**A Context Dependent Role for DNA Methylation in Bivalves**

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Abstract: The function of DNA methylation in species such as bivalves where the limited amount of DNA methylation is predominantly found in gene bodies remains unclear. An emerging possible explanation is that the role of gene body DNA methylation is dependent on gene function, a potential phenomenon that has arisen from selective pressure on lineage-specific life history traits. In genes contributing to phenotypes that benefit from increased plasticity, the absence of DNA methylation could contribute to stochastic transcriptional opportunities and increased transposable element activity. In genes where regulated control of activity is essential, DNA methylation may also play a role in targeted, predictable genome regulation. Here we review the current knowledge concerning DNA methylation in bivalves and explore the putative role of DNA methylation in both an evolutionary and ecological context.

The variability observed in DNA methylation landscapes and functionality in invertebrates is fascinating from both a molecular and evolutionary perspective. At the molecular level we are still uncovering the many nuances associated with the functional mechanism of methylation, which in turn should eventually provide insight into the evolution of this prevalent epigenetic mark. Although we continue to understand more about DNA methylation in invertebrates, relatively limited information exists concerning the role of DNA methylation in molluscs. The phylum Mollusca encompasses xxx species represents diverse evolutionary branch and stuff. Bivalves in particular are economically (commercial relevance in aquaculture) and ecologically important (sentinel organisms) group. Here we review the current knowledge concerning DNA methylation in bivalves and explore the putative role of DNA methylation in both an evolutionary and ecological context.

The presence of DNA methylation has been confirmed in several bivalves including the Japanese scallop, *Chlamys farreri* [1], the salt water clam, *Donax trunculus* [2], and the Pacific oyster *Crassostrea gigas* [3]. Using high-throughput sequencing of bisulfite treated DNA (BS-Seq) it was recently determined 15% of CpG dinucleotides (1.8% of total cytosines) in the *C. gigas* genome are methylated (Gavery & Roberts in review), similar to the 2% total methylation for a gastropod (snail) as measured by LC-MS [4]. Methylation levels reported for the Pacific oyster were characterized in adult gill tissue but it is important to note that methylation levels are likely to vary among life history stages and among tissue types. This point is clearly indicated by Riviere et al [5] where they used an ELISA to quantify relative DNA methylation in developing oysters. Although the ELISA approach does not provide comparable values with respect to the extent of absolute DNA methylation levels, methylation almost doubled during the morula and blastula stage as compared to oocyte and then decreased again during later developmental stages [5].

DNA methylation in bivalves appears to be predominantly found in gene bodies (Gavery & Roberts, in review). The observation that gene bodies are the primary methylated genomic feature is consistent with what has been described in other invertebrates (e.g. [6–8] There is increasing evidence that this form of methylation is the ancestral pattern [9] as gene body methylation is observed not only in invertebrates and vertebrate species [10] but also in plants [11]. The function of DNA methylation in species such as bivalves where the limited amount of DNA methylation is predominantly found in gene bodies remains unclear. One possible explanation that is emerging is that the role of gene body DNA methylation is dependent on the gene function, a potential phenomenon that has arisen from selective pressure on lineage-specific life history traits. In genes whose function may benefit from increased variability (e.g. immune response), the absence of DNA methylation contributes to stochastic ‘transcriptional opportunities’, whereas genes considered core to survival (e.g. housekeeping genes) are protected from this type of transcriptional variation by the presence of DNA methylation [12]. This theory of beneficial stochastic variation as a result of hypomethylation could also be extended to other regions of the genome such as transposable elements. Further, and not mutually exclusively, DNA methylation may also play a role in a directed and targeted genome regulation. Here we will explore studies of both stochastic and targeted methylation functions that are emerging as potential roles for DNA methylation in bivalves.

**Stochastic Variation**

A classical explanation of gene body methylation is that it reduces transcriptional noise by preventing initiation of transcription outside of traditional transcription start sites (TSS) [14]. There are data to support this explanation in mammals [15], though to our knowledge, this idea has not been tested directly in an invertebrate. The implication of this explanation is that unmethylated regions would be inherently ‘noisier’. It has been proposed that this type of ‘noise’ could result in more diverse transcriptional opportunities [12], which may be beneficial for organisms such as marine bivalves that live in unpredictable and variable nearshore habitats, and as a result, have unpredictable and variable reproduction and recruitment success. As such, oysters may use epigenetic systems to maintain the genomic and phenotypic diversity necessary for a species that undergoes this type of ‘sweepstakes reproduction’ [16] where chance events dictate which individuals are successful each spawning season. The lack of methylation, by allowing more transcriptional opportunities in genes functionally associated with environmental response, may contribute to phenotypic plasticity by providing access for transcription factors to bind to alternative transcription start sites, facilitating exon skipping or other alternative splicing mechanisms, and/or through unknown mechanisms supporting increased sequencing variation [12]. Although direct evidence is currently lacking to support the idea that hypo-methylation is correlated with increased transcriptional opportunities in bivalves, recent evidence is concordant with this possibility in insects. Specifically, in the honeybee *A. mellifera*, knockdown of global methylation was associated with increased transcriptional opportunities in the form of the generation of splice variants [17] .

