DNA methylation variation in gametes and larvae of the Pacific oyster, *Crassostrea gigas*

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**Abstract**

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Epigenetics describes DNA modifications that change gene expression without altering the underlying nucleotide sequence. Epigenetic mechanisms such as DNA methylation can change genome function under external influences. The focus of this project is examining one epigenetic modification, DNA methylation, in oysters. DNA methylation has been well studied in vertebrates, but remains understudied in invertebrates. Furthermore, the amounts and functions of DNA methylation in organisms are extremely diverse and variable across taxa. This thesis determines patterns of DNA methylation in *C. gigas* to elucidate the functional role of DNA methylation. The first chapter examines the genome-wide DNA methylation profile in *C. gigas* male gamete cells using whole-genome bisulfite sequencing. RNA-Seq analysis was also performed on the same tissue to provide insight into the mechanisms by which DNA methylation impacts transcriptional processes. The work presented in Chapter 2 examines methylation patterns of *C. gigas* during early oyster developmental stages (spermatozoa and larvae). Together these data were used to test the predictions that DNA methylation is involved in gene regulatory activity and is heritable. This work also describes individual variation, parental transmission and developmental patterns of DNA methylation in oysters. Our results indicate a positive relationship between DNA methylation and gene expression, and that DNA methylation patterns are inherited in oysters.

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**Chapter I: Genome-wide profiling of DNA methylation and gene expression in *Crassostrea gigas* male gametes**

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***Abstract***

DNA methylation patterns and functions are variable across invertebrate taxa. In order to provide a better understanding of DNA methylation in the Pacific oyster (*Crassostrea gigas*), we characterized the genome-wide DNA methylation profile in male gamete cells using whole-genome bisulfite sequencing. RNA-Seq analysis was performed to examine the relationship between DNA methylation and transcript expression. Methylation status of over 7.6 million CpG dinucleotides was described with a majority of methylated regions occurring among intragenic regions. Overall, 15% of the CpG dinucleotides were determined to be methylated and the mitochondrial genome lacked DNA methylation. Integrative analysis of DNA methylation and RNA-Seq data revealed a positive association between methylation status, both in gene bodies and putative promoter regions, and expression. This study provides a comprehensive characterization of the distribution of DNA methylation in the oyster male gamete tissue and suggests that DNA methylation is involved in gene regulatory activity.

***Introduction***

DNA methylation is an important epigenetic process that varies in genomic distribution and biological function across taxa. DNA methylation involves the addition of a methyl group to a cytosine pyrimidine ring and most often occurs as part of C-G nucleotide pairs, frequently referred to as CpG dinucleotides. Mammals exhibit a pattern commonly referred to as global methylation, in which 70–80% of CpG dinucleotides are methylated (Bird, 1980). In contrast, invertebrates display relatively low levels of DNA methylation, from almost no methylation in Drosophila melanogaster (Gowher et al., 2000) to intermediate levels in the sea urchin Echinus esculentus (Bird et al., 1979). In mammals, a primary function of DNA methylation is to suppress gene expression through increased promoter DNA methylation (Bell and Felsenfeld, 2000). However, the function of DNA methylation in invertebrates is variable and likely differs among invertebrate taxa. The roles of methylation include regulation of transcriptional activity (Suzuki and Bird, 2008), alternatively exon splicing (Lyko et al., 2010), and developmental activity (Riviere et al., 2013).

While in general there is a limited amount of comprehensive information regarding DNA methylation in non-mammalian taxa, some recent studies have focused on DNA methylation in the Pacific oyster. The Pacific oyster is an excellent model for studying epigenetic modifications because its life history characteristics make it an important aquaculture species (Glude and Chew, 1982) and genomic resources for this species have recently become available (Zhang et al., 2012). Gavery and Roberts (2010) first reported the presence of DNA methylation in the Pacific oyster. In the same study, in silico analysis revealed a significant correlation between gene function and methylation level (Gavery and Roberts, 2010). The relationship between gene methylation and gene function was experimentally corroborated with high-throughput sequencing and it has been proposed that limited methylation in select genes may contribute to increased phenotypic plasticity in highly fluctuating environments (Roberts and Gavery, 2012). More recently, methylation enrichment and bisulfite sequencing were used to describe high-resolution DNA methylation patterns in pooled oyster gill tissue (Gavery and Roberts, 2013). A characterization of DNA methylation during oyster larval development has also been performed, revealing that DNA methylation varies through early development and treatment with 5-Aza-cytidine, a DNA methyltransferase (DNMT) inhibitor, leads to developmental alterations (Riviere et al., 2013). In the same study, researchers found an inverse correlation between methylation proximal to the transcription start site and expression of hox genes (Riviere et al., 2013). In addition to studies that investigate putative function of DNA methylation, other research has begun to evaluate relationships between epigenetic and genetic variations in C. gigas mass selection procedures (Jiang et al., 2013).

