.7%)
PC 1 (18.1%)
PC 2 (9.5%)
PC 1 (47.6%)
600
400
200
0
−200
−400
40
20
0
−20
−400
−200
0
200
−20
−10
0
10
20
Control
Elevated


a
Number of DML
150
100
50
0
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1
2
3
4
5
6
7
8
9
10
Chromosome
b
Number of Genes with DML
450
0
1
2
3
4
5
Number of DML
c
Number of Genes
250
0
1,0
0,1
d
20
0
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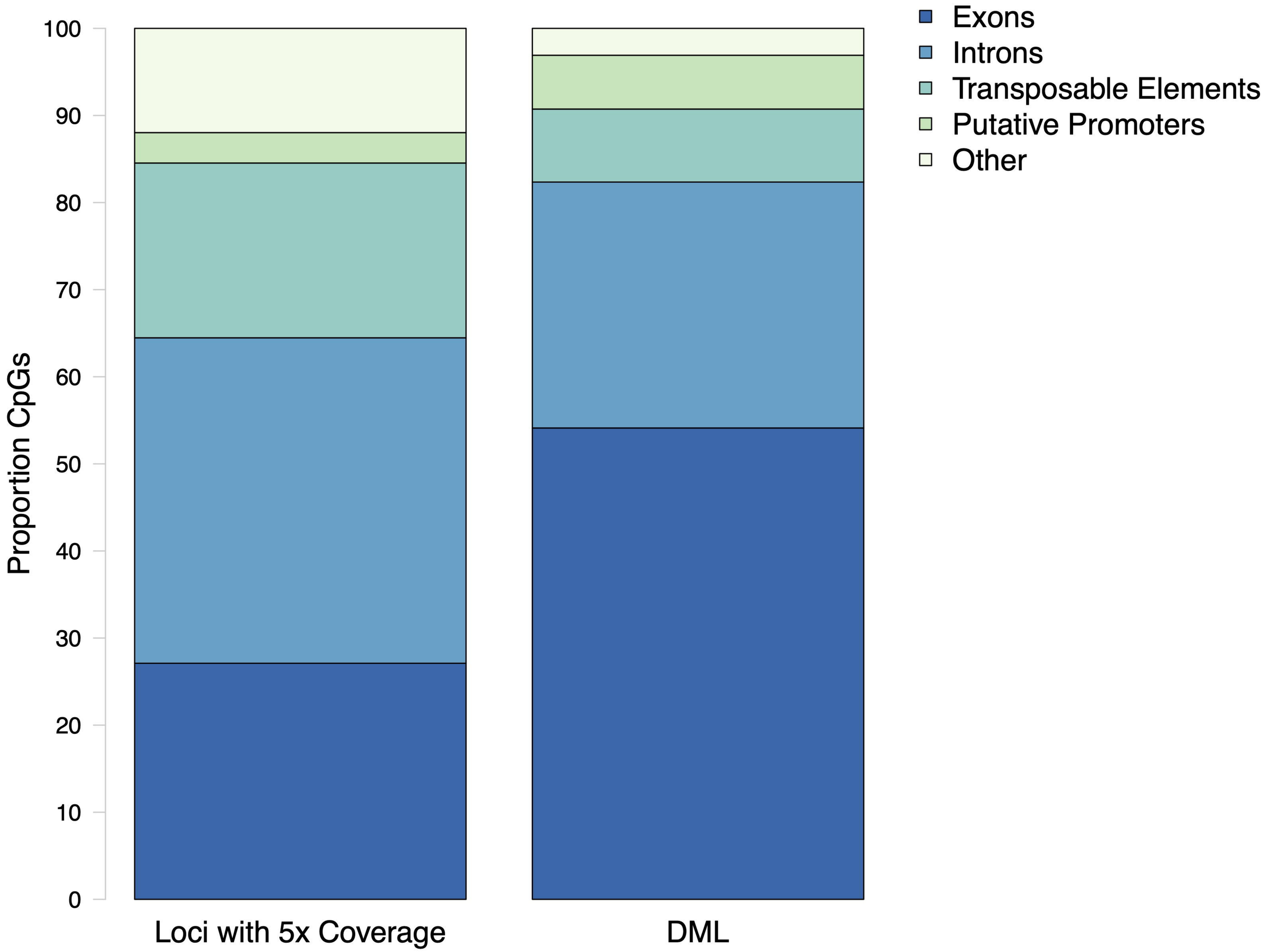
