Gene regulation by DNA methylation is contingent on chromatin accessibility during transgenerational plasticity in the purple sea urchin

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Abstract

Epigenetic processes are proposed to contribute to phenotypic plasticity. In invertebrates, DNA methylation commonly varies across environments and can correlate or causally associate with phenotype, but its role in transcriptional responses to the environment remains unclear. Maternal environments experienced by the sea urchin *Strongylocentrotus purpuratus* induce 3 – 6x greater differential CpG methylation in offspring larvae relative to larval developmental environments, suggesting a role for DNA methylation in transgenerational plasticity (TGP). However, a negligible association has been observed between differentially methylated and differentially expressed genes. What gene regulatory roles does invertebrate DNA methylation possess under environmental change, if any? We quantified DNA methylation and gene expression in *S. purpuratus* larvae exposed to different ecologically relevant conditions during gametogenesis (maternal conditioning) or embryogenesis (developmental conditioning). We modeled differential gene expression and differential splicing under maternal conditioning as functions of DNA methylation, incorporating variables for genomic feature and chromatin accessibility. We detected significant interactions between differential methylation, chromatin accessibility, and genic architecture associated with differential expression and splicing. Observed transcriptional responses to maternal conditioning were also 4 – 13x more likely when accounting for interactions between methylation and chromatin accessibility. Our results provide evidence that DNA methylation possesses multiple functional roles during TGP in *S. purpuratus,* but its effects are contingent upon other genomic and epigenomic states. Singularly unpredictive of transcription, DNA methylation is likely one cog in the epigenomic machinery contributing to environmental responses and phenotypic plasticity in *S. purpuratus* and other invertebrates.

1. Introduction

Phenotypic plasticity, variation of a trait's expression in the absence of genetic variation, can elicit both adaptive and maladaptive responses to rapid environmental change on ecological timescales (Donelan et al., 2020; Marshall & Uller, 2007). However, the molecular processes that mediate plasticity remain poorly understood. Uncovering these mechanisms will inform our understanding of physiological and evolutionary responses to changing environments (Herman & Sultan, 2011; Jones & Robinson, 2018). Epigenetic modifications to the genome such as DNA methylation are one suite of regulatory factors that, in some cases, underpin plasticity by driving changes in transcription and subsequent phenotypes (Xu et al., 2019). Our understanding of differential DNA methylation's effects on gene expression and phenotype in metazoans is mostly derived from studies in vertebrates, but research in invertebrates finds correlations between changes in DNA methylation and phenotype across distinct environments, suggesting that methylation may influence acclimatization (Eirin-Lopez & Putnam, 2019; Hofmann, 2017). Connections between DNA methylation, plasticity, and acclimatization hinge on how and whether DNA methylation modulates gene regulation. Invertebrate DNA methylation frequently exhibits negligible relationships with differential expression (DE) and other modes of gene regulation in environmental studies, posing an obstacle to assessing the epigenetic basis of acclimatization to current and future environments. However, DNA methylation does not influence gene expression independent of other epigenetic and genetic factors. Using the purple sea urchin *Strongylocentrotus purpuratus* as a model invertebrate, we tested the hypothesis that the effects of differential methylation (DM) on gene regulation (differential expression and alternative splicing) are contingent upon additional epigenomic and genomic states such as chromatin accessibility and genic architecture. This integrated epigenomic approach allowed us

to determine what regulatory roles DNA methylation in *S. purpuratus* possesses, if any, during plastic responses to ecologically relevant stressors that are worsening under climate change.

Multiple lines of evidence support DNA methylation's influence on invertebrate ecology and biology. In broadly dispersing marine invertebrates, high connectivity reduces structure across populations inhabiting distinct environments, and interpopulation epigenetic divergence can exceed genetic divergence (Ardura, Zaiko, Moran, Planes, & Garcia-Vazquez, 2017; Johnson & Kelly, 2020; Ni et al., 2018; Watson, Baldanzi, Pérez-Figueroa, Gouws, & Porri, 2018; Zhang, Li, Kong, & Yu, 2018). *In situ* temporospatial environmental variation has been linked to modifications in invertebrate methylomes independent of genetic variation, demonstrating a potential role for DM during acclimatization. (Clark et al., 2018; Dimond & Roberts, 2020; Rodríguez-Casariego et al., 2020; Wang, Li, et al., 2021). DM of genes, gene modules, or whole genomes induced by environmental variation are associated with performance traits in stony corals, molluscs, crustaceans, and insects (Arsenault, Hunt, & Rehan, 2018; Clark et al., 2018; Li et al., 2018; Norouzitallab et al., 2014; Putnam, Davidson, & Gates, 2016). Causative tests of DNA methylation's effect on phenotype have also been conducted. In the crustacean *Daphnia manga*, inhibition of *de novo* methyltransferases in the P generation induced genome-wide hypomethylation and DE of essential metabolic pathways, proceeding to impact performance and expression in F1 and F2 generations while leaving histone modifications unaffected (Lindeman et al., 2019; Vandegehuchte, Lemiere, Vanhaecke, Vanden Berghe, & Janssen, 2010). Environmentally induced changes and intraspecific variation in DNA methylation can also be inherited across the germline in some invertebrate taxa (Liew et al., 2020; Wang, Werren, & Clark, 2016). Testing the gene regulatory roles of DNA methylation will

uncover its potential functions in a diversity of biological processes, particularly mechanisms of developmental and transgenerational plasticity (TGP) which drive acclimatization.

DNA methylation's effects on gene regulation and its interactions with other epigenetic factors are both highly multiplicative. Most invertebrate phyla exhibit sparsely methylated genomes punctuated by high levels of CpG methylation within gene bodies (Keller, Han, & Yi, 2016; Suzuki, Kerr, De Sousa, & Bird, 2007; Zemach, McDaniel, Silva, & Zilberman, 2010). Patterns of invertebrate gene body methylation (GBM) vary across (i) genic features such as promoters, introns, exons, and UTRs (Li et al., 2018; Riviere et al., 2017) and (ii) phylogeny (de Mendoza et al., 2019; Keller et al., 2016; Sarda, Zeng, Hunt, & Yi, 2012). GBM positively correlates with gene expression in cnidarians (Dixon, Liao, Bay, & Matz, 2018; Li et al., 2018; Zemach et al., 2010), bivalve molluscs of *Crassostrea sp*. (Downey-Wall et al., 2020; Johnson, Sirovy, Casas, La Peyre, & Kelly, 2020), and arthropods (Bonasio et al., 2012; Gatzmann et al., 2018; Glastad, Gokhale, Liebig, & Goodisman, 2016; Kvist et al., 2018) with exceptions to this pattern evident in some species and cell types (de Mendoza et al., 2019; Flores et al., 2012; Wang, Song, et al., 2021). By contrast, inducible changes in invertebrate gene expression in response to environmental variation have frequently possessed insignificant relationships with differential GBM (Arsenault et al., 2018; Dixon et al., 2018; Downey-Wall et al., 2020; Johnson et al., 2020; Strader et al., 2020). In some arthropods, molluscs, and nematodes, there is evidence that GBM aids in regulating alternative splicing and exon skipping (Flores et al., 2012; Gao et al., 2012; Li-Byarlay et al., 2013; Libbrecht, Oxley, Keller, & Kronauer, 2016; Song, Li, & Zhang, 2017). Among these taxa, changes in alternative splicing under environmental change have shown relationships with differential GBM of varying strengths (Arsenault et al., 2018; Glastad et al., 2016). Invertebrate DNA methylation is also associated with chromatin state

(Gatzmann et al., 2018; Nanty et al., 2011) and the suppression of spurious intragenic transcription (Li et al., 2018). Determining the function of DM during transcriptional responses to the environment thus requires an integrated approach that considers genic architecture, additional epigenetic features, and multiple modes of gene regulation (Moler et al., 2018).

The purple sea urchin *Strongylocentrotus purpuratus* is a uniquely poised model invertebrate in which to conduct an integrative test of DNA methylation's regulatory roles during phenotypic plasticity. *S. purpuratus* is an abundant herbivore distributed throughout North America's Pacific subtidal kelp forests and rocky intertidal. Populations inhabiting environmental gradients or mosaics exhibit genetic evidence of local adaptation and interpopulation variation in performance and gene expression under ecologically relevant stress (Evans, Chan, Menge, & Hofmann, 2013; Evans, Pespeni, Hofmann, Palumbi, & Sanford, 2017; Kelly, Padilla-Gamino, & Hofmann, 2013; Pespeni, Chan, Menge, & Palumbi, 2013; Pespeni & Palumbi, 2013). CpG methylation is more abundant in *S. purpuratus* relative to most invertebrates, likely because of its phylogenetic position as a basal deuterostome (Regev, Lamb, & Jablonka, 1998). TGP linked to maternal effects have been observed in *S. purpuratus* for traits including egg protein content, larval body size, gene expression, and DNA methylation (Hoshijima & Hofmann, 2019; Strader et al., 2020; Strader, Wong, Kozal, Leach, & Hofmann, 2019; Wong, Johnson, Kelly, & Hofmann, 2018; Wong, Kozal, Leach, Hoshijima, & Hofmann, 2019) alongside similar observations in congeneric *Strongylocentrotus spp.* (Ding et al., 2019) and other urchin genera (Clark et al., 2019; Karelitz, Lamare, Patel, Gemmell, & Uthicke, 2019; Wong & Hofmann, 2020; Wong & Hofmann, 2021). Maternal conditioning of *S. purpuratus* to abiotic conditions mimicking coastal upwelling can induce 3 – 6x greater DM in offspring larvae relative to the effects of larval development under upwelling (Strader et al., 2020; Strader et al.,

2019). Maternal conditioning of *S. purpuratus* can also trigger DE of a larger number of genes than progeny conditioning (Wong et al., 2018). These results suggest a function for DM in facilitating TGP's effects on gene expression, but negligible overlap between DM CpGs and DE genes has left that role ambiguous (Strader et al., 2020). Accounting for interactions between DNA methylation and additional epigenomic states in *S. purpuratus*, which may better explain epigenetic effects on transcription, is made possible by the species' use as a model of deuterostome embryology, yielding developmental time series of chromatin accessibility (e.g., ATAC-seq) spanning the two-cell embryo to late prism larva.

To elucidate the gene regulatory roles of differential methylation during TGP, we quantified changes in DNA methylation, gene expression, and alternative splicing in prism larvae induced by maternal exposure to ecologically relevant, abiotic stress in *S. purpuratus* using data from Strader *et al.,* 2020 initially exhibiting limited overlap between DM and DE genes and robust annotations of chromatin accessibility during the *S. purpuratus* prism stage (Kudtarkar & Cameron, 2017). We then modeled differential expression and splicing as functions of DM, genic feature type, and chromatin accessibility to test the hypothesis that invertebrate DNA methylation's regulatory role is contingent upon additional genomic and epigenomic factors and reveal epigenetic interactions influencing gene expression during TGP.

2. Methods

2.1. Data sources – To investigate the potential gene regulatory role of DNA methylation on transcription, we used previously published and publicly available datasets. For RNA-seq and bisulfite sequencing datasets, a controlled transgenerational experiment was performed (Strader et al. 2020). Briefly, adult urchins were conditioned to two treatments, non-upwelling (631 ± 106

μatm *p*CO2 and 16.8 ± 0.2 °C) and upwelling (1390 ± 307 μatm *p*CO2 and 12.7 ± 0.5 °C), mimicking variation in their natural environment (Hoshijima & Hofmann, 2019). Temperature and pCO_2 conditions were maintained by a flow-through CO_2 system (Fangue et al., 2010) and described in detail by Strader et al. 2020. Briefly, treated seawater was evenly pumped from two reservoir tanks to conditioning tanks at a rate of 20 L/hr. Adult urchins were induced to spawn and fertilizations were performed in ambient seawater conditions. Embryos were reared in either the same conditions as their parents or the reciprocal condition in triplicate using a flowthrough system with seawater treated as described above and by Strader et al. 2020.

