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Parental environments alter DNA methylation in offspring of the purple sea urchin, *Strongylocentrotus purpuratus*

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ABSTRACT

Phenotypic plasticity, within and across generations, is a strategy by which organisms can respond rapidly to environmental change. Epigenetic modifications, such as DNA methylation, have been proposed to be involved in phenotypic plasticity. We examined the potential for the environment to mediate both transgenerational and intragenerational plasticity in DNA methylation and phenotypes in early stages of the purple sea urchin, *Strongylocentrotus purpuratus*, an ecologically important herbivore in kelp forest ecosystems. This approach involved a controlled laboratory experiment where adult urchins were acclimated during gametogenesis to upwelling (~1300 µatm $p\text{CO}_2$ & 13 °C) or non-upwelling (~650 µatm $p\text{CO}_2$ & 17 °C) conditions that are representative of their kelp forest habitat. Progeny from these adults were raised in either high (~1050 µatm) or low (~450 µatm) $p\text{CO}_2$ treatments and sampled at three developmental stages. Differences in condition experienced by mothers were associated with differentially methylated genes in the offspring. However, differences in developmental conditions corresponded to little observable effects on gene methylation in the progeny. Variation in gene body methylation across treatments was correlated with body size of the embryos and larvae, illustrating a potential link between transgenerational phenotypic plasticity and DNA methylation. Overall, our results suggest that epigenetic factors such as DNA methylation have the potential to contribute to phenotypic plasticity in a transgenerational framework, and further, that epigenetic processes may act as a mechanism of rapid response to environmental change.

1. Introduction

As the global climate shifts towards higher annual average temperatures and increased incidences of extreme climactic events, many organisms will likely be pushed beyond their range of physiological tolerances (Hofmann and Todgham, 2010; Sunday et al., 2012). These rapid environmental changes have focused attention on understanding ways in which organisms can respond across relatively short time scales. Although studies have shown evidence for the capacity for rapid adaptation when populations have high standing genetic variation (Barrick and Lenski, 2013; Dixon et al., 2015; Matz et al., 2018; Messer and Petrov, 2013; Torda et al., 2017), it is also likely that phenotypic plasticity is an important mechanism enabling organisms to cope with environmental change (Buckley and Kingsolver, 2012; Munday et al., 2013). In well-studied mammalian and plant systems, there is evidence that epigenetic mechanisms mediate some forms of phenotypic plasticity, however, this link is not substantiated in other taxa (Eirin-Lopez and Putnam, 2019; Feil and Fraga, 2012; Herman and Sultan, 2011; Richards et al., 2017). In this study, we examine the potential for plasticity in patterns of DNA methylation, an epigenetic mark, in offspring that could be influenced by the environmental history of the parents, using purple sea urchins (*Strongylocentrotus purpuratus)* from a temperate kelp forest environment.

In many plant and animal taxa, parental exposure to stressful environments can influence phenotypes of their offspring (Agrawal et al., 1999; Herman and Sultan, 2011; Marshall, 2008). This inheritance of environmental effects across generations is often referred to as transgenerational plasticity (TGP), and can include parental provisioning of a number of non-genetic factors including nutrients, hormones, maternal RNAs, microbial communities, and epigenetic marks (Bonduriansky et al., 2012; Gavery and Roberts, 2017; Torda et al., 2017). Transgenerational responses to environmental change can be due to context-dependent effects, where plastic changes in phenotypes associated with the environment persist across generations because both generations experience the same environmental pressures, or germline-dependent, where environmentally driven changes in the

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epigenome of the parents are carried over to the offspring through the germline, a mechanism that remains contentious (Eirin-Lopez and Putnam, 2019). Despite the well-recognized role of maternal effects in evolutionary studies (Kirkpatrick and Lande, 1989; Mousseau and Fox, 1998), the impact and extent of TGP in marine systems has only recently been examined in a global change context (Donelson et al., 2017; Hofmann, 2017; Munday, 2014).

Emerging studies suggest that TGP could be occurring frequently in organisms experiencing stressors associated with global change; however, this is likely a complex situation where cost and consequences will vary in different systems. Transgenerational effects can sometimes be non-adaptive, such as when parents exposed to stressful environments can produce lower quality offspring (Mccormick, 2006; Shama and Wegner, 2014). However, the opposite could occur as well, where parental exposure to environmental stress results in offspring better acclimated to that stress (Donelson et al., 2017, 2012; Miller et al., 2012). TGP and its potential to increase offspring fitness is more likely to occur when the parental environment is a good predictor of offspring environment (Burgess and Marshall, 2014; Mousseau and Fox, 1998). Therefore, priming offspring to certain environmental conditions can be associated with negative consequences if environments or stressors shift, making TGP and maternal effects complex, and context-dependent. Despite the inherent trade-off risks, occurrences of TGP have been observed in a diversity of marine organisms both in situ and in laboratory experiments (Donelson et al., 2017; Munday, 2014; Murray et al., 2014). Overall, it is clear that the complexity of TGP requires knowledge of the natural variation of the environment and how these conditions might vary between life-history stages (Burgess and Marshall, 2014; Donelson et al., 2017).

As field experiments intended to investigate TGP in marine environments are not always feasible, laboratory experiments, particularly those designed to mimic natural environmental conditions under a controlled setting, can provide useful insights. Indeed, TGP has been widely observed in laboratory experiments on marine species (Beal et al., 2018; Donelson et al., 2017; Ross et al., 2016). TGP has been documented in response to elevated $p\mathrm{CO}_2$ conditions in oysters (Parker et al., 2015; Parker et al., 2012), clams (Zhao et al., 2018), sea urchins (Dupont et al., 2013; Wong et al., 2018), calcifying tubeworms (Lane et al., 2015), copepods (Pedersen et al., 2014; Thor and Dupont, 2015), and coral (Putnam et al., 2016; Putnam and Gates, 2015). In addition, TGP in marine systems has been observed in response to temperature (Donelson and Munday, 2015; Marsh and Pasqualone, 2014; Metzger and Schulte, 2017; Norouzitallab et al., 2014; Shama et al., 2016; Veilleux et al., 2015), salinity stress (Jensen et al., 2014), and toxicants (Marshall, 2008; Vandegehuchte et al., 2010).