While a better understanding of DNA methylation is emerging for this species, there are still several questions that remain. Importantly we still do not fully understand the relationship between DNA methylation and gene expression, nor DNA methylation patterns in a single cell type. Examining a single cell type is important as methylation levels and patterns may differ between multiple cell types and life history stages, and our research attempted to limit this potential variability. Spermatozoa are an ideal resource for studying a single cell type and also provide the secondary benefit of understanding more about oyster spermatogenesis. The oyster male gonad consists of numerous gonadal tubules that grow during tissue development (Franco et al., 2008) and evolve according to four successive reproductive stages annually (Berthelin et al., 2000). These gonadal stages include undifferentiated (stage 0), mitosis of spermatogonia and differentiation of germ cells (stage 1), visible spermatogenesis (stage 2), and mature gametes (stage 3) (Franco et al., 2008). This is the first time DNA methylation has been characterized in Pacific oyster gametes, however spermatozoa methylation has been previously examined in other marine invertebrates. For example, spermatozoa DNA methylation has been described in both the marine annelid worm Chaetopterus variopedatus (del Gaudio et al., 1997) and Ciona intestinalis (Suzuki et al., 2013).

This research represents the first high-resolution characterization of DNA methylation patterns from a single cell type in a mollusc, including an examination of the relationship between gene expression and promoter region methylation. Our results demonstrate that DNA methylation is predominant in intragenic regions (exons and introns) and that there is a positive relationship between methylation and gene expression in C. gigas. Furthermore, we were surprised to find similar patterns of tissue-specific methylation in male gametes as has been previously described in oyster gill tissue, thus suggesting that overall methylation levels do not dramatically vary between tissue types, and specifically between gametic and somatic cells.

***Methods***

*Bisulfite treated DNA sequencing (BS-Seq)*

A single male adult oyster was collected from Thorndyke Bay, WA and thermally conditioned and fed for 6 weeks in the laboratory. Male gamete tissue was scored with a razor blade, gametes rinsed with sterile seawater, centrifuged, and immediately placed on dry ice then stored at −80°C until further processing. Genomic DNA was extracted using DNAzol according to the manufacturer's protocol (Molecular Research Center, Inc. Cincinnati, OH). High molecular weight genomic DNA (6 ug) was used to prepare a library for whole-genome bisulfite sequencing. Lambda DNA (Promega Co. Madison, WI) was added to the sample prior to fragmentation and library construction to serve as a measure of bisulfite conversion efficiency. Extracted DNA was fragmented to an average length of 250 bp using a Covaris S2 (Covaris Inc. Woburn, MA) and fragment size was confirmed by gel electrophoresis. The library was constructed using the Paired-End DNA Sample Prep Kit (Illumina, San Diego, CA) with standard protocols. DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA) and 72 bp paired-end sequencing was performed on the Illumina HiSeq 2000 system. Library construction and sequencing was performed by the High Throughput Genomics Center (htSEQ, Seattle, WA).

DNA sequence reads were mapped to all genomic scaffolds from the *Crassostrea gigas* draft genome (Fang et al., 2012). Sequences were mapped using Bisulfite Sequencing Mapping Program BSMAP v2.73 (Xi and Li, 2009). Resulting data from mapping bisulfite treated reads was analyzed with *methratio*, a Python script that accompanies BSMAP to calculate and extract methylation ratios. Parameters for *methratio* included reporting loci with zero methylation ratios (-z), combining CpG methylation ratios on both strands (-g) and only using unique mappings (-u). The same mapping procedure was also performed with the *Crassostrea gigas* mitochondrial genome (Accession # AF177226). The resulting *methratio* outputs were uploaded to SQLShare (Howe et al., 2011) and queried to examine distribution of methylation. Methylation characteristics were initially calculated for all cytosines.

*Genomic features and CpG dinucleotide methylation*

Methylation of CpG dinucleotides was characterized in relation to genomic features. A CpG locus was considered methylated if it had at least 5× coverage and at least half the reads remained unconverted after bisulfite treatment. Methylation ratios were calculated for individual loci as well as for full-length genes and intragenic regions (introns and exons). Methylation on a per gene basis was determined by obtaining the number of methylated cytosines divided by the total number of CpG dinucleotides per region. Genome feature tracks were generated in order to characterize the distribution of methylation in the male gamete tissue. All CpG dinucleotides were identified using the EMBOSS tool fuzznuc (Rice et al., 2000). Methylated CpGs (5× coverage, ≥50% unconverted), sparsely methylated CpGs (5× coverage, 0–50% unconverted) and unmethylated CpG loci (5x coverage, 0% unconverted) within genomic regions were determined using Bedtools (i.e., *intersectBED*) (Quinlan and Hall, 2010). Methylation was examined within exons and introns (Fang et al., 2012), promoter regions (characterized as 1 kb regions upstream from transcription start sites), and putative transposable elements identified using RepeatMasker and the Transposable Element Protein Database (Smit et al., 1996-2010).

*Transcriptome sequencing*

Total RNA was isolated using TRI reagent (Molecular Research Center) from the same oyster gamete tissue used for bisulfite sequencing. RNA was enriched for mRNA using Sera-Mag oligo dT beads (Thermo Scientific). A shotgun library was constructed from double stranded cDNA for paired end sequencing by end-polishing, A-tailing and ligation of sequencing adaptors. Sequencing and library preparation were performed on the Illumina HiSeq 2000 platform at the Northwest Genomics Center at the University of Washington (Seattle, WA). RNA-Seq analysis was performed using CLC Genomics Workbench version 6.5 (CLC Bio, Aarhus, Denmark) with high-throughput reads (50 bp paired end) mapped back to the oyster transcriptome (Fang et al., 2012). Initially, sequences were trimmed based on quality scores of 0.05 (Phred; Ewing and Green, 1998; Ewing et al., 1998), and the number of ambiguous nucleotides (>2 on ends). Sequences smaller than 20 bp were also removed. For RNA-Seq analysis, expression values for each gene (28,027) were measured as RPKM (reads per kilobase of exon model per million mapped reads) (Mortazavi et al., 2008) with an unspecific match limit of 10 and maximum number of 2 mismatches.