Once larval development progressed to the early prism stage, replicate samples of 6,000 larvae were collected for RNA-seq and reduce representation bisulfite sequencing (RRBS) and flash frozen in liquid nitrogen before storage at -80 °C. Libraries for polyA-enriched RNA-seq and RRBS were constructed at the UC Davis genome center and sequenced on the Illumina 4000 (BioProject: PRJNA548926). The use of polyA-enriched RNA-seq libraries is beneficial for analyzing alternative splicing as it mitigates the contribution of unprocessed RNA to quantification of differential exon use (Sultan et al., 2014). RRBS poses fewer biases on CpG representation across genomic feature type relative to other reduced representation BS-seq methods (Trigg et al., 2021). ATAC-seq data was obtained through the GEO expression omnibus (BioProject: PRJNA377768). This dataset represents a developmental time course of Tn5 transposon chromatin accessibility regions in the *S. purpuratus* genome.

For comparison with the Strader *et al.*, 2020 datasets, we chose ATAC-seq profiles for animals at 39 hours post-fertilization, the closest developmental time point for early prism larvae, for which 3 pooled samples were sequenced (GSM2520650, GSM2520651, GSM2520652). ATAC-seq bed files were concatenated and summarized using the R package

184 *ChIPSeeker* v1.22.1 to quantify chromatin accessibility, expressed as the mean density of Tn5
185 ATAC-seq reads, across intra- and intergenic and genomic features. Mean chromatin
186 accessibility of ± 500 bp transcriptional start sites (TSS), introns, and exons were each calculated
187 in both gene- and feature-wise manners for analysis.

188 *2.2. Gene expression analyses* – RNA-seq reads were trimmed of adaptor sequences and
189 filtered for quality using *TrimGalore*. Cleaned reads were mapped to the *S. purpuratus* genome
190 (v3.1) using *hisat2* (Kim, Langmead, & Salzberg, 2015). Gene and exon counts were compiled
191 with *featureCounts* (Liao et al. 2014) and analyzed in *edgeR* v3.28.1 (Robinson, McCarthy, &
192 Smyth, 2010) for analyses of DE and differential exon use (DEU), a measure of exon inclusion
193 or exclusion attributable to skipping and splicing. Gene-level and exon-level read counts were
194 filtered to retain genes with > 0.5 counts per million (CPM) across at least 75% of all samples.

195 In order to test for DE and DEU, gene- and exon-level counts were modeled as a function
196 of maternal environment, developmental environment, and their interaction using the robust
197 iteration of the *edgeR* glmQLfit function to fit negative binomial generalized linear models
198 (GLMs). Robust negative binomial dispersion estimates were calculated using empirical
199 Bayesian shrinkage with the *edgeR* function estimateGLMRobustDisp. Log_2 foldchanges
200 (logFC), F-statistic scores, and p-values for genewise DE between maternal and developmental
201 treatments were estimated using the *edgeR* function glmQLFTest to account for uncertainty of
202 tagwise dispersion estimates and improve type I error control. Significant DE was determined
203 using FDR-adjusted p-values (alpha = 0.05). DEU was assessed by applying the edgeR function
204 diffSpliceDGE to exon-level negative binomial GLMs which output exon use coefficients
205 denoted as ΔlogFC (exon logFC – gene logFC) as well as likelihood coefficients and FDR-

adjusted p-values (alpha = 0.05) for LRTs of gene- and exon-level DEU (McCarthy, Chen, & Smyth, 2012; Robinson et al., 2010).

Quantifying DEU attributable to alternative splicing and exon skipping required the removal of genes exhibiting patterns of exon use consistent with spurious intragenic transcription and alternative TSS. Genes that are spuriously transcribed or exhibit alternative TSS possess exons with progressively lower inclusion toward 5' ends (Li et al., 2018). Filtering out such genes from exon-level read counts used in DEU analysis required the fitting linear models to exon-use data of each gene and removing genes with positive slopes and a y-intercept of DEU > -0.25. Without this filtering step, 56.0% of genes that exhibited significant DEU under maternal upwelling would likely have been attributed to alternative TSS or spurious transcription while such genes would have composed 64.9% of significant DEU under developmental upwelling. While this approach honed in on DEU attributed to splicing and exon skipping, it is likely to remove genes with few exons in which a 5' exon removed during splicing. Plots of DEU trends demonstrative of spurious transcription or alternative TSS are available in the GitHub repository https://github.com/snbogan/Sp_RRBS_ATAC.

Enriched gene ontologies (GO) were identified among genes exhibiting DE or DEU with Mann–Whitney U-tests input with signed, -log p-values using rank-based gene ontology analysis with adaptive clustering (Wright, Aglyamova, Meyer, & Matz, 2015) parameterized with alpha value = 0.05 and minimum GO-term group size = 5 genes for gene-level enrichment. Alpha = 0.01 and min. Minimum GO-term group size = 25 genes for exon-level enrichment to account for a mean exon count of ~5 per gene in the *S. purpuratus* genome.

2.3. DNA methylation analyses – RRBS sequences were trimmed and filtered with *TrimGalore* specifying the –rrbs option. Trimmed RRBS reads were mapped to the genome

using *Bismark* (Krueger & Andrews, 2011), and methylation calls were determined using the bismark_methylation_extractor command using default settings. Coverage files were used for subsequent DM analysis using an adapted *edgeR* workflow for RRBS data (Chen, Pal, Visvader, & Smyth, 2017). To examine feature-specific responses by DNA methylation to environmental treatments, DM was estimated as the logFC of summed methylation scores across all CpGs within the -1 kb promoters, introns, and exons of a given gene. For each feature type, summed counts were filtered to include only genes represented by ≥10 reads across all samples. edgeR was selected for DM analysis to provide a statistical framework unified with estimations of DE and DEU. Functional enrichment of GO terms among differentially methylated genes was assessed using Mann–Whitney U-tests input with signed, -log p-values using rank-based Gene Ontology analysis (Wright et al., 2015).

2.4. Modeling gene regulation as a function of epigenomic variation – Using a Bayesian framework, Gaussian linear models were fitted in order to predict baseline gene expression (log_2 counts per million or logCPM) and binomial generalized linear models were fitted to binary values for the presence of alternative transcripts (e.g., splicing) as functions of mean CpG methylation and mean chromatin accessibility of promoters, introns, exons, and interactions between these predictors. Linear models of DE were fitted to predict logFC as a function of DM in -1 kb promoters, introns, and exons, as well as logCPM, chromatin accessibility across genic features, and components of genic architecture such as the total length of genic feature types. Linear models of DEU included predictors for DM of the corresponding exon, DM of all exons and introns of the associated gene, exon number, logCPM, chromatin accessibility across genic features, and genic architecture.

All linear and generalized linear models were fitted using the R package *brms* v2.14.0, an R interface to the Stan programming language for specifying Bayesian models (Bürkner, 2017). All models were fitted with scaled Z-score transformations of continuous variables. Linear models of DE and DEU were fit with studentized model families to reduce prediction of artificially high or low outcome variables and were specified with weakly informative normal priors (mean = 0; SD = 0.5) for both slope (β) and intercept parameters. Z-score transformations were used in order to improve model convergence and compare posterior distributions of β parameters for predictors of different dependent variables such as DE and DEU. Weakly informative priors expressing a low probability of DNA methylation affecting gene regulation accounted for knowledge that DM associated with plasticity has exhibited negligible singular effects on gene regulation in most invertebrates. Posterior distributions were sampled using 4 chains at 20,000 iterations each, including 5,000 warmup iterations.

Model selection was performed by (i) applying Bayes factors using the bayesfactor_models function in *bayestestR* v0.9.0 (Makowski, Ben-Shachar, & Lüdecke, 2019) to compare the likelihoods of models fit with iterative combinations of predictor variables excluding >3rd order interactions and (ii) comparing the selected model to two additional, alternative models using k-fold cross validation via *rstanarm* v2.21.1 (Goodrich, Gabry, Ali, & Brilleman, 2020): a model of the outcome predicted by differential methylation alone and the selected model without its highest-order interaction term. Bayes factors are less likely to select complex models (Gronau & Wagenmakers, 2019) and were applied to a large number of varyingly complex models before the selected model's predictive strength was evaluated with k-fold cross validation. To account for variation in RRBS read coverage across the data, the selected model was then refit to include an error parameter for estimated methylation and

differential methylation that equaled the inverse CpG coverage of each gene or feature in the dataset. RRBS CpG coverage per feature is described in Supplemental Results for reported models. Posterior predictive checks were used to evaluate selected model predictions according to observed data. Effect significance was tested using probability of direction, a Bayesian corollary of the p-value (Makowski, Ben-Shachar, Chen, & Ludecke, 2019); fixed effects were deemed significant if 95% of their posterior distribution fell above or below 0. Inclusion Bayes factors were employed to test for the explanatory power of an effect by estimating the likelihood of observed data when fitted with a parameter relative to a null model excluding it (Hinne, Gronau, van den Bergh, & Wagenmakers, 2020). Diagnostic plots, QC information, and predictions of selected models are available in Supplemental Results. The specifications and relative likelihoods of selected and unselected models are available in the following GitHub repository: https://github.com/snbogan/Sp_RRBS_ATAC.

3. Results

The results of our study demonstrate (i) that differential DNA methylation likely bears gene regulatory effects during TGP in *Strongylocentrotus purpuratus* and (ii) that these effects are conditional upon chromatin accessibility and genic architecture. We observed positive correlations between baseline DNA methylation, transcript abundance, and the presence of alternative splice forms within genes. With regard to plastic changes in DNA methylation and gene regulation, differential GBM interacted with chromatin accessibility and genic architecture to affect both differential gene expression and differential exon use/splicing such that the strength and direction of DM's effects were contingent upon these additional genomic and epigenomic states. We describe these results in three sections below, focusing first on baseline

297 relationships between DNA methylation and transcription, followed by epigenetic and gene
298 regulatory responses to experimental upwelling. Finally, we present the results of integrated
299 epigenomic models of DNA methylation's gene regulatory effects during TGP.

300 *3.1. Associations between constitutive epigenomic states and transcription* – GBM in *S.*
301 *purpuratus* prism larvae showed significant and positive correlations with gene expression level
302 and the probability of associated alternative transcriptional variants. CpGs within -1 kb
303 promoters, exons, and introns exhibited mean methylation levels of 34.70%, 43.21%, and
304 44.64%, respectively. Mean promoter methylation demonstrated a significant, albeit weak,
305 negative effect on the expression (logCPM) of corresponding genes. By contrast, mean exon and
306 intron methylation both exhibited stronger, positive effects on expression. Genes that were
307 highly methylated in either exons or introns were ~2x more expressed than unmethylated genes
308 (Fig. 1A). Exon and intron methylation also exhibited a significant, antagonistic interaction such
309 that genes with high methylation at both introns and exons were not more expressed than genes
310 with high methylation at only introns or exons. Interestingly, accounting for TSS accessibility in
311 models of logCPM resulted in the loss of a significant effect of intron methylation on gene
312 expression. Lastly, genes with high levels of intron or exon methylation were more likely as to
313 exhibit transcript variants consistent with alternative splicing, alternative TSS, and/or exon
314 skipping. The relationship between the probability of transcript variants and methylation at
315 promoters was insignificant (Fig. 1B).

