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Differential DNA methylation across environments has no effect on gene expression in the eastern oyster

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Abstract

1. It has been hypothesized that environmentally induced changes to gene body methylation could facilitate adaptive transgenerational responses to changing environments.
2. We compared patterns of global gene expression (Tag-seq) and gene body methylation (reduced representation bisulfite sequencing) in 80 eastern oysters (*Crassostrea virginica*) from six full-sib families, common gardened for 14 months at two sites in the northern Gulf of Mexico that differed in mean salinity.
3. At the time of sampling, oysters from the two sites differed in mass by 60% and in parasite loads by nearly two orders of magnitude. They also differentially expressed 35% of measured transcripts. However, we observed differential methylation at only 1.4% of potentially methylated loci in comparisons between individuals from these different environments, and little correspondence between differential methylation and differential gene expression.
4. Instead, methylation patterns were largely driven by genetic differences among families, with a PERMANOVA analysis indicating nearly a two orders of magnitude greater number of genes differentially methylated between families than between environments.
5. An analysis of CpG observed/expected values (CpG O/E) across the *C. virginica* genome showed a distinct bimodal distribution, with genes from the first cluster showing the lower CpG O/E values, greater methylation, and higher, and more stable gene expression, while genes from the second cluster showed lower methylation, and lower and more variable gene expression.
6. Taken together, the differential methylation results suggest that only a small portion of the *C. virginica* genome is affected by environmentally induced changes in methylation. At this point, there is little evidence to suggest that environmentally induced methylation states would play a leading role in regulating gene expression responses to new environments.

Introduction

Rapid environmental change is putting many species and populations at risk of extinction, and there is an urgent need to understand which will be most vulnerable. One way to approach this question is

to quantify the impacts of changing environmental conditions on organismal physiology, and test how these physiological changes are translated into population and ecosystem level effects of environmental change. On short time scales, these physiological changes are mediated by environmentally responsive gene expression (Evans & Hofmann, 2012). Over longer time periods, and possibly even across generations, these responses may be modulated by changes in the epigenome (Eirin-Lopez & Putnam, 2019). If epigenetic changes were stably inherited, they could add to the more widely recognized effects of evolutionary genetic change in contributing to phenotypic changes in populations over time. As a result, there is substantial interest in understanding interactions among epigenetic, genomic and environmental variation, and ultimately, how changes in the epigenome contribute to environmentally responsive physiology.

The links between environmental variation, changes in gene expression, and changes in organismal physiology are increasingly well documented by comparative transcriptomics studies (Alvarez, Schrey, & Richards, 2014; DeBiasse & Kelly, 2016). A growing number of studies also document environmental effects on the epigenome (Dixon, Liao, Bay, & Matz, 2018; Eirin-Lopez & Putnam, 2019), however, the phenotypic consequences of environmental alterations to the epigenome remain unclear. One of the best studied sources of epigenetic variation is DNA methylation, which in animals is mostly associated with CpG motifs (Eirin-Lopez & Putnam, 2019). Historically, DNA methylation has been best studied in mammals, where it is highly dynamic, and implicated in gene regulation and silencing, genomic imprinting, and X-chromosome inactivation (Bird, 1986; Jones and Takai, 2001). Invertebrate genomes generally have low levels of methylation, with methylation tending to be concentrated in gene bodies (introns and exons). In both plants and invertebrates, genes with higher levels of gene body methylation tend to be more highly and stably expressed, whereas those with lower levels of methylation tend to show lower, but inducible expression (Dimond & Roberts, 2016; G. B. Dixon, Bay, & Matz, 2016, 2014a; Gavery & Roberts, 2013; Sarda, Zeng, Hunt, & Yi, 2012; Zemach & Zilberman, 2010). Some evidence suggests that methylation may also help to direct alternative splicing of mRNA transcripts (Flores et al., 2012; Neri et al., 2017). Environmental differences can also produce changes in methylation patterns (Bogan, Johnson, & Hofmann, 2020; Johnson & Kelly, 2020; Johnson, Sirovy, Casas, La Peyre, & Kelly, 2020) including convergence in methylation patterns among common-gardened individuals in corals (Dimond & Roberts, 2020). Other studies have also found evidence for heritable variation in methylation

patterns (Dimond & Roberts, 2020; Liew et al., 2020; Rondon et al., 2017; Sharma et al., 2021; Strader, Wong, Kozal, Leach, & Hofmann, 2019). However the critical knowledge gap in this body of research is that we still know very little about how environmentally induced and heritable differences in methylation translate into phenotypic effects (McGuigan, Hoffmann, & Sgrò, 2021). Most studies have failed to document a causal link between gene body methylation and environmentally responsive regulation of gene expression (Bewick et al., 2016; Bewick, Zhang, Wendte, Zhang, & Schmitz, 2019; Choi, Lyons, Kim, Moore, & Zilberman, 2020; Harris, Lloyd, Domb, Zilberman, & Zemach, 2019; Zilberman, 2017). Nevertheless, in the coral *Acropora millepora*, variation in methylation was a better predictor of fitness in transplanted corals than either SNPs or gene expression (Dixon et al., 2018).

In this study, we investigated effects of environment and genotype on DNA methylation patterns in eastern oysters (*Crassostrea virginica*), and examined the association between methylation and environmentally induced gene expression. The effect of environmental salinity on oyster physiology is of particular interest in the northern Gulf of Mexico, where changes in land use and climate are impacting salinity regimes, driving local declines in oyster populations (La Peyre, Eberline, Soniat, & La Peyre, 2013; Lowe, Sehlinger, Soniat, & La Peyre, 2017). The role of methylation in environmental responses of oysters is of particular interest because this species faces a rapidly changing environment, and epigenetic methylation is a possible mechanism for transgenerational inheritance of environmentally responsive phenotypes. Our previous work with *C. virginica* provides evidence both for environmentally induced methylation differences (Johnson & Kelly, 2020) and for transgenerational inheritance in oysters, with parental acclimation to sites with differing salinity conditions contributing to variation in larval traits (Griffiths et al., 2021).