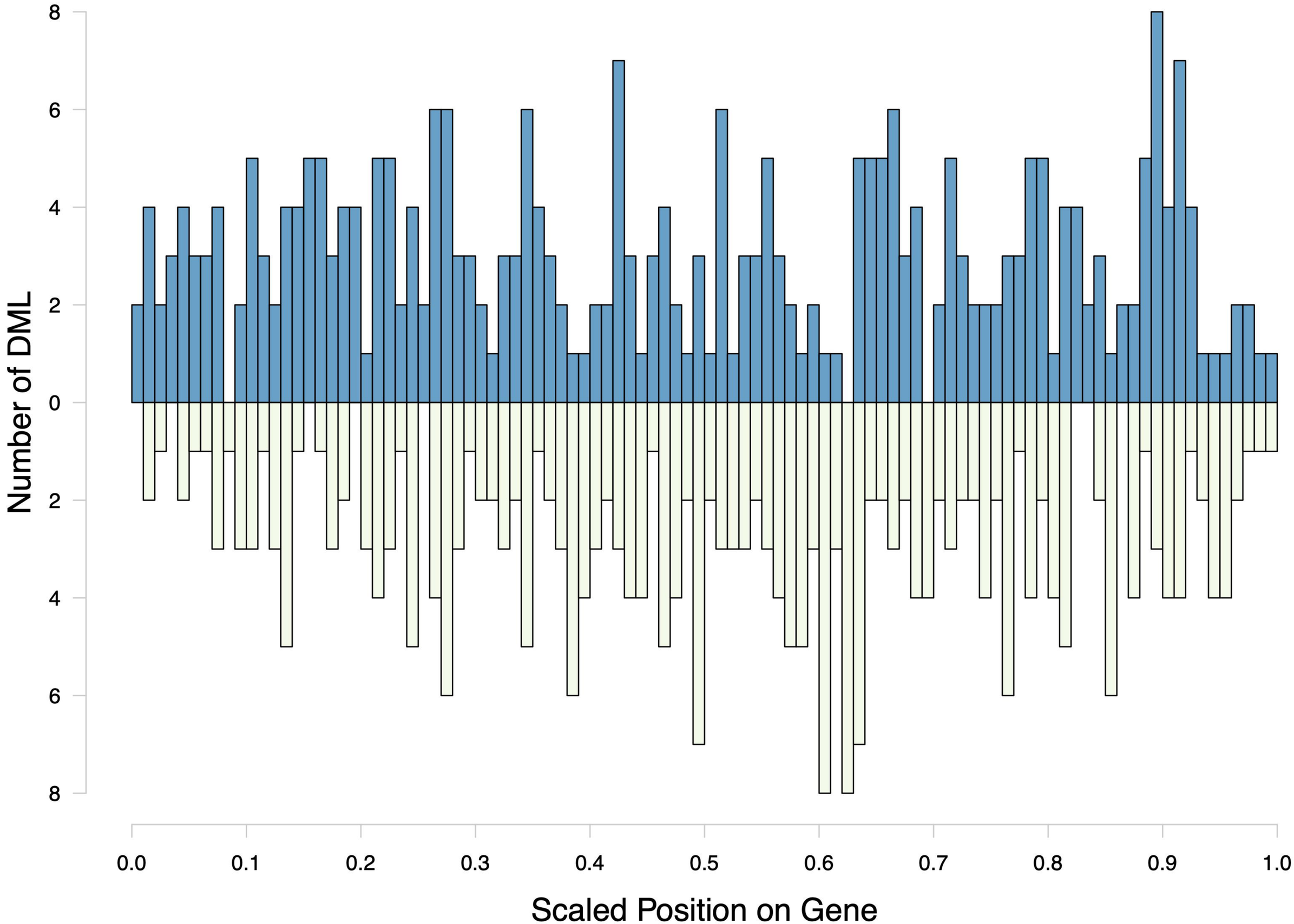
Hyper, Hypo DML


Exons
Introns
Transposable Elements
Putative Promoters
Other
Proportion CpGs
100
90
80
70
60
50
40
30
20
10
0
Loci with 5x Coverage
DML


Number of DML
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6
4
2
0
2
4
6
8
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Scaled Position on Gene