316 Chromatin accessibility at TSS, exons, and introns were also correlated with gene
317 expression level, but not with the probability of transcriptional variants. However, selected
318 models of logCPM did not include parameters related to chromatin accessibility and such
319 parameters also yielded low inclusion Bayes factors. Chromatin accessibility was enriched

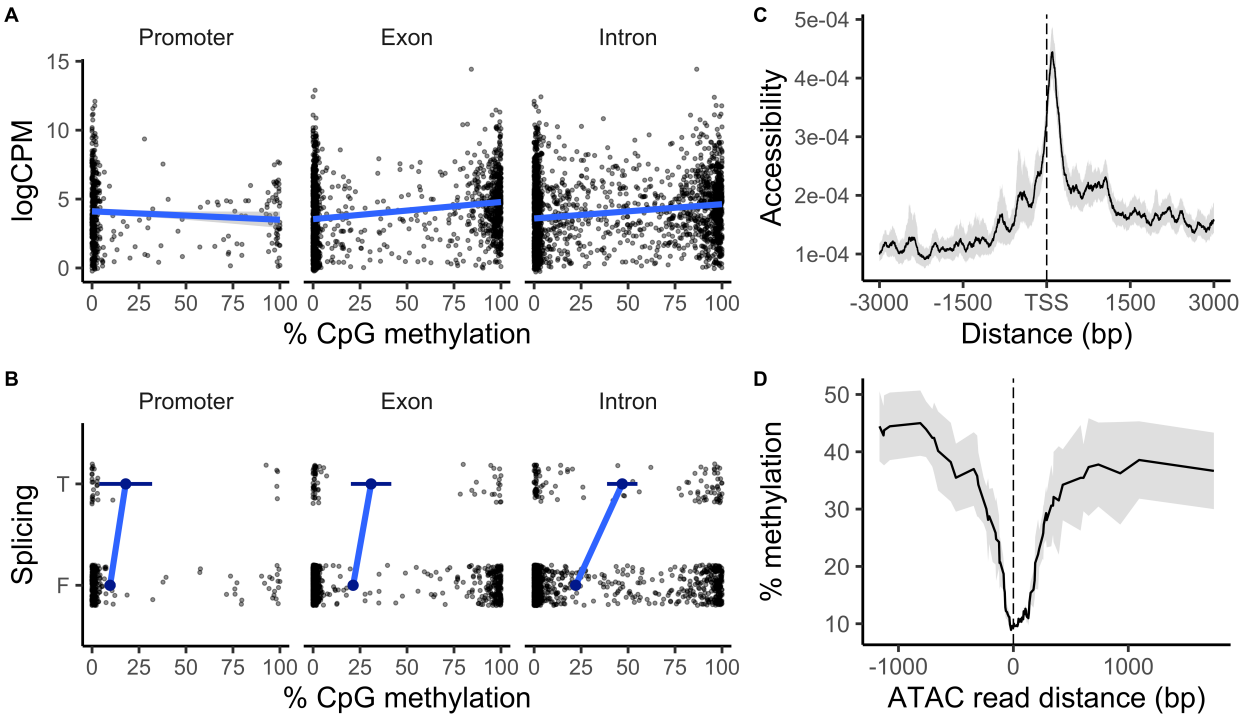

Figure 1 | Relationships between constitutive DNA methylation, expression, and chromatin accessibility in larval *Strongylocentrotus purpuratus*. (a) Median methylation averaged across CpGs in the promoter, introns, and exons per gene are plotted against transcript abundance and (b) whether a gene exhibited transcript variants. Error bars depict ± 95% CI. (c) Loess trend of mean ATAC-seq read density (i.e., chromatin accessibility) ± 95% CI is plotted across distance to TSS sites. (d) 50 bp sliding window averages of CpG methylation ± 95% CI are plotted across distance to accessible chromatin regions for which ATAC-seq reads intersect from all replicate samples.

proximal to TSS (Fig. 1C) but was greatest in introns, which exhibited a mean of 0.19 ± 0.11 ATAC-seq reads per bp compared to 0.043 ± 0.001 and 0.039 ± 0.043 in TSS and exons, respectively. Open chromatin regions showed ~30% less CpG methylation than inaccessible regions (Fig. 1D). Gene-level intron and exon methylation showed no relationship with chromatin accessibility of either genic feature (Fig. S1A). Chromatin accessibility within ± 500 bp of TSS and gene bodies both showed significant and positive correlations with gene expression, with TSS accessibility exhibiting an effect that was 32.98% stronger than gene body accessibility (Fig. S1B). TSS accessibility did exhibit a significant and positive correlation with the probability of alternative transcriptional variants. This effect was insignificant after

337 accounting for intron methylation however. Thus, intron methylation was the only significant
338 predictor of alternative splicing events.

339 *3.2. Transcriptional and epigenetic responses to environmental variation* – Maternal and
340 developmental exposure to experimental upwelling induced DE, as well as DEU consistent with
341 alternative splicing and exon skipping in prism larvae of *S. purpuratus*. As reported by Strader et
342 al. 2020, differential CpG methylation was observed in response to maternal upwelling, but no
343 DM was observable under developmental upwelling. This distinction between maternal and
344 developmental effects remained constant after quantifying DM averaged across genes'
345 promoters, exons, and introns.

346 Developmental upwelling exposure induced 2,263 upregulated and 2,459 downregulated,
347 differentially expressed genes (DEGs). Maternal exposure induced 1,380 upregulated and 1,025
348 downregulated DEGs (Fig. 2). After applying a $\log_2$FC cutoff of > 1.0, 309 significant
349 developmental DEGs were retained while 245 maternal DEGs were retained. Although the
350 developmental treatment gave rise to a greater number of DEGs, absolute logFCs of DE among
351 maternal DEGs were significantly higher than absolute logFCs of developmental DEGs by
352 10.45%. Functional enrichment of biological process, molecular function, cellular component
353 GO terms among DEGs was observed in response to maternal and developmental treatments and
354 is extensively reported by Strader et al. 2020.

355 Substantially less DEU occurred in response to experimental upwelling relative to DE.
356 Significant DEU was evaluated using both gene- and exon-level tests. Developmental upwelling
357 induced 78 differentially spliced genes (DSGs) while maternal upwelling induced 121 DSGs,
358 with 16 DSG genes shared between treatments. 43 and 49 genes were both differentially
359 expressed and differentially spliced in response to developmental upwelling and maternal

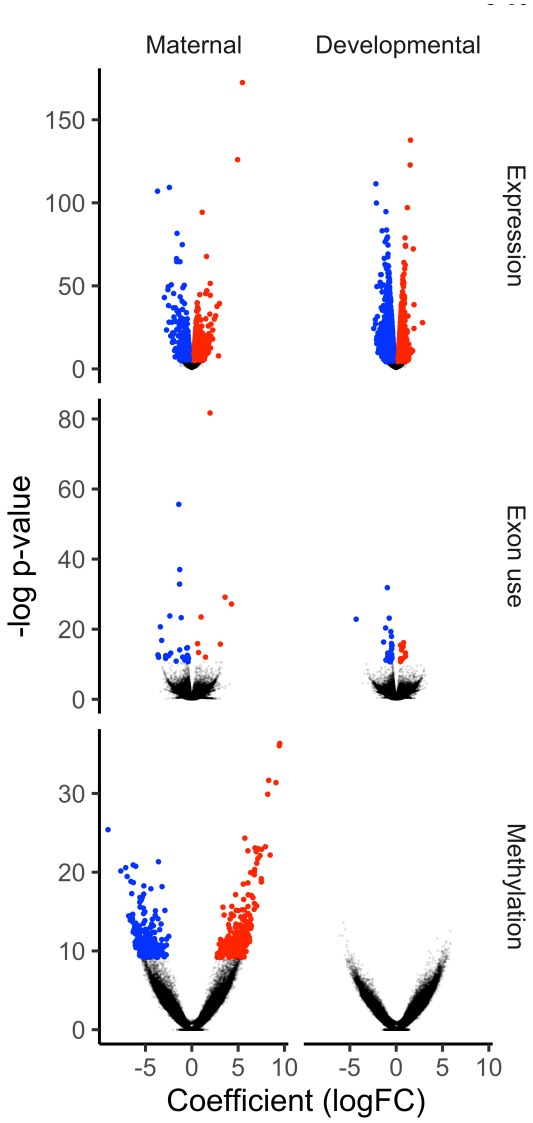

Figure 2 | Molecular responses to developmental and maternal upwelling exposure. (top) Volcano plots of differential expression depicting genewise -$\log_2$ p-values, $\log_2$FC, and significant differential expression (color). (middle) Volcano plots of differential exon use or DEU (e.g., splicing) depicting exon-level -$\log_2$ p-values, DEU coefficients, and significant DEU (color). (bottom) Volcano plots of differential CpG methylation depicting CpG-level -$\log_2$ p-values, $\log_2$FC of differential methylation, and significant differential methylation (color). Red and blue points depict significant positive and negative coefficients, respectively (FDR < 0.05).

upwelling, respectively (Fig. S2). Significant DEU was detected among 44 exons in response to developmental upwelling: 14 upregulated or "included" exons and 30 downregulated or "dropped" exons. DEU in response to maternal upwelling occurred in 47 exons: 12 included and 35 dropped exons (Fig. 2). The molecular function (MF) GO terms 'structural molecule activity' and 'structural constituent of ribosome' and the biological process terms 'obsolete GTP catabolic process', 'small GTPase mediated signal transduction', and 'cellular amide metabolic process' were enriched among differentially spliced exons in response to both maternal and developmental treatments. Exons differentially spliced under the maternal treatment were also enriched for the biological processes (BP) 'cellular localization' and 'nuclear transport' among others (see Supplemental Material).

389 In response to maternal upwelling, 288 CpGs were hypermethylated and 233 were
390 hypomethylated. Zero CpGs were differentially methylated in response to developmental
391 upwelling (Fig. 2). This effect was distinct from DE and DEU, which both exhibited greater or
392 equal variation in response to developmental upwelling compared to maternal upwelling.
393 Functional enrichment among differentially methylated genes is described in detail by Strader et
394 al 2020.

395 *3.3. Chromatin state, genic architecture, and differential methylation interactively*
396 *influenced transcriptional responses to environmental variation* – The strength of differential
397 GBM's effect on DE was conditional upon chromatin accessibility and transcript abundance.
398 Differential GBM across whole genes did not affect DE, while intron DM was significantly
399 associated with DE. The selected model of DE as a function of intron DM under maternal
400 upwelling included a significant three-way interaction between intron DM, TSS accessibility,
401 and logCPM. Intron DM had higher absolute effects on DE among genes with poorly accessible
402 TSS. These effects were positive among genes with low expression and negative for highly
403 expressed genes (Fig. 3A). The inclusion Bayes factor for the interaction between intron DM,
404 logCPM, and TSS accessibility was 3.84 (Fig. 3B). A Fishers exact test demonstrated that genes
405 in the lowest logCPM quartile and lowest quartile of TSS accessibility were enriched with MF
406 GO terms that included 'nucleotidyltransferase activity' and 'cytoskeletal motor activity', two
407 MF terms that were also enriched among genes with CpGs that were differentially methylated
408 under maternal upwelling (Strader et al., 2020). The biological process GO terms 'plasma
409 membrane bounded cell projection assembly' and 'movement of cell or subcellular component'
410 were also enriched among genes with low TSS accessibility and expression, among others (see
411 Supplemental Material). Genes in the lowest TSS accessibility quartile and highest logCPM
412

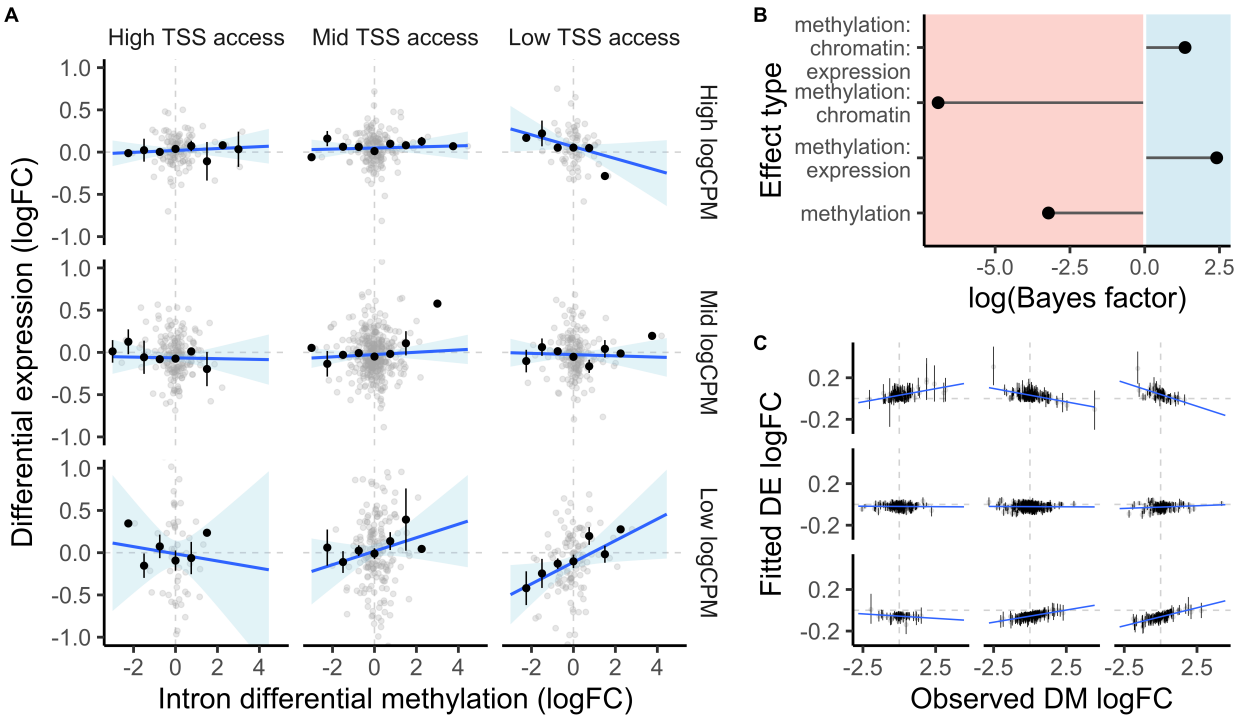

Figure 3 | Differential intron methylation affected expression conditional upon TSS accessibility and transcript abundance. (a) Differential gene expression under maternal upwelling is plotted against mean intron differential methylation. Rows and columns group data based on transcript abundance and TSS accessibility quartiles, respectively. First and last rows/columns denote highest and lowest quartiles. Linear regressions are fitted across observed values. Average logFC across binned intron differential methylation is plotted as black points ± SE. (b) Log-scale inclusion Bayes factors depicting the probability of observed data under a parameter, including the interaction plotted in panel A. (c) logFC values fitted by the selected model of differential expression as a function of intron differential methylation. Error bars represent ± 95% credibility intervals. Columns, rows, and the x-axis depict binned TSS accessibility, logCPM, and observed intron differential methylation as presented in panel A.