As with other traits, the complex links between epigenetic, genetic, and environmental variation can be disentangled using controlled breeding and common garden experiments (Clark et al., 2018; Dimond & Roberts, 2020; Putnam, Davidson, & Gates, 2016; Wang et al., 2021). In this study, we used a family-based approach, with 80 oysters from 6 full-sib families reared in two sites with differing salinity conditions for 14 months. These oysters were part of a larger study, where we observed effects of outplant site on growth rates, infection load (*Perkinsus marinus* parasites), and global gene expression patterns (Fig. 1, Sirovy et al., 2021). Our approach in this study allowed us to

test the plasticity of DNA methylation and its association with changes in gene expression in response to variation in the abiotic environment. Using measurements of methylation and gene expression made in gill tissue, we quantified variation in DNA methylation between outplant sites, variation in DNA methylation among families, and the association between DNA methylation and gene expression. This approach allows us to investigate the genetic vs environmental drivers of DNA methylation and associated changes in gene expression. We also investigated the methylation history of individual genes by measuring the distribution of CpG observed/expected ratios (CpG O/E) across the genome. The CpG O/E ratio will decrease in highly methylated genes over evolutionary time because methylation is mutagenic and will lead to an increase in C-T transition mutations, decreasing the CpG O/E ratio(Coulondre, Miller, Farabaugh, & Gilbert, 1978).

Our study provides a fine-scaled examination of the joint effects of genotype and environment on methylation and gene expression in a marine invertebrate reared in two common garden environments, and our results suggest that the methylome is shaped more strongly by genotype than by the environment. We observed only a weak connection between environmentally induced methylation and gene expression, suggesting that changes to the methylome do not play an important role in directing environmentally responsive gene expression in oysters.

Methods

In May 2016, adult oysters (*C. virginica*) were collected by dredging from Sister Lake, LA (29˚14’57” N, 90˚55’16” W, LDWF saltwater collecting permit #1904). These oysters were transported to the Louisiana Department of Wildlife and Fisheries Michael C. Voisin Oyster Hatchery in Grand Isle, LA (29°14'20.3" N, 90°00'11.2" W) and placed into off-bottom mesh cages for long-term acclimation. In October 2016, after six months of acclimation, the oysters were spawned at the MCV oyster hatchery using 3 males and 2 females. Oyster spat were reared in an upwelling system, individually tagged, and outplanted in one of three adjustable long-line mesh bags at both the Grand Isle Hatchery farm and near the Louisiana Universities Marine Consortium (LUMCON) (29°15'12.6" N, 90°39'45.9" W) on February 20th, 2017. Because larvae from all six families were combined for culturing, parentage assignments were unknown at the time of outplant, and as such families were unequally outplanted between sites. Oysters within each bag were monitored for mortality and cleaned of epibionts approximately every 3 months over a 14-month period. On April 24th, 2018, after 14-months at the

two outplant sites, 40 individuals were haphazardly chosen from each site. Shell height of each individual was measured from shell umbo to distal edge using a digital caliper (ABS Coolant Proof Calipers, Mituyoto Corporation, Japan). Approximately 1 cm^2 piece of gill tissue was sampled in the field from each individual and preserved with either Invitrogen RNAlater (gene expression) or 95% ethanol (DNA methylation). The remaining whole animal was placed in a pre-weighed 50 ml test tube and used to measure wet meat weight and *Perkinsus marinus* infection intensities. No approval for from an institutional animal ethics committee was required for this research.

Gene expression

Total RNA was extracted using a E.Z.N.A.® Total RNA Kit I (Omega BIO-TEK Inc., Norcross, GA, USA) following the manufacturer's instructions. The yield and quantity were initially assessed using a NanoDrop 2000 spectrophotometer. Total RNA extracted from the 80 individuals was sent to the University of Texas at Austin's Genomic Sequencing and Analysis Facility where RNA quality control was confirmed using a 2100 Agilent Bioanalyzer on a Eukaryote Total RNA Nano chip and libraries were produced using the Tag-Sequencing approach (Meyer, Aglyamova, & Matz, 2011). The resulting 80 libraries were sequenced on two lanes of an Illumina HiSeq 2500 platform, with 100 base pair single-end reads.

Sequencing reads were trimmed of adapter sequences using Trimmomatic (version 0.39) (Bolger, Lohse, & Usadel, 2014) and base pairs with quality scores below 30 were removed (Table S2). The trimmed reads were mapped to the *C. virginica* reference genome (Gómez-Chiarri, Warren, Guo, & Proestou, 2015) with known haplotigs removed (https://github.com/jpuritz/OysterGenomeProject/tree/master/Haplotig_Masked_Genome) using the single pass option for STAR RNA-seq aligner (version 2.6.0a) (Dobin et al., 2013). Reads were mapped to gene features with the options (--quantMode GeneCounts --outFilterScoreMinOverLread 0.50 --outFilterMatchNminOverLread 0.50) specified to adjust for poly-A tail contamination. A count matrix was generated from the ReadsPerGene.out.tab output from STAR.

Assigning genotypes from RNAseq reads

Genotypes for each individual were called from the RNAseq data using angsd (version 0.931) to produce an identity-by-state (IBS) matrix that uses probabilistic uncertainty to robustly genotype

individuals from sequencing reads with variable or low depths of coverage (Korneliussen et al., 2014). The filters used for assigning IBS scores included removing sites with allele frequency lower than 0.05, requiring a minimum read mapping quality score of 30, a minimum base mapping quality above 20, and removing SNPs with a p-value > 2e-6. These filters allow for high stringency and align with previously published work that has used ANGSD to assess genotypes in wild populations (Sturm et al., 2020). Genotype clusters were identified by plotting the first two axes from a distance-based redundancy analysis with the capscale function in the R program vegan (version 2.5-6).