A Chi-squared test was performed to determine if the degree of gene methylation with respect to gene expression levels (RPKM) was different from what would be expected from a random distribution of methylation levels in promoter regions (p-value < 0.05 was considered significant). For promoter region analysis, these regions were determined to be the 1 kb regions upstream from transcription start sites that did not overlap with neighboring genes. In addition, only promoter regions with at least 10 CpG dinucleotides were considered. Oyster genes were classified as either heavily methylated (methylation ratio ≥0.5), sparsely methylated (methylation ratio 0–0.5) or unmethylated (methylation ratio = 0).

***Results***

*Bisulfite treated DNA sequencing (BS-Seq)*

Bisulfite treated DNA sequence reads (171.5 million) were produced and are available (NCBI Sequence Read Archive: accession number SRX386228). A total of 90 million paired end reads (53% of total reads) and 32 million single end reads (9.6%) mapped to the *Crassostrea gigas* genome. Sodium bisulfite conversion efficiency was estimated to be 99.72% based on analysis of lambda phage DNA. All cytosine dinucleotide motifs were examined and a majority of methylated cytosines were reported in CpG dinucleotides. We found that 15% of the CpG dinucleotides were methylated while the next highest motif (CpA) methylated at 0.14%, which falls within the sodium bisulfite conversion efficiency margin of error (0.28%).

*DNA methylation and genomic features*

The bisulfite sequencing effort provided ≥1x coverage for 8.52 million of the 9.98 million CpGs (85%) in the oyster nuclear genome. Using a 5x coverage threshold, which corresponds to 7.64 million CpGs (77%), the majority of CpGs were not methylated (Figure 1).

The proportion of CpG methylation occurring in specific regions of the oyster genomic landscape were characterized. Methylation occurs predominantly in intragenic regions, with 74% of methylated CpGs found in exons and introns. A total of 30% of CpGs in exons were methylated and 18% of CpGs in introns were methylated (Figure 2). These are particularly high levels of methylation when compared to methylation levels of other oyster genomic regions, wherein between 4 and 7% of CpGs were methylated.

The oyster mitochondrial genome is predominantly unmethylated. With an average coverage of 39.76-fold, of the 2518 cytosines with at least 5x coverage, 2316 cytosines were converted upon bisulfite treatment and no cytosines were considered methylated.

*Whole transcriptome sequencing expression patterns*

After quality trimming, 50.3 million reads (paired end 50 bp) remained (NCBI Sequence Read Archive: accession number SRX390346). Expression (RPKM) was detected in a majority of the genes (17,093 genes or 63%). Median expression level was 0.749 and expression level ranged from 0 to 35637.3 RPKM. The relationship between gene methylation and expression levels was examined by determining methylation on a per gene basis.

A minimum of one methylated CpG dinucleotide with ≥ 5× coverage was observed for every 14,517 genes in the C. gigas genome, or 53% of genes. The proportion of methylated CpGs was characterized with respect to RNA-Seq data on expression levels for full-length genes. Specifically, within genes and putative promoter regions we found a greater proportion of fully methylated CpGs for genes that have elevated expression levels (>1 RPKM) (Figure 3). The observed distributions of methylated CpGs within genes (X2 = 5493.85, df = 2, p < 0.0001) and promoter regions (X2 = 1765.56, df = 2, p < 0.0001) were significantly different than what would be expected if methylated CpGs were randomly distributed among genes and promoter regions.

Additionally, our transcriptome data was compared to a comprehensive microarray experiment focused on gametogenesis in *C. gigas* (Dheilly et al., 2012). Elevated gene expression was observed in genes identified by Dheilly et al. (2012) to increase in expression over spermatogenesis (clusters 5 and 6) (Figure 4). Based on this analysis, we suspect our sample was likely from a fully mature male gonad.

***Discussion***

Genome-wide shotgun bisulfite sequencing was conducted on gametes from a male oyster to illustrate the role of DNA methylation in a single cell type. While reduced representation CpG DNA methylation has been previously quantified for this species, this study represents the first examination of other dinucleotide motifs. Although CpG DNA methylation is ubiquitous across organisms, types and levels of methylation vary considerably among invertebrates. For example, Caenorhabditis elegans essentially lacks DNA methylation in its genome where neither 5-methylcytosine nor DNA methyltransferase are present (Bird, 2002). The Drosophila melanogaster genome contains extremely low 5-methylcytosine levels (Gowher et al., 2000; Lyko et al., 2000) and mostly in the CpT dinucleotide context rather than CpG (Lyko et al., 2000). In this study, DNA methylation was only observed in CpG motifs.