quartile were enriched with the MF terms ‘threonine-type endopeptidase activity’, ‘transcription regulator activity’, and ‘calcium ion binding’. An Intron DM standard deviation of +1.00 corresponded to a DE standard deviation of +0.26 ± 0.12 among genes in the lowest quartile of TSS accessibility and lowest logCPM quartile and -0.14 ± 0.09 for low TSS accessibility, high logCPM genes. Lastly, some model iterations in the top 25% of marginal likelihoods indicated that intron length interacted with intron DM to affect expression. Intron hypermethylation of genes in the top quartile of intron length silenced expression while genes in the middle-to-lowest

quartiles of intron length showed enhanced expression as a result of intron hypermethylation (Fig. S3), though the inclusion Bayes factor for this interaction was negligible (BF = 1.67).

Differential exon methylation responding to maternal upwelling interacted with gene body accessibility and genic architecture to affect DEU attributable to variation in alternative splicing and/or exon skipping. Selected models of DEU yielded a significant three-way interaction between exon DM, exon accessibility, and the total genic intron length. Both the strength and direction of exon DM's effect on DEU was conditional upon exon accessibility and intron length. Positive correlations between exon DM and DEU were observed among exons from genes with poor exon accessibility while negative correlations were observed among exons from genes with highly accessible exons. Absolute effect strengths of exon DM on DEU were stronger among genes with longer introns (Fig. 4A). The inclusion Bayes factor of exon DM, exon accessibility, and intron length's interactive effect on DEU equaled 13.03 (Fig. 4B). Exons from genes with long introns in the lowest quartile of exon accessibility exhibited a Z-score β of $+0.43 \pm 0.20$. Exons from genes with long introns in the highest quartile of exon accessibility bore an effect strength of -0.12 ± 0.14. Genes in the lowest quartile of exon accessibility and highest intron length quartile were enriched with the MF GO terms 'calcium ion binding', 'cytoskeletal motor activity', and 'ATPase activity', among others, and the BP terms 'purine-containing compound metabolic process', 'microtubule-based movement', and 'cell adhesion'. Genes with long introns and high exon accessibility were also enriched with the MF term 'calcium ion binding', as well as 'transporter activity', 'small molecule binding', and others, and enriched BP terms including 'cell adhesion', 'localization', 'regulation of intracellular signal transduction' (see Supplemental Material).

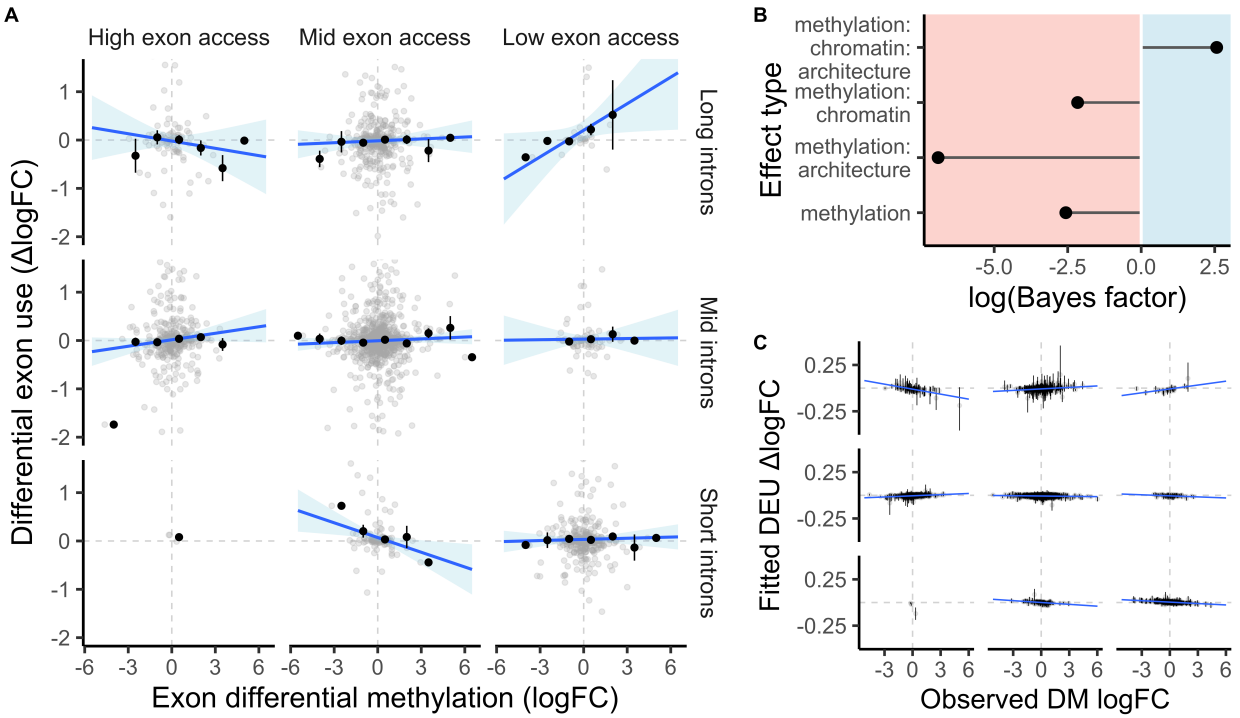

Figure 4 | Differential exon methylation affected splicing conditional upon exon accessibility and genic architecture. (a) Differential exon use under maternal upwelling is plotted against mean exon differential methylation. Rows and columns group data based on total genic intron length and exon accessibility quintiles. First and last rows/columns denote highest and lowest quintiles. Linear regressions are fitted across observed values and are absent in the bottom left panel due to a low representation of observations. Average ΔlogFC across binned exon differential methylation is plotted as black points ± SE. (b) Log-scale inclusion Bayes factors depicting the probability of observed data under a parameter, including the interaction plotted in panel A. (c) ΔlogFC values fitted by the selected model of differential exon use as a function of exon differential methylation. Error bars represent ± 95% credibility intervals. Columns, rows, and the x-axis depict binned exon accessibility, intron length, and observed exon differential methylation as presented in panel A. Points represent exons in panels A and C.

4. Discussion

We sought to characterize relationships between differential methylation and transcriptional plasticity in the purple urchin *Strongylocentrotus purpuratus*, a species for which DNA methylation appears to play a role in transgenerational plasticity (Strader et al., 2020; Strader et al., 2019; Wong et al., 2019). *S. purpuratus* induces both significant differential expression and differential exon use (e.g., alternative splicing) in response to developmental and maternal exposure to experimental upwelling. Furthermore, differential gene body methylation in

S. purpuratus larvae induced by maternal exposure to this ecologically relevant stressor exhibits significant and strong effects on DE and DEU among subsets of genes contingent upon chromatin accessibility and genic architecture (e.g., genomic feature type and genic feature length). Observed changes in expression and exon use under maternal conditioning were 4x and 13x more likely, respectively, when accounting for interactions between differential GBM, chromatin accessibility, and genic architecture. These results support the hypotheses that DM induced during TGP elicits multiple gene regulatory effects in *S. purpuratus* and, secondly, that these effects are conditional upon genomic and epigenomic features extrinsic of DNA methylation. Here we (i) discuss potential mechanisms to explain interactions between DNA methylation, chromatin accessibility, and genic architecture, (ii) interpret our results in the context of *S. purpuratus* and invertebrate physiological ecology, and (iii) highlight questions to be pursued in future studies of ecological epigenomics in metazoans.

4.1. Relationships between DNA methylation, chromatin accessibility, and gene expression in Strongylocentrotus purpuratus *and other invertebrates* – Baseline patterns of genomic methylation, chromatin accessibility, transcription, and the relationships between these processes in *S. purpuratus* were consistent with those typically observed in other invertebrate lineages (Bonasio et al., 2012; Dixon et al., 2018; Downey-Wall et al., 2020; Flores et al., 2012; Gao et al., 2012; Gatzmann et al., 2018; Glastad et al., 2016; Johnson et al., 2020; Kvist et al., 2018; Li et al., 2018; Li-Byarlay et al., 2013; Libbrecht et al., 2016; Song et al., 2017; Zemach et al., 2010). Our results also further illuminate the relationship between baseline intragenic methylation and gene expression in metazoans; we observed an antagonistic relationship between intron and exon methylation in *S. purpuratus* such that a saturation of methylation in one genic feature reduced the additive effect of methylation on expression by the other. Exon

methylation was significantly correlated with gene expression regardless of intron methylation, but the effect of intron methylation was conditional upon low exon methylation. Several competing and non-competing hypotheses have been put forward to explain positive correlations between GBM and gene expression. Firstly, GBM inhibition in metazoans can reduce gene expression, causally linking GBM and expression (Lindeman et al., 2019; Yang et al., 2014). Recent advances in invertebrate epigenomics suggest that intragenic DNA methylation is bound by methyl-DNA-binding domain protein 2/3, which recruits acetyltransferases to promote H3K27 acetylation and initiate transcriptional elongation (Xu et al., 2021). Non-competing hypotheses posit that GBM may correlate with gene expression because it supports sequence conservation and transcriptional homeostasis. For example, genes with intragenic hypermethylation (i) are less accessible on average in at least some invertebrates (Gatzmann et al., 2018), which can protect them from mutation (Shi et al., 2016), and (ii) evolve at a slower rate across invertebrate lineages (Hunt, Brisson, Yi, & Goodisman, 2010; Park et al., 2011; Sarda et al., 2012). Intragenic methylation can also ensure a reduction in spurious transcription at noncanonical TSS, promoting transcriptional homeostasis (Neri et al., 2017). The singular effect of exon methylation on baseline expression in *S. purpuratus,* and the absence of a singular effect attributed to introns, suggest a unique association between exon methylation and expression. Conversely, the antagonistic interaction between exon and intron methylation may be indicative of functional redundancy for a secondary association between GBM and gene expression. For example, it is possible that exon methylation is more strongly associated with reduced intragenic accessibility, preventing mutation in coding regions of conserved genes, while exon and intron methylation may contribute relatively equally in directing transcriptional elongation to canonical TSS by preventing intragenic accessibility.

Intron methylation was also positively correlated with the prevalence of transcript variants among *S. purpuratus* genes consistent with associations between GBM and alternative splicing in other metazoans. Intragenic hypermethylation recruits methyl CpG binding protein 2 to splice junctions, which promotes exon recognition and, in some cases, intron retention via alterations to the elongation rate of RNA pol II (Maunakea, Chepelev, Cui, & Zhao, 2013; Wong et al., 2017). Considering existing evidence that exon methylation is positively associated with exon inclusion, it is surprising that intron methylation was associated with splicing in *S. purpuratus* while exon methylation was not. Relationships between the inclusion of specific exons or introns and their baseline methylation may still exist in *S. purpuratus*. We chose to model genewise averages of median CpG methylation at exons and introns as predictors of splice variation rather than fitting models of exon- or intron-specific use. This decision was made because our short read RNA-seq approach allowed for analyses of DEU between treatment groups, but not baseline exon use independent of treatment; we could not determine whether a methylated exon or intron was more likely to be constitutively skipped or included in a transcript. A more resolute quantification of GBM's effect on alternative splicing in *S. purpuratus* or other invertebrates would be achieved by incorporating BS-seq with long read RNA-seq to enable isoform-specific read counting.

