

UNIVERSITY OF CALIFORNIA

Santa Barbara

Investigating the Response of Sea Urchin Early Developmental Stages to Multiple Stressors
Related to Climate Change

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Ecology, Evolution and Marine Biology

by

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by

Juliet Marie Wong

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ABSTRACT

Investigating the Response of Sea Urchin Early Developmental Stages to Multiple Stressors Related to Climate Change

by

Juliet Marie Wong

Within climate change biology, the red sea urchin *Mesocentrotus franciscanus* has remained relatively overlooked despite its sizeable ecological and economic importance, particularly within the context of multi-stressor effects. I assembled and described a developmental transcriptome for *M. franciscanus*, providing a useful molecular resource with which to study this organism. I then examined both the physiological and molecular mechanisms that underlie the response of early developmental stage (EDS) *M. franciscanus* to different combinations of pH levels and temperatures that represented ecologically relevant present and future ocean conditions. Elevated $p\text{CO}_2$ levels decreased embryo body size, but at the prism embryo stage, warmer temperatures helped to offset this via an increase in body size. Warmer temperatures also slightly increased the thermal tolerance of prism stage embryos. Neither $p\text{CO}_2$ nor temperature stressors affected prism metabolic rate as measured by rate of oxygen consumption. Gene expression patterns differed by developmental stage and by temperature exposure. Elevated temperatures led to an up-regulation of cellular stress response genes. Under colder temperatures, the embryos exhibited an up-regulation of epigenetic genes related to histone modifications. There was a

comparatively minimal transcriptomic response to different $p\text{CO}_2$ levels. Examining the physiological and molecular responses of EDS *M. franciscanus* to multiple stressors provided much needed information regarding a species of significant ecological and economic value by examining its capacity to respond to stressors related to climate change and ocean acidification under an ecologically relevant context.

I also investigated the role of transgenerational plasticity (TGP), in which the environmental conditions experienced by parents affect progeny phenotypes. TGP may provide a valuable mechanism by which organisms can keep pace with relatively rapid environmental change. Adult *S. purpuratus* were conditioned to two divergent, but ecologically relevant pH levels and temperatures throughout gametogenesis. The adults were spawned and crossed, and their progeny were raised under different pH levels to determine if maternal conditioning impacted the response of the progeny to low pH stress. I investigated maternal provisioning, a mechanism of TGP, by measuring the size, total protein content, and total lipid content of the eggs that they produced. Acclimatization of the adult urchins to simulated upwelling conditions (combined low pH, low temperature) appeared to increase maternal provisioning of lipids to the eggs but did not affect egg size or protein content. I also investigated the physiology and gene expression of progeny responding to low pH stress, which were affected more by maternal conditioning than by offspring pH treatment. Maternal conditioning to simulated upwelling resulted in larger offspring body sizes. Additionally, I found the progeny expressed differential regulatory patterns of genes related to epigenetic modifications, ion transport, metabolic processes and ATP production. This work showed that adult exposure to upwelling conditions can improve the resilience of EDS progeny to low pH levels.

TABLE OF CONTENTS

I. Introduction.....	1
Statement of the problem.....	1
Biological responses to ocean change.....	3
Sea urchins as a study system.....	5
The objective.....	7
Chapter II: Transcriptional profiles of early stage red sea urchins (<i>Mesocentrotus franciscanus</i>) reveal differential regulation of gene expression across development	9
Chapter III: The effects of temperature and $p\text{CO}_2$ on the size, thermal tolerance and metabolic rate of the red sea urchin (<i>Mesocentrotus</i> <i>franciscanus</i>) during early development.....	9
Chapter IV: Gene expression patterns of red sea urchins (<i>Mesocentrotus</i> <i>franciscanus</i>) exposed to different combinations of temperature and $p\text{CO}_2$ during early development	10
Chapter V: Transgenerational effects in an ecological context: Conditioning of adult sea urchins to upwelling conditions alters maternal provisioning and progeny phenotype	11
Chapter VI: Transcriptomics reveal transgenerational effects in purple sea urchins exposed to upwelling conditions, and the response of their progeny to differential $p\text{CO}_2$ levels	11
II. Transcriptional profiles of early stage red sea urchins (<i>Mesocentrotus franciscanus</i>) reveal differential regulation of gene expression across development.....	13

Abstract.....	13
Overview	13
Introduction.....	14
Materials and methods.....	18
Animal collection and culturing.....	18
RNA extractions and sequencing	20
<i>De novo</i> transcriptome assembly.....	21
Gene prediction and functional annotation.....	22
Expression quantitation and differential expression analyses.....	23
Survey of the maternal-to-zygotic transition.....	24
Results and discussion.....	27
Culturing conditions	27
Summary statistics of the transcriptome assembly and annotation.....	27
Gene expression patterns in eggs and throughout early development.....	30
The maternal-to-zygotic transition	40
Conclusions.....	47
Data deposition	48
Acknowledgments.....	48
Supplementary material.....	48

III. The effects of temperature and $p\text{CO}_2$ on the size, thermal tolerance and metabolic rate of the red sea urchin (*Mesocentrotus franciscanus*) during early development..... 50

Abstract.....	50
Introduction.....	50

Materials and methods.....	53
Animal collection and culturing.....	53
Sampling	55
Morphometrics	55
Thermal tolerance.....	56
Respirometry	58
Results	59
Culturing of embryos.....	59
Thermal tolerance.....	61
Respiration rate.....	64
Discussion.....	66
Only $p\text{CO}_2$ impacts gastrula size.....	66
Temperature and $p\text{CO}_2$ impact prism size	67
Temperature impacts thermal tolerance.....	69
Metabolic rate is unaffected by experiment treatments.....	70
Conclusions.....	71
Acknowledgements	73

IV. Gene expression patterns of red sea urchins (*Mesocentrotus franciscanus*) exposed to different combinations of temperature and $p\text{CO}_2$ during early development..... 74

Abstract.....	74
Overview	74
Introduction.....	75
Materials and methods.....	76

Sample generation	76
Results	79
Summary statistics and overview of RNA-seq	79
Expression differences driven primarily by developmental stage.....	79
Differential expression and WGCNA results.....	82
Temperature affected gastrula gene expression patterns	85
Discussion.....	91
Transcriptomic patterns varied by developmental stage.....	91
Temperature influences cellular stress response	92
Temperature influences epigenetic processes	94
Temperature influences prism metabolism and motility	96
Limited transcriptomic response to $p\text{CO}_2$ conditions.....	97
Acknowledgements	101
Supplementary material.....	101
V. Transgenerational effects in an ecological context: Conditioning of adult sea urchins to upwelling conditions alters maternal provisioning and progeny phenotype.....	102
Abstract.....	102
Overview	103
Introduction.....	104
Material and methods	109
Animal collection and conditioning	109
Spawning and egg assessment.....	110
Egg morphometric analyses	111

Protein quantification.....	112
Lipid content	113
Principal component analysis.....	115
Embryo and larval culturing.....	116
Results	121
Outcome of the adult conditioning phase and raising cultures of offspring	121
Assessment of the eggs.....	124
Egg morphometric analyses	124
Protein quantification.....	128
Lipid content	128
Principal component analysis.....	133
Morphometric analyses of embryos and larvae.....	135
Discussion.....	137
Impact of differential maternal conditioning on eggs.....	138
Impact of differential maternal conditioning on early developmental stages	
.....	142
Mechanisms of transgenerational plasticity.....	146
Acknowledgements	149
Data accessibility	149

VI. Transcriptomics reveal transgenerational effects in purple sea urchins exposed to upwelling conditions, and the response of their progeny to differential *p*CO₂ levels.. 150