This is the first characterization of DNA methylation in bivalve mitochondrial DNA. A lack of methylation in oyster mitochondrial DNA in our study is consistent with previous invertebrate research, which found that genes encoding mitochondrial DNA were unmethylated in the brain tissue of the honey bee Apis mellifera (Lyko et al., 2010). Recent research has also demonstrated the absence of CpG methylation in the mitochondrial genome of the sea squirt Ciona intestinalis (Suzuki et al., 2013). Studies on the mammalian system have found similar results, confirming a lack of methylation among CpGs in human mitochondrial DNA (Hong et al., 2013).

Overall, approximately 15% of the CpGs are methylated in the oyster male gamete nuclear genome. This is the same estimated proportion from analysis of DNA methylation in oyster gill tissue (Gavery and Roberts, 2013). Similarly, (Suzuki et al., 2013) found identical gene groups to be methylated in tissues from C. intestinalis sperm and muscle cells. In their study 23% of the genome was determined to be methylated (Suzuki et al., 2013). The degree of methylation we found in oyster male gamete tissue falls within those previously described for other molluscs. For instance, recent research examining DNA methylation in multiple tissues of the uropea Chlamys farreri found methylation levels in the genome to be around 21% using a methylation-sensitive amplification polymorphism approach (Sun et al., 2014). Studies of DNA methylation in the foot tissue of the gastropod Biomphalaria glabrata found approximately 2% of the CpG dinucleotides to be methylated (Fneich et al., 2013). Our findings, in agreement with previous studies in C. gigas, corroborates that overall genome methylation in C. gigas is at an intermediate level and suggests that DNA methylation does not significantly vary among tissue type.

DNA methylation is predominantly found in exons and introns in oyster male gametes (Figure 2). These findings are consistent with previous work characterizing DNA methylation in Crassostrea gigas gill tissue (Gavery and Roberts, 2013). In several animal and plant genomes, transcribed regions of genes, including intragenic regions, have higher levels of DNA methylation than neighboring regions (Suzuki et al., 2007; Zilberman et al., 2007). DNA methylation in insects, however, appears to be primarily confined to exons (Lyko et al., 2000). Recent work examining DNA methylation in C. intestinalis sperm found that methylated domains of the genome are primarily contained within transcription units and promoter regions, with intergenic regions completely unmethylated (Suzuki et al., 2013). Together these studies demonstrate that the distribution of DNA methylation in invertebrate genomes is diverse and that most invertebrate genomes exhibit interspersed regions of methylated and unmethylated DNA. Although we have a relatively comprehensive profile of where DNA methylation occurs across the oyster genome, there is no definitive evidence of a link between DNA methylation and function. However, based on RNA-Seq data there is likely an association between methylation status and gene expression in C. gigas.

Recently, Riviere et al. (2013) proposed that proximal promoter and first exon methylation in C. gigas larvae have similar functions than those in mammalian systems, where increased methylation in promoter regions corresponds to decreased gene expression. Specifically, Riviere et al. (2013) reported a negative correlation between DNA methylation and expression of some homeobox gene orthologs during early oyster development. Our results indicate the opposite- a positive correlation between DNA methylation and gene expression (Figure 3), in which genes with high expression levels had high methylation levels. This is similar to what has been reported for oyster gill tissue (Gavery and Roberts, 2013). Riviere et al. (2013) suggested that a lack of DNA methylation influences gene expression specifically by controlling the transcription level of homeobox orthologs in the proximal promoter and first exon. Similarly, the presence of promoter methylation has specifically been associated with transcriptional silencing among many organisms (Suzuki and Bird, 2008). Here we found a significant difference in promoter methylation in high versus low gene expression. It is likely that the relationship of DNA methylation and gene expression is complex and dependent on several factors (Gavery and Roberts, 2014). For example, it has been shown that methylation pattern is dependent on gene function (Roberts and Gavery, 2012) and the current study focused on the entire transcriptome where Riviere et al. (2013) specifically looked at homeobox orthologs. The differences we observed between DNA methylation and expression than was previously reported (Riviere et al., 2013) could also be attributable to differences in cell type or a differing role of methylation throughout C. gigas life history stages. It should also be noted both studies have limitations as they rely on accurate genome annotation, and, as recently reported (Elsik et al., 2014), this can significantly alter research findings.

Here we provide the first genome-wide characterization of DNA methylation in a bivalve uropea that focused on a single-cell type. We found that DNA methylation levels in male gametes are similar to those previously reported in gill tissue (Gavery and Roberts, 2013). In examining the relationship between DNA methylation and transcript expression, we observed a pattern suggesting that promoter regions with a higher proportion of CpG methylation are associated with highly expressed genes. Further explorations into the roles of miRNA and histone modifications will help to elucidate our understanding of epigenetic regulatory functions in oysters.

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***Figures***



**Figure 1.** *Frequency of CpG methylation ratios in C. gigas male gamete tissue.* A total of 7,642,816 CpG dinucleotides with at least 5x coverage were examined.



**Figure 2.** *Genome-wide distribution of CpG methylation in C. gigas male gamete tissue within genomic regions.* The proportion of CpG unmethylated (ratio = 0), sparsely methylated (ratio between 0 and 0.5), heavily methylated (ratio ≥ 0.5), or CpG dinucleotides with less than 5x coverage (“No data”) are shown as percentage contributions to specific oyster genomic regions. Genomic regions are scaled according to their relative CpG contribution to the genome.