Patterns of chromatin accessibility and its relationship with gene expression in *S. purpuratus* were typical of other invertebrates and metazoans. Models of logCPM that included TSS or exon accessibility as predictors demonstrated significant, positive correlations with gene expression. However, variables related to chromatin accessibility were not included in the selected model of logCPM and yielded low Bayes factors for inclusion. Indeed, chromatin accessibility is necessary for the initiation of transcriptional elongation at canonical TSS

(Klemm, Shipony, & Greenleaf, 2019). No relationships were observed between chromatin accessibility at genic features and the presence of transcript variants. Alternatively spliced exons exhibit more accessibility and less nucleosome occupancy than constitutively retained exons (Naftelberg, Schor, Ast, & Kornblihtt, 2015), inconsistent with the lack of relationship between chromatin accessibility and the prevalence of transcript variants in *S. purpuratus*. Finally, our observation that CpG methylation precipitously declined proximal to open regions of chromatin is consistent with findings in other invertebrates, vertebrates, and plants (Gatzmann et al., 2018; Lhoumaud et al., 2019; Zhong et al., 2021) and is characteristic of methylation CpG binding protein's interactions with repressive chromatin factors (Klose & Bird, 2006).

4.2. Differential methylation's effect on expression depends on chromatin state and genic architecture – Our finding that DM induced by maternal environment affected DE and DEU conditional upon chromatin accessibility and genic architecture supports the hypotheses (i) that DM possesses gene regulatory roles in invertebrates during plastic responses to the environment and (ii) that these effects are contingent upon additional epigenomic and genomic states. This degree of complexity underlying differential GBM's functions juxtaposes the simple relationship between promoter DM and expression exhibited across vertebrates (Boyes & Bird, 1992). Such complexity is expected, however. In model species for which GBM has been frequently investigated, its potential effects are numerous, interrelated, and remain a point of active debate (Zilberman, 2017). Our results provide additional evidence that GBM has multiple non-mutually exclusive functions in metazoans, extending current knowledge in that GBM's multivariate effects may be shaped by a multifactorial epigenomic space. While a great deal remains to be uncovered about the gene regulatory roles of GBM in metazoans, several key results arose from

563 our study that may aid in understanding the physiological significance of epigenomic regulation
564 associated with responses to environmental stress and phenotypic plasticity.

565 The effect of GBM on DE was strongest among genes with high absolute intron DM and
566 low TSS accessibility, with transcript abundance influencing the direction of effect (Fig. 3A).
567 We also observed that introns were significantly more accessible than exons and promoters and
568 that intron methylation shared stronger correlations with the occurrence alternative splicing
569 events than exon methylation (Fig 1B). These feature-dependent effects on both baseline and
570 plastic patterns of gene regulation underscore the unique roles that exon and intron methylation
571 may possess. While our analyses indicate, with strong likelihood, that the regulatory role of
572 intron DM is dependent on TSS accessibility and expression level, the mechanisms by which
573 intron methylation contributes to gene regulation, and how those mechanisms differ from exon
574 DM, remain unresolved.

575 Previous studies have identified effects of intron DM on DE outside of invertebrates and
576 interactions between GBM and TSS accessibility in their effects on baseline expression, but there
577 is strong potential for such effects to phylogenetically vary. GBM is positively correlated with
578 TSS accessibility measured using a putative approach for several arthropods (Lewis et al., 2020),
579 negatively correlated with TSS accessibility as demonstrated by ATAC-seq in the crustacean
580 *Procrambus virginalis* (Gatzmann et al., 2018) and uncorrelated with TSS accessibility in *S.*
581 *purpuratus* (Fig. S4). With regard to intron methylation, CpG methylation of large introns can be
582 required for gene expression (Rigal, Kevei, Pelissier, & Mathieu, 2012). Conversely, methylation
583 of first introns can be negatively correlated with gene expression, suggesting a distinct role
584 relative to introns 2 – *n* in plants and vertebrates (Anastasiadi, Esteve-Codina, & Piferrer, 2018;
585 Rose, 2008; Tan, 2010). The mean intron length of *S. purpuratus* is 1.753 kb (Tu, Cameron,

586 Worley, Gibbs, & Davidson, 2012) and, as we have demonstrated, average % CpG methylation
587 is relatively even across introns and exons. Basal invertebrates such as placozoans and sponges
588 exhibit shorter intron lengths than higher order invertebrates such as deuterostomes (McCoy &
589 Fire, 2020). Furthermore, the evenness of CpG methylation between introns and exons of *S.*
590 *purpuratus* is distinct from most other invertebrates for which exon methylation is greater
591 (Downey-Wall et al., 2020; Lewis et al., 2020; Li et al., 2018) but comparable to other
592 echinoderms (Yang, Zheng, Sun, & Chen, 2020). Despite our support in *S. purpuratus* for the
593 hypothesis that DNA methylation's gene regulatory roles during plasticity are dependent
594 additional epigenomic and genomic states, it is important to underscore that the nature of intron
595 methylation's effect on DE and its interactions with TSS accessibility may vary across
596 invertebrate phyla.

597 DE induced by maternal stress was most positively affected by intron DM among lowly
598 expressed genes with low TSS accessibility that were enriched for molecular functions including
599 'nucleotidyltransferase' and 'cytoskeletal motor activity', molecular functions that were also
600 enriched among genes that were differentially methylated following maternal upwelling.
601 'Nucleotidyltransferase activity' was also enriched among genes that were downregulated in
602 response to maternal upwelling. (Strader et al., 2020). Therefore, it is possible that the DM and
603 DE of some gene families were attributed to interactions between methylation, chromatin
604 accessibility, and expression level.

605 Importantly, intron DM exhibited a negative correlation with DE among genes with low
606 TSS accessibility, but high expression. Bidirectional effects of differential GBM on DE could
607 help explain why past associations between differential GBM and expression have been
608 negligible in invertebrates. Multiple hypotheses may explain how the directionality of intron

609 DM's association with DE changed according to expression level. Introns possess both
610 enhancing and silencing effects on gene expression across eukaryota (Rose, 2008; Rose, 2018).
611 Intragenic enhancers are predominantly located in introns and their methylation can be
612 negatively correlated with expression in some eukaryotic lineages (Blattler et al., 2014).
613 Enhancer profiling in developing *S. purpuratus* has revealed correlations between enhancer
614 activity and gene expression, indicating that a proportion of highly expressed genes in early
615 prism-stage larvae may correspond to a greater number of activated enhancers (Khor, Guerrero-
616 Santoro, Douglas, & Ettensohn, 2021). Long introns are also enriched with conserved regulatory
617 elements such as transcriptional enhancers (Haddrill, Charlesworth, Halligan, & Andolfatto,
618 2005; Park et al., 2011), potentially explaining the silencing effect of intron DM we observed
619 among *S. purpuratus* genes with long intron lengths (Fig. S3). Future work should test whether
620 intron hypermethylation induces downregulation of transcripts enriched with intragenic
621 enhancers and whether positive associations between intron DM and DE are attributable to
622 MBD-binding at methylated gene bodies, promoting histone H3K27 acetylation and gene
623 expression (Xu et al., 2021).

624 *4.3. Effects of differential methylation on splicing are conditional upon chromatin and*
625 *genic architecture states* – Alternative splicing diversifies the proteome and is an essential and
626 conserved gene regulatory mechanism (Keren, Lev-Maor, & Ast, 2010). Changes in exon use
627 under stress can be attributable to alterations in both constitutive and alternative splicing events
628 (Biamonti & Caceres, 2009). In response to maternal upwelling, differential GBM in *S.*
629 *purpuratus* larvae interacted with chromatin state and genic architecture to potentially influence
630 alternative splicing and/or exon skipping. A three-way interaction between exon DM, exon
631 accessibility, and genic intron length affected DEU such that (i) the absolute effect of exon DM

was strongest among genes with greater total intron lengths and (ii) DEU of poorly accessible exons positively correlated with exon DM while the DEU of accessible exons negatively correlated with exon DM. To our knowledge, our results mark the first evidence in an invertebrate of a significant association between DM and exon inclusion responding to environmental variation.

The effect of total intron length on DEU aligns with observations that alternative splicing is more pervasive among long genes (Flores et al., 2012; Grishkevich & Yanai, 2014), whose size is largely attributable to intron length in *S. purpuratus* (Tu et al., 2012). Long genes involved in cellular structure and cell adhesion are targets of alternative splicing, producing a diversity of protein isoforms that can modify protein-protein complexes that construct or regulate the cytoskeleton, organelle organization, and the extracellular matrix across cell types, developmental stages, and physiological states as evidenced in multiple model systems (Belkin et al., 1997; Exposito, D'Alessio, & Ramirez, 1992; Kalsotra & Cooper, 2011; Leung, Zheng, Prater, & Liem, 2001; O'Leary, Lasda, & Bayer, 2006). Long genes with poorly accessible exons in *S. purpuratus* were enriched with 'protein-protein dimerization' MF GO terms and BP terms such as 'cellular component assembly' and 'organelle organization'. Similar to genes whose DE was most affected by DM, these genes were also enriched with signaling receptor and signal transduction functions. ~65% of exons with the strongest positive associations between DM and DEU (e.g., high genic intron length and low exon accessibility) came from genes involved in organelle organization, cytoskeletal structure, and the extracellular matrix.

The positive correlation between exon DM and DEU in genes with inaccessible exons was expected as hypermethylation at alternatively spliced exons has generally been associated with their inclusion in transcripts (Flores et al., 2012; Shayevitch, Askayo, Keydar, & Ast, 2018).

As stated however, the direction of exon methylation's effect on splicing was influenced by chromatin accessibility; accessible exons exhibited a negative correlation between DEU and exon DM. While our study cannot derive the exact mechanism by which exon DM and accessibility interacted to affect exon use, several lines of evidence demonstrate their joint influence on alternative splicing. For example, the methyl-binding protein MeCP2 links splicing, DNA methylation, and chromatin state. MeCP2 aids in the recognition of alternatively included, methylated exons resulting in a positive correlation between methylation and inclusion among MeCP2-regulated exons (Lev Maor, Yearim, & Ast, 2015). MeCP2 also interacts with nucleosomes, and its genomic positioning is associated with H3K27Me3, a histone mark related to chromatin inaccessibility (Yin et al., 2021). Thus, the negative correlation between exon DM and DEU among genes with inaccessible exons could result from MeCP2 regulation. Methylated exons that are regulated by the transcriptional repressor CTCF experience skipping rather than inclusion. CTCF induces exon inclusion, but its binding to exons is inhibited by DNA methylation, resulting in a negative correlation between methylation and inclusion (Lev Maor et al., 2015). CTCF binding motifs are associated with increased chromatin accessibility (Jain, Ba, Zhang, Dai, & Alt, 2018). Thus, genes with accessible exons for which a positive correlation exists between exon DM and DEU may be regulated by CTCF. CTCF is conserved and expressed in *S. purpuratus* (Gomez-Marin et al., 2015) and MeCP2 is present in the *S. purpuratus* genome assembly and transcriptome.

5. Conclusion

Variation in DNA methylation appears to be a component of molecular responses by many invertebrates to predicted global change including ecologically critical, threatened groups such as stony corals (Putnam et al., 2016) and pteropods (Bogan, Johnson, & Hofmann, 2020) or detrimental invasive species (Hawes et al., 2018). Given the heritability of DNA methylation in some invertebrate clades (Liew et al., 2020), exacting its transcriptional and phenotypic consequences is critical for understanding the mechanistic basis of TGP. Our findings (i) provide quantitative support for the hypothesis that gene regulation by differential GBM in *S. purpuratus* is affected by additional epigenomic and genomic states and (ii) indicate that these effects influence both gene expression and mRNA splicing. The majority of ecological epigenomic studies in metazoans have focused on the singular effects of DM on expression, likely due to the predictive power of promoter methylation in vertebrates (Boyes & Bird, 1992). However, DNA methylation is not a silver bullet to predict transcriptional changes by *S. purpuratus* in response to environmental variation. Rather, it is likely one cog in the epigenomic machinery contributing to plasticity in gene expression and alternative splicing. A shift toward integrated studies combining DNA methylation, chromatin accessibility, and genomic/genic architecture may be necessary to accurately quantify non-genetic sources of transcriptional and phenotypic variation in invertebrates and other eukaryotes.