Abstract.....	150
Overview	151

Introduction.....	151
Materials and methods.....	157
Animal collection and conditioning	157
Spawning and culturing	158
Sample collections.....	162
Morphometrics	162
RNA extractions and sequencing	163
Data processing and DE analyses.....	164
WGCNA	165
Functional gene enrichment.....	166
KEGG analyses	166
Results	167
Parental conditioning and carbonate chemistry	167
Morphometric analyses.....	169
Summary statistics and overview of RNA-seq	172
Influence of adult conditioning on the gastrula transcriptome.....	172
Pairwise comparisons of the gastrula transcriptomes.....	175
Comparison I: Different maternal conditions + progeny raised under low <i>p</i> CO ₂ – UL vs. NL.....	175
Comparison II: Different maternal conditions + progeny raised under high <i>p</i> CO ₂ – UH vs. NH	178
Comparison III: Mothers conditioned to non-upwelling + different progeny <i>p</i> CO ₂ conditions – NH vs. NL	178

Comparison IV: Mothers conditioned to upwelling + different progeny $p\text{CO}_2$ conditions – UH vs. UL	179
WGCNA – data summary and module overview.....	182
Module eigengene, “red”	184
Module eigengene, “green”.....	184
Module eigengene, “blue”.....	184
Module eigengene, “black”.....	185
KEGG analyses	187
Discussion.....	189
Maternal influence on epigenetic processes.....	191
Maternal influence on metabolic processes	192
TGP in the response to high $p\text{CO}_2$	193
Data accessibility	198
Acknowledgements	198
Supplementary material.....	198
References	199

LIST OF FIGURES

- Figure 1.** Diagram of major dissertation goals and concepts. This research incorporated a combination of molecular and physiological approaches to investigate the effects of multiple stressors on the early development of the red sea urchin, *Mesocentrotus franciscanus*, and the purple sea urchin, *Strongylocentrotus franciscanus*. This dissertation also investigated the role of transgenerational plasticity and its impacts on how early developmental stages respond to environmental stress..... 8
- Figure 2.** PCA of *Mesocentrotus franciscanus* eggs and early developmental stages showing the first two dimensions and hierarchical clustering of the samples. Sample colors denote the different stages, which include: egg (EG), 8- to 16-cell (CL), morula (MO), blastula (BL), gastrula (GA), prism (PR), and pluteus (PL). Hierarchical clustering show two main clusters (1 and 2) that each contain two clusters (a and b). 32
- Figure 3.** WGCNA identified significant correlations between module eigengenes (rows) and stages (columns). The stages are: egg (EG), 8- to 16-cell (CL), morula (MO), blastula (BL), gastrula (GA), prism (PR), and pluteus (PL). The number of genes within each module eigengene is noted in parentheses following each color name. The red-blue color scale represents the strength of the correlation (1 to -1). Each correlation value (r^2) is followed by a *p*-value in parentheses. Hierarchical clustering of the module eigengenes revealed three primary clusters of gene expression (1-3)..... 33
- Figure 4.** The expression of putative genes that play a functional role during the MZT. These genes **A.** regulate the removal of mRNA, and **B.** regulate zygotic development. The data are in \log_2 counts per million reads (\log_2 CPM) expressed at each stage of development:

egg (EG), 8- to 16-cell (CL), morula (MO), blastula (BL), gastrula (GA), prism (PR), and pluteus (PL)..... 43

Figure 5. Heatmaps of relative expression profiles for transcripts that are or are not maternally expressed. The rows are transcripts and the columns are in order of developmental stage: egg (EG), 8- to 16-cell (CL), morula (MO), blastula (BL), gastrula (GA), prism (PR), and pluteus (PL). Transcript expression data are in log₂ counts per million reads (log₂ CPM), and the data are scaled by row. The top heatmap contains the top 500 maternal transcripts expressed at EG (i.e., transcripts that are maternally expressed). The bottom heatmap contains 19,044 transcripts with a negative expression value of log₂ CPM (log₂ CPM < 0) at EG (i.e., transcripts that are not maternally expressed). 44

Figure 6. Body size measurements of embryos under different temperature and pCO₂ treatment conditions, including **A.** the length of the gastrula stage, **B.** the 2D area of the gastrula stage, and **C.** the length of the prism stage. Bars show standard error. 60

Figure 7. Thermal tolerance of prism stage embryos following 1-hour temperature exposures. Points are plotted as percent survivorship with the lines showing the logistic regression for each treatment..... 63

Figure 8. Respiration rates (expressed as pmol O₂ per hour per individual) for prism embryos raised under different temperature and pCO₂ treatment conditions. Error bars show standard error. 65

Figure 9. Principal component analysis (PCA) plot showing distances between all samples. 81

Figure 10. Principal component analysis (PCA) plots showing sample-to-sample distances at the **A.** gastrula and **B.** prism stages. 81

Figure 11. Histogram displaying the number of differentially expressed genes (gene counts) for each pairwise comparison of interest determined using LIMMA (FDR = 0.05)... 83

Figure 12. The WGCNA identified significant correlations between module eigengenes (rows) and traits (columns). The traits of interest included temperature and $p\text{CO}_2$ treatments for each of the two developmental stages. The red-blue color scale represents the strength of the correlation (1 to -1). Each correlation value (r^2) is followed by a p -value in parentheses. For the temperature traits, correlations >0 are positively correlated to 17 °C and negatively correlated to 13 °C (and vice versa for correlations <0). For the $p\text{CO}_2$ traits, correlations >0 are positively correlated to 1,050 μatm and negatively correlated to 475 μatm (and vice versa for correlations <0). The number of genes within each module eigengene is noted in parentheses following each color name. 84

Figure 13. Body size was measured using ImageJ (National Institutes of Health, USA). Eggs size was determined by **A.** taking the average of three independent diameter measurements, and **B.** measuring the 2-dimensional (2D) area. The average diameter was subsequently used to calculate egg volume by assuming the eggs were spherical in shape. Body size of the hatched blastula stage was determined by **C.** measuring the maximum length, from the animal to vegetal end, and **D.** measuring the 2D area. Body size of the gastrula stage was determined by **E.** measuring the maximum length, from the anterior to posterior end, when measured across the center of the archenteron, and **F.** measuring the 2D area. Body size of the prism stage was determined by **G.** measuring the tip of the body rod to the tip of the postoral rod. Body size of the echinopluteus stage was determined by **H.** measuring the length of the left postoral arm, from the spicule tip of the postoral arm to the spicule tip of the aboral point..... 112

Figure 14. Cross design of adult urchins conditioned under treatments that mimic non-upwelling (N: ~650 $\mu\text{atm } p\text{CO}_2$, ~17 °C) or upwelling (U: ~1,300 $\mu\text{atm } p\text{CO}_2$, ~13 °C) conditions observed in the kelp forest ecosystem (Kapsenberg and Hofmann 2016; Rivest et al. 2016). Embryos were raised at the same temperature (~15 °C) under low $p\text{CO}_2$ (L: ~450 μatm) or high $p\text{CO}_2$ (H: ~1,050 μatm) conditions. This resulted in four embryo treatments: 1) progeny of females that experienced non-upwelling conditions raised under low $p\text{CO}_2$ levels (NL), (2) progeny of females that experienced non-upwelling conditions raised under high $p\text{CO}_2$ levels (NH), (3) progeny of females that experienced upwelling conditions raised under low $p\text{CO}_2$ levels (UL), and (4) progeny of females that experienced upwelling conditions raised under high $p\text{CO}_2$ levels (UH). 117

Figure 15. Temperature and $p\text{CO}_2$ conditions for the duration of the adult and embryo/larval conditioning periods. The arrow denotes an anomaly due to an unexpected campus-wide seawater pump failure during system maintenance, in which all conditions experienced >3,000 $\mu\text{atm } p\text{CO}_2$ for less than 24 hours. Error bars represent +/- the standard deviation across the three replicate tanks (maternal conditioning) or cultures (larval conditioning). 123

Figure 16. Size differences across developmental stages of offspring, with error bars showing standard error. **A.** The average diameter of eggs from female urchins conditioned under either non-upwelling (N) or upwelling (U) treatments. The average length of developmental stages, including **B.** hatched blastula, **C.** gastrula, **D.** prism, and **E.** echinopluteus larvae stages of each of the four treatment types (i.e., NL, NH, UL, and UH). 126