In general, despite accumulating evidence for TGP, the precise molecular-level mechanisms that underlie these transgenerational effects remain unexplained in most organismal systems. There is a strong interest to understand these mechanisms as it has been proposed that epigenetic modifications could contribute to rapid acclimatization and adaptation (Beal et al., 2018; Eirin-Lopez and Putnam, 2019). Recently, our understanding of these processes has expanded in plants and mammals with evidence that epigenetic modifications can induce changes in phenotype associated with the environment (Feil and Fraga, 2012). These epigenetic modifications include DNA methylation, histone posttranslational modifications as well as non-coding RNAs (Beal et al., 2018; Eirin-Lopez and Putnam, 2019). Of these modifications, DNA methylation has been the most explored as a means of transcriptional regulation shifting phenotypes across short time scales (Beal et al., 2018). For example, differences in methylation at key loci are associated with transgenerational acclimatization to temperature in both a coral reef fish (Ryu et al., 2018) and in stickleback (Metzger and Schulte, 2017). Although DNA methylation is conserved across eukaryotes, patterns of genome methylation vary greatly between vertebrates and invertebrates, where vertebrate genomes are globally methylated and invertebrates exhibit mosaic patterns of methylation (Feng et al., 2010). How these inherent differences in genome methylation patterns relate to function, transcriptional regulation, and phenotypes is beginning to be characterized. There is emerging evidence that DNA methylation is associated with transcriptional differences in invertebrates. In oysters, methylated gene bodies are associated with highly expressed transcripts that are stably expressed across cell types (Gavery and Roberts, 2013), an effect also observed in corals (Dimond and Roberts, 2016; Dixon et al., 2018; Dixon et al., 2016; Dixon et al., 2014; Liew et al., 2018), suggesting gene body methylation has an evolutionarily ancient function involved with the stability of gene expression and controlling transcriptional noise. The extent to which DNA methylation mediates transcriptional flexibility in response to the environment in other animals remains an open question, although there is extensive work investigating this in plants (Herman and Sultan, 2011; Huang and Ecker, 2017). In this study, we explore if DNA methylation is associated with differences in offspring phenotypes after parental conditioning during gametogenesis in the purple sea urchin.

In changing marine environments it is predicted that planktonic early life-history stages will be especially sensitive as they have limited mechanisms for defense (Byrne, 2011). However, there is strong selection pressure to develop means of tolerating abiotic stressors during this phase. Purple sea urchins, *Strongylocentrotus purpuratus*, are ecologically important herbivores in kelp forest ecosystems and have a planktonic larval stage (Pearse, 2006). *S. purpuratus* inhabits coastal ecosystems characterized by highly fluctuating water conditions and seasonal upwelling typical of the California Current System. Upwelling brings deep, cold, nutrient-rich water low in pH up to surface waters, interacting directly with coastal communities and is predicted to intensify in the future (Bakun et al., 2015; Gruber et al., 2012). In the Santa Barbara Channel, *S. purpuratus* adults experience upwelling events regularly (Chan et al., 2017), which could impact gametogenesis and subsequent tolerance of early life-history stages in situ in the kelp forest environment (Wong et al., 2019; Hoshijima and Hofmann, 2019).

In early stage *S. purpuratus* embryos and larvae, high $p\mathrm{CO}_2$ has been shown to impact body size, metabolic rates, gene expression, internal acid-base balance, and growth (Evans et al., 2017; Kelly et al., 2013; Padilla-Gamino et al., 2013; Sheppard Brennand et al., 2010; Stumpp et al., 2011). Adult exposure to upwelling conditions, however, may mitigate the effects of high $p\mathrm{CO}_2$ on early developmental stages. In a previous study, when adult *S. purpuratus* were exposed to upwelling or non-upwelling conditions during gametogenesis, progeny showed divergence in both body size and expression of the transcriptome associated with maternal exposure (Wong et al., 2018), suggesting that TGP can play a role in offspring traits in *S. purpuratus*. Furthermore, transcriptome patterns that differed between the offspring of the differentially conditioned adults were found to include genes related to epigenetic processes. The mechanisms enabling the maternal environment to impact phenotypes in the progeny are yet to be explored, but we posit that epigenetic modifications could contribute a means for quick adjustments and the perpetuation of TGP.

Here, we examined the possibility of transgenerational plasticity of DNA methylation in purple sea urchins to begin to address if epigenetic processes might contribute to changes in progeny traits that occur in a transgenerational context. The analysis presented in this study used samples generated from a laboratory experiment that is described both here and in a companion paper Wong et al. (2019). Wong et al. (2019) found maternal provisioning differences in the eggs and morphometric differences in the progeny based on maternal conditioning. In the current study, we found evidence that maternal conditioning to upwelling or non-upwelling temperature and $p\mathrm{CO}_2$ treatments impacted DNA methylation patterns in the progeny, suggesting epigenetic mechanisms could be involved in maternal priming of offspring in *S. purpuratus*. Overall, this study begins to unravel the potential for epigenetic mechanisms to play a role in rapid responses to environmental change in an ecologically important benthic marine invertebrate.

Table 1
Carbonate chemistry parameters. For adult conditioning (N & U), pCO_2 and Ω_{Arag} were calculated from other values using daily salinity values (33.1 ± 0.1) and total alkalinity values measured every three days during these exposures (2211.25 ± 7.10 µmol kg^{-1}). For embryo and larval culturing (H & L), pCO_2 and Ω_{Arag} were calculated from other values using daily salinity values (33 ± 0.0) and total alkalinity values measured twice during these exposures (2220.25 ± 4.06 µmol kg^{-1}). Variance reported is standard deviation.

	pH_{total}	pCO_2 (µatm)	Ω_{Arag}	Temperature (°C)
Non-Upwelling (N)	7.87 ± 0.11	651 ± 242	1.74 ± 0.34	17.0 ± 0.1
Upwelling (U)	7.57 ± 0.08	1330 ± 300	0.79 ± 0.14	12.9 ± 0.2
Low pCO_2 (L)	7.99 ± 0.01	446 ± 12.4	2.03 ± 0.05	15.0 ± 0.1
High pCO_2 (H)	7.66 ± 0.01	1050 ± 37.7	1.02 ± 0.03	14.9 ± 0.1