**Figure 3.** *Proportion of methylation on a per gene basis for putative promoter regions and gene bodies (exons and introns) for high and low expression levels indicate that DNA methylation is positively correlated to gene expression in C. gigas male gamete tissue.* Two classifications shown include genes with low expression (Promoter regions = 13,919 and genes = 14,377; RPKM ≤ 1, including no expression) and high expression (Promoter regions = 12,477 and genes = 12,877; RPKM > 1). The proportion of corresponding genes that are unmethylated (methylation ratio = 0), sparsely methylated (methylation ratio 0-0.5), and heavily methylated (methylation ratio ≥ 0.5) are shown.



**Figure 4.** *Boxplots of expression levels (RPKM) from RNA-Seq data relative to the 10 clusters of similar gene expression patterns during oyster gonad development identified by Dheilly et al. 2012.* The number of genes represented are 511 (Cluster 1), 222 (Cluster 2), 312 (Cluster 3), 197 (Cluster 4), 226 (Cluster 5), 146 (Cluster 6), 200 (Cluster 7), 295 (Cluster 8), 332 (Cluster 9), and 41 (Cluster 10). For a full description and all files please see:

Olson C., Roberts S. (2014). *Crassostrea gigas* male gonad transcriptome data comparison.Figshare. <http://dx.doi.org/10.6084/m9.figshare.1004464>

**Chapter II: Indication of lineage-specific DNA methylation patterns and extragenic variation in developing oysters**

***Abstract***

The roles of DNA methylation during development remain enigmatic in numerous invertebrate species. This includes understanding whether DNA methylation is inherited and to what extent methylation varies during development. To identify individual variation, paternal transmission, and developmental patterns of DNA methylation, high-resolution methylome characterization of *Crassostrea gigas* sperm and larvae was performed. Our data suggest that DNA methylation patterns are inherited, as methylation patterns are similar between males and their offspring among two lineages. To examine methylation differences between two oyster families, lineage-specific differentially methylated loci (189) were identified and found throughout the genome though primarily in transposable elements. Similarly, a high proportion of loci differing in methylation status during developmental stages (160) were found within transposable elements. Differences in methylation among transposable elements is likely related to the assumption that changes in methylation in genes and promoter regions are selected against as they would compromise survival.  Further, differentially methylated loci in transposable elements could contribute to increased diversity. Together our study indicates that oyster lineages are more similar in their DNA methylation patterns, which could have evolutionary implications, particularly if these epigenetic patterns are independent of genetic structure.

***Introduction***

DNA methylation is an epigenetic modification that is ubiquitous across many eukaryotes, with variable patterns and functions across taxa. This epigenetic mechanism involves the addition of a methyl group to cytosines, usually in a CpG dinucleotide context, catalyzed by DNA methyltransferases. Epigenetic modifications such as DNA methylation can alter gene expression without modifying the underlying nucleotide sequence, and functions in mammals to suppress transcription through increased methylation in promoter regions (Bell and Felsenfeld, 2000).  In mammals, DNA methylation is essential for development and differentiation of organs and tissues (Okano et al. 1999). Likewise, mutations of DNA methyltransferase in mammals may result in developmental delays and mortality (Li et al. 1992).

In contrast to the densely methylated mammalian genomes, several invertebrate species display low to intermediate levels of methylation. In invertebrates, it has been proposed that DNA methylation of genes may be associated with alternative splicing events (i.e. honey bee (Lyko et al. 2010) and *Nasonia* (Park et al. 2011)). Methylation of gene bodies has also been shown to have a positive relationship with transcriptional activity in oysters (Gavery and Roberts, 2013; Olson and Roberts, 2014a). Currently there is an incomplete understanding of the regulation of gene expression by DNA methylation in invertebrates, though it appears to be distinct from mechanisms observed in mammals and likely varies across species.

            From the limited studies that have focused on invertebrates, research has shown that similar to mammals, DNA methylation has important regulatory functions during early development. For example, research on the honey bee *Apis mellifera* found DNA methylation to be abundant in the genome, with methylation being associated with altered gene expression resulting in bee caste differentiation (Elango et al. 2009; Kucharski et al. 2008). Furthermore, DNA methylation has been shown to regulate gene expression during *Octopus vulgaris* development, particularly during the first paralarval stage that includes significant morphological changes (Diaz-Freije et al. 2014). The first indication that methylation was an important regulator of development in *C. gigas* was by Riviere et al. (2013), who found treatment with 5-Aza-cytidine leads to developmental alterations and abnormal phenotypes in oysters.

Despite the essential role of methylation in development, there is limited information on individual variation in DNA methylation patterns among invertebrates and particularly how any methylation information might be passed on to offspring.  Furthermore, we do not have a full understanding of ontogenetic changes in DNA methylation. In order to better understand to what degree DNA methylation patterns are heritable, variable between individuals, and changing during *C. gigas* development, we analyzed genome-wide DNA methylation in gametes and larval oysters (72 and 120 hours post-fertilization) from two lineages.

***Methods***

*Experimental Design*

Oysters (two males and a single female) were collected from Oakland Bay, South Puget Sound, WA. Oysters were strip spawned by scoring the gonad tissue with a sterile razor blade and rinsing out gametes with sterile seawater. Oocytes were incubated for 30 minutes in sterile seawater and 2 million oocytes each were placed into two separate plastic containers. Spermatozoa diluted in sterile seawater (1L) from each male were used to fertilize oocytes. Fertilization was confirmed by examining polar bodies in cells under a compound microscope.