Figure 17. Size differences across eggs and developmental stages of offspring, with error bars showing standard error. **A.** The average 2D area of eggs from female urchins conditioned under either non-upwelling (N) or upwelling (U) treatments, and the average 2D area of **B.** hatched blastula and **C.** gastrula stages of each of the four treatment types (i.e., NL, NH, UL, and UH)..... 127

Figure 18. Lipid content (ng/egg) of eggs from females conditioned to the non-upwelling treatment (N) and females conditioned to the upwelling treatment (U), with error bars showing standard error. Three lipid classes were measured including triacylglycerols (TAG; Tripalmitin), sterol (ST; cholesterol) and phospholipids (PL; L- α -phosphatidylcholine). Total is the total of all three lipid classes. Any significant difference between treatments is marked with an asterisk (*)..... 131

Figure 19. Per egg lipid density (ng/nl) of eggs produced by females conditioned to the non-upwelling treatment (N) and females conditioned to the upwelling treatment (U), with error bars showing standard error. Three lipid classes were measured including triacylglycerols (TAG; Tripalmitin), sterol (ST; cholesterol) and phospholipids (PL; L- α -phosphatidylcholine). Total is the total of all three lipid classes (TAG + ST + PL)... 132

Figure 20. A PCA biplot of the eggs from females conditioned to the non-upwelling treatment (N) and from females conditioned to upwelling treatment (U), using a combination of the egg data, including: 1) size (average egg diameter), 2) protein (the average ng of protein per egg), and 3) lipid (the average ng of total lipid per egg)..... 134

Figure 21. Experimental design showing crosses of non-upwelling- and upwelling-conditioned females with a single non-upwelling-conditioned male. The embryos from

each cross were placed into either low or high $p\text{CO}_2$ culturing vessels (with three replicates each), resulting in four treatment types: NL, NH, UL, and UH..... 161

Figure 22. Average diameter of eggs from each female urchin, with error bars showing standard error. Eggs were collected from five females conditioned under the non-upwelling treatment (i.e., N1 – N5) and from seven females conditioned under the upwelling treatment (i.e., U1 – U7). 170

Figure 23. Average length of gastrulae from each of the four treatment types (i.e., NL, NH, UL, and UH), with error bars showing standard error. Any treatments that do not share assigned letters (i.e., a, b, or c) are significantly different from one another. 171

Figure 24. Global RNA-seq patterns of the gastrula stage progeny. **A.** Principal Component Analysis (PCA) plot showing distances between samples. The PCA was performed on gene counts that had undergone filtering and voom transformation using *limma*. **B.** Histogram and Venn diagram displaying the amount of differentially expressed genes (FDR = 0.05) for each treatment pairwise comparison of interest determined using *limma*. 174

Figure 25. Log-fold change in expression of differentially expressed genes in response to high versus low $p\text{CO}_2$ treatment of the offspring, separated by maternal treatment. A line with slope = 1 is shown. 181

Figure 26. WGCNA, including module generation and their correlation to experiment treatments. **A.** Cluster dendrogram of all 13,967 genes, with dissimilarities based on topological overlap. Modules are assigned below, including modules remaining after merging based on a cut height of 0.26. **B.** Significant relationships between module eigengenes (rows) and treatments (columns) in which the color scale (red-blue)

represents the strength of the correlation (1 to -1). Each correlation is represented by a r^2 value and p-value in parenthesis..... 183

LIST OF TABLES

Table 1. Transcripts that play a functional role during the MZT.	26
Table 2. Statistics of <i>de novo</i> transcriptome assembly.	29
Table 3. Top 10 KEGG pathways in the transcriptome.	29
Table 4. Select GO term results from functional enrichment analyses of WGCNA module eigengenes.	38
Table 5. Seawater conditions throughout embryological development. All values are given as mean \pm standard deviation.	60
Table 6. LT ₁₀ , LT ₂₅ , and LT ₅₀ values for each treatment. All values are given as mean \pm standard error.	63
Table 7. Select GO term results from functional enrichment analyses of genes that were differentially expressed in each pairwise comparison of interest and in each WGCNA module eigengene.	88
Table 8. Average (\pm standard deviation) carbonate chemistry parameters and temperatures for adult conditioning (N or U) and offspring culturing (L or H). The $p\text{CO}_2$ and Ω_{Arag} were calculated from measured pH, salinity (33.1 ± 0.1), and total alkalinity values ($2211.25 \pm 7.10 \mu\text{mol kg}^{-1}$).	122
Table 9. Average (\pm standard deviation) sizes, protein contents and densities, lipid contents and densities, and energy equivalents and densities of eggs produced by females conditioned to the Non-upwelling treatment (N) and females conditioned to the Upwelling treatment (U).	126

Table 10. Seawater conditions during the experiment. Adult conditioning included additional temperature differences to mimic non-upwelling and upwelling conditions. Values are given as mean \pm standard deviation. 168

Table 11. Select GO term results from functional enrichment analyses of genes that were differentially expressed in each pairwise comparison of interest. 176

Table 12. Select GO term results from functional enrichment analyses of genes within WGCNA module eigengenes. 186

I. Introduction

Statement of the problem

Atmospheric concentrations of carbon dioxide (CO₂) have risen steadily since the Industrial Revolution (Godbold and Calosi 2013; IPCC 2013). Recent global atmospheric CO₂ levels have reached historic modern-day highs of 411.84 ppm as recorded at Mauna Loa (Dlugokencky and Tans 2016), and these levels are predicted to continue to rise (Godbold and Calosi 2013; IPCC 2013). Ocean warming and ocean acidification (OA) are both major consequences of increasing atmospheric CO₂. Indications of warming seas and reductions in pH have already been recorded across the world's oceans, and these changes in ocean conditions are predicted to continue (IPCC 2013). As ocean conditions are altered, marine organisms must face the changing environments that they inhabit.

Surface ocean temperatures are predicted to increase by 0.6 – 2.0 °C by the end of this century (IPCC 2013). Additionally, prolonged warming events (i.e., marine heat waves) are predicted to increase in frequency, duration, and intensity (Oliver et al. 2018). The global area affected by marine heat waves has risen in recent decades (Oliver et al. 2018). Already, marine heat waves have been associated with large changes in community composition, species range shifts, and mass mortalities (Frölicher and Laufkötter 2018; Ummenhofer and Meehl 2017). For example, a warming event in 2015 to 2016 led to bleaching in 91.1% of corals surveyed on the Great Barrier Reef (Hughes et al. 2017). In the northeast Pacific Ocean, warming events associated with “the Blob” occurred between 2013 and 2016 (Gentemann et al. 2017; Hu et al. 2017), leading to northward geographical shifts of several species as well as mass strandings of many marine birds and mammals (Bond et al. 2015; Cavole et al. 2016). Ocean warming can also have significant economic impacts if important

marine fisheries are negatively impacted by rising temperatures (Bond et al. 2015; Cavole et al. 2016; Frölicher and Laufkötter 2018).

In addition to ocean warming, pH levels are predicted to decrease by 0.1 – 0.4 units by the end of the 21st century as a result of OA (IPCC 2013). Decreasing pH conditions can negatively impact marine organisms across a variety of taxa via a reduction in calcium carbonate available for calcification, acid-base imbalance, and a reduction in capacity for oxygen transport (Doney et al. 2009; Fabry et al. 2008). Furthermore, while OA is the primary process affecting pH in the open ocean, coastal systems can be highly dynamic with much more complex trends in changing pH levels (Doney et al. 2009; Duarte et al. 2013; Fabry et al. 2008; Feely et al. 2010; Feely et al. 2008; Salisbury et al. 2008; Wootton et al. 2008). For example, upwelling processes that bring low pH waters to the surface can cause the pH in certain coastal areas to be highly variable across both time and space (Feely et al. 2008; Gruber et al. 2012; Hofmann et al. 2010).