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Author Contributions

GEH conceived the original design and scope of the experiment. SNB, MES, and GEH conceived hypotheses and analytical approaches. MES led experimental execution and prepared DNA and RNA for sequencing. SNB and MES performed bioinformatic analyses. SNB wrote the manuscript with contributions from MES and input from GEH. All authors have read and approved the final manuscript.

724 **Data Availability**

725 Raw RNA-seq and RRBS fastq files associated with this study are available through the
726 NCBI Short Read Archive under the accession PRJNA548926. Scripts associated with trimming,
727 mapping, and counting RNA-seq reads and CpG methylation are available in the following
728 GitHub repository: https://github.com/mariestrader/S.purp_RRBS_RNAseq_2019. Code
729 corresponding to all analyses reported in this study, their intermediate files, and outputs can be
730 found in the following GitHub repository: https://github.com/snbogan/Sp_RRBS_ATAC.

731

732 **Competing Interests**

733 The authors declare that they have no competing interests.

734

735 **Supplemental Material**

736 Supplemental Results – PDF of supplemental figures and tables

737 Datasheet 1 – Test statistics for differential expression and exon use

738 Datasheet 2 – Test statistics for differential methylation across CpGs and genomic features

739 Datasheet 3 – Parameters of exon use ~ exon number regressions evaluating spurious
740 transcription and alternative TSS

741 Datasheet 4 – Enriched GO terms according to Mann Whitney U or Fisher's exact tests

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References

Anastasiadi, D., Esteve-Codina, A., & Piferrer, F. (2018). Consistent inverse correlation between DNA methylation of the first intron and gene expression across tissues and species. *Epigenetics & Chromatin, 11*(1), 37. doi:10.1186/s13072-018-0205-1

Ardura, A., Zaiko, A., Moran, P., Planes, S., & Garcia-Vazquez, E. (2017). Epigenetic signatures of invasive status in populations of marine invertebrates. *Scientific Reports, 7*, 42193. doi:10.1038/srep42193

Arsenault, S. V., Hunt, B. G., & Rehan, S. M. (2018). The effect of maternal care on gene expression and DNA methylation in a subsocial bee. *Nature Communications, 9*(1), 3468. doi:10.1038/s41467-018-05903-0

Belkin, A. M., Retta, S. F., Pletjushkina, O. Y., Balzac, F., Silengo, L., Fassler, R., . . . Tarone, G. (1997). Muscle β1D integrin reinforces the cytoskeleton–matrix link: modulation of integrin adhesive function by alternative splicing. *The Journal of Cell Biology, 139*, 1583-1595.

Biamonti, G., & Caceres, J. F. (2009). Cellular stress and RNA splicing. *Trends in Biochemical Sciences, 34*(3), 146-153. doi:10.1016/j.tibs.2008.11.004

Blattler, A., Yao, L., Witt, H., Guo, Y., Nicolet, C. M., Berman, B. P., & Farnham, P. J. (2014). Global loss of DNA methylation uncovers intronic enhancers in genes showing expression changes. *Genome Biology, 15*, 469.

Bogan, S. N., Johnson, K. M., & Hofmann, G. E. (2020). Changes in genome-wide methylation and gene expression in response to future *p*CO2 extremes in the Antarctic pteropod *Limacina helicina antarctica*. *Frontiers in Marine Science, 6*. doi:10.3389/fmars.2019.00788

770 Bonasio, R., Li, Q., Lian, J., Mutti, N. S., Jin, L., Zhao, H., . . . Reinberg, D. (2012). Genome-
771 wide and caste-specific DNA methylomes of the ants *Camponotus floridanus* and
772 *Harpegnathos saltator*. *Current Biology, 22*(19), 1755-1764.
773 doi:10.1016/j.cub.2012.07.042
774 Boyes, J., & Bird, A. (1992). Repression of genes by DNA methylation depends on CpG density
775 and promoter strength: evidence for involvement of a methyl-CpG binding protein. *The*
776 *EMBO Journal, 11*, 327-333.
777 Bürkner, P.-C. (2017). brms: an R package for Bayesian multilevel models using Stan. *Journal*
778 *of Statistical Software, 80*(1). doi:10.18637/jss.v080.i01
779 Chen, Y., Pal, B., Visvader, J. E., & Smyth, G. K. (2017). Differential methylation analysis of
780 reduced representation bisulfite sequencing experiments using edgeR. *F1000Research, 6*,
781 2055. doi:10.12688/f1000research.13196.2
782 Clark, M. S., Suckling, C. C., Cavallo, A., Mackenzie, C. L., Thorne, M. A. S., Davies, A. J., &
783 Peck, L. S. (2019). Molecular mechanisms underpinning transgenerational plasticity in
784 the green sea urchin *Psammechinus miliaris*. *Scientific Reports, 9*(1), 952.
785 doi:10.1038/s41598-018-37255-6
786 Clark, M. S., Thorne, M. A. S., King, M., Hipperson, H., Hoffman, J. I., Peck, L. S., & Pfrender,
787 M. (2018). Life in the intertidal: cellular responses, methylation and epigenetics.
788 *Functional Ecology, 32*(8), 1982-1994. doi:10.1111/1365-2435.13077
789 de Mendoza, A., Hatleberg, W. L., Pang, K., Leininger, S., Bogdanovic, O., Pflueger, J., . . .
790 Lister, R. (2019). Convergent evolution of a vertebrate-like methylome in a marine
791 sponge. *Nature Ecology & Evolution, 3*(10), 1464-1473. doi:10.1038/s41559-019-0983-2

792 Dimond, J. L., & Roberts, S. B. (2020). Convergence of DNA methylation profiles of the reef
793 coral *Porites astreoides* in a novel environment. *Frontiers in Marine Science, 6*.
794 doi:10.3389/fmars.2019.00792
795 Ding, J., Zhang, L., Sun, J., Shi, D., Chi, X., Yang, M., . . . Zhao, C. (2019). Transgenerational
796 effects of UV-B radiation on egg size, fertilization, hatching and larval size of sea urchins
797 *Strongylocentrotus intermedius*. *PeerJ, 7*, e7598. doi:10.7717/peerj.7598
798 Dixon, G., Liao, Y., Bay, L. K., & Matz, M. V. (2018). Role of gene body methylation in
799 acclimatization and adaptation in a basal metazoan. *Proceedings of the National Academy*
800 *of Sciences U.S.A., 115*(52), 13342-13346. doi:10.1073/pnas.1813749115
801 Donelan, S. C., Hellmann, J. K., Bell, A. M., Luttbeg, B., Orrock, J. L., Sheriff, M. J., & Sih, A.
802 (2020). Transgenerational plasticity in human-altered environments. *Trends in Ecology &*
803 *Evolution, 35*(2), 115-124. doi:10.1016/j.tree.2019.09.003
804 Downey-Wall, A. M., Cameron, L. P., Ford, B. M., McNally, E. M., Venkataraman, Y. R.,
805 Roberts, S. B., . . . Lotterhos, K. E. (2020). Ocean acidification induces subtle shifts in
806 gene expression and DNA methylation in mantle tissue of the Eastern oyster (*Crassostrea*
807 *virginica*). *Frontiers in Marine Science, 7*, 566419. doi:10.3389/fmars.2020.566419
808 Eirin-Lopez, J. M., & Putnam, H. M. (2019). Marine environmental epigenetics. *Annual Review*
809 *of Marine Science, 11*, 335-368. doi:10.1146/annurev-marine-010318-095114
810 Evans, T. G., Chan, F., Menge, B. A., & Hofmann, G. E. (2013). Transcriptomic responses to
811 ocean acidification in larval sea urchins from a naturally variable pH environment.
812 *Molecular Ecology, 22*(6), 1609-1625. doi:10.1111/mec.12188
813 Evans, T. G., Pespeni, M. H., Hofmann, G. E., Palumbi, S. R., & Sanford, E. (2017).
814 Transcriptomic responses to seawater acidification among sea urchin populations

inhabiting a natural pH mosaic. *Molecular Ecology, 26*(8), 2257-2275. doi:10.1111/mec.14038

Exposito, J. Y., D'Alessio, M., & Ramirez, F. (1992). Novel amino-terminal propeptide configuration in a fibrillar procollagen undergoing alternative splicing. *Journal of Biological Chemistry, 267*(24), 17404-17408. doi:10.1016/s0021-9258(18)41940-0

Fangue, N. A., O'Donnell, M. J., Sewell, M. A., Matson, P. G., MacPherson, A. C., & Hofmann, G. E. (2010). A laboratory-based, experimental system for the study of ocean acidification effects on marine invertebrate larvae. *Limnology and Oceanography: Methods, 8*(8), 441-452. doi:10.4319/lom.2010.8.441

Flores, K., Wolschin, F., Corneveaux, J. J., Allen, A. N., Huentelman, M. J., & Amdam, G. V. (2012). Genome-wide association between DNA methylation and alternative spicing in an invertebrate. *BMC Genomics, 13*, 480. doi:10.1186/1471-2164-13-480

Gao, F., Liu, X., Wu, X.-P., Wang, X.-L., Gong, D., Lu, H., . . . Liu, M. (2012). Differential DNA methylation in discrete developmental stages of the parasitic nematode *Trichinella piralis*. *Genome Biology, 13*, R100. doi:10.5524/100043

Gatzmann, F., Falckenhayn, C., Gutekunst, J., Hanna, K., Raddatz, G., Carneiro, V. C., & Lyko, F. (2018). The methylome of the marbled crayfish links gene body methylation to stable expression of poorly accessible genes. *Epigenetics & Chromatin, 11*(1), 57. doi:10.1186/s13072-018-0229-6

Glastad, K. M., Gokhale, K., Liebig, J., & Goodisman, M. A. (2016). The caste- and sex-specific DNA methylome of the termite *Zootermopsis nevadensis*. *Scientific Reports, 6*, 37110. doi:10.1038/srep37110

837 Gomez-Marin, C., Tena, J. J., Acemel, R. D., Lopez-Mayorga, M., Naranjo, S., de la Calle-
838 Mustienes, E., . . . Gomez-Skarmeta, J. L. (2015). Evolutionary comparison reveals that
839 diverging CTCF sites are signatures of ancestral topological associating domains borders.
840 *Proceedings of the National Academy of Sciences U.S.A, 112*(24), 7542-7547.
841 doi:10.1073/pnas.1505463112
842 Goodrich, B., Gabry, J., Ali, I., & Brilleman, S. (2020). rstanarm: Bayesian applied regression
843 modeling via Stan (Version R package version 2.21.1). Retrieved from https://mc-
844 stan.org/rstanarm
845 Grishkevich, V., & Yanai, I. (2014). Gene length and expression level shape genomic novelties.
846 *Genome Research, 24*(9), 1497-1503. doi:10.1101/gr.169722.113
847 Gronau, Q. F., & Wagenmakers, E. J. (2019). Limitations of Bayesian leave-one-out cross-
848 validation for model selection. *Computational Brain & Behavior, 2*(1), 1-11.
849 doi:10.1007/s42113-018-0011-7
850 Haddrill, P. R., Charlesworth, B., Halligan, D. L., & Andolfatto, P. (2005). Patterns of intron
851 sequence evolution in *Drosophila* are dependent upon length and GC content. *Genome*
852 *Biology, 6*(8), R67. doi:10.1186/gb-2005-6-8-r67
853 Hawes, N. A., Tremblay, L. A., Pochon, X., Dunphy, B., Fidler, A. E., & Smith, K. F. (2018).
854 Effects of temperature and salinity stress on DNA methylation in a highly invasive
855 marine invertebrate, the colonial ascidian *Didemnum vexillum*. *PeerJ, 6*, e5003.
856 doi:10.7717/peerj.5003
857 Herman, J. J., & Sultan, S. E. (2011). Adaptive transgenerational plasticity in plants: case
858 studies, mechanisms, and implications for natural populations. *Frontiers in Plant Science,*
859 *2*, 102. doi:10.3389/fpls.2011.00102