Larvae were kept in static tanks (100L) up to 120 hours post-fertilization (hpf).  Counts of oyster larvae were performed at 120 hpf to confirm normal development. Two samples for DNA methylation analyses were taken from spermatozoa prior to fertilization, and four oyster larvae samples collected at days 72 hpf and 120 hpf.  Larvae samples were taken by filtering on a 20µm screen. All samples were preserved in 95% ethanol. For simplicity the spermatozoa and corresponding larvae samples are referred to as lineage #1 and lineage #3 based on paternity.

*Bisulfite treated DNA Sequencing (BS-Seq)*

Genomic DNA was extracted using DNAzol according to the manufacturer’s protocol (Molecular Research Center, Inc., Cincinnati, OH). High molecular weight genomic DNA (6 ug per sample), was used to prepare six libraries for whole-genome bisulfite sequencing. Briefly, DNA was fragmented to an average length of 250 bp in an AFA microtube using a Covaris S2 (Covaris Inc Woburn, MA) with the following settings: duty cycle 20%, intensity of 4.0, cycles per burst 200, for 60 seconds. Fragment size resulting from DNA shearing was confirmed by gel electrophoresis. Libraries were constructed using the Paired-End DNA Sample Prep Kit (Illumina, San Diego, CA) with standard protocols. Unmethylated Lambda DNA (0.5%) (Promega Co. Madison, WI) was added to the each sample prior to fragmentation and library construction to serve as a measure of bisulfite conversion efficiency**.** DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA) and 72 bp paired-end sequencing was performed on the Illumina HiSeq 2000 system. Library construction and sequencing was performed by the High Throughput Genomics Center (htSEQ, Seattle, WA).

Bisulfite sequencing reads from the six libraries were quality filtered to remove adapter sequences and separately mapped to the *Crassostrea gigas* genome (version GCA\_000297895.1; Zhang et al. 2012) using Bisulfite Sequencing Mapping Program BSMAP v2.74 (Xi and Li 2009). Resulting alignment from mapping bisulfite treated reads was analyzed with *methratio*, a Python script that accompanies BSMAP. Parameters for *methratio* included reporting loci with zero methylation ratios (-z), combining CpG methylation ratios on both strands (-g) and only using unique mappings (-u). Only CpG loci covered by at least 3 sequenced reads were considered for further analysis. Data can be accessed and computational analysis performed as described via the GitHub repository (https://github.com/che625/olson-thesis-nb). This IPython notebook includes all steps necessary for download and analyses of DNA methylation data.

*Global DNA Methylation Comparison*

Whole-genome DNA methylation correlation and clustering were performed using the program methylKit 0.9.2 (Akalin et al. 2012) in R v3.0.3.  Pairwise coefficients by Pearson’s correlation scores were calculated based on the percent methylation profiles across all pairs of samples to create a matrix. Hierarchical clustering was performed using 1-Pearson’s correlation distance of the six methylation profiles.

*Differentially Methylated Loci*

In order to examine lineage-specific DNA methylation patterns we identified similarities of the male gamete sample to its larvae, and then compared methylation differences between the two lineages (Lineages 1 and 3). For this method, samples from the same lineage were pooled (i.e. sperm, 72 hpf, 120 hpf) and differential methylation at each loci were determined using Fisher’s exact test in methylKit. A CpG loci was determined to be different between lineages when the difference of methylation was more than 25% and *p*-value <0.01.

To characterize DNA methylation patterns that changed during development we first identified loci that 1) were the same across biological replicates (i.e. lineage #1 and #3) and 2) differed in methylation levels among the sperm and two larval samples (72 hpf and 120 hpf). Specifically, differences through development were defined using Fisher’s exact test in methylKit. Differentially methylated loci were identified as any CpG where the differences in methylation of biological replicates was greater than 25% and *p*-value <0.01 for any comparison.

All loci determined to be different across lineages and during development were characterized with respect to overlapping genomic features (intron, exon, promoter region, transposable element) using Bedtools (i.e., *intersectBed*) (Quinlan and Hall, 2010). Putative promoters were defined as 1kb regions upstream from transcription start sites and transposable elements were identified using RepeatMasker and the Transposable Element Protein Database (Smit et al., 1996-2010). A Chi-squared test was performed to determine if the distribution of differentially methylated loci among genomic regions (intron, exon, promoter region, transposable element) is significantly different than the distribution of all CpGs in the oyster genome (*p*-value <0.05 was considered significant).

***Results***

*Bisulfite treated DNA Sequencing (BS-Seq)*

Bisulfite treated DNA sequence reads are available (NCBI Sequence Read Archive: accession numbers SRX795174, SRX795175, SRX795176, SRX795177, SRX795178, SRX795179). Sodium bisulfite conversion efficiency was estimated to be 99.9% based on analysis of lambda phage DNA. The number of coverage obtained for cytosines in the oyster genome ranged from 2.6x107 to 5.3x107 across libraries. Using a 3x coverage threshold, a majority of cytosines (75-78%) were determined to be unmethylated (methylation ratio = 0), with 15-18% of the CpG dinucleotides methylated (methylation ratio ≥ 0.5).