Methylation

We quantified variation in methylation via reduced representation bisulfite sequencing (Van Gurp et al., 2016) which cost-effectively provides locus-specific methylation states across a large proportion of the genome (G. Dixon & Matz, 2021; Trigg et al., 2021). DNA was extracted using the OMEGA E.Z.N.A. Tissue DNA Kit (D3396-01; Omega bio-tek) with a 2 min RNase A digestion to remove co-purified RNA. DNA purity was assessed based on 260/280 and 260/230 ratios using a nanodrop spectrophotometer (ND1000; Thermofisher Scientific). Presence of high molecular weight DNA was confirmed using a 1.5 % agarose gel, and DNA concentration was verified using a Qubit 3.0 Fluorometric dsDNA BR assay kit (Q32850; Life Technologies). The epiGBS library preparation followed previously published methods (Johnson & Kelly, 2020; Van Gurp et al., 2016). Briefly, a total of 500 ng of purified genomic DNA was double digested using the two frequent cutter enzymes AseI and NsiI (NEB-R0127L and NEB-R0526L; Van Gurp 2016). Digested DNA was ligated to custom y-yoked methylated sequencing adapters using a T4 DNA ligase (B9000S; New England Biolabs) with additional rATP to ensure ligation of custom adapters (Glenn et al., 2019). The adapter ligated DNA was bisulfite converted in a 96 well plate using the Zymo Research EZ DNA Methylation-Lightning kit (D5031; Zymo Research) with a 15 min L-desulphonation step. This bisulfite converted DNA was tagged and amplified with Illumina adapters using 16 cycles of PCR. Amplified libraries were size selected to 300-600 base-pairs (bp) using the Zymo Research Select-A-Size DNA clean & Concentrator (D4080; Zymo Research). Size selection was confirmed using the Agilent Bioanalyzer DNA high sensitivity chip (5067-4626; Agilent Technologies). Libraries were pooled and sequenced by NovoGene Inc (R) with a 10% PhiX spike-in on a full flow cell of the Illumina HiseqX with 100 bp paired-end reads.

The epiGBS sequencing reads were adapter trimmed and base pairs with a phred score less than 30 were removed using Trimmomatic (version 0.39) (Bolger, Lohse, & Usadel, 2014). Trimmed reads were mapped to the reference genome (NCBI GCF_002022765.2) and CpG methylation was called using the software package bismark (v0.19.0) (Krueger and Andrews, 2011). The bismark commands used in the mapping allowed for 1 mismatch in a seed alignment of 10 with a minimum alignment score setting of -0.6 (--score_min L, 0, -0.6). These settings were selected to account for genomic variations between *C. virginica* collected from the northern Gulf of Mexico (nGOM, this study) and the disease-resistant inbred line from the U.S. East Coast used for the construction of the reference genome (Gómez-Chiarri, Guo, Tanguy, He, & Proestou, 2015). CpG methylation was extracted from the non-deduplicated mapped reads using the bismark command bismark_methylation_extractor with the following commands; --ignore_r2 2, --bedGraph, --zero_based, --no_overlap, --cytosine_report, and –report.

Statistical analysis

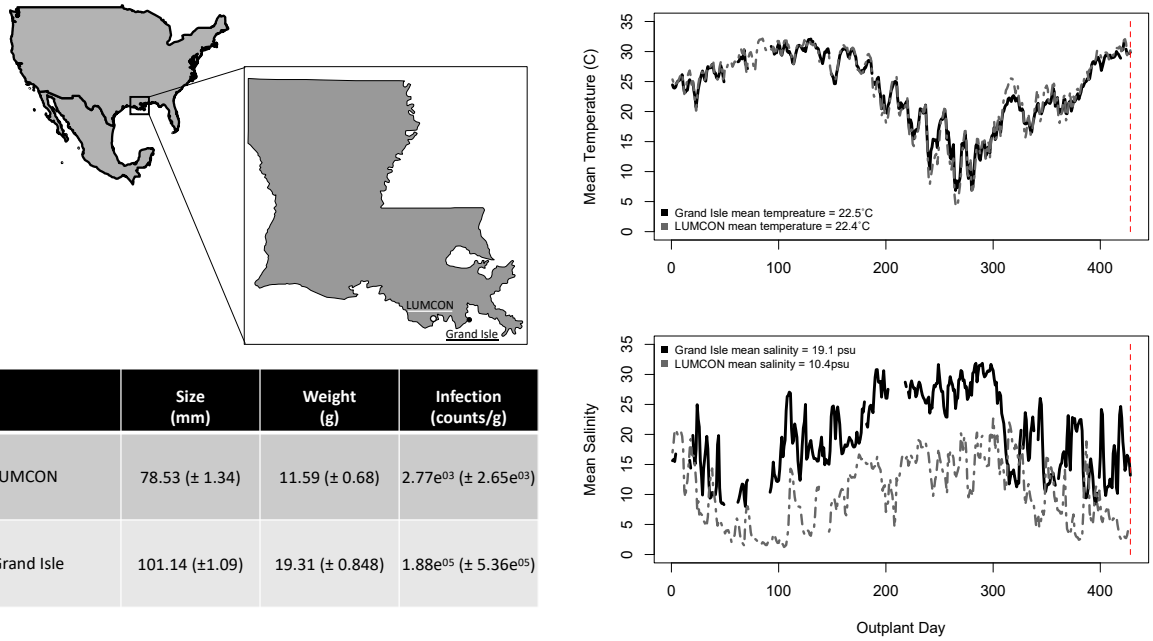
Differential methylation analysis was conducted using two methods with CpG features collected from the bismark coverage files imported and analyzed using the R program MethylKit (v.1.2.4) (Akalin et al., 2012). The first approach focused on CpG methylation using a non-overlapping tiled window approach with a tile size of 1000 bp (1kb) and a step size of 1kb. The 1kb regions were filtered using the filterByCoverage command to require coverage greater than 10x in at least 10 individuals. These tiles were tested for differential methylation in MethylKit. This approach was used to identify tiles that were differentially methylated between environments. Results from this analysis were consolidated to gene level mean percent methylation and tested for functional enrichment using a Mann-Whiteny U-Test (Wright et al., 2015; https://github. com/z0on/GO_MWU). For this analysis, the background list for GO enrichment was restricted to only those gene regions for which any methylation was measured (n=18,773). This method calculates enrichment across three gene ontology (GO) categories; Molecular Function (MF), Biological Processes (BP), and Cellular Component (CC). The second approach first measured percent methylation for every CpG captured in the analysis removing reads that did not have at least 1x coverage for all individuals. These CpG's were further filtered into groups to only retain those that overlapped with annotated exons, introns, promoter regions, or transposable elements. This filtering was conducted using the R package 'ChIPpeakAnno' (Zhu et al., 2010) using the function findOverlapsOfPeaks to find genomic regions

fully overlapping each annotated element. Genomic regions were converted to Grange objects for this analysis using he R package 'GenomicRanges' (Lawrence et al., 2013). For each group, the percent methylation of CpG's within each of these regions was averaged across all CpG's present (and restricted to only include regions that had more than 1 CpG was present, and a variance greater than 0 across all samples). This dataset was used to identify methylation patterns associated with genotype, environment, or GxE interaction using a PERMANOVA. This was performed using the R function 'adonis2' within the 'vegan' package (version 2.5-6). The PERMANOVA examined the effect of percent methylation~sire + dam + outplant + sire*outplant + dam*outplant + bag for each group of genomic features independently with 9,999 permutations. The resulting p-values were then corrected for multiple comparisons using the benjamini-hochberg method (Benjamini & Hochberg, 1995), and overlap with differential expression data was done by overlapping the peak lists using the R program 'ChIPpeakAnno'.