While many studies have focused on single stressor effects on marine organisms, it is important to consider the effects of multiple, simultaneous stressors. Factors such as ocean temperature and pH will both be altered as a result of climate change, and as a result, marine organisms may be exposed to simultaneous high temperatures and high $p\text{CO}_2$ levels. At other times, such as during upwelling events, organisms may be exposed to a combination of low temperatures with high $p\text{CO}_2$ levels. Regardless, failing to consider continuing ocean warming and acidification, and the dynamic nature of marine environments, may lead to inaccurate assessments regarding how marine organisms are responding to their current environments, and predictions of how they will respond to future ocean conditions (Bockmon et al. 2013; Boyd et al. 2018; Byrne and Przeslawski 2013). Therefore, it is important to

examine the effects of multiple stressors in an ecologically relevant context that reflect realistic current and future ocean conditions (Frieder et al. 2014; Przeslawski et al. 2015).

Biological responses to ocean change

Gaining an understanding of how organisms are currently responding to environmental stressors as well as their potential to respond to future environmental conditions is necessary for predicting how populations, communities, and ecosystems will be impacted by continuing climate change. A given environmental stressor can have variable impacts on an organism depending on the species, its ontogeny, the population location in which it is found and its given evolutionary history, and the intensity and duration of exposure (Breitburg et al. 2015). Effects may be either lethal or sublethal (Przeslawski et al. 2015). Furthermore, different stressors may have different effects, particularly when they occur simultaneously. In general, there are three major classifications of stressor interactions: (1) additive effects in which the combined effect is equal to the sum of the individual effects, (2) synergistic effects in which the combined effect is greater than the sum of the individual effects, and (3) antagonistic effects in which the combined effect is less than the sum of the individual effects (Folt et al. 1999; Griffith et al. 2011). Additive effects may occur when each individual stressor does not interact with one another, or if one or more of the stressors has no significant effect (Przeslawski et al. 2015). Synergistic effects occur when stressors interact in combination (Chen and Stillman 2012; Folt et al. 1999; Melzner et al. 2013). For instance, hypercapnia may cause an organism to be more susceptible to warmer temperatures or vice versa, resulting in greater vulnerability than if the exposure was to either low pH levels or high temperatures alone (Kroeker et al. 2013; O'Donnell et al. 2009; Zippay and Hofmann 2010). Antagonistic effects occur when one stressor offsets another (Crain et al.

2008; Folt et al. 1999). In some organisms, for example, low pH may hamper development, but warmer temperatures that stimulate development may help compensate for this effect (Byrne and Przeslawski 2013).

Upon investigating the response of marine organisms to environmental stress, it is important to consider the life history of the study species in question. An organism's phenology will influence the timing, intensity, and type of stressor it is exposed to as well as its stage of life during exposure (e.g., embryological, larval, juvenile, or reproductive adult). The early developmental stages (EDSs) of many marine animals, such as during embryological and larval stages, may be particularly vulnerable to environmental stressors (Byrne 2011; Dupont and Thorndyke 2009; Gosselin and Qian 1997; Kurihara 2008). Detrimental effects that occur early in an organism's life history can negatively carry over into later life stages or lead to population bottlenecks if mortality occurs (Byrne 2012; Byrne and Przeslawski 2013; Kurihara 2008). Therefore, it is valuable to examine the capacity of these potentially vulnerable EDSs to respond to environmental stressors such as changing temperatures or pH conditions.

The environmental history of the parental generation and the potential for transgenerational plasticity (TGP) are factors that may influence EDS performance under stress. Although these factors are often overlooked, understanding transgenerational effects may be necessary to comprehending early stage tolerance to multiple stressors and accurately predicting future responses. Most multistressor studies, often limited by long generation times, only involve one generation (Chen and Stillman 2012). However, the experience of the parental generation can shape the phenotype of their offspring (Bondurianski and Day 2009; Munday 2014; Parker et al. 2015). Transgenerational effects occur across generations, but do

not involve the direct transmission of DNA sequences (Bondurianski and Day 2009). Thus, transgenerational effects of the parent's environment exposures on the development of the offspring may be transmitted somatically, cytoplasmically, nutritionally, or epigenetically (Munday 2014). This transgenerational acclimation, or transgenerational plasticity, may provide a valuable mechanism by which species may cope and keep pace with rapid environmental changes (Bossdorf et al. 2008; Salinas and Munch 2012). In contrast, transgenerational effects may be maladaptive if there is a negative parental response (Putnam and Gates 2015), such as a reduction in gamete quality, or if offspring are poorly prepared due to a mismatch between the parent's and the progeny's environmental conditions (Marshall 2008; Munday 2014).

Sea urchins as a study system

In this dissertation, I investigated two temperate sea urchin species, the red urchin, *Mesocentrotus franciscanus*, and the purple urchin, *Strongylocentrotus purpuratus*, that are both found in kelp forest ecosystems along the western coast of North America. These species are excellent study systems with which to investigate the effects of multiple stressors related to climate change on EDSs because they: 1) have important ecological roles, particularly within kelp forest ecosystems, 2) are economically valuable as fisheries species, as is the case for red urchins, 3) are excellent models for examining early development 4) are vulnerable to changes in temperature and pH during their early development, and 5) are regularly exposed to combined high $p\text{CO}_2$ and low temperature conditions as a result of living in an upwelling regime. Red and purple sea urchins are important grazers in algal communities and are capable of creating urchin barrens (Pearse 2006; Rogers-Bennett 2007).

These urchins also affect community diversity by providing shelter for invertebrates and fishes that live under and around their spines. Furthermore, these urchins act as an important food source to a variety of predators such as sea otters and lobsters.

Although both urchin species are harvested for human consumption, red sea urchins in particular support a lucrative wild fishery in the United States (Rogers-Bennett 2007). According to data reported by the Pacific Fisheries Information Network (PacFIN), the revenue generated from the red urchin fisheries in Washington, Oregon, and California was estimated to be over \$6.4 million USD in 2018 (<https://pacfin.psmfc.org>; accessed 4 June 2019). In addition to their ecological and economic value, sea urchins have also been used as developmental model systems for a wide variety of studies (Ernst 1997). Spawning of urchins is easy to initiate, allowing for straightforward collection of large quantities of gametes and synchronously developing embryos (Foltz et al. 2004; Strathmann 1987). The embryos develop relatively rapidly and are easily observed due to their high transparency. Furthermore, there are extensive molecular resources available across a diversity of urchin species (Kudtarkar and Cameron 2017), including an annotated genome and transcriptome for *S. purpuratus* (Sodergren et al. 2006; Tu et al. 2012).

Lastly, changes in both temperature and pH conditions are likely to impact populations of both *M. franciscanus* and *S. purpuratus*. The waters these species inhabit are characterized by highly variable temperature and pH conditions (Chan et al. 2017; Hoshijima and Hofmann 2019; Kapsenberg and Hofmann 2016). Their habitat region is dominated by seasonal upwelling that brings sudden decreases in water temperature and pH. However, this region is also vulnerable to prolonged warming events (Gentemann et al. 2017; Hu et al. 2017). The timing of gametogenesis and spawning in red and purple sea urchin populations

largely overlaps with high seasonal upwelling (Strathmann 1987), and therefore, the EDSs may experience a variety of temperature and pH conditions, depending on if they develop during or between upwelling events. Furthermore, as species with calcifying larvae, urchins may be particularly susceptible to low pH conditions. Overall, *M. franciscanus* and *S. purpuratus* are ideal study organisms for the focus of this dissertation due to their ecological and economic value, usefulness as a developmental model, and vulnerability to temperature and pH stress in their natural environments.

The objective

The conceptual focus of this dissertation research was to explore organism-environment interactions with an emphasis on ecological developmental biology (Figure 1). Here, I have investigated (1) the response of the EDSs of organisms to multiple environmental stressors, (2) the connection between the environment and phenotype by exploring the transcriptome as a trait, and (3) transgenerational plasticity, from the organismal to molecular level. The study organisms for this research were red urchins, *M. franciscanus*, and purple urchins, *S. purpuratus*, due to their large ecological roles in kelp forest ecosystems as well as their economic impacts to fisheries. The peak spawning periods of these sea urchins can coincide with times of high upwelling and therefore, large fluctuations in pH and water temperature. Furthermore, with continuing climate change, these organisms will experience progressive ocean warming and acidification. Using multiple, integrated physiological and molecular approaches, I examined the response mechanisms of urchin EDSs raised under different pH and temperature conditions, including the role of transgenerational effects on offspring phenotypes.