*Genome-wide DNA Methylation Comparison*

Sample correlation and clustering was performed to assess relationships on a genome-wide scale. Sperm and respective progeny exhibited high sample correlations with a pair-wise Pearson’s correlation coefficient (*r*) of 0.84 for both lineages. These relationships are also evident in hierarchical clustering (Figure 1). Both male gamete samples were more similar in their methylation profiles to respective 120 hpf larvae.

*Lineage-specific and Developmental Differences*

A total of 189 lineage-specific differentially methylated loci (DMLs) were identified. These loci were classified with respect to oyster genomic regions (exon, intron, promoter region, transposable element) and compared to the frequency of CpG dinucleotides located among these regions (Figure 2). A majority of differentially methylated CpG loci among oyster lineages were found among transposable elements relative to the distribution of CpG dinucleotides in the oyster genome. The observed distribution of lineage-specific differentially methylated loci among transposable elements (2= 18.84, df= 1, p < 0.0001) is significantly different than the distribution of all CpGs in the oyster genome.

Differences among oyster developmental stages were also identified revealing a total of 160 differentially methylated CpG loci. These loci were classified with respect to oyster genomic regions (exon, intron, promoter region, transposable element) and compared to the distribution of all CpG dinucleotides in the oyster genome (Figure 2). Similarly, a majority of loci differing in methylation levels among oyster developmental stages were present among transposable elements, when compared to the distribution of CpG dinucleotides. The observed distribution of differentially methylated loci during development among transposable elements ( 2= 9.76, df= 1, p < 0.0018) was significantly different than the distribution of all CpGs in the oyster genome.

***Discussion***

This study represents the first single-base pair resolution DNA methylomes for both oyster spermatozoa and larval samples from multiple spawns. This research not only provides new information on DNA methylation patterns during oyster development, but also examines the degree of inheritance and methylation changes during early development. Interestingly, our research indicates that different epigenetic patterns may differ among oyster families.

Methylation levels in oyster spermatozoa and larvae samples ranged from 15-18% with interspersed regions of both methylated and unmethylated DNA in both male gamete and larval samples. This proportion of CpG methylation falls within the range of that previously reported for oyster male gonad tissue (Olson and Roberts, 2014a) and oyster gill tissue (Gavery and Roberts, 2013). Overall methylation levels are also comparable to those reported among multiple developmental stages of the Pearl oyster *Pinctada fucata* (Li et al. 2014). These findings indicate that overall genome methylation in *C. gigas* is at an intermediate level and suggests that DNA methylation levels do not significantly vary between multiple cell types and life history stages. This is similar to what has been described in global 5-methylcytosine content during different stages of *Ciona intestinalis* development (Suzuki et al. 2013). However, it contrasts with research on mammals and vertebrates which exhibit the presence of tissue and developmental stage specific methylation profiles, as that seen in zebrafish (McGaughey et al. 2014). DNA methylation primarily occurs among CpG dinucleotides and was predominantly located in gene bodies in both adult and larval oysters, which is consistent with other invertebrates (i.e. Suzuki et al. 2007, Lyko et al. 2010, Suzuki et al. 2013, Sun et al. 2014, Fneich et al. 2013).

Hierarchical clustering indicated oyster families are more similar in their DNA methylation patterns, as opposed to distinct developmental stages having similar methylation profiles. These findings suggest that DNA methylation patterns are inherited from parents to their progeny, as methylation profiles were specific to oyster lineages. If epigenetic marks are indeed heritable, this mechanism has significant implications for evolutionary change and epigenetic variation could potentially provide a new basis for selection. It has been proposed that epigenetic variation may compensate for a decrease in genetic variation in species such as sparrows (Shrey et al. 2012). It is therefore worth assessing relationships between genetic and epigenetic variation, and several studies have done this in vertebrate and plant populations (Liu et al. 2012; Herrera and Bazaga 2010). Limited studies have focused on invertebrates, but research by Jiang et al. 2013 has investigated the relationship between genetic and epigenetic variations in two groups of *C. gigas*, a base population and third generation mass selection population. This study demonstrated genetic differentiation between the oyster mass selection populations, but did not find overall epigenetic variation (Jiang et al. 2013). Despite this, a significant correlation was observed between genetic and epigenetic profiles, with few individuals having similar genetic but distinct epigenetic profiles (Jiang et al. 2013). Additional studies are required in invertebrates to determine if epigenetic variation is independent from genetic variation.

Genome-wide methylation comparisons revealed that oyster sperm and larvae have similar methylation profiles that were distinguishable among oyster lineages (Figure 1). This is indicative of paternal transmission of methylation patterns and suggestive of parental genomic imprinting as a feature during oyster embryogenesis. Genomic imprinting describes the epigenetic mechanism in which specific genes are differentially expressed in a parent-of-origin manner. In many species, it has been shown that DNA methylation regulates development by imprinting expression of genes in a parent-specific manner (i.e. Reik et al. 1987; Li et al. 1993; Reik and Walter, 2001). The phenomenon of genomic imprinting and lineage-specific methylation patterns has been described in few other invertebrates. For example, recent research by Drewell et al. (2014) found extensive germline-specific methylation patterns in the honey bee *Apis mellifera*, suggesting epigenetic marks in gametes are specific to parents. Similarly, DNA methylation was found to be responsible for genomic imprinting and parent-of-origin-specific DNA methylation patterns in mealybugs (*Planococcus citri)* (Bongiorni and Prantera, 2003). Interestingly, research on the Pearl oyster found DNA methylation inheritance is mainly influenced by oocytes, rather than spermatozoa (Li et al. 2014). However, studies on zebrafish development have indicated that embryos inherit the methylation profile of spermatozoa rather than oocyte (Jiang et al. 2013, Potok et al. 2013). Although our study did not examine the oocyte methylome, our results are consistent with these findings in which we found parent-of-origin effects, in which larvae inherited the methylation profile of spermatozoa during oyster development.