We also examined the CpG observed/expected (O/E) ratios in order to test the relationship between CpG O/E, percent methylation, and gene expression. CpG observed ratios were counted for all genes in the published genome using python scripts written by Dimond and colleagues https://github.com/jldimond/Coral-CpG (Dimond and Roberts, 2016). The CpG O/E ratios showed the expected bimodal distribution (Sarda et al., 2012). To understand the significance of these two groups we separated these genes into either a lower or upper distributions using a k-means clustering around 2 centers using the R package 'stats' (version 4.1.0; R Code Team 2021). Functional enrichment for each CpG O/E cluster was also tested using a Fishers exact test. Finally, we tested the association between percent methylation and gene expression with CpG O/E values for each gene examining each CpG cluster independently. This analysis was also conducted for both level of gene expression (CPM) and variation in gene expression (coefficient of variation, CV) for the 11,795 genes with data for both percent methylation and gene expression.

Differential gene expression was analyzed and described by Sirovy et al., (2021), these methods are briefly summarized here. We filtered the gene list using the (filterByExpr) function and normalized the remaining reads using the trimmed mean of M-values (TMM) normalization method (Robinson & Oshlack, 2010). Global expression patterns were analyzed using a PCoA conducted with the R program vegan and Euclidean distances calculated from log2 +1 transformed normalized counts obtained from the cpm() function in edgeR. Our differential expression analysis used two

approaches: a pairwise assessment of expression between outplant sites, and a PERMANOVA to identify genes associated with genotype, environment, or GxE interaction. The pairwise assessment of differential gene expression between outplant sites (regardless of family) were measured using a genewise negative binomial generalized linear model implemented in the edgeR package using the function glmQLFit. Significantly differentially expressed genes (DEGs) were identified based on FDR rates calculated using benjamini-hochberg method (Benjamini & Hochberg, 1995). Our second approach used a PERMANOVA performed using the R function 'adonis2' within the 'vegan' package (version 2.5-6). For this approach we used the log-transformed counts (cpm) from edgeR as our count matrix. The PERMANOVA examined the effect of gene expression~sire + dam + outplant + sire*outplant + dam*outplant +bag for each gene independently with 10^5 permutations. The resulting p-values were corrected using the benjamini-hochberg method (Benjamini & Hochberg, 1995). Functional enrichment of differentially expressed genes and PERMANOVA significant genes was tested using a Fisher's Exact Test. This method calculates enrichment across three gene ontology (GO) categories; Molecular Function (MF), Biological Processes (BP), and Cellular Component (CC).



	Size (mm)	Weight (g)	Infection (counts/g)
LUMCON	78.53 (± 1.34)	11.59 (± 0.68)	$2.77e^{03}$ (± $2.65e^{03}$)
Grand Isle	101.14 (±1.09)	19.31 (± 0.848)	$1.88e^{05}$ (± $5.36e^{05}$)

Figure 1. Environmental data for outplant sites: Louisiana Department of Wildlife and Fisheries Michael C. Voisin Oyster Hatchery farm in Grand Isle, LA (29°14'20.3" N, 90°00'11.2" W) and Louisiana Universities Marine Consortium (LUMCON) (29°15'12.6" N, 90°39'45.9" W), used for 14-month outplant of 80 oysters from six full-sib families used to measure effects of genotype and environmental conditions on gene expression and genome-wide methylation patterns.

Phenotypic data (size, weight and infection with *Perkinsus marinus*) are shown for the time of collection.

Results

Site specific differences in environmental conditions

Throughout the duration of the outplant, the two sites experienced similar temperature trends, but Grand Isle was consistently higher in mean daily salinity (Figure 1). These differences in environmental conditions (i.e. high freshwater input – LUMCON vs low freshwater input -Grand Isle) influenced the growth rate and dermo infection intensities leading to more growth but more infection in Grand Isle (Figure 1). These differences were shown to be statistically significant using a Kruskal-Wallis Rank Sum test (p-value < 0.05) as described by Sirovey et al., (2021).

Methylation Sequencing

Methylation sequencing of the 80 individuals was only considered successful for 73 samples with greater than 2 million reads. These 73 samples had a median of 9.9 million reads per sample after quality trimming; of these, 84.6% of reads mapped to the reference genome resulting in a median of 8.5 million mapped reads per sample. These reads were distributed across 74,541 1kb tiles that overlapped a total of 18,773 gene regions.

Transcriptome Sequencing

Transcriptome sequencing produced a total of 408 million reads, with an average of 5.1 million reads per sample. Trimming of those reads led to a final read count of 4.9 million per sample. Star mapping resulted in 91% of reads mapping to the reference genome distributed across 21,388 gene features. Test for differential gene expression conducted using edgeR identified 4,525 differentially expressed genes between sites with 1,871 up-regulated and 2,654 down-regulated genes in Grand Isle .

Assigning families

Genotypes for each oyster were determined using ANGSD (version 0.931). A total of 6 clusters were identified using a redundancy analysis that likely represent the parentage of 2 females and 3 males (supplemental 1). Unfortunately, distribution of families between sites was not even. This result allowed us to only examine pairwise differences in DNA methylation between sites, but still allowed

us to explore the role of genotype and outplant environment on DNA methylation in our non-parametric PERMANOVA analysis.