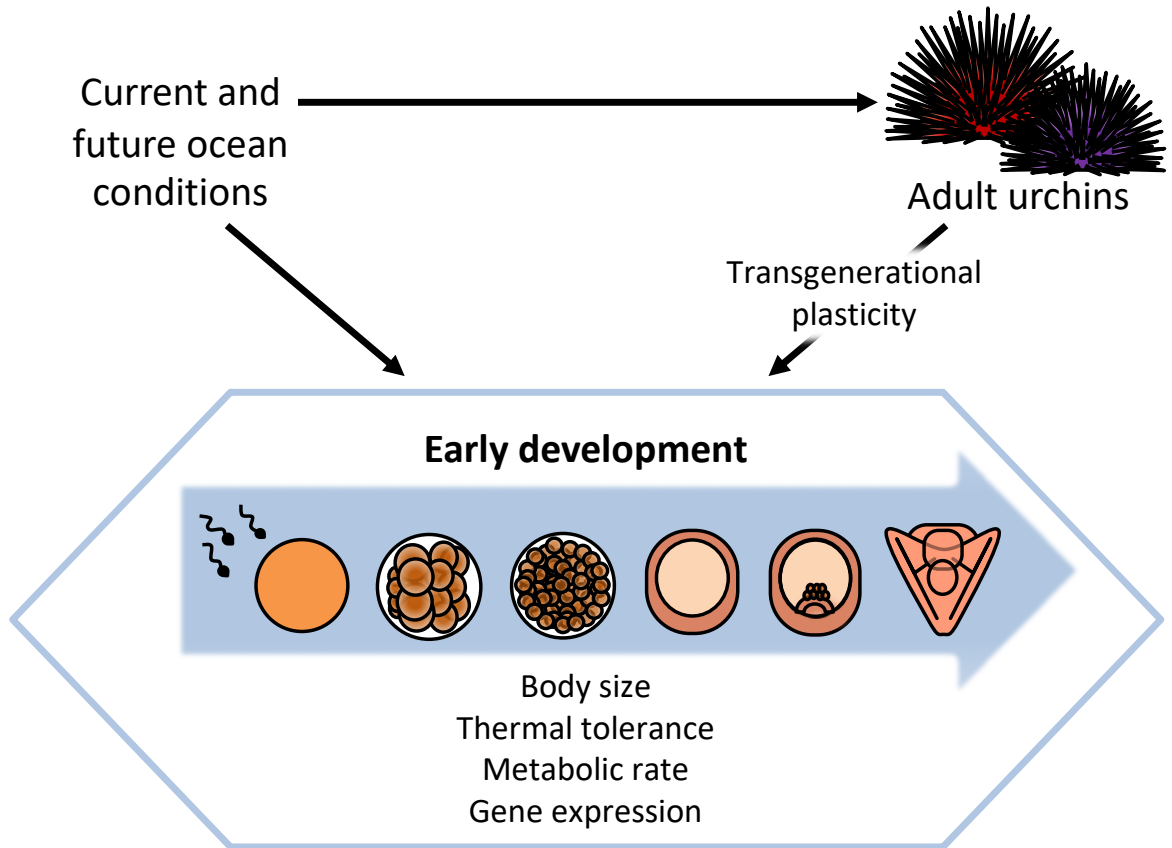


Figure 1. Diagram of major dissertation goals and concepts. This research incorporated a combination of molecular and physiological approaches to investigate the effects of multiple stressors on the early development of the red sea urchin, *Mesocentrotus franciscanus*, and the purple sea urchin, *Strongylocentrotus franciscanus*. This dissertation also investigated the role of transgenerational plasticity and its impacts on how early developmental stages respond to environmental stress.

This body of work has been organized into five chapters to investigate how multiple environmental stressors related to temperature and $p\text{CO}_2$ impact the early development of two ecologically important marine species that inhabit kelp forest ecosystems and contribute to economically valuable marine fisheries.

Chapter II: Transcriptional profiles of early stage red sea urchins (Mesocentrotus franciscanus) reveal differential regulation of gene expression across development

In this chapter, I assembled, annotated and described a developmental transcriptome for *M. franciscanus*. This transcriptome was representative of unfertilized eggs and six early developmental stages: 8- to 16-cell, morula (~64-cell), hatched blastula, early gastrula, prism, and pluteus. I found that the variation in the transcriptome coincided with major developmental and morphological changes and processes (Wong et al. 2019a). I also identified the maternal-to-zygotic transition, the period during which transcriptional control shifts from the maternal genome to the zygotic genome, in *M. franciscanus*. The purpose of this transcriptome was to provide a molecular resource that could be used to study gene expression patterns in this important fishery species.

Chapter III: The effects of temperature and $p\text{CO}_2$ on the size, thermal tolerance and metabolic rate of the red sea urchin (Mesocentrotus franciscanus) during early development

In this chapter, I examined the effects of multiple stressors on the early developmental stages of *M. franciscanus*. Very little is known regarding how this species responds to stressors, particularly those related to climate change. I raised *M. franciscanus* embryos under different combinations of temperature and $p\text{CO}_2$ and examined the effects of

the treatment conditions of embryo body size, thermal tolerance, and metabolic rate.

Development under different temperature and $p\text{CO}_2$ conditions impacted body size, although the effect differed by developmental stage. Embryos raised under warmer temperatures also appeared to exhibit a slightly greater thermal tolerance at the prism stage. This work is significant as it increases our predictive capacity of how *M. franciscanus* populations will respond to future ocean warming and acidification, with important implications for the fishery that the species supports.

Chapter IV: Gene expression patterns of red sea urchins (Mesocentrotus franciscanus) exposed to different combinations of temperature and $p\text{CO}_2$ during early development

In this chapter, I used the developmental transcriptome of *M. franciscanus* that was assembled in Chapter II to examine the gene expression patterns of embryos raised under different combinations of temperature and $p\text{CO}_2$ conditions. Specifically, I assessed the transcriptome of embryos as a trait to examine their transcriptomic response to temperature and $p\text{CO}_2$ conditions during the gastrula and prism stages. Gene expression patterns were also paired with observations from Chapter III to examine the phenotypic changes as well as molecular changes in *M. franciscanus* embryos as a result of their developmental treatment conditions. Gene expression patterns varied by stage and revealed a much greater transcriptomic response to elevated temperatures than to elevated $p\text{CO}_2$ levels.

Chapter V: Transgenerational effects in an ecological context: Conditioning of adult sea urchins to upwelling conditions alters maternal provisioning and progeny phenotype

In this chapter, I acclimated adult *S. purpuratus* to simulated upwelling conditions (combined low temperature and high $p\text{CO}_2$ level) and non-upwelling conditions (average temperature and $p\text{CO}_2$ level) representative of their kelp forest habitats. I investigated how differential conditioning of adult urchins during gametogenesis impacted maternal provisioning. This was measured by examining the size, protein content, and lipid content of the eggs produced by females conditioned to the upwelling and non-upwelling treatment. The offspring of the differentially conditioned mothers were then raised under high and low $p\text{CO}_2$ treatments to examine how the conditions experienced by the adults during gametogenesis impacted the offspring's phenotype and their response to $p\text{CO}_2$ stress. Specifically, I assessed the body size of embryos and larvae that differed in: 1) the conditions their mothers experienced during gametogenesis (i.e., simulated upwelling versus non-upwelling) and 2) the developmental $p\text{CO}_2$ conditions under which the progeny were raised (i.e., low versus high $p\text{CO}_2$ levels). Adult conditioning to simulated upwelling conditions appeared to increase maternal provisioning of lipids to the eggs (Wong et al. 2019b). Additionally, offspring from females conditioned to the upwelling treatment were larger in body size during early development.