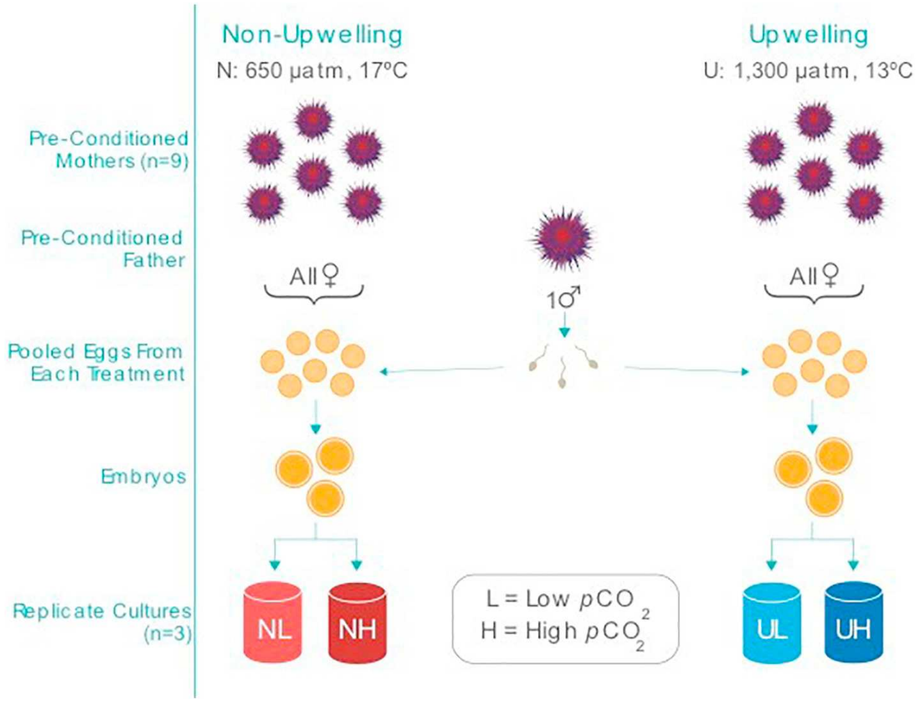

Fig. 1. Experimental Design. Adult purple sea urchins were conditioned in either upwelling (U) or non-upwelling (N) conditions for four months during gametogenesis. Eggs from each treatment were pooled ($N = 9$ females per treatment) and fertilized with sperm from one non-upwelling treatment male. Embryos from each cross were reared in either high (H) or low (L) pCO_2 conditions, resulting in four treatment types: UH, UL, NH & NL. Each larval culture was replicated three times.

2. Materials and methods

2.1. Preconditioning of adult urchins

Adult purple sea urchins (*Strongylocentrotus purpuratus*, Stimpson, 1857, N = ~90) were collected by SCUBA at a subtidal site near Goleta Beach, Goleta, CA (34° 24.840′ N, 119° 49.742′ W) on October 5, 2016 under permit (California Scientific Collecting permit SC - 1223). Adults urchins were transported back to the aquarium facilities at University of California, Santa Barbara, held in flow-through seawater tanks for ~2.5 weeks and acutely transferred to 20-gal flow-through aquarium tanks containing local seawater that had been adjusted in pCO_2 and temperature (Table 1, Fig. S1). Adult urchins were separated equally into two treatments (3 tanks/treatment, 8–13 urchins in each tank), each reflecting upwelling (~1330 µatm & 13 °C) and non-upwelling (~651 µatm & 17 °C) conditions experienced in their natural environment (Table 1, Fig. S1) (Kapsenberg and Hofmann, 2016; Rivest et al., 2016). Adults were maintained at these conditions for ~4 months, encompassing the length of gametogenesis for this species and were fed kelp (*Macrocystis pyrifera*) once a week.

The pCO_2 conditions were controlled in the treatment tanks using a flow-through CO_2-mixing system modified from Fangue et al. (2010). Briefly, for each treatment, two 20-gal glass reservoir tanks fed 0.35um filtered, UV sterilized seawater (FSW) into three 20-gal replicate glass aquaria. The flow rate was set at 10 L/h from each of two reservoir tanks, for a total of 20 L/h to each treatment tank. To establish target pCO_2 levels in reservoir tanks, dry air was filtered and CO_2 scrubbed before being mixed with pure CO_2 using Mass Flow Controllers (Sierra Instruments, USA). Each 20-gal treatment tank was fitted with a small pump to aid mixing and temperature was regulated using Delta Star heat pumps connected to Nema 4× digital temperature controllers (AquaLogic, San Diego, CA, USA).

Seawater chemistry measurements (temperature, salinity and pH) were taken for each treatment tank every 1–2 days of the 4-month adult conditioning for each treatment tank. Temperature and salinity were measured using a digital thermometer (Omega HH81A) and a conductivity meter (YSI-3100), respectively. Measurements of pH were determined using *m*-cresol purple indicator dye (Sigma-Aldrich) and a spectrophotometer (Bio Spec-1601, Shimadzu), following SOP6b (Dickson et al., 2007a). Total alkalinity measurements were taken every 3–5 days as in SOP3b (Dickson et al., 2007b). Calculations of $pCO2$ and $\Omega_{aragonite}$ were performed using CO_2calc (Robbins et al., 2010), with carbonic acid dissociation constants defined by (Mehrbach et al., 1973) and refit by (Dickson and Millero, 1987).

2.2. Spawning and culturing of embryos and larvae

After adult conditioning, all urchins from each treatment were pooled in an ambient seawater environment. Spawning of adult preconditioned urchins was induced with intracoelomic injections of 0.53 M KCl. Of the 38–39 urchins from each treatment, nine females from each of the two treatment conditions (upwelling and non-upwelling) released a high quantity of quality eggs after visual inspection. Sperm quality was assessed visually and one male urchin from the non-upwelling treatment was chosen to fertilize pools of eggs from both treatments. Prior to pooling the eggs, individual fertilization trials were performed using small aliquots of eggs ($N = 9$ dams per condition) and sperm ($N = 1$ sire) and a 95% fertilization success rate was verified, providing additional support that gametes were of sufficient quality. One cross was performed for each condition; eggs from the same condition were evenly pooled, and each pool of eggs (non-upwelling and upwelling) was fertilized by sperm from a single male (non-upwelling) (Fig. 1). Fertilization occurred at ambient seawater conditions. We prioritized pooling as many females as possible to maximize the likelihood of identifying average maternal effects for each treatment regardless of genotypic differences between mothers. One male was used to minimize genetic variance due to male genotype and ensure that all larvae in the experiment were genetic full or half-siblings. After fertilization, zygotes from each cross were divided equally into culture vessels set at the two different $p\mathrm{CO}_2$ treatment conditions (Table 1, Fig. S1), and reared at a density of 10 embryos/mL. Embryo cultures were maintained within two nested 5-gal buckets equipped with a small paddle to enable gentle mixing and a flow-rate of 6 L/h. Developmental treatments reflected high and low $p\mathrm{CO}_2$ levels experienced in the wild (Kapsenberg and Hofmann, 2016) (Table 1, Fig. S1) but with a constant temperature of 15 °C. This temperature was maintained to ensure synchronous development and consistency when sampling across treatments. This design yielded four treatment conditions, each replicated three times: offspring of mothers conditioned to non-upwelling conditions and developed in either high (NH) or low (NL) pCO2 conditions and offspring of mothers conditioned to upwelling conditions and developed in either high (UH) or low (UL) $p\mathrm{CO}_2$ conditions (Fig. 1). Temperature, salinity and $p\mathrm{CO}_2$ measurements were taken daily for larval cultures (Table 1, Fig. S1) in the same manner as described above.

Embryos and larvae were sampled at three stages of development: hatched blastula (BL), gastrula (G), and prism (PR), a very early echinopluteus stage, to investigate potential variation in TGP effects across development. Details and photographs of stage morphology are described in a companion paper (Wong et al., 2019) and there was no evidence of developmental delay based on differences in maternal temperature. Embryos and larvae were sampled and concentrated using a 35 μm mesh filter. For each sampling timepoint, ~5000 embryos from each culture were transferred to a 1.5 mL tube and briefly pelleted. Excess seawater was removed and the sample was flash frozen in liquid nitrogen and stored at −80 °C.