CpG O/E analysis

CpG O/E values for the *C. virginica* genome show a distinct bimodal distribution. Genes from across the genome were broken into either the lower or upper distributions using a k-means clustering around 2 centers using the R package *stats* (Figure 2A). This analysis found a total of 16,726 genes to be in cluster 1, the leftmost cluster with a mean CpG O/E of 0.421; and, 22,112 genes in cluster 2, the right most cluster with a mean CpG O/E of 0.837. We explored functional enrichment for each CpG O/E cluster using a Fishers exact test. This analysis revealed that genes within cluster 1 (n=16,726) were enriched for 132 gene ontologies across all 3 categories (MF = 46, BP = 82, and CC =4, see supplemental Figure 2). In contrast, genes within cluster 2 (n=22,112) were only enriched for 10 ontologies across all three categories (MF =5, BP=3, CC=0, see supplemental Figure 3).

When we examined the relationship between gene CpG O/E values and percent methylation, we observed a sharp drop in mean percent methylation between clusters 1 and 2 (Figure 2B).We tested the association between gene expression and CpG O/E values for both level of expression (CPM) and variation in expression (coefficient of variation, CV) calculated for each gene from the transcriptomic data, examining each cluster independently (Figure 2C,D). This analysis showed the expected association between high methylation and both increased gene expression (CPM) and low variance (CV) of expression only for cluster 1 genes. This pattern broke down for cluster 2 genes with no relationship between percent methylation and either level of expression or variation in expression.

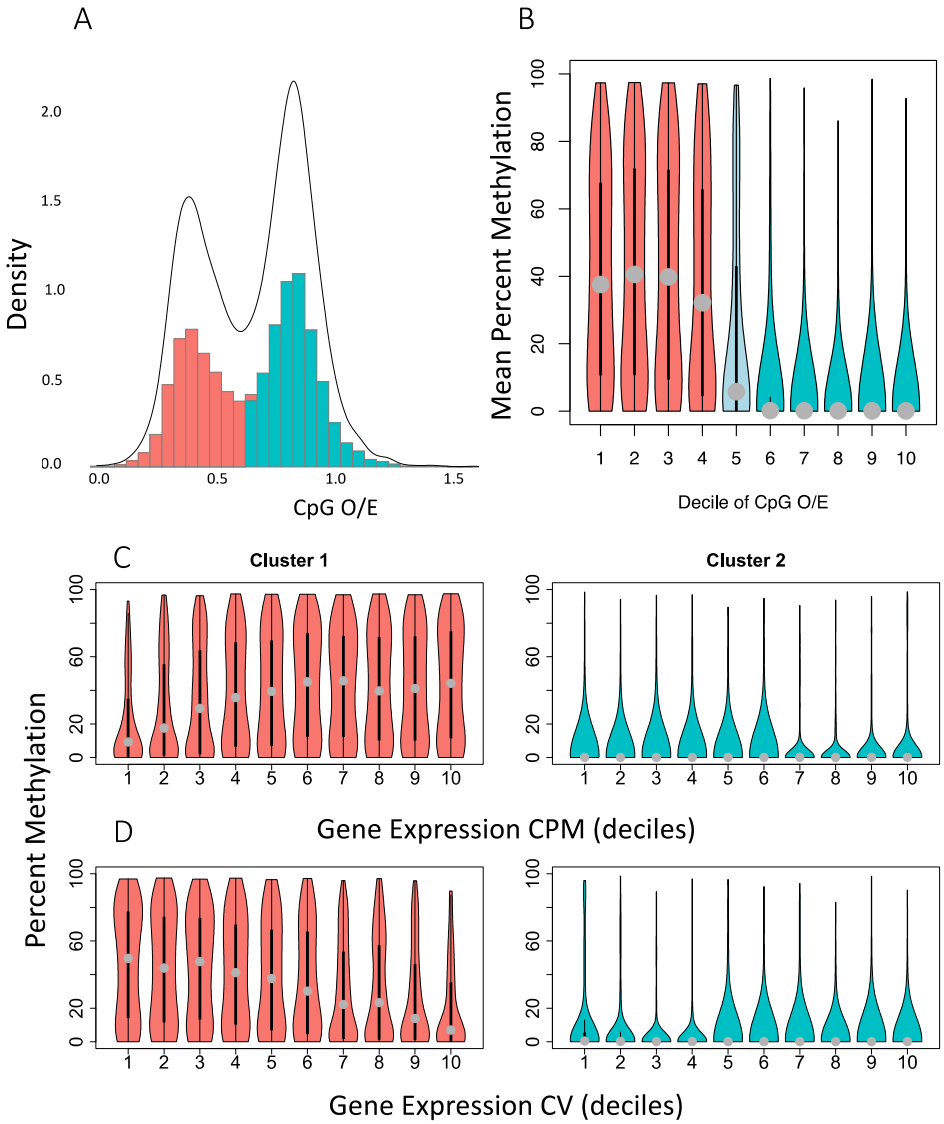


Figure 2. Characteristics of genes in relation to their CpG observed/expected ratios (CpG O/E) across the *C. virginica* genome. (A) Histogram showing distribution of CpG O/E values for all genes in the *C. virginica* genome, with bimodal distribution identified via k-means clustering. (B) Violin plot showing distribution of methylation levels across deciles of CpG O/E. (C) Violin plots showing distribution of methylation levels across levels of expression for each gene (measured via Tag-seq) for each of two CpG O/E clusters identified via k-means clustering. (D) Violin plots showing distribution of methylation levels across variation in expression for each gene (measured via Tag-seq) for each of two CpG O/E clusters identified via k-means clustering. The mean and median methylation and gene expression data are also available in supplemental table 4.

Global Methylation and Expression Patterns

We measured variation in both the percent methylation data and the log-transformed gene expression data with a PERMANOVA. For the methylation data, this analysis was restricted to 1,289 1kb loci that had no missing data for any individual. This approach identified an effect of site (p-value = 0.048) and family (p-value $\leq 1e^{-6}$), no interaction of family-by-site (p-value = 0.595; Figure 3), and no bag effect (p-value $\leq$ 0.765). This relationship can be seen along the first two principal components for the methylation data that describe 12.2 % of the variance (Figure 3). This same analysis identified a much larger effect of site on gene expression with significant influences of site (p-value $\leq 1e^{-6}$) and family (p-value $\leq 1e^{-6}$), but not the interaction of family-by-site (p-value = 0.114) or bag (p-value =0.14) on global gene expression patterns. This relationship can also be seen along the first two principal components that described 27.1 % of the variance for the gene expression data (Figure 3).