Chapter VI: Transcriptomics reveal transgenerational effects in purple sea urchins exposed to upwelling conditions, and the response of their progeny to differential $p\text{CO}_2$ levels

In this chapter, I examined how the conditions experienced by adult *S. purpuratus* affected the gene expression patterns of their progeny and their transcriptomic response to

$p\text{CO}_2$ stress. After adult *S. purpuratus* were conditioned to simulated upwelling (combined low temperature and high $p\text{CO}_2$ level) and non-upwelling (average temperature and $p\text{CO}_2$ level) treatments during gametogenesis, they were spawned, and their offspring were raised under high or low $p\text{CO}_2$ conditions. I examined the transcriptomes of gastrula stage embryos to assess how parental experience influenced the gene expression patterns. The transcriptomic response to elevated $p\text{CO}_2$ conditions differed greatly by parental conditioning treatments, revealing that the conditions the adults experienced during gametogenesis was capable of impacting the gene expression patterns of the offspring (Wong et al. 2018).

II. Transcriptional profiles of early stage red sea urchins (*Mesocentrotus franciscanus*) reveal differential regulation of gene expression across development

Abstract

The red sea urchin, *Mesocentrotus franciscanus*, is an ecologically important kelp forest species that also serves as a valuable fisheries resource. In this study, we have assembled and annotated a developmental transcriptome for *M. franciscanus* that represents eggs and six stages of early development (8- to 16-cell, morula, hatched blastula, early gastrula, prism and early pluteus). Characterization of the transcriptome revealed distinct patterns of gene expression that corresponded to major developmental and morphological processes. In addition, the period during which maternally-controlled transcription was terminated and the zygotic genome was activated, the maternal-to-zygotic transition (MZT), was found to begin during early cleavage and persist through the hatched blastula stage, an observation that is similar to the timing of the MZT in other sea urchin species. The presented developmental transcriptome will serve as a useful resource for investigating, in both an ecological and fisheries context, how the early developmental stages of this species respond to environmental stressors.

Overview

In this chapter, I present a developmental transcriptome for the red sea urchin, *Mesocentrotus franciscanus*. This research was supported by a UC Climate Champion award to my advisor, Dr. Gretchen Hofmann. This work used computing resources supported by

NSF under grant No. ABI-1458641 to Indiana University. This work was also supported by resources from the Santa Barbara Coastal Long Term Ecological Research program (PI: Dr. Daniel Reed: NSF award OCE-1232779). I would also like to acknowledge the use of the UCSB and UCOP-supported Biological Nanostructures Laboratory within the California NanoSystems Institute. The research presented in this chapter was conducted in collaboration with Dr. Gretchen Hofmann, and a collaborating researcher at the University of Hong Kong, Dr. Juan Diego Gaitán-Espitia. This chapter has been published in *Marine Genomics*.

Introduction

The red sea urchin *Mesocentrotus franciscanus* (A. Agassiz, 1863) is found along the West Coast of North America, ranging from Baja California, Mexico to Kodiak, Alaska, USA (Ebert et al. 1999). *M. franciscanus* is harvested for its gonads (i.e., roe), and in recent decades has suffered overfishing and exploitation as a high-demand wild fishery species (Andrew et al. 2002; Keesing and Hall 1998). In terms of worth, the export value of roe in the United States was estimated to be approximately \$28.7 million in 2011 (Rogers-Bennett 2013). However, the price of red sea urchins harvested in the states of California, Oregon, and Washington has risen in recent years, increasing from \$0.70 USD per pound in 2011 to \$1.73 USD per pound in 2018 according to data reported by the Pacific Fisheries Information Network (PacFIN) (www.pacfin.psmfc.org; accessed 5 April 2019).

As a valuable wild fishery, red sea urchins are found in temperate rocky reefs in nearshore coastal regions. As benthic marine invertebrates in coastal marine ecosystems, they are threatened by ocean change (Sato et al. 2018), including future ocean acidification in regions dominated by episodic upwelling (Chan et al. 2017) and from marine heat waves

(Gentemann et al. 2017). For example, between 2013 to 2016, anomalous warming events associated with “the Blob” led to dramatically increased sea surface temperatures in the northeast Pacific Ocean (Bond et al. 2015; Gentemann et al. 2017; Hu et al. 2017). While a direct cause-effect relationship between these warming events and red urchin harvests has not been formally established, in California alone, the weight of harvested *M. franciscanus* decreased from 6,320 to 2,669 metric tons per year between 2013 and 2016 (www.pacfin.psmfc.org; accessed 5 April 2019). Importantly, marine heat waves like “the Blob” are predicted to increase in frequency and intensity in the future (Oliver et al. 2018); these events will likely have an impact on marine systems, including organism physiology, species abundances, and population biogeographic distributions (Frölicher and Laufkötter 2018; Leung et al. 2017; Sanford et al. 2019). Ecologically, *M. franciscanus* acts as an important ecosystem engineer by controlling algae populations, particularly in kelp forest ecosystems, and are capable of transforming algal communities into urchin barrens (Leighton et al. 1966; Rogers-Bennett 2007). Given the high economic and ecological importance of *M. franciscanus*, as well as its potential vulnerability to environmental change, genomic resources are very useful for studying and monitoring this species.

The introduction of next-generation sequencing and increasing affordability of associated technologies has expanded the molecular resources and knowledge available for non-model species, particularly those of high value to fisheries and aquaculture. Annotated and assembled *de novo* transcriptomes have been published across a variety of valuable fishery and aquaculture species, including mollusks (Coppe et al. 2012; De Wit and Palumbi 2013; Tian et al. 2018; Zhao et al. 2012), crustaceans (Ghaffari et al. 2014; Lv et al. 2014; Souza et al. 2018), echinoderms (Gaitán-Espitia et al. 2016; Gillard et al. 2014; Jo et al.

2016), and fishes (Carruthers et al. 2018; Ji et al. 2012; Liao et al. 2013). These transcriptomes are useful for investigating parameters important to fisheries health and management, such as population dynamics, evolutionary processes, the effects of abiotic stress, disease susceptibility and resilience, and stock assessments (Valenzuela-Quiñonez 2016; Wenne et al. 2007). For example, assembled transcriptomes have been used to investigate salinity stress in the Pacific oyster *Crassostrea gigas* (Zhao et al. 2012), and viral infection in the Pacific whiteleg shrimp *Litopenaeus vannamei* (Chen et al. 2013). Transcriptomic data have also been used to investigate patterns of gene flow and local adaptation in the red abalone *Haliotis rufescens* (De Wit and Palumbi 2013). Here, we used RNA sequencing (RNA-seq) to assemble and annotate a developmental transcriptome for the economically and ecologically important sea urchin, *M. franciscanus*, and assessed patterns of gene expression throughout early development. This tool could be used to assess the response of early stages to climate-changed related stressors.

Numerous studies have used transcriptomics to investigate how marine organisms respond to changes in their environment that are related to climate change, such as elevated temperatures and lowered pH and oxygen concentrations (Ekblom and Galindo 2011; Franks and Hoffmann 2012; Reusch and Wood 2007; Strader et al. 2019). Describing the transcriptional dynamics of *M. franciscanus* during its early development is particularly pertinent, as the early stages of development are believed to be the most vulnerable times during the life history of many marine organisms (Byrne 2011; Dupont and Thorndyke 2009; Gosselin and Qian 1997; Kurihara 2008). A reduction in fitness at the embryological and larval stages leading to poor recruitment could have devastating impacts on marine population dynamics. As rapid environmental change continues, the early life stages may act

as a bottleneck that dictates whether a species will be successful in the future (Byrne 2012; Byrne and Przeslawski 2013; Kurihara 2008).

Several recent studies have reported developmental transcriptomes for marine invertebrates (Brekhman et al. 2015; Gildor et al. 2016; Heyland et al. 2011; Lenz et al. 2014; Zeng et al. 2011), and have described stage-specific expression of many transcription factors. Such transcriptomes help to identify which genes are important for development as well as the timing of expression of these genes. One group of processes that occur during development involves the change of control from the maternal to the zygotic genome, identified as the maternal-to-zygotic transition (MZT), when there is a shift in expression from maternal to zygotic transcripts (Shier 2007; Tadros and Lipshitz 2009). Understanding the timing of the MZT is important for interpreting expression dynamics during early development. In general, the generation and characterization of developmental transcriptomes provide useful genomic resources that offer the opportunity to unveil mechanisms underlying developmental plasticity and its role in buffering different abiotic stressors across ontogeny.