2.3. Body size of embryos and larvae

Morphometric analysis is detailed in a companion paper (Wong et al., 2019). In brief, images of embryos and larvae (≥30 individuals per culture replicate) were analyzed using ImageJ (National Institutes of Health, USA) to measure the body size of each developmental stage. Body sizes of the hatched blastula and gastrula stages were assessed by measuring the maximum body length from the animal to the vegetal pole. The prism stage was measured by determining skeletal rod length, from the tip of the body rod to the tip of the postoral rod (Wong et al., 2019).

2.4. Preparation of MeDIP libraries and sequencing

Genomic DNA was isolated using an established CTAB extraction protocol with a phenol chloroform purification. Each sample consisted of ~5000 embryos or larvae from each culture replicate (3 replicates per treatment) for three developmental stages. DNA (600-1000 ng per sample) was sheared to a range of 150-500 bp using a Covaris M220 Focused-ultrasonicator and the recommended program for producing an average fragment length of 350 bp in 50 μL: 75 W peak incident power, 200 cycles per burst, 70s. Sheared DNA was quantified fluorometrically with a Qubit 3.0 Fluorometer (Invitrogen). Samples were enriched for DNA containing 5-mC using the methylated-DNA IP kit (Zymo Cat. No. D5101) following manufacturer's instructions using the maximum initial input, 500 ng of sheared DNA. Immunoprecipitated samples were prepared for sequencing using the Pico Methyl-Seq library prep kit (Zymo Cat. No. D5455), and the 36 samples were uniquely dual indexed using primers specified by Illumina (Illumina Adapter Sequences (1,000,000,002,694 v06)). Final libraries were quantified fluorometrically using a Qubit 3.0 Fluorometer (Invitrogen), and the distribution of fragment sizes was confirmed using an Agilent Tape Station. Libraries were sent to the University of California, Davis Genome Center (http://dnatech.genomecenter.ucdavis.edu/) for final pooling and Single end-100 reads were sequenced on the Illumina HiSeq4000 across three lanes.

2.5. Pre-processing of MEDIP sequences

One of the samples had insufficient quantity and quality prior to pooling (NH2_Gastrula), therefore only 35 libraries were sequenced. The following steps were used to pre-process the reads: (1) adaptor sequences were removed, and base pairs with quality < 33 were removed using cutadapt (Martin, 2011), (2) reads were verified for quality using FASTQC, (3) trimmed and filtered reads were mapped to the *Strongylocentrotus purpuratus* genome (V3.1) using hisat2 (V2.0) (Kim et al., 2015), (4) read duplicates from alignment files were removed using the *Mark Duplicates* command in Picard tools (V1.141), and (5) counts of mapped reads that overlapped gene regions were performed using *featureCounts*, a component of the *subread* package (V1.6.2) (Liao et al., 2014) and the *Strongylocentrotus purpuratus_3.1.39* GTF file from *Ensembl*. Putative promoters were defined as being 1 kb upstream from the gene region. Counts of mapped reads overlapping with regions 1 kb upstream from the gene were also performed using *featureCounts*.

2.6. Analysis of differentially methylated regions (DMRs)

All statistical analyses were performed in R (V3.4.2). Invertebrates show characteristic bimodal patterns of gene body methylation (GBM), where genes fall into two classes: highly methylated or lowly methylated. GBM class values for each gene were defined as -log(mean methylation counts/gene length). Normalization and statistical analysis of gene and regions 1 kb upstream from the gene counts were performed using DESeq2 (Love et al., 2014), and genome features with mean counts < 3 were removed. Sample outliers were identified using *arrayQualityMetrics* (Kauffmann et al., 2009) and removed from subsequent analysis. A wald test was performed with a full model specifying three fixed factors: stage (blastula, gastrula, prism), maternal conditioning (upwelling, non-upwelling) and developmental conditioning (high $p\mathrm{CO}_2$, low $p\mathrm{CO}_2$) (formula ~ maternal treatment + developmental treatment + stage). For each fixed factor, pair-wise contrasts were run to identify specific DMRs. Contrasts for maternal conditioning were between larvae that came from upwelling mothers (UL & UH, Fig. 1) and larvae that came from non-upwelling mothers (NL & NH, Fig. 1). Contrasts for developmental conditioning were between larvae reared at low $p\mathrm{CO}_2$ (UL & NL, Fig. 1) and larvae reared at high $p\mathrm{CO}_2$ (UH & NH, Fig. 1). To further explore the potential for effects of developmental conditioning, comparisons between high and low $p\mathrm{CO}_2$ treatments were performed on subsets of the data (NL vs. NH and UL vs. UH) to eliminate potential maternal effects. Contrasts for developmental stages included all pair-wise comparisons between the three stages, hatched blastula (BL) vs. gastrula (G), gastrula (G) vs. prism (PR) and hatched blastula (BL) vs. prism (PR).

2.7. Multivariate analysis of DMRs

To assess broad differences in methylation patterns, normalized count data files for gene regions and regions 1 kb upstream from the gene were regularized-log transformed using the *rlog* function in DESeq2 (Love et al., 2014). Principal Coordinates Analysis (PCoA) analysis was performed using the package *adegenet* (Jombart, 2008), specifying Manhattan distances and significance was assessed using the package *vegan* and the multivariate analysis of variance function *adonis* (Dixon, 2003).

A discriminant function analysis of principal components (DAPC) was performed using normalized count data for genes, to further discriminate the subtle impacts of maternal and developmental condition on overall methylation patterns in genes (Jombart et al., 2010). This multivariate analysis identifies the axis of variation that best discriminates between two specified groups, in this case between NL (non-upwelling maternal condition, low $p\mathrm{CO}_2$ developmental condition) and UH (upwelling maternal condition, high $p\mathrm{CO}_2$ developmental condition) treatments. This discriminant function was then applied to groups, NH (non-upwelling maternal condition, high $p\mathrm{CO}_2$ developmental condition) and UL (upwelling maternal condition, low $p\mathrm{CO}_2$ developmental condition), to assess variation in the context of the predefined groups (NL and UH). This analysis allows us to quantify the extent to which the reciprocal treatment's methylation patterns matched that of the equivalent maternal/developmental condition. The discriminant function was correlated to body size measurements.

2.8. Gene ontology (GO) analysis

For each pair-wise contrast, Gene Ontology (GO) enrichment tests were performed using the GOMWU package, which ranks stat values (equivalent to z-scores) for the entire set of genes (24,347) using a Mann-Whitney U (MWU) test (Voolstra et al., 2011; Wright et al., 2015). Each pairwise comparison was performed using these same 24,347 genes. Unlike traditional GO enrichment tests that compare a short list of significant genes to a background dataset, this test assesses if GO categories are enriched on either end of a distribution of values, eliminating the need to set arbitrary significance cut-off values prior to testing for enrichment (Voolstra et al., 2011; Wright et al., 2015). MWU GO enrichment tests were also performed on two classes of methylated genes (highly methylated and lowly methylated genes), which were identified using size normalized raw count data, defined as -log(mean methylation counts/gene length).