Consistent with the theory that limited methylation allows for a variety of transcriptional opportunities is the possibility that transposable element (TE) mobilization may be facilitated by the lack of repressive DNA methylation in bivalves. In vertebrates and some plants, extensive methylation of TEs suppresses their activity in the genome [18]. In invertebrates, species such as *A. mellifera* show very little methylation in TEs [8]. Likewise, in oysters there appears to be no preponderance of TE methylation (Gavery and Roberts, in review). The finding that TEs are not methylated in oysters is consistent with the theory outlined above regarding the ability of a population to present a variety of phenotypes in response to environment change (i.e. phenotypic plasticity). Thus the absence of TE methylation may indicate an evolutionary pressure to retain the variation generated by TE mobilization to maintain genetic diversity in a species inhabiting heterogeneous environments [19].

It is worth considering the relationships between DNA methylation, TEs and transcriptional / genomic variation in light of recent evidence coming from studies of DNA methylation and stress response in plants. For example, it has recently been reported that DNA methylation is involved in regulating the defense response of *Arabidposis thaliana* to the pathogen *Pseudomonas syringae* [20]. Using mutant strains of *A. thaliana* defective in the various types of DNA methylation, Dowen and colleagues were able to show that genome-wide hypomethylation increased plant resistance to the pathogen and was associated with mobilization of TEs and disregulaton of several immune response genes. This was further examined by Yu and colleagues [21] where *Arabidposis* subject to bacterial challenge exhibited globally reduced DNA methylation. This resulting hypomethylation was associated with the reactivation of previously silent TEs. The authors conclude that the defense response in *A. thaliana* is negatively regulated by DNA methylation, and propose that hypomethylation is a part of the plant immune response that acts by priming transcriptional activation of defense genes that are linked to TEs.  Considering these studies as a whole, it is interesting that oysters, like plants, which are immobile and face intense selection at early life stages, may benefit from these ‘noisy’ or ‘unstable’ genomes. It is important to note that the lack of DNA methylation does not preclude TE silencing, which can be repressed by a variety of epigenetic mechanisms (reviewed [22]). Future investigation in bivalves should focus on characterizing these additional epigenetic marks (e.g. histone modification, noncoding RNAs) to determine what roles they might play in stabilizing bivalve genomes, and examining the relationship between TE activity and environmental stress.

**Targeted Regulation**

A second explanation regarding a role for DNA methylation as it pertains to gene body methylation in bivalves is that the epigenetic mark regulates transcriptional activity in a targeted, predictable manner. Evidence is emerging linking gene body methylation to a potential function in regulating alternative splicing [23,24]. The production of both constitutive and alternative isoforms by alternative splicing is important for developmental processes and tissue-specific functions. In oysters, alternative splicing regulates the generation of both tissue-specific [25] and stress activated [26] isoforms of genes. The relationship between methylation and splicing has been examined in a number of studies performed in *Apis mellifera* [8,17,27]. Mechanistically, it has been proposed that exon specific DNA methylation may impact exon-skipping through interactions with DNA binding proteins (CTCF) and subsequent effects on RNA polymerase II pausing [24]. Interestingly, although intronic methylation is rare in *A. mellifera*, Foret et al. [27] identified a relationship between differential methylation in an intron upstream of a differentially expressed cassette exon of the ALK gene. Specifically, they reported that low methylation was correlated with high inclusion of the upstream exon [27].

Additional support for a targeted role in regulating transcription in bivalves is the recent work of Riviere et al [5]. Investigators examined the relationship between expression and methylation in homeobox (hox) genes, a family of genes that are critical developmental genes. Riviere et al. [5] observed an inverse relationship with gene body methylation and expression, and hypothesized that the apparent suppression of hox expression by DNA methylation was due to a “CpG island-like” repression by DNA methylation proximal to the transcription start site (TSS) in these genes. Results were obtained using methylated DNA immunoprecipitation (MeDIP) qPCR, so the context of the region investigated was known. When possible (6 out of 10 genes) the region examined was in the 1st exon or 5’UTR. The trend is similar to repression in proximal promoter/1st exon repression as seen in mammals. Riviere et al [5] not only provide evidence of active regulation of transcription via gene body methylation, but their work also suggests mechanism similar to the conventional repressive nature of promoter methylation in vertebrates. While little research exists on the relationship of promoter methylation and expression in invertebrates, there is at least one report in molluscs. In *Aplysia*, Rajasethupathy et al [28] found that serotonin exposure induced an increase in methylation in the promoter of the CREB2 gene, which is also associated with the downregulation of CREB2 mRNA in neurons. In general, CpG island containing promoter methylation is not typical in invertebrates [7]; however it is possible that depending on the context of the methylation (i.e. whether gene body or promoter methylation) it may play either a repressive or expressive role. This is known as the DNA methylation paradox [29] and has been observed in a wide range of taxa.