Here, we present a developmental transcriptome for *M. franciscanus* that represents eggs as well as embryos and larvae from six stages of development: 8- to 16-cell, morula (composed of approximately 64 cells), hatched blastula, early gastrula, prism, and early pluteus. The developmental transcriptome presented here will provide insight into the timing of the MZT and will help identify genes and regulatory pathways that are important for successful development at each stage. Overall, this is an important resource for transcriptomic analyses of this species, including ecological or fisheries studies that use gene expression to assess the response of early developmental stages to stress.

Materials and methods

Animal collection and culturing

Adult sea urchins were collected in May 2016 at Mohawk Reef, CA, USA (34° 23.606' N, 119° 43.807' W) under California Scientific Collection permit SC-1223. Urchins were immediately transported to the Marine Science Institute at the University of California, Santa Barbara (UCSB) (Santa Barbara, CA), and maintained in flow-through seawater tanks for approximately one week prior to spawning. Spawning was induced via intracoelomic injection of 0.53 M KCl. Egg samples (EG) were collected by gently transferring ~5,000 eggs into a 1.5 mL microcentrifuge tube, quickly pelleting the sample by centrifugation, removing the excess seawater, and flash freezing the sample using liquid nitrogen. All samples were stored at -80 °C. Test fertilizations were performed to verify egg-sperm compatibility. The eggs from two females were gently pooled together and were fertilized using sperm from a single male. To avoid polyspermy, dilute sperm was slowly added to the eggs until at least 95% fertilization success was reached. The newly fertilized embryos were then placed into each of three replicate culture vessels (total volume = 12 L) at a concentration of ~9 embryos per mL of seawater.

All *M. franciscanus* early developmental stage (EDS) cultures were raised in 0.35 µm filtered, UV-sterilized seawater (FSW). The EDS cultures were raised at ~15 °C and ~425 µatm $p\text{CO}_2$. These conditions were chosen to represent average, “normal” ambient abiotic conditions that populations of *M. franciscanus* have been observed to experience *in situ* near Mohawk reef; these observations are made via sensor arrays deployed by the Santa Barbara Coastal LTER (Hofmann and Washburn 2015). During the EDS culturing, water temperature

was controlled using a Delta Star® heat pump with a Nema 4x digital temperature controller (AquaLogic, San Diego, CA, USA), which maintained culturing temperatures at 15 °C. A flow-through CO₂-mixing system modified from Fanguie et al. (2010) was used to ensure stable carbonate chemistry conditions throughout development. The CO₂ system was used to establish a 5-gallon reservoir tank, in which water was treated to the target *p*CO₂ level prior to delivering the treated water to each culture vessel.

Each culture vessel was composed of two, nested 5-gallon buckets (12 L capacity). The inner bucket had a dozen holes 5.5 cm in diameter, each fitted with 64-micron mesh to prevent the loss of embryos or larvae while allowing for a flow-through of seawater. Seawater flow to each vessel was controlled using irrigation button drippers (DIG Corporation), which regulated the flow to a rate of 4 L/hr. Each vessel contained a 15 cm x 15 cm plastic paddle driven by a 12-V motor to allow for continuous, gentle mixing and to prevent early embryos from settling to the bottom of the bucket.

Embryos and larvae were sampled at six developmental stages: 8- to 16- cell (CL; ~4 hours post-fertilization (hpf)), morula (MO; ~7 hpf), hatched blastula (BL; ~16 hpf), early gastrula (GA; ~29 hpf), prism (PR; ~44 hpf), and early pluteus (PL; ~64 hpf). While the development of *M. franciscanus* in culture is generally synchronous, during early cellular divisions, it is unlikely to collect a large batch of embryos that are exhibiting identical timing. As such, the samples collected at the 8- to 16-cell stage (CL) were composed of a mixture of embryos undergoing their third and fourth cleavage divisions. At the morula stage (MO), all embryos were composed of approximately 64 or more cells. The blastula stage (BL) was designated by the enzymatic digestion of the fertilization envelope and emergence of swimming blastula. The early gastrula (GA) stage was designated by the formation of

mesenchyme cells and an archenteron extended to approximately one-half the body length. The prism stage (PR) was identified by the formation of the pyramid-like prism shape, the archenteron becoming tripartite, and the early development of skeletal rods. Lastly, the early pluteus state (PL) was defined as having internal structures, including the mouth, esophagus, stomach and anus, as well as anterolateral and postoral skeletal body rods and the early formation of feeding arms. Samples from each of the three culture vessels were taken at each developmental stage. All samples were preserved using the same methods for preserving the eggs.

Temperature, salinity, pH, and total alkalinity (TA) were recorded daily to monitor the culturing conditions throughout development. Temperature was measured using a wire thermocouple (Thermolyne PM 20700 / Series 1218), and salinity was measured using a conductivity meter (YSI 3100). Daily pH measurements were conducted by following the standard operating procedure (SOP) 6b (Dickson et al. 2007b), using a spectrophotometer (Bio Spec-1601, Shimadzu) and *m*-cresol purple (Sigma-Aldrich) indicator dye. Water samples for TA were poisoned with saturated 0.02% mercuric chloride. TA was estimated using SOP 3b (Dickson et al. 2007a). Using the carbonic acid dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987), parameters of $p\text{CO}_2$, Ω_{ara} , and Ω_{cal} were calculated using CO₂calc (Robbins et al. 2010).

RNA extractions and sequencing

Total RNA was extracted using 500 μL of Trizol[®] reagent, following the manufacturer's instructions (Invitrogen). Briefly, each sample was homogenized in Trizol[®] reagent by passing the sample three times through decreasing sizes of needles (21-gauge, 23-

gauge, and then 25-gauge). A chloroform addition and centrifugation were used to isolate the RNA-containing upper aqueous phase. The RNA was precipitated in isopropyl alcohol, washed using ethanol, and resuspended in DEPC-treated water. RNA purity, quantity, and quality were verified using a NanoDrop® ND100, a Qubit® fluorometer, and a Tapestation 2200 system (Agilent Technologies).

Three libraries were generated from triplicate samples of eggs. For each developmental stage, one library was generated for each of the three replicate culture vessels. This resulted in a total of 21 libraries. Libraries were generated using high quality total RNA (RIN values > 9.1) using a TruSeq Stranded mRNA Library Preparation Kit (Illumina) following the manufacturer's instructions. The quantity and quality of each library were verified using a Qubit® fluorometer and a Tapestation 2200 system (Agilent). The libraries were submitted to the Genome Center at the University of California, Davis for sequencing on an Illumina HiSeq 4000 sequencer on two lanes with 150 base-pair (bp) paired-end reads.

De novo transcriptome assembly

Additional *M. franciscanus* raw sequence data from Gaitán-Espitia and Hofmann (2017) were included with sequence data from our 21 libraries to generate the *de novo* transcriptome. These data represented gastrula stage embryos (GenBank accession numbers SRS823202 and SRS823216) and pluteus larvae (accession numbers SRS823218 and SRS82322) of Bioproject PRJNA272924. Any potential adapter sequence contamination as well as any base pairs with quality scores below 30 were removed from all raw sequence data using Trim Galore! (version 0.4.1) (Krueger 2015). Sequence quality was verified using FastQC (version 0.11.5) (Andrews 2010).