3. Results

3.1. Processing of MEDIP sequences

Overall, the sequencing effort was successful, yielding > 1 billion reads, an average of 30,034,082 reads per sample across the 35 samples. After removing adaptors, reads were mapped to the *Strongylocentrotus purpuratus* genome (V3.1) (Cameron et al., 2009) with an average mapping efficiency of 48.6%. An average of 58.45% of mapped reads were marked as duplicates and removed. The remaining mapped reads were converted to counts of genes and regions 1 kb upstream from the gene. Counts of genes averaged 4,274,867 per sample across 30,240 genes with mean and median count values of 141.36 and 31.07, respectively, averaged across all samples. Outlier analysis using *arrayQualityMetrics* identified 3 samples (NL1_BL, NL2_G, UH2_G) as being significant outliers, and these samples were removed from the subsequent analysis. These outlier samples were either noticeably under sequenced relative to other samples or had a large proportion of read duplicates. Lowly methylated transcripts (mean counts across samples < 3) were removed from subsequent analysis, leaving 24,347 methylated genes. Putative promoter regions were defined as 1 kb upstream of the transcription start site for all annotated genes. Mapped reads were converted to promoter counts, averaging 473,915 counts per sample across 29,923 promoter regions. Putative promoter regions with mean counts < 3 across all samples were removed, leaving 14,553 methylated putative promoter regions. Outlier analysis identified the same 3 samples that were identified in the gene analysis; here too, counts from these 3 samples were removed from subsequent analysis.

3.2. Classes of gene body methylation

To begin the analysis, we examined whether GBM exhibited a bimodal distribution that has commonly been observed in invertebrates (Dimond and Roberts, 2016; Dixon et al., 2018; Dixon et al., 2014; Elango et al., 2009; Gavery and Roberts, 2010; Park et al., 2011; Suzuki et al., 2016). We found that the density of GBM across the *S. purpuratus* genome formed a bimodal distribution with two distinct classes across a range of values (methylation density per gene) (Fig. S2). Due to our inability to report absolute levels of methylation, these results on a gene-by-gene level should be interpreted with caution as it is possible that highly methylated but CpG poor regions with low coverage could be misinterpreted as hypomethylated. However, the bimodal pattern produced by our data (Fig. S2) is similar to what has been reported in this species before based on CpG observed/expected values across the genome (Keller et al., 2016). Gene Ontology (GO) enrichment indicated that highly methylated genes included those involved in structural components of the ribosome, helicases, enzyme regulators, RNA and DNA binding proteins and ATPase, among others, while lowly methylated genes were enriched for processes involving ion transport and channels, signal transduction, G-protein coupled receptors, and carbohydrate binding proteins (Fig. S2).

3.3. Variance in DNA methylation across genome features

In order to explore the pattern of DNA methylation in the experimental samples, variance in overall DNA methylation patterns in genes and regions 1 kb upstream from genes was assessed using a Principle Coordinates Analysis (PCoA). PCoA of gene methylation revealed no significant difference in overall methylation by developmental stage, maternal condition, or condition under which the embryos were raised (Fig. S3). This same pattern was observed across methylation in regions 1 kb upstream from the gene (Fig. S3). Next, we examined how much of the variance in the observed DNA methylation was explained by the experimental factors, developmental stage, maternal condition and developmental condition (Fig. 2A, E). In general, partitioning of variance across the 3 factors (developmental stage, maternal condition and developmental condition) revealed modest effects of each of these factors when considering both GBM and methylation in regions 1 kb upstream from the gene. (Fig. 2A, E). For variance in GBM, stage accounted for 6.5%, maternal condition accounted for 6.6%, and developmental condition accounted for 2.6%, with the majority of variance explained by residuals (Fig. 2A). For variance in methylation in regions 1 kb upstream from the gene, stage accounted for 7.0%, maternal condition accounted for 5.8%, and developmental condition accounted for 3.4% (Fig. 2E). A permutational multivariate analysis of variance revealed only maternal variance was significant for both GBM and methylation in regions 1 kb upstream from the gene ($p < .05$).

3.4. Differentially methylated regions (DMRs) between treatments

In order to assess whether transcripts or regions 1 kb upstream from the gene were differentially methylated with respect to transgenerational or intragenerational plasticity, we ran models in DESeq2 (Love et al., 2014). DMRs varied as a function of maternal condition (upwelling vs. non-upwelling), but not as a function of developmental condition (high vs. low $p\mathrm{CO}_2$) (Fig. 2). When comparing maternal conditioning environments (upwelling vs. non-upwelling), we identified 605 differentially methylated genes (FDR < 0.01) (Fig. 2B, Table S1). In comparing developmental condition (high vs. low $p\mathrm{CO}_2$), we identified 0 differentially methylated genes (FDR < 0.01) (Fig. 2C, Table S1). Additional comparisons between developmental treatments within each maternal treatment (NL vs. NH and UL vs. UH) yielded only