860 Hinne, M., Gronau, Q. F., van den Bergh, D., & Wagenmakers, E. J. (2020). A conceptual
861 introduction to Bayesian model averaging. *Advances in Methods and Practices in*
862 *Psychological Sciences, 3*(2), 200-215.
863 Hofmann, G. E. (2017). Ecological epigenetics in marine metazoans. *Frontiers in Marine*
864 *Science, 4*. doi:10.3389/fmars.2017.00004
865 Hoshijima, U., & Hofmann, G. E. (2019). Variability of Seawater Chemistry in a Kelp Forest
866 Environment Is Linked to in situ Transgenerational Effects in the Purple Sea Urchin,
867 Strongylocentrotus purpuratus. *Frontiers in Marine Science, 6*.
868 doi:10.3389/fmars.2019.00062
869 Hunt, B. G., Brisson, J. A., Yi, S. V., & Goodisman, M. A. (2010). Functional conservation of
870 DNA methylation in the pea aphid and the honeybee. *Genome Biology and Evolution, 2*,
871 719-728. doi:10.1093/gbe/evq057
872 Jain, S., Ba, Z., Zhang, Y., Dai, H. Q., & Alt, F. W. (2018). CTCF-binding elements mediate
873 accessibility of RAG substrates during chromatin scanning. *Cell, 174*(1), 102-116 e114.
874 doi:10.1016/j.cell.2018.04.035
875 Johnson, K. M., & Kelly, M. W. (2020). Population epigenetic divergence exceeds genetic
876 divergence in the Eastern oyster *Crassostrea virginica* in the Northern Gulf of Mexico.
877 *Evolutionary Applications, 13*(5), 945-959. doi:10.1111/eva.12912
878 Johnson, K. M., Sirovy, K. A., Casas, S. M., La Peyre, J. F., & Kelly, M. W. (2020).
879 Characterizing the epigenetic and transcriptomic responses to *Perkinsus marinus*
880 infection in the eastern oyster *Crassostrea virginica*. *Frontiers in Marine Science, 6*, 598.
881 doi:10.3389/fmars.2020.00598

882 Jones, B. M., & Robinson, G. E. (2018). Genetic accommodation and the role of ancestral
883 plasticity in the evolution of insect eusociality. *Journal of Experimental Biology, 221*(Pt
884 23). doi:10.1242/jeb.153163
885 Kalsotra, A., & Cooper, T. A. (2011). Functional consequences of developmentally regulated
886 alternative splicing. *Nature Reviews Genetics, 12*(10), 715-729. doi:10.1038/nrg3052
887 Karelitz, S., Lamare, M., Patel, F., Gemmell, N., & Uthicke, S. (2019). Parental acclimation to
888 future ocean conditions increases development rates but decreases survival in sea urchin
889 larvae. *Marine Biology, 167*(1). doi:10.1007/s00227-019-3610-5
890 Keller, T. E., Han, P., & Yi, S. V. (2016). Evolutionary transition of promoter and gene body
891 DNA methylation across invertebrate-vertebrate boundary. *Molecular Biology and*
892 *Evolution, 33*(4), 1019-1028. doi:10.1093/molbev/msv345
893 Kelly, M. W., Padilla-Gamino, J. L., & Hofmann, G. E. (2013). Natural variation and the
894 capacity to adapt to ocean acidification in the keystone sea urchin *Strongylocentrotus*
895 *purpuratus*. *Global Change Biology, 19*(8), 2536-2546. doi:10.1111/gcb.12251
896 Keren, H., Lev-Maor, G., & Ast, G. (2010). Alternative splicing and evolution: Diversification,
897 exon definition and function. *Nature Reviews Genetics, 11*(5), 345-355.
898 doi:10.1038/nrg2776
899 Khor, J. M., Guerrero-Santoro, J., Douglas, W., & Ettensohn, C. A. (2021). Global patterns of
900 enhancer activity during sea urchin embryogenesis assessed by eRNA profiling. *Genome*
901 *Research*. doi:10.1101/gr.275684.121
902 Kim, D., Langmead, B., & Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low
903 memory requirements. *Nature Methods, 12*(4), 357-360. doi:10.1038/nmeth.3317

Klemm, S. L., Shipony, Z., & Greenleaf, W. J. (2019). Chromatin accessibility and the regulatory epigenome. *Nature Reviews Genetics, 20*(4), 207-220. doi:10.1038/s41576-018-0089-8

Klose, R. J., & Bird, A. P. (2006). Genomic DNA methylation: the mark and its mediators. *Trends in Biochemical Sciences, 31*(2), 89-97. doi:10.1016/j.tibs.2005.12.008

Krueger, F., & Andrews, S. R. (2011). Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics, 27*(11), 1571-1572. doi:10.1093/bioinformatics/btr167

Kudtarkar, P., & Cameron, R. A. (2017). Echinobase: an expanding resource for echinoderm genomic information. *Database (Oxford), 2017*. doi:10.1093/database/bax074

Kvist, J., Goncalves Athanasio, C., Shams Solari, O., Brown, J. B., Colbourne, J. K., Pfrender, M. E., & Mirbahai, L. (2018). Pattern of DNA methylation in *Daphnia*: evolutionary perspective. *Genome Biology and Evolution, 10*(8), 1988-2007. doi:10.1093/gbe/evy155

Leung, C. L., Zheng, M., Prater, S. M., & Liem, R. K. (2001). The BPAG1 locus: Alternative splicing produces multiple isoforms with distinct cytoskeletal linker domains, including predominant isoforms in neurons and muscles. *The Journal of Cell Biology, 154*(4), 691-697. doi:10.1083/jcb.200012098

Lev Maor, G., Yearim, A., & Ast, G. (2015). The alternative role of DNA methylation in splicing regulation. *Trends in Genetics, 31*(5), 274-280. doi:10.1016/j.tig.2015.03.002

Lewis, S. H., Ross, L., Bain, S. A., Pahita, E., Smith, S. A., Cordaux, R., . . . Sarkies, P. (2020). Widespread conservation and lineage-specific diversification of genome-wide DNA methylation patterns across arthropods. *PLoS Genetics, 16*(6), e1008864. doi:10.1371/journal.pgen.1008864

927 Lhoumaud, P., Sethia, G., Izzo, F., Sakellaropoulos, T., Snetkova, V., Vidal, S., . . . Skok, J.
928 (2019). EpiMethylTag: simultaneous detection of ATAC-seq or ChIP-seq signals with
929 DNA methylation. *Genome Biology, 20*(1), 248. doi:10.1186/s13059-019-1853-6
930 Li, Y., Liew, Y. J., Cui, G., Cziesielski, M. J., Zahran, N., Michell, C. T., . . . Aranda, M. (2018).
931 DNA methylation regulates transcriptional homeostasis of algal endosymbiosis in the
932 coral model *Aiptasia*. *Science Advances, 4*, eaat2142. doi:10.1126/sciadv.aat2142
933 Li-Byarlay, H., Li, Y., Stroud, H., Feng, S., Newman, T. C., Kaneda, M., . . . Robinson, G. E.
934 (2013). RNA interference knockdown of DNA methyl-transferase 3 affects gene
935 alternative splicing in the honey bee. *Proceedings of the National Academy of Sciences*
936 *U.S.A, 110*(31), 12750-12755. doi:10.1073/pnas.1310735110
937 Libbrecht, R., Oxley, P. R., Keller, L., & Kronauer, D. J. (2016). Robust DNA methylation in the
938 clonal raider ant brain. *Current Biology, 26*(3), 391-395. doi:10.1016/j.cub.2015.12.040
939 Liew, Y. J., Howells, E. J., Wang, X., Michell, C. T., Burt, J. A., Idaghdour, Y., & Aranda, M.
940 (2020). Intergenerational epigenetic inheritance in reef-building corals. *Nature Climate*
941 *Change, 10*(3), 254-259. doi:10.1038/s41558-019-0687-2
942 Lindeman, L. C., Thaulow, J., Song, Y., Kamstra, J. H., Xie, L., Asselman, J., . . . Tollefsen, K.
943 E. (2019). Epigenetic, transcriptional and phenotypic responses in two generations of
944 *Daphnia magna* exposed to the DNA methylation inhibitor 5-azacytidine. *Environmental*
945 *Epigenetics, 5*(3), dvz016. doi:10.1093/eep/dvz016
946 Makowski, D., Ben-Shachar, M., & Lüdecke, D. (2019). bayestestR: describing effects and their
947 uncertainty, existence and significance within the Bayesian framework. *Journal of Open*
948 *Source Software, 4*(40). doi:10.21105/joss.01541

949 Makowski, D., Ben-Shachar, M. S., Chen, S. H. A., & Ludecke, D. (2019). Indices of effect
950 existence and significance in the Bayesian framework. *Frontiers in Psychology, 10*, 2767.
951 doi:10.3389/fpsyg.2019.02767
952 Marshall, D. J., & Uller, T. (2007). When is a maternal effect adaptive? *Oikos, 116*(12), 1957-
953 1963. doi:10.1111/j.2007.0030-1299.16203.x
954 Maunakea, A. K., Chepelev, I., Cui, K., & Zhao, K. (2013). Intragenic DNA methylation
955 modulates alternative splicing by recruiting MeCP2 to promote exon recognition. *Cell*
956 *Research, 23*(11), 1256-1269. doi:10.1038/cr.2013.110
957 McCarthy, D. J., Chen, Y., & Smyth, G. K. (2012). Differential expression analysis of
958 multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids*
959 *Research, 40*(10), 4288-4297. doi:10.1093/nar/gks042
960 McCoy, M. J., & Fire, A. Z. (2020). Intron and gene size expansion during nervous system
961 evolution. *BMC Genomics, 21*(1), 360. doi:10.1186/s12864-020-6760-4
962 Moler, E. R. V., Abakir, A., Eleftheriou, M., Johnson, J. S., Krutovsky, K. V., Lewis, L. C., . . .
963 Rajora, O. P. (2018). Population epigenomics: advancing understanding of phenotypic
964 plasticity, acclimation, adaptation and diseases. In O. P. Rajora (Ed.), *Population*
965 *Genomics*: Springer.
966 Naftelberg, S., Schor, I. E., Ast, G., & Kornblihtt, A. R. (2015). Regulation of alternative
967 splicing through coupling with transcription and chromatin structure. *Annual Reviews of*
968 *Biochemistry, 84*, 165-198. doi:10.1146/annurev-biochem-060614-034242
969 Nanty, L., Carbajosa, G., Heap, G. A., Ratnieks, F., van Heel, D. A., Down, T. A., & Rakyan, V.
970 K. (2011). Comparative methylomics reveals gene-body H3K36me3 in *Drosophila*

predicts DNA methylation and CpG landscapes in other invertebrates. *Genome Research, 21*(11), 1841-1850. doi:10.1101/gr.121640.111

Neri, F., Rapelli, S., Krepelova, A., Incarnato, D., Parlato, C., Basile, G., . . . Oliviero, S. (2017). Intragenic DNA methylation prevents spurious transcription initiation. *Nature, 543*(7643), 72-77. doi:10.1038/nature21373

Ni, P., Li, S., Lin, Y., Xiong, W., Huang, X., & Zhan, A. (2018). Methylation divergence of invasive *Ciona ascidians*: significant population structure and local environmental influence. *Ecology and Evolution, 8*(20), 10272-10287. doi:10.1002/ece3.4504

Norouzitallab, P., Baruah, K., Vandegehuchte, M., Van Stappen, G., Catania, F., Vanden Bussche, J., . . . Bossier, P. (2014). Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic *Artemia* model. *The FASEB Journal, 28*(8), 3552-3563. doi:10.1096/fj.14-252049

O'Leary, H., Lasda, E., & Bayer, K. U. (2006). CaMKII*β* association with the actin cytoskeleton is regulated by alternative splicing. *Molecular Biology of the Cell, 17*, 4656-4665. doi:10.1091/mbc.E06

Park, J., Peng, Z., Zeng, J., Elango, N., Park, T., Wheeler, D., . . . Yi, S. V. (2011). Comparative analyses of DNA methylation and sequence evolution using *Nasonia* genomes. *Molecular Biology and Evolution, 28*(12), 3345-3354. doi:10.1093/molbev/msr168

Pespeni, M. H., Chan, F., Menge, B. A., & Palumbi, S. R. (2013). Signs of adaptation to local pH conditions across an environmental mosaic in the California Current Ecosystem. *Integrative and Comparative Biology, 53*(5), 857-870. doi:10.1093/icb/ict094