Our research identified CpG loci responsible for lineage differences in methylation, and these DMLs were classified with respect to oyster genomic regions. Interestingly, a high proportion of differentially methylated loci among lineages were found within transposable elements (Figure 2). Transposable elements are commonly known to be silenced by epigenetic mechanisms such as DNA methylation (Yoder et al. 1997), as methylation will help silence both the expression and mobility of transposable elements (Liu and Schmid 1993). Conversely, a lack of methylation will result in transposable elements being transcriptionally active, causing rearrangements in the genome and potentially generating new genetic variation. This finding has been demonstrated in a number of plant species, in which DNA methylation has been associated with transcriptional silencing in the truffle plant *Tuber malanosporum* (Montanini et al. 2014) and in maize DNA methylation plays a role in epigenetically silencing plant transposons in order to protect their genes (Rabinowicz et al. 2003). However, the mechanism of DNA methylation silencing transposons remains underexplored among invertebrates. Limited information is available about the methylation status of transposable elements in invertebrate species, and what is available suggests transposons are generally unmethylated and contain similar levels of methylation to neighboring DNA (Suzuki and Bird, 2008). DNA methylation among transposable elements is also known to be dynamic during development, and several studies have examined this in detail among vertebrates. For example, recent research on zebrafish found embryonic and fully developed tissue types with distinct methylation profiles, particularly among repetitive elements (McGaughey et al. 2014). Although we did not find repetitive elements to have different methylation patterns among tissues, we did observe a majority of DMLs among transposable elements.

Differentially methylated loci during oyster developmental stages were also identified. A significant proportion of DMLs during oyster development were found within transposable elements, relative to the distribution of CpG dinucleotides in the oyster genome (Figure 2). Interestingly, we did not observe a high proportion of differentially methylated loci among promoter regions, as would be expected if promoter methylation was regulating gene expression to play a role in oyster development. Recent research has found that DNA methylation of promoter regions specifically controls expression of *Hox* genes during oyster development, in which increased methylation in promoter regions corresponds to decreased gene expression (Riviere et al. 2013). This was further supported by identifying considerable stage-specific differences in total methylation levels during oyster early development, indicating that DNA methylation plays a crucial role in oyster embryogenesis (Riviere et al. 2013). While our study didn’t examine gene expression levels, we previously found variation in expression levels depending on the amount of promoter region methylation (Olson and Roberts, 2014a). Surprisingly we did not observe any dramatic differences in overall methylation levels during oyster development, nor differential methylation of promoter regions. This is likely due to the different analysis time points as Riviere et al. (2013) examined the first 24 hours post-fertilization, and our first larval sample was taken at 72 hpf. It is also possible that the function of DNA methylation will vary depending on developmental stage of oysters.

A high proportion of loci differing in methylation levels between lineages and during development were found among transposable elements, when compared to the distribution of all CpG dinucleotides in the oyster genome. Conversely, the distribution of differentially methylated loci among genomic regions such as exons, introns, and promoter regions, was not significantly different than the distribution of all the CpGs in the oyster genome. We propose that these differences in methylation are random, but a change in methylation among transposable elements is less likely to result in lethality than a change in methylation among functional genes (exons, introns, and promoter regions). Specifically, a change in DNA methylation, or transcriptional activity, among functional genes core to *C. gigas* survival would likely result in a lethal situation, and it is therefore beneficial for genes (exons, introns, and promoter regions) to have consistent methylation patterns rather than being differentially methylated. However, a change in methylation among transposable elements could potentially be lethal if a transposable element insertion occurs in a functional gene and disrupts gene expression, but transposable element mobility could also generate new genetic variation. This mechanism would be important for organisms like oysters, in which populations are living in stressful intertidal areas and may experience abrupt changes in environmental conditions. Therefore, we propose that differential methylation is occurring among transposable elements because differences in DNA methylation can be random, so it isn’t crucial if transposable elements are either methylated or unmethylated, however it is crucial for the methylation status of functional genes (exons, introns, and promoter regions) to be consistent. While this is a plausible explanation, more research is needed to examine transposable element mobility and DNA methylation in oysters.  While many of our findings were unexpected, together these results indicate new avenues of research and suggest there could be larger evolutionary implications for DNA methylation in some invertebrates.

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***Figures***



**Figure 1.** *Dendogram of the male spermatozoa and oyster larvae genome-wide methylation profiles using Pearson’s correlation distance*. Numeric prefix refers to lineage.



**Figure 2.** *Comparison of the DMLs versus all CpGs in the oyster genome by genomic feature.* The proportion of loci located within a genomic region (Intron, Exon, Promoter Region, Transposable Element) for differentially methylated loci between lineages (n= 189), differentially methylated loci during development (n= 160), and all CpGs in the oyster genome (n= 10035701) are displayed.