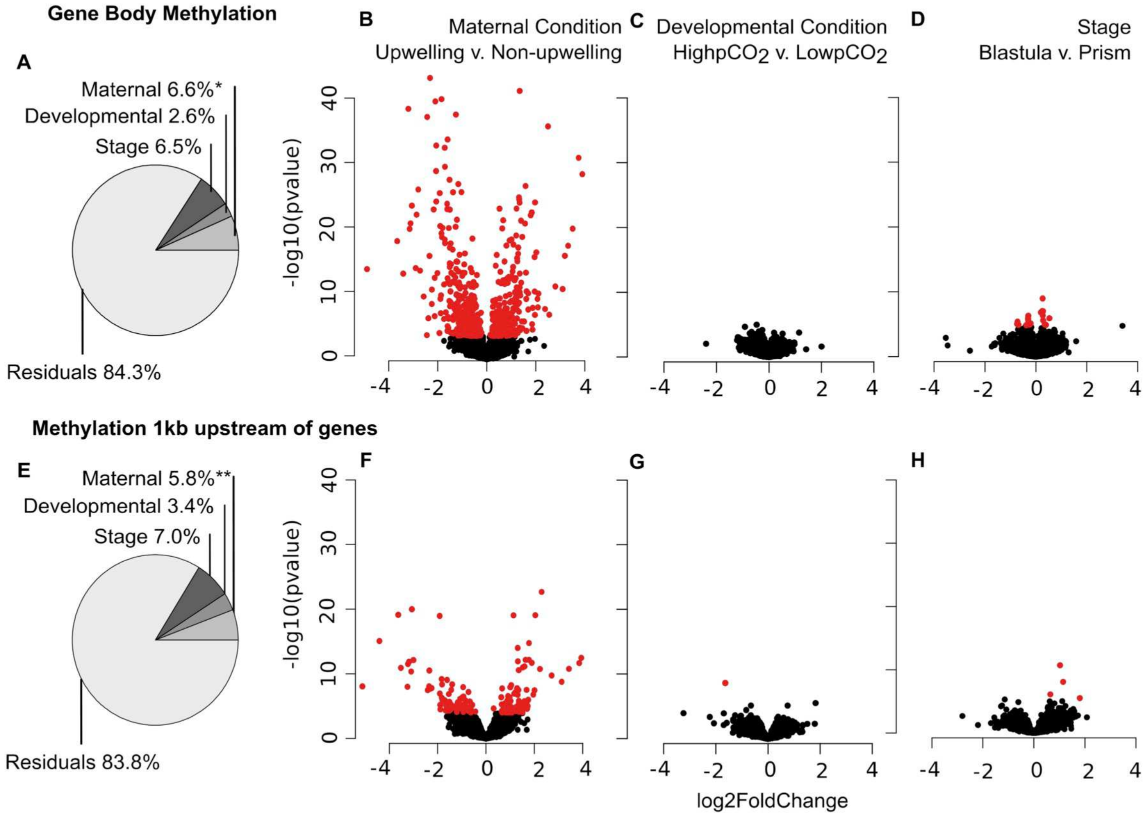

Fig. 2. DNA methylation varies by maternal condition, but not developmental condition or stage. Percentage of variation in gene body methylation (A) or regions 1 kb upstream of genes (putative promoter methylation) (E) explained by maternal condition, developmental condition or stage using a permutational multivariable ANOVA. Significantly differentially methylated genes (FDR < 0.01, in red) among pairwise comparisons of maternal conditions (B), developmental conditions (C) and blastula vs. prism stages (D). Significantly differentially methylated putative promoter regions (FDR < 0.01, in red) among pairwise comparisons of maternal conditions (F), developmental conditions (G), and blastula vs. prism stages (H). *$p < .05$, **$p < .01$.

1 differentially methylated gene for the comparison of non-upwelling samples, lending further support for no effect of developmental treatment on GBM. The same overall pattern was found in regions 1 kb upstream from the gene, with 156 and 1 differentially methylated regions by maternal condition and developmental condition, respectively (FDR < 0.01) (Fig. 2F, G, Table S2).

Although not all pairwise comparisons were found to have significant differentially methylated genes using DESeq2, an MWU GO test was performed for the entire set of genes (24,347) for each pairwise test of interest, revealing significantly enriched categories for maternal or developmental condition (Fig. S5). We saw that genes involved in macromolecular biosynthesis processes, phosphorylation and lipid metabolic processes were more methylated in offspring from upwelling females, while offspring from non-upwelling females showed higher methylation of categories involving cell surface receptor signaling, cell adhesion and chromosome organization (Fig. S5). In comparing offspring reared in different developmental conditions, we found that offspring exposed to high $p\mathrm{CO}_2$ had higher methylation of cell surface receptor signaling while offspring exposed to low $p\mathrm{CO}_2$ show higher methylation of DNA replication genes and macromolecule biosynthetic processes (Fig. S5).

3.5. DMRs observed between stages

In our assessment of DNA methylation patterns in early stage purple sea urchins we examined whether DNA methylation varied as a function of developmental stage. Pairwise contrasts between each developmental stage revealed differences in GBM only when comparing hatched blastula and prism (22 genes) or gastrula and prism (1 gene) (FDR < 0.01) (Fig. 2D, Table S1). When considering methylation in regions 1 kb upstream from the gene, only the comparison between hatched blastula and prism revealed 4 differentially methylated regions (Fig. 2H, Table S2). Despite relatively low levels of differentially methylated genes and regions 1 kb upstream from the gene between stages, there were numerous significantly enriched GO categories between developmental stage comparisons (Fig. S4). These could represent differences in methylation of broad functional categories between developmental stages. In comparing the two earlier stages, blastula and gastrula, we see significant enrichment of categories involving ion transport mechanisms, cell surface receptor signaling, responses to stimulus and regulation of metabolic processes (Fig. S4). Differentially enriched processes between blastula and prism include ion transport and lipid metabolic processes, carbohydrate metabolic processes as well as DNA replication (Fig. S4). Comparing gastrula and prism revealed differentially enriched categories such as phosphorylation and proteolysis mechanisms, immune response, oxidation-reduction processes, cell adhesion, microtubule-based processes, cell surface receptors and response to stimulus (Fig. S4).

3.6. Relationship between GBM and body size

Lastly, another goal of the study was to examine whether GBM correlated to other response variables in the early life history stages of *S. purpuratus*. Body size data and morphometric analysis is presented in detail in a companion paper (Wong et al., 2019). This study found that females conditioned to upwelling conditions had progeny with larger body sizes (Wong et al., 2019).

A discriminant analysis of principal components (DAPC) was performed to quantify the effects of maternal and developmental conditions on GBM and relate it to body size of embryos. The DAPC was

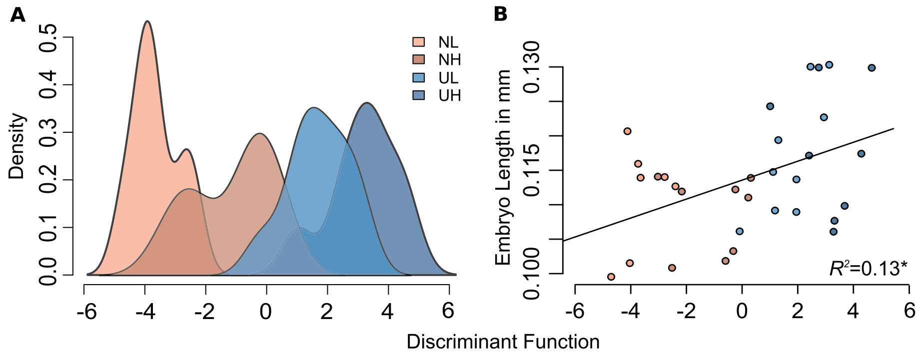

Fig. 3. Relationship between gene body methylation and body size. (A) Density plots for discriminant analysis of principal components (DAPC). The discriminant function was built using normalized gene body methylation (GBM) count data from UH and NL cultures. GBM count data for UL and NH was applied to the function. (B) Discriminant function values were correlated to embryo body size measurements. $*p < .05$. NL (non-upwelling maternal condition, low $p\text{CO}_2$ developmental condition), NH (non-upwelling maternal condition, high $p\text{CO}_2$ developmental condition), UL (upwelling maternal condition, low $p\text{CO}_2$ developmental condition), UH (upwelling maternal condition, high $p\text{CO}_2$ developmental condition).

specified using the two defined groups that did not experience a shift in $p\text{CO}_2$ environments across generations (UH and NL), presumably reflecting the most variation between treatments and baseline levels between the two conditions (Figs. 1 & 3A). Additional samples, in this case the UL and the NH treatments, were applied to the already determined discriminant function to assess variation of these treatments (larvae that experienced different $p\text{CO}_2$ levels than their mothers) in the context of the baseline (UH and NL) groups (Fig. 3A). This analysis showed that the treatments characterized by a transgenerational shift in $p\text{CO}_2$ exposure (UH and NL) had intermediate shifts along the discriminant axis, emphasizing a subtle shift in methylation variation in these treatments. To further assess if there was a relationship between methylation variation and embryo phenotypes, we found that embryo size positively correlated with the DAPC axis ($R^2 = 0.13$, $p_{lm} = 0.025$) (Fig. 3B). This increase in embryo size along the discriminant function axis was not associated with the size increase as embryos developed (across stages). Overall, we found that variation in GBM between experimental conditions showed the same pattern as embryo body size, suggesting a potential link between transgenerationally-mediated changes in phenotype and GBM changes.