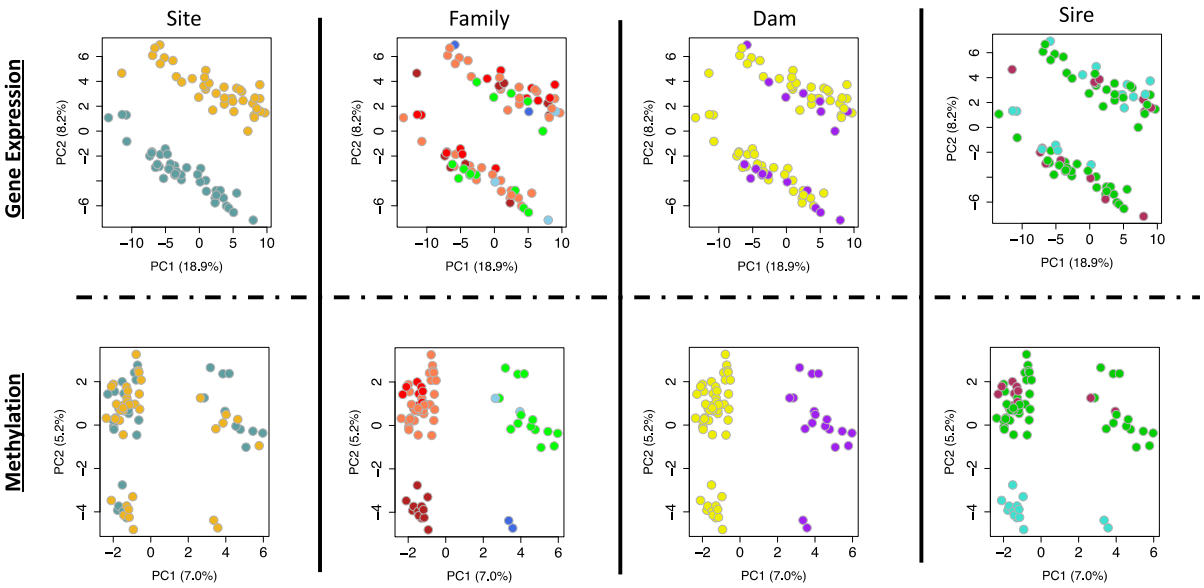


Figure 3: Principal coordinate analysis (PCoA) plot showing distances between samples for measurements of gene expression and methylation. Colors represent outplant site, full-sib family, sire or dam.

The PERMANOVA results from both DNA methylation and gene expression revealed distinct patterns. The DNA methylation data revealed a strong relationship between genotypes (sire/dam) and not outplant site across all four genomic regions tested (exons, introns, promoters, TEs) (Table 2). Across

all four regions introns and transposable elements were found to have the highest association with genotype (10-15%) while only 7 CpG's (across all four genomic regions) were found to be associated with outplant site. This is in contrast with the gene expression data that found 34.8% of genes (n= 7,454) were associated with outplant site. In addition, the methylated loci among gene body regions (exons and introns) that showed a significant effect of genotype (n = 1,127), 73.6% (n= 830) were genes found in CpG O/E cluster 1, representing a significant enrichment for cluster 1 genes (Fisher's exact test P<0.0001) This is again different from what was observed in the gene expression data where only 38.9% of DEGs (n=1,777) were from cluster 1, which was slightly lower than be expected by chance, given that 43% of genes were found in cluster 1 (Fisher's exact test, P=0.0003).

Table 1. PERMANOVA results for both DNA methylation and gene expression, testing effects of outplant site, sire and dam.

Number Significant (FDR < 0.05)	Methylation Exons (n=1,719)	Methylation Promoters(n=1,585)	Methylation Introns (n=4,870)	Methylation TE's (n=3,444)	Gene Expression (n=21,395)
Outplant	1 (0.06%)	1 (0.06%)	4 (0.08%)	1 (0.03%)	7454 (34.8%)
Sire	93 (5.4%)	57 (3.6%)	446 (9.2%)	523 (15.2%)	3009 (14.1%)
Dam	98 (5.7%)	74 (4.7%)	490 (10.1%)	451 (13.1%)	1963 (9.2%)
Sire_x_Outplant	2 (0.12%)	0	0	1	0
Dam_x_Outplant	0	0	0	0	0
Mean R^2 of Significant (FDR<0.05)	**Methylation Exons (n=1,719)**	**Methylation Promoters(n=1,585)**	**Methylation Introns (n=4,870)**	**Methylation TE's (n=3,444)**	**Gene Expression (n=21,395)**
Outplant	0.201	0.195	0.179	0.143	0.214
Sire	0.243	0.243	0.212	0.206	0.147
Dam	0.168	0.184	0.171	0.167	0.173
Sire_x_Outplant	0.184	0	0	0.292	0
Dam_x_Outplant	0	0	0	0	0

Differential Methylation: High vs Low Salinity

Differential methylation analysis between outplant sites (regardless of genotype) identified 1,039 differentially methylated 1kb tiled loci with at least a 20% difference in methylation and an adjusted p-value (q-value) less than 0.05. These regions represent approximately 1.4% of the potential methylated tiles in the genome and were distributed across 730 genes. Functional enrichment of these genes identified 53 enriched terms (35 MF, 16 BP, and 2 CC, see supplemental Figure 4).

Interestingly, 441 of these differentially methylated genes were hypermethylated in the low salinity site compared to the high salinity site and 520 (71%) were found in cluster 1, characterized by a low CpG O/E ratio (cluster, Figure 2a). This represents a significant enrichment for cluster 1 genes, given that cluster 1 makes up only 43% of the genome (Fisher's exact test P<0.001). Comparing the differential gene expression results from edgeR to the differential methylation results identified 16 genes that were both differentially methylated and differentially expressed (Table 1). All of the 16 genes were found to be hypermethylated in the low salinity site, but differential expression was found to be downregulated for 5 of the 16 genes. This overlap between differentially methylated and differentially expressed genes was no greater than expected by chance (Fisher's exact test P>0.05).