**Future Direction**

Continued endeavors exploring the role of DNA methylation in invertebrates will certainly shed light on general similarities and lineage specific nuances. There remains a multitude of research questions and phenomena that need attention; among them are some of the ideas presented here. The only direct evidence available relating DNA methylation and expression in bivalves focuses on a single family of genes [5]. A draft genome of *C. gigas* is now available [30] and new bivalve genomic resources are increasingly available to the scientific community, allowing us to characterize stochastic versus targeted roles for DNA methylation in bivalves. To ultimately gain a better understanding of this, future studies are needed to characterize genome wide methylation and gene expression on individuals with consideration towards cell-type, developmental stage, and environmental condition. Future investigations into other epigenetic phenomena, including histone modifications and non-coding RNAs, will provide a fuller picture regarding genome regulation in bivalves.

Another important question is the extent that the environment influences DNA methylation in bivalves. In other species it has been clearly shown that DNA methylation can be influenced by the environment [31–33]. Interestingly, one of the best examples of this phenomenon comes from findings in an invertebrate. In honeybees, larvae fed royal jelly become queens, which are phenotypically distinct from workers. It has been shown that DNA methylation serves as an intermediary between this environmental signal (nutrition) and the developmental outcome into a queen or a worker [34]. It is a likely generality that the environment influences DNA methylation in bivalves, though possibly in a different fashion, in light of the ideas presented here with respect to the stochastic nature of new transcriptional opportunities and local adaptation.

It remains to be determined to what extent transgenerational epigenetic inheritance occurs in invertebrate taxa. In mammals, evidence exists of transgenerational inheritance of DNA methylation patterns and phenotypes in response to certain xenobiotics (e.g. [35,36]). Transgenerational inheritance of DNA methylation patterns associated with phenotypes (epialleles) have also been observed in plants [37,38], including evidence that environmental stress induces heritable changes [39]. C. elegans [40]. Transgenerational epigenetic effects have not been investigated in bivalves, but o

If epigenetic marks are heritable, they may play a role in evolutionary processes. To address the question of heritability, we need to compare levels of existing epigenetic variation in natural populations with genetic variation. This indeed could be a game changer, as epigenetic variation may offer a new platform for selection. There has been some work done in vertebrates and plants [41–43], though information in invertebrates is limited. Researchers have started to address this fact in oyster aquaculture settings in response to mass selection protocols. Jiang et al [44] used a methylation-sensitive amplified polymorphism (MSAP) methodology to identify epigenetic variation between a base population and a fourth generation mass selection population. They also used AFLP to look at genetic variation. Jiang et al [44] found genetic variation with no epigenetic variation over all, though specific differences were observed. The authors observed a correlation between epigenetic and genetic variation. Despite the limitations of this study in using a relatively small number of random markers, it is the first study comparing epigenetic and genetic variation in bivalves and illuminates an interesting direction for future work.

The relationship of heritable transmission of genome patterns and epigenetic resetting is another research avenue that should be explored. In mammalian systems epigenetic resetting, a clearing and re-establishment of DNA methylation with each generation, is thought to be necessary to induce pluripotency of cells (reviewed - [45]). Nevertheless, there are certain loci (e.g. imprinted genes) where the clearing of epigenetic marks is incomplete resulting in meiotic inheritance of DNA methylation patterns. This type of transgenerational inheritance has been studied in plants and mammals, but to our knowledge has yet to be addressed in invertebrates. As mentioned above, oysters show temporal changes in the total amount of DNA methylation during embryonic development, with lower methylation in the 2-4 cell stages and increasing in morula and blastula [5]. This observation may be indicative of an epigenetic resetting event. However, characterizing epigenetic changes at finer temporal time scales are needed.

Exploring these questions of epigenetic flexibility to environmental cues, natural variation, heritability, as well as the possibility of epigenetic resetting in bivalves will inform the direction of much larger research questions. Maybe add sentence about experimental methodology? While we are gaining a better understanding of invertebrate epigenetics, we certainly have a lot more to learn, which could considerably change our comprehension of organismal and ecosystem responses to environmental change.

**Key Points**

* DNA methylation is found throughout the genome and is predominant in gene bodies in bivalves.
* The role of gene body DNA methylation could be dependent on the gene function and serve to both reduce stochastic transcriptional noise as well as regulate activity in a targeted manner.
* Several important research questions remain unanswered with respect to DNA methylation in bivalves related to environmental influence, relationship with genetic variation, and transgenerational inheritance.

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