4. Discussion

We sought to examine if abiotic conditions experienced by female purple urchins during gametogenesis, or those encountered by embryos during development, would impact DNA methylation patterns across the genome of the progeny, and if this was correlated to changes in phenotype. Ultimately, we hope these lab observations will inform experiments examining TGP in natural populations. Further, in marine systems it remains to be seen how much observed variation in methylation state of the DNA is driven by environmental factors as opposed to other mechanisms, such as genotype specific patterns of DNA methylation and spontaneous epimutation. Our study presents 3 salient findings: (1) there was an effect of maternal environment on DNA methylation (transgenerational effects), but little effect of developmental environment (intragenerational effects), (2) differences in DNA methylation across developmental stages were minimal, and (3) differences in GBM across maternal treatments were associated with body size of the embryos, providing a potential link between transgenerational phenotypic plasticity and DNA methylation.

4.1. Transgenerational plasticity and DNA methylation

In this study, we found that maternal conditioning influenced DNA methylation patterns in the offspring (transgenerational plasticity) while conditions experienced during development had comparatively minimal influence (intragenerational plasticity) (Figs. 2 & 3). Although many studies report maternal carry-over effects based on environmental conditions (Jensen et al., 2014; Marshall et al., 2010; Vandegehuchte et al., 2010), few have begun to tease apart the role epigenetic processes could play in mediating phenotypes across generations and these studies are restricted to plants and vertebrates (Mirbahai and Chipman, 2014). In general, transgenerational epigenetic inheritance in animals remains a controversial topic due to methylation erasure in primordial germ cells and zygotes (Miyoshi et al., 2016). However, studies in some vertebrates suggest highly heritable patterns of DNA methylation despite embryonic erasure (Potok et al., 2013), and heritable epigenetic lines are well known in plants (Herman and Sultan, 2011), emphasizing that DNA methylation patterns are often under genetic control (Riviere et al., 2017; Verhoeven et al., 2016). Therefore, in order to assess the evolutionary potential of epigenetic marks, we need to understand the links between DNA methylation and phenotypic plasticity as well as tease apart the extent that variation in DNA methylation is under genetic or environmental control (Verhoeven et al., 2016). Although differences in DNA methylation reported in our study could be due to different genotypes of the mothers between treatments or stochastic epimutation, our results maintain that the environment can impact DNA methylation variation across generations (Fig. 2); and further, that these molecular changes are associated with a change in phenotype (Fig. 3). Specifically, in the companion study (Wong et al., 2019), maternal conditioning affected body size of early stages. In addition, the transcriptome exhibits a similar overall pattern to the transgenerational effect on DNA methylation (Fig. 2), where maternal environment explained large shifts in the transcriptome of the offspring, which was also correlated with differences in body size (Wong et al., 2018). While these results suggest that DNA methylation could play a role in modulating the phenotypes of offspring in sea urchins, additional investigations are required to demonstrate the cause-effect relationships between DNA methylation, gene expression and phenotype. Additionally, it is likely that other parental factors such as genomic background, lipid content, proteins and small RNAs are involved as well (Hamdoun and Epel, 2007; McAlister and Moran, 2012; Moran and Emlet, 2001; Torda et al., 2017).

In general, there is growing interest in the role of epigenetic mechanisms in modulating phenotypes in response to the environment, particularly in response to stress. DNA methylation changes have been associated with exposure to high $p\text{CO}_2$ in corals (Liew et al., 2018), harmful algal blooms in oysters (Gonzalez-Romero et al., 2017), and thermal stress in fish (Metzger and Schulte, 2017; Ryu et al., 2018). In addition, there is growing literature that associates DNA methylation changes with flexibility in the transcriptome (Anastasiadi et al., 2018; Liew et al., 2018; Roberts and Gavery, 2012). In vertebrate systems, promoter methylation is associated with transcriptional silencing (Stancheva et al., 2002; Weber et al., 2007). However, promoter methylation is not commonly found across invertebrates. In the current study, we see differential methylation of putative promoter regions (regions 1 kb upstream of the gene) (Fig. 2E & F). Across the invertebrate/vertebrate boundary, it has been shown that the effect of promoter methylation on transcription depends on the DNA methylation of nearby regions (Keller et al., 2016). This suggests that the effect of promoter DNA methylation on transcription in basal deuterostomes such as urchins may be complicated and context-dependent. However, our results provide a valuable first assessment of putative promoter methylation in an environmental context in *S. purpuratus*, suggesting this is another potential mechanism that could influence phenotypes. This would need to be thoroughly investigated with follow-up studies.

GBM, on the other hand, is observed across most metazoan taxa. How GBM regulates transcription is not straightforward, although there is a clear relationship between GBM and gene function. Highly methylated genes tend to be more highly and stably expressed, and lowly methylated genes are those that are more transcriptionally flexible, and associated with functions well-known to be involved in responses to the environment (Dimond and Roberts, 2016; Dixon et al., 2014; Gavery and Roberts, 2014; Liew et al., 2018; Roberts and Gavery, 2012). Importantly for environmental epigenetics, studies are emerging that demonstrate a link between the extent that genes are methylated, and how transcription is regulated. Research on the scleractinian coral, *Stylophora pistillata*, demonstrated that DNA methylation within gene bodies reduced transcriptional noise and levels of spurious transcription (Liew et al., 2018), an observation also found in mammals (Neri et al., 2017), suggesting the function of DNA methylation within gene bodies could be broadly conserved across animals. In other work on scleractinian coral species, DNA methylation within gene bodies also impacted codon usage and was associated with substitution rates, illuminating other possible ancestral evolutionary functions of DNA methylation (Dixon et al., 2016). As the research community continues to unravel the functions of GBM, the role of the environment in inducing DNA methylation variation is beginning to be understood. The study presented here suggests that the environment experienced by mothers could modulate DNA methylation across a generation, potentially impacting phenotypes in a transgenerational framework.