Table 2. Gene IDs, percent methylation, and change in expression for 16 transcripts that were both differentially methylated and differentially expressed between outplant sites. The direction of differential expression and methylation are comparisons of low salinity vs high salinity, with positive values indicating greater methylation and/or expression at the low salinity site.

Gene ID	Protein name	Percent Methylation Difference	Methylation qvalue	Expression logFold Change	Expression FDR
LOC111100600	protein RFT1 homolog isoform X2	+21.5	0.0E+00	+1.71	2.9E-19
LOC111103668	hsp90 co-chaperone Cdc37-like	+25.1	1.2E-13	+1.03	5.5E-14
LOC111104123	gastric triacylglycerol lipase-like	+22.3	1.4E-117	+1.05	1.0E-07
LOC111106173	pre-mRNA-splicing factor ISY1 homolog	+22.7	7.2E-34	+1.03	1.2E-10
LOC111108356	serine/threonine-protein phosphatase 4 regulatory subunit 2-A-like	+22.0	1.9E-204	+1.07	2.2E-11
LOC111108404	heat shock 70 kDa protein 12A-like	+22.8	2.0E-175	-1.00	3.0E-03
LOC111109620	nudix hydrolase 20, chloroplastic-like	+45.2	3.4E-285	+1.42	1.4E-11
LOC111112920	4-trimethylaminobutyraldehyde dehydrogenase-like	+23.4	1.6E-23	+1.53	1.9E-10
LOC111115975	protein disulfide-isomerase A6-like	+21.6	1.3E-204	-1.42	1.2E-08
LOC111122305	LOW QUALITY PROTEIN: ferric-chelate reductase 1-like	+20.4	4.9E-91	-1.31	1.1E-02
LOC111126139	LOW QUALITY PROTEIN: tumor protein D54-like	+20.1	6.5E-28	+1.52	5.6E-16
LOC111134504	uncharacterized protein LOC111134504 isoform X2	+20.3	2.4E-19	-1.01	3.6E-02
LOC111135004	uncharacterized protein LOC111135004 isoform X2	+27.1	0.0E+00	+1.60	3.6E-17
LOC111136020	proline dehydrogenase 1, mitochondrial-like isoform X4	+30.5	3.8E-14	+1.96	3.1E-16
LOC111136399	delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial-like	+25.1	0.0E+00	+1.19	3.0E-10
LOC111136652	multidrug resistance-associated protein 4-like	+27.5	6.7E-272	-1.05	1.5E-03

Discussion

Changes in DNA methylation have been proposed as a mechanism for long-term environmental acclimation and transgenerational plasticity, however the precise influence of the environment on DNA methylation, and the phenotypic consequences of this epigenomic modification are unclear for most species. We subjected six full-sib families of eastern oysters to common garden acclimation

under divergent field conditions and observed large differences in gene expression, growth, and disease prevalence, but relatively modest modifications to the methylome. Instead, we found that patterns of methylation were largely driven by genotype, and showed little correspondence to the environmentally responsive differences in gene expression.

Changes in Methylation Do Not Direct Environmentally Responsive Gene Expression.

We observed only a weak connection between environmentally induced methylation and environmentally-responsive gene expression. While there were 4,525 differentially expressed (DE) genes and 730 differentially methylated (DM) genes between sites, the overlap between these two sets was only 16 genes, which is no greater than would be expected by chance. Our results are in line with a similar analysis of differentially expressed and methylated genes between intertidal and subtidal Pacific oysters, where only 13% of genes that were DE were also DM (Wang et al., 2021). Similarly, methylation changes induced by ocean acidification conditions in eastern oysters were small, and uncorrelated with changes in gene expression under the same conditions (Downey-Wall et al., 2020). This trend also has been observed in the purple sea urchin (*Strongylocentrotus purpuratus*) where there were small overlaps between genes showing differential methylation and differential expression for either maternal environment (12 of 136 differentially methylated genes also differentially expressed) or developmental environment (22 of 136 differentially methylated genes also differentially expressed; Strader et al. 2020).

Nevertheless, there were a number of notable genes that fell into the list of DE/DM overlap in our study. Two of the DE/DM genes, *proline dehydrogenase* and *pyrroline dehydrogenase*, are part of a free amino acid metabolic pathway known to be heavily involved in cell volume regulation during salinity stress in oysters (Meng et al., 2013). Another DM/DE gene was a *serine/threonine phosphatase*, which is part of a pathway involved in response to immune challenge in *C. gigas* (Nguyen, Alfaro, & Merien, 2019). This gene was also one of only eight observed to be differentially methylated between natural populations of oysters that differed in *Perkinsus* disease pressure, with hypermethylation of this gene in the site with greater disease pressure (Johnson & Kelly, 2020). Finally, two of the 16 DE/DM genes were heat shock proteins, which were also heavily represented among the genes that were both DE and DM in a comparison between tidal regions in Pacific oysters (Wang et al., 2021).

Methylation Is Heritable

Our analysis indicates that genotype has a much stronger effect than the environment in shaping variation in oyster methylomes, with the number of differentially methylated loci between offspring of different parents (dam or sire) being two orders of magnitude greater than between environments. To our knowledge, ours is one of only a few studies to directly compare genetic vs. environmental influences on CpG methylation patterns in any invertebrate (but see Dixon et al., 2018; Yagound et al., 2019, Hearn et al., 2021). However, our results are in line with data from humans, where variation in CpG methylation is under strong genetic control, with a higher heritability for variation in methylation than for variation in gene expression (McRae et al., 2014).

More Differential Methylation in TEs and Introns

The largest number of loci that were differentially methylated among genotypes fell into introns and transposable elements (TEs). This is consistent with what is likely to be the most important function of methylation in metazoans: control of alternative splicing and suppression of TEs (Dahlet et al., 2020; Mlura et al., 2001). Our results are consistent with results from *C. gigas*, where differential methylation between families is concentrated in TEs (Olson & Roberts, 2015). The role of differential methylation in TE suppression seems to be taxonomically variable: for example there is currently little evidence to support differential methylation of TEs in insects (Glastad et al., 2019). In addition, previous work has found that exon methylation is positively correlated with inclusion in mRNA transcripts in *C. gigas* (Song, Li, & Zhang, 2017), and while our data does not find an association between differential gene expression and differential methylation, our TAG-seq approach for measuring gene expression does not allow us to detect alternative splicing events that may be occurring.