992 Pespeni, M. H., & Palumbi, S. R. (2013). Signals of selection in outlier loci in a widely
993 dispersing species across an environmental mosaic. *Molecular Ecology, 22*(13), 3580-
994 3597. doi:10.1111/mec.12337
995 Putnam, H. M., Davidson, J. M., & Gates, R. D. (2016). Ocean acidification influences host
996 DNA methylation and phenotypic plasticity in environmentally susceptible corals.
997 *Evolutionary Applications, 9*(9), 1165-1178. doi:10.1111/eva.12408
998 Regev, A., Lamb, M. J., & Jablonka, E. (1998). The role of DNA methylation in invertebrates:
999 developmental regulation or genome defense? *Molecular Biology and Evolution, 15*(7),
1000 880-891. doi:10.1093/oxfordjournals.molbev.a025992
1001 Rigal, M., Kevei, Z., Pelissier, T., & Mathieu, O. (2012). DNA methylation in an intron of the
1002 IBM1 histone demethylase gene stabilizes chromatin modification patterns. *The EMBO*
1003 *Journal, 31*(13), 2981-2993. doi:10.1038/emboj.2012.141
1004 Riviere, G., He, Y., Tecchio, S., Crowell, E., Gras, M., Sourdaine, P., . . . Favrel, P. (2017).
1005 Dynamics of DNA methylomes underlie oyster development. *PLoS Genetics, 13*(6),
1006 e1006807. doi:10.1371/journal.pgen.1006807
1007 Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package for
1008 differential expression analysis of digital gene expression data. *Bioinformatics, 26*(1),
1009 139-140. doi:10.1093/bioinformatics/btp616
1010 Rodríguez-Casariego, J. A., Mercado-Molina, A. E., Garcia-Souto, D., Ortiz-Rivera, I. M.,
1011 Lopes, C., Baums, I. B., . . . Eirin-Lopez, J. M. (2020). Genome-wide DNA methylation
1012 analysis reveals a conserved epigenetic response to seasonal environmental variation in
1013 the staghorn coral *Acropora cervicornis*. *Frontiers in Marine Science, 7*.
1014 doi:10.3389/fmars.2020.560424

1015 Rose, A. B. (2008). Intron-mediated regulation of gene expression. In A. S. N. Reddy & M.
1016 Golovkin (Eds.), *Nuclear pre-mRNA Processing in Plants. Current Topics in*
1017 *Microbiology and Immunology*. Berlin Heidelberg: Springer.
1018 Rose, A. B. (2018). Introns as gene regulators: a brick on the accelerator. *Frontiers in Genetics,*
1019 *9*, 672. doi:10.3389/fgene.2018.00672
1020 Sarda, S., Zeng, J., Hunt, B. G., & Yi, S. V. (2012). The evolution of invertebrate gene body
1021 methylation. *Molecular Biology and Evolution, 29*(8), 1907-1916.
1022 doi:10.1093/molbev/mss062
1023 Shayevitch, R., Askayo, D., Keydar, I., & Ast, G. (2018). The importance of DNA methylation
1024 of exons on alternative splicing. *RNA, 24*, 1351-1362. doi:10.1261/rna
1025 Shi, Y., Su, X. B., He, K. Y., Wu, B. H., Zhang, B. Y., & Han, Z. G. (2016). Chromatin
1026 accessibility contributes to simultaneous mutations of cancer genes. *Scientific Reports, 6*,
1027 35270. doi:10.1038/srep35270
1028 Song, K., Li, L., & Zhang, G. (2017). The association between DNA methylation and exon
1029 expression in the Pacific oyster *Crassostrea gigas*. *PLoS One, 12*(9), e0185224.
1030 doi:10.1371/journal.pone.0185224
1031 Strader, M. E., Kozal, L. C., Leach, T. S., Wong, J. M., Chamorro, J. D., Housh, M. J., &
1032 Hofmann, G. E. (2020). Examining the role of DNA methylation in transcriptomic
1033 plasticity of early stage sea urchins: developmental and maternal effects in a kelp forest
1034 herbivore. *Frontiers in Marine Science, 7*. doi:10.3389/fmars.2020.00205
1035 Strader, M. E., Wong, J. M., Kozal, L. C., Leach, T. S., & Hofmann, G. E. (2019). Parental
1036 environments alter DNA methylation in offspring of the purple sea urchin,

Strongylocentrotus purpuratus. *Journal of Experimental Marine Biology and Ecology, 517*, 54-64. doi:10.1016/j.jembe.2019.03.002

Sultan, M., Amstislavskiy, V., Risch, T., Schuette, M., Dökel, S., Ralser, M., . . . Yaspo, M. (2014). Influence of RNA extraction methods and library selection schemes on RNA-seq data. *BMC Genomics, 15*, 675.

Suzuki, M. M., Kerr, A. R., De Sousa, D., & Bird, A. (2007). CpG methylation is targeted to transcription units in an invertebrate genome. *Genome Research, 17*(5), 625-631. doi:10.1101/gr.6163007

Tan, M. (2010). Analysis of DNA methylation of maize in response to osmotic and salt stress based on methylation-sensitive amplified polymorphism. *Plant Physiology and Biochemistry, 48*, 21-26. doi:10.1016/j.plaphy.20

Trigg, S. A., Venkataraman, Y. R., Gavery, M. R., Roberts, S. B., Bhattacharya, D., Downey-Wall, A., . . . Putnam, H. M. (2021). Invetebrate methylomes provide insight into mechanisms of environmental tolerance and reveal methodological biases. *biorXiv*. doi:10.1101/2021.03.29.437539

Tu, Q., Cameron, R. A., Worley, K. C., Gibbs, R. A., & Davidson, E. H. (2012). Gene structure in the sea urchin *Strongylocentrotus purpuratus* based on transcriptome analysis. *Genome Research, 22*(10), 2079-2087. doi:10.1101/gr.139170.112

Vandegehuchte, M. B., Lemiere, F., Vanhaecke, L., Vanden Berghe, W., & Janssen, C. R. (2010). Direct and transgenerational impact on *Daphnia magna* of chemicals with a known effect on DNA methylation. *Comparative Biochemistry and Physiolgy C: Toxicology & Pharmacology, 151*(3), 278-285. doi:10.1016/j.cbpc.2009.11.007

1059 Wang, X., Li, A., Wang, W., Que, H., Zhang, G., & Li, L. (2021). DNA methylation mediates
1060 differentiation in thermal responses of Pacific oyster (*Crassostrea gigas*) derived from
1061 different tidal levels. *Heredity (Edinb.), 126*(1), 10-22. doi:10.1038/s41437-020-0351-7
1062 Wang, X., Song, W., Ji, G., Song, Y., Liu, X., Luo, X., . . . Sun, S. (2021). Regulation of DNA
1063 methylation on key parasitism genes of Cysticercus cellulosae revealed by integrative
1064 epigenomic-transcriptomic analyses. *Hereditas, 158*(1), 28. doi:10.1186/s41065-021-
1065 00195-9
1066 Wang, X., Werren, J. H., & Clark, A. G. (2016). Allele-specific transcriptome and methylome
1067 analysis reveals stable inheritance and *cis*-regulation of DNA methylation in *Nasonia*.
1068 *PLoS Biology, 14*(7), e1002500. doi:10.1371/journal.pbio.1002500
1069 Watson, R. G. A., Baldanzi, S., Pérez-Figueroa, A., Gouws, G., & Porri, F. (2018).
1070 Morphological and epigenetic variation in mussels from contrasting environments.
1071 *Marine Biology, 165*(3). doi:10.1007/s00227-018-3310-6
1072 Wong, J. J., Gao, D., Nguyen, T. V., Kwok, C. T., van Geldermalsen, M., Middleton, R., . . .
1073 Rasko, J. E. J. (2017). Intron retention is regulated by altered MeCP2-mediated splicing
1074 factor recruitment. *Nature Communications, 8*, 15134. doi:10.1038/ncomms15134
1075 Wong, J. M., & Hofmann, G. E. (2020). The effects of temperature and *p*CO2 on the size,
1076 thermal tolerance and metabolic rate of the red sea urchin (*Mesocentrotus franciscanus*)
1077 during early development. *Marine Biology, 167*(3). doi:10.1007/s00227-019-3633-y
1078 Wong, J. M., & Hofmann, G. E. (2021). Gene expression patterns of red sea urchins
1079 (*Mesocentrotus franciscanus*) exposed to different combinations of temperature and
1080 *p*CO2 during early development. *BMC Genomics, 22*(1), 32. doi:10.1186/s12864-020-
1081 07327-x

1082 Wong, J. M., Johnson, K. M., Kelly, M. W., & Hofmann, G. E. (2018). Transcriptomics reveal
1083 transgenerational effects in purple sea urchin embryos: adult acclimation to upwelling
1084 conditions alters the response of their progeny to differential *p*CO2 levels. *Molecular*
1085 *Ecology, 27*(5), 1120-1137. doi:10.1111/mec.14503
1086 Wong, J. M., Kozal, L. C., Leach, T. S., Hoshijima, U., & Hofmann, G. E. (2019).
1087 Transgenerational effects in an ecological context: conditioning of adult sea urchins to
1088 upwelling conditions alters maternal provisioning and progeny phenotype. *Journal of*
1089 *Experimental Marine Biology and Ecology, 517*, 65-77. doi:10.1016/j.jembe.2019.04.006
1090 Wright, R. M., Aglyamova, G. V., Meyer, E., & Matz, M. V. (2015). Gene expression associated
1091 with white syndromes in a reef building coral, *Acropora hyacinthus*. *BMC Genomics, 16*,
1092 371. doi:10.1186/s12864-015-1540-2
1093 Xu, G., Lyu, H., Yi, Y., Peng, Y., Feng, Q., Song, Q., . . . Zheng, S. (2021). Intragenic DNA
1094 methylation regulates insect gene expression and reproduction through the MBD/Tip60
1095 complex. *iScience, 24*(2), 102040. doi:10.1016/j.isci.2021.102040
1096 Xu, J., Chen, G., Hermanson, P. J., Xu, Q., Sun, C., Chen, W., . . . Li, Q. (2019). Population-
1097 level analysis reveals the widespread occurrence and phenotypic consequence of DNA
1098 methylation variation not tagged by genetic variation in maize. *Genome Biology, 20*(1),
1099 243. doi:10.1186/s13059-019-1859-0
1100 Yang, X., Han, H., De Carvalho, D. D., Lay, F. D., Jones, P. A., & Liang, G. (2014). Gene body
1101 methylation can alter gene expression and is a therapeutic target in cancer. *Cancer Cell,*
1102 *26*(4), 577-590. doi:10.1016/j.ccr.2014.07.028

1103 Yang, Y., Zheng, Y., Sun, L., & Chen, M. (2020). Genome-wide DNA methylation signatures of
1104 sea cucumber *Apostichopus japonicus* during environmental induced aestivation. *Genes*
1105 *(Basel), 11*(9). doi:10.3390/genes11091020
1106 Yin, X., Romero-Campero, F. J., de Los Reyes, P., Yan, P., Yang, J., Tian, G., . . . Zhou, Y.
1107 (2021). H2AK121ub in *Arabidopsis* associates with a less accessible chromatin state at
1108 transcriptional regulation hotspots. *Nature Communications, 12*(1), 315.
1109 doi:10.1038/s41467-020-20614-1
1110 Zemach, A., McDaniel, I. E., Silva, P., & Zilberman, D. (2010). Genome-wide evolutionary
1111 analysis of eukaryotic DNA methylation. *Science, 328*(5980), 916-919.
1112 doi:10.1126/science.1186366
1113 Zhang, X., Li, Q., Kong, L., & Yu, H. (2018). Epigenetic variation of wild populations of the
1114 Pacific oyster *Crassostrea gigas* determined by methylation-sensitive amplified
1115 polymorphism analysis. *Fisheries Science, 84*(1), 61-70. doi:10.1007/s12562-017-1154-5
1116 Zhong, Z., Feng, S., Duttke, S. H., Potok, M. E., Zhang, Y., Gallego-Bartolome, J., . . . Jacobsen,
1117 S. E. (2021). DNA methylation-linked chromatin accessibility affects genomic
1118 architecture in *Arabidopsis*. *Proceedings of the National Academy of Sciences U.S.A.,*
1119 *118*(5). doi:10.1073/pnas.2023347118
1120 Zilberman, D. (2017). An evolutionary case for functional gene body methylation in plants and
1121 animals. *Genome Biology, 18*(1), 87. doi:10.1186/s13059-017-1230-2
1122