4.2. DNA methylation in the context of development

We found very little differential methylation between developmental stages, despite the dramatic morphological changes that occur across development from blastula to prism (Fig. 2D, H). We did however see significant enrichment of GO categories in pairwise comparisons between stages (Fig. S4). These somewhat confounding results could suggest there are broad categories of genes that show differences in GBM across these life-history stages, but not enough differences in single genes to be detected with the tests in DESeq2. In other deuterostomes, such as mammals, DNA methylation is essential for development, shaping cell differentiation by silencing pluripotency factors and germline-specific cells (Smith and Meissner, 2013). In invertebrates much less is known about how DNA methylation regulates development, let alone how epigenetic mechanisms could be involved in the well-characterized gene regulatory networks underlying echinoderm development (Cary and Hinman, 2017; Davidson, 2006). To complicate matters, patterns of DNA methylation during development have been investigated in only a small subset of invertebrate taxa: mollusks (García-Fernández et al., 2017; Riviere et al., 2017,2013) and insects (Bonasio et al., 2012; Elango et al., 2009; Glastad et al., 2016; Smith et al., 2012). In oysters, patterns of methylation in oocytes are maintained throughout development, implying that these patterns are inherited (Riviere et al., 2017). In addition, dynamic restructuring of DNA methylation occurs during early cleavage events and metamorphosis, with little differences in DNA methylation patterns across developmental stages ranging from morula to D-stage larvae (Riviere et al., 2017). These findings, coupled with our own results showing minimal differences in GBM between blastula and prism, highlight research areas that could potentially shed more light onto the relationship between epigenetics and the gene regulatory networks responsible for regulating large scale developmental processes. Firstly, future work examining developmental DNA methylation patterns should widen its scope to include early cleavage events or metamorphic transitions. In addition, future investigations of any developmental transition may be better served by assaying other epigenetic mechanisms (histone modifications, small RNAs, etc.) in addition to DNA methylation. Our data suggests that DNA methylation during development is robust to environmental differences in pH. However, differential methylation in developmental genes was observed in oysters exposed to toxic levels of copper, suggesting DNA methylation could have very targeted and specific effects on developmental processes when experiencing specific types of environmental stressors (Sussarellu et al., 2017).

4.3. Maternal influences on DNA methylation and phenotypes in urchins

The maternal conditioning environment impacted DNA methylation of specific functional groups of genes (Fig. S5). We see differential methylation of genes involved in signaling pathways, modification of macromolecules and lipid metabolic processes (Fig. S5). This suggests that differences in DNA methylation induced by maternal condition may have impacts on physiological and sensory phenotypes in offspring. Further, we see correlations between maternal conditioning effects on DNA methylation and body size of the offspring (Fig. 3B), an important phenotype in early life history stages of marine invertebrates (Marshall et al., 2003; Marshall and Keough, 2003). A previous study with a similar experimental design found differential gene expression in offspring of functional categories associated with metabolic processes when mothers were conditioned to upwelling or non-upwelling conditions (Wong et al., 2018). Despite broad similarities between the functional enrichments of transcriptomic and DNA methylation differences, how transcription and DNA methylation interact to influence phenotypes will need to be investigated further on a gene-by-gene basis. However, this study highlights that DNA methylation may be playing a role in mediating phenotypes in response to the environment in urchins.

DNA methylation and epigenetic mechanisms in general are rarely studied as they are here, that is, in a transgenerational context in response to environmentally relevant abiotic conditions. However, transgenerational effects alone are common in marine invertebrates, and have been observed before in sea urchins (Ross et al., 2016). In green urchins (*Stronglocentrotus droebachiensis)*, adult exposure to high $p\mathrm{CO}_2$ reduced female fecundity and survivorship of the progeny over acute adult exposures, a result that dissipated after a long-term adult exposure (Dupont et al., 2013). A similar result was also observed in the Antarctic sea urchin (*Sterechinus neumayeri*), where short-term adult acclimations to different temperature regimes resulted in variation in percentage of hatching and larval survival, but these negative stress impacts dissipated with longer adult conditioning (Suckling et al., 2015). Lastly, in a tropical sea urchin, *Echinometra mathaei*, adult exposure to elevated $p\mathrm{CO}_2$ for seven weeks did not increase larval resilience to stressful $p\mathrm{CO}_2$ conditions (Uthicke et al., 2013). These studies highlight that preconditioning adult urchins to stressful conditions can cause detrimental carry-over effects on offspring as opposed to priming them to tolerate stress better than previous generations, as is seen in behavioral experiments in coral reef fish (Donelson et al., 2011; Miller et al., 2012). In our experimental design, adult urchins were conditioned to combinations of temperature and $p\mathrm{CO}_2$ periodically or chronically experienced in their natural environments, therefore potentially illuminating TGP in a more natural context. For example, in *S. purpuratus*, it has been shown that differences in maternal environment (upwelling versus non-upwelling) impacted body size and expression of the embryonic transcriptome (Wong et al., 2018). In addition to maternally driven differences in expression of metabolic and ion transport genes in the offspring, the authors observed differences in expression of genes related to epigenetic processes and suggested changes in the epigenome could be responsible for TGP effects in sea urchins (Wong et al., 2018). Our results indicate that the maternal environment did in fact lead to differences in DNA methylation in the progeny, suggesting epigenetic processes may be involved in modulating gene expression in a transgenerational framework in *S. purpuratus*.

5. Summary

The role of epigenetics in phenotypic plasticity is being explored in marine invertebrates in an environmental context (Beal et al., 2018; Eirin-Lopez and Putnam, 2019; Hofmann, 2017; Torda et al., 2017). In a study on an Antarctic marine polychaete, Marsh and Pasqualone (2014) found that DNA methylation was associated with metabolic shifts in response to temperature change. In oysters, global hypomethylation was observed after exposure to toxic algal blooms (Gonzalez-Romero et al., 2017), potentially influencing higher transcriptional flexibility. In corals, there is a correlation between GBM and gene expression flexibility in genotypes that were reciprocally transplanted across sites in the Great Barrier Reef (Dixon et al., 2014). These studies emphasize that DNA methylation likely plays a role in mediating diverse organism-environment interactions in marine invertebrates. Our study contributes knowledge regarding environmental impacts on DNA methylation in a transgenerational context, and its possible implications concerning phenotypic plasticity. Future work will aim to further illuminate the connection between DNA methylation, regulation of gene expression, and stress-resistant phenotypes.

We are just beginning to understand mechanisms of phenotypic plasticity in organisms with complex life-histories in a global change context. Advancing our understanding requires more knowledge on the degree to which environmental change impacts different life-history stages, and how transmittable these effects are across the life-cycle and across generations (Burgess and Marshall, 2014). For example, in intertidal gastropods, early stages have lower tolerance to extreme temperature stresses, but have better survivorship when exposed to moderate thermal stress when compared to adults (Truebano et al., 2018). Such outcomes highlight that it is critical to consider how flexible organism-environment interactions are between life-history stages, an eco-devo perspective (Sultan, 2015). In this study, we found evidence of transgenerational effects that could be explained by differences in DNA methylation driven by adult conditioning in *S. purpuratus*. This result begins to address the extent to which the epigenome mediates rapid responses to variable abiotic conditions, urging further investigation into the transgenerational transmission of epigenetic information, as this could be an important mechanism enabling the transfer of environmental information to subsequent generations on short time scales.

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Competing interests

The authors declare no competing interests.

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Data availability

Scripts and analysis walkthrough are available on a Github repository (https://github.com/mariestrader/S.purp_MeDIP) and fastq sequences are avaliable through the NCBI Short Read Archive under the accession PRJNA525826.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2019.03.002.

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