Given that the suppression of TEs is expected to be beneficial, it is somewhat puzzling that we observed variation in the methylation of TEs among families, since positive selection should tend to fix beneficial heritable methylation patterns over time. One possibility is that due to their high mutation rates, methylation states of TEs are in a state of flux: as new TEs form, there is selection for epimutations to suppress them, but the newest TEs in the genome have not yet fixed those beneficial epimutations, leading to a concentration of segregating epimutations on TEs.

Higher Methylation, and Higher and More Stable Gene Expression For CpG O/E Cluster 1

Across the *C. virginica* genome, CpG O/E values followed a bimodal distribution, a pattern that has been observed in many taxa, including other marine invertebrates (Bogan et al., 2020; Dimond & Roberts, 2016; G. B. Dixon, Bay, & Matz, 2014b). Cluster 1, which contained 43% of the genes, was characterized by higher levels of methylation and higher and more stable gene expression, while cluster 2, with 57% of the genes had lower methylation and lower and more variable gene expression. A strong positive correlation between gene body methylation, and higher and more stable gene expression has been observed across many taxa, including Pacific oysters (Gavery & Roberts, 2013; Wang et al., 2021). What is striking in our results is that methylation, gene expression and expression variation do not decline continuously in relation to CpG O/E values. Instead, the shape of this relationship is sigmoidal with a sharp decline in all three metrics between clusters. The previously observed positive correlation between methylation and the level and stability of gene expression is in fact only evident in cluster 1, which represents fewer than half of the genes in the *C. virginica* genome. Taken together, the bimodal CpG O/E ratios and the distinctive gene expression patterns of cluster 1 and 2 suggest that these clusters represent two distinct categories of genes. Methylation is mutagenic, with an increase in C-T transition mutations at methylated sites leading to a decrease in the CpG O/E ratio over time in more highly methylated genes (Coulondre, Miller, Farabaugh, & Gilbert, 1978). Thus, the lower CpG O/E ratio in cluster 1 is a signature of the higher average methylation of these gene over evolutionary time.

Conclusion

There has been substantial excitement in recent years about the potential for epigenetic DNA methylation to facilitate adaptive transgenerational responses to changing environments, leading some to call for the inclusion of epigenetic data in conservation plans. However, our results cause us to be skeptical that DNA methylation data will provide useful information about physiological responses to environmental change in oysters. Salinity is the most important environmental variable shaping oyster growth and health in the northern gulf of Mexico. As a result, oysters common gardened for 14 months at the two sites used in this study differed in size by 60% and in parasite loads by nearly two orders of magnitude. At the time of sampling, they differentially expressed 35% of their genes. All of these measurements indicate that oysters experience these two sites as very different environments. And yet we observed differential methylation between environments at only 1.4% of potentially methylated loci and little connection between differential methylation and

519 differential gene expression. Instead, methylation patterns were largely driven by genetic differences
520 among families. Furthermore, the bimodal clustering of CpG O/E ratios suggest that methylation
521 states for most of the genome have been stable over evolutionary time. While these changes in DNA
522 methylation may play a role in alternative splicing or possibly interact with other epigenetic features;
523 at this point, there is little evidence to suggest that environmentally induced methylation states, if
524 they occur in oysters, would have appreciable transgenerational effects on gene expression.

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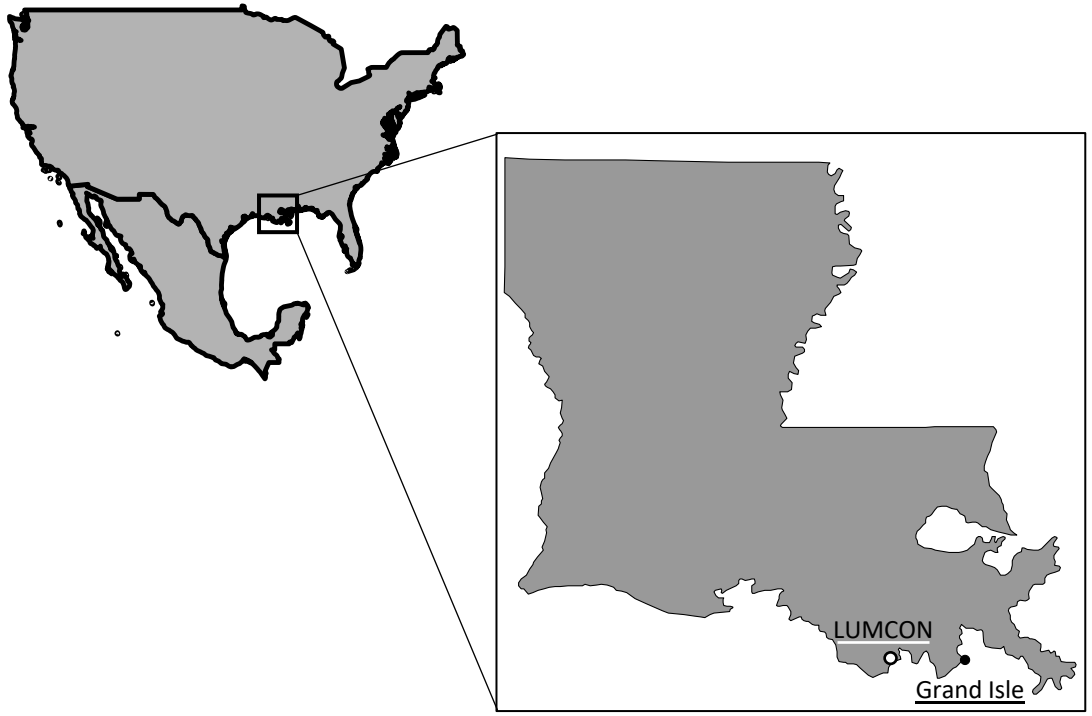

	Size (mm)	Weight (g)	Infection (counts/g)
LUMCON	78.53 (± 1.34)	11.59 (± 0.68)	$2.77e^{03}$ ($\pm$ $2.65e^{03}$)
Grand Isle	101.14 (±1.09)	19.31 (± 0.848)	$1.88e^{05}$ ($\pm$ $5.36e^{05}$)