The transcriptome was assembled following a pipeline available from the National Center for Genome Analysis Support (NCGAS) at Indiana University (<https://github.com/NCGAS/de-novo-transcriptome-assembly-pipeline>). This workflow generates a combined *de novo* assembly that uses multiple assemblers with multiple parameters. Prior to assembly, the data were normalized using the in silico read normalization function in Trinity (version 2.6.6) (Grabherr et al. 2011). Multiple *de novo* assemblies were created using different assemblers with different selections of kmer lengths: Trinity (version 2.6.6) (kmer = 25), SOAPdenovo-Trans (version 1.03) (Xie et al. 2014) (kmers = 35, 45, 55, 65, 75, and 85), Velvet (version 1.2.10) (Zerbino and Birney 2008) and Oases (version 0.2.09) (Schulz et al. 2012) (kmers = 35, 45, 55, 65, 75, and 85), and Trans-ABYSS (version 2.0.1) (Robertson et al. 2010) (kmers = 35, 45, 55, 65, 75, and 85). These 19 assemblies were then combined using EvidentialGene (version 2013.07.27) (Gilbert 2013), which removes perfect redundancy and fragments to reduce false transcripts while predicting unique transcripts within the final assembly. Quast (version 5.0.0) (Gurevich et al. 2013) was used to generate basic quality metrics of the final assembly. BUSCO (version 3.0.2) (Simão et al. 2015) was used to assess completeness of the final assembly using the single-copy ortholog reference for metazoans.

Gene prediction and functional annotation

Gene models from the *de novo* assembled transcriptome were inferred and annotated using the BLASTP (against the nr database), BLASTN (against the eukaryotic nt database) and BLASTX (against the Uniprot database, Swiss-Prot and TrEMBL) algorithms with an e-value cutoff of $1e^{-5}$. Annotated sequences were further searched for Gene Ontology (GO)

terms using Blast2GO software (version 5.2.5) according to the main categories of Gene Ontology (GO; molecular functions, biological processes and cellular components) (Ashburner et al. 2000). Complementary annotations were done with the InterProScan v.5 software (Jones et al. 2014). Finally, the annotation results were further fine-tuned with the Annex and GO slim functions and the enzyme code annotation tool of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000) implemented in Blast2GO.

Expression quantitation and differential expression analyses

Trimmed sequence data from the 21 libraries were mapped onto the *de novo* reference transcriptome and expression values were calculated using RSEM (version 1.3.0) (Li and Dewey 2011) and bowtie2 (version 2.3.2) (Langmead and Salzberg 2012). Using the LIMMA package (Ritchie et al. 2015) in R (version 3.4.4), the data were filtered to sequences that have more than 0.5 counts per million mapped reads across at least three of the 21 samples. A trimmed mean of M-values (TMM) normalization method (Robinson and Oshlack 2010) was used to apply scale normalization to the read counts. The data were voom-transformed using LIMMA to convert the read counts to log-counts per million while accounting for sample-specific quality weights and blocking design (i.e., technical replicates). The filtered, normalized and voom-transformed data were used to perform a principal component analysis (PCA) using the *prcomp* function in R. An unweighted pair group method with arithmetic mean (UPGMA) method using Euclidean distances was implemented to perform hierarchical clustering on the principal components using the *HCPC* function of the *FactoMineR* package (Le et al. 2008) in R.

Using the WGCNA package (Langfelder and Horvath 2008) in R, a Weighted Gene Co-Expression Network Analysis (WGCNA) was performed on the same filtered, normalized and voom-transformed data to identify clusters of similarly expressed genes into modules, in which each module contained a minimum of 30 genes. Modules with highly correlated eigengenes were merged using a threshold of 0.27 (i.e., a height cut-off of 0.27 and a correlation of 0.73 for merging). Eigengene expression was correlated with eggs and each developmental stage (i.e., EG, CL, MO, BL, GA, PR, and PL), and a heatmap was generated to visualize significant correlations between each stage and module.

Functional enrichment analyses were performed on lists of genes within modules with significant correlations to a developmental stage (r^2 correlation ≥ 0.50 and p -value ≤ 0.05). Enrichment analyses were performed in Blast2GO (version 5.2.5) using a Fisher's Exact Test with an FDR filter value of 0.05 to identify gene ontology (GO) terms within the GO categories: biological process, molecular function, and cellular component.

Survey of the maternal-to-zygotic transition

To investigate the timing of the maternal-to-zygotic transition (MZT), two aspects of the data were probed: 1) the loss of maternally-derived transcripts, and 2) the initiation of zygotic transcription. To examine the loss of maternally-derived transcripts, putative genes related to the removal of maternal RNAs were identified. These included putative genes for the *microprocessor complex subunit DGCR8* (*dgcr8*), *endoribonuclease dicer* (*dicer*), *smaug* (*smaug1*), and *nonsense mediated mRNA decay* (*smg7*, *smg8*, and *smg9*) (Gildor et al. 2016; Marlow 2010; Tadros and Lipshitz 2009) (Table 1). The expression levels of these transcripts were examined across the eggs and early development. Additionally, a heatmap was

constructed using Euclidean distances to visualize the expression of maternal transcripts throughout development. Transcripts expressed in the eggs were considered to be maternal. The heatmap was constructed using the top 500 transcripts expressed in unfertilized eggs (EG).

To examine the initiation of zygotic transcription, transcripts associated with zygotic development were targeted. Putative genes for *aristaless-like homeobox (alx)*, *brachyury (bra)*, *dead ringer (dri)*, *glial cells missing (gcm)*, *goosecoid (gsc)*, *homeobox 11/13b (hox11/13b)*, *left-right determination factor 2 (lefty2)*, *nodal*, and *wnt8* were targeted within *M. franciscanus* (Tadros and Lipshitz 2009; Wei et al. 2006) (Table 1). The expression levels of these transcripts were examined in eggs and as early development progressed. A heatmap was also constructed using Euclidean distances to visualize the expression of transcripts that were not maternally expressed (i.e., not expressed in unfertilized eggs). Transcripts with a negative expression value of \log_2 counts per million reads ($\log_2 \text{CPM} < 0$) at the EG stage were selected, for a total of 19,044 transcripts. Using these transcripts, a heatmap was constructed to visualize at what stages throughout development these transcripts were expressed.

Table 1. Transcripts that play a functional role during the MZT.

Transcript ID	Name	Description
058424	<i>dgcr8</i>	microprocessor complex subunit DGCR8-like
103747	<i>dicer</i>	endoribonuclease Dicer
035070	<i>smaug1</i>	protein Smaug homolog 1 isoform X1
089348	<i>smg7</i>	protein SMG7 isoform X2
052093	<i>smg8</i>	protein smg8
035665	<i>smg9</i>	protein SMG9-like
089934	<i>alx</i>	aristaless-like homeobox protein
082528	<i>bra</i>	transcription factor Brachyury
015822	<i>dri</i>	protein dead ringer homolog
036581	<i>gcm</i>	glial cells missing transcription factor
035045	<i>gsc</i>	homeobox protein goosecoid-like
047087	<i>hox11/13b</i>	transcription factor Hox11/13b
104463	<i>lefty2</i>	left-right determination factor 2-like
047266	<i>nodal</i>	nodal homolog 2-A-like
076942	<i>wnt8</i>	Wnt8

Results and discussion

Culturing conditions

The embryo and larval cultures developed normally with little to no mortality observed throughout development. Temperature and seawater chemistry conditions were stable throughout the ~64 hour culturing period. Across all replicate culture vessels, the temperature was 15.3 ± 0.1 °C, the salinity was 33.4 ± 0.04 , the pH was 8.00 ± 0.03 , the $p\text{CO}_2$ level was 438 ± 33.1 μatm , and the TA was 2228.97 ± 3.19 $\mu\text{mol kg}^{-1}$.

Summary statistics of the transcriptome assembly and annotation

Sequencing of the 21 libraries yielded a total of 751,578,474 150-bp paired-end reads. After trimming to remove any adapter contamination or low quality reads, an average of 35.4 ± 5.8 million reads remained per library. FastQC reports of the trimmed reads from all libraries showed high sequence quality (scores >30) with limited adapter contamination or presence of overrepresented sequences. The transcriptome generated by the NCGAS pipeline was 96.74 megabases (Mb) with 115,719 contigs, a N50 of 3,292 bp, and a GC content of 42.58% (Table 2). The BUSCO analysis that used metazoan as the single-copy ortholog reference showed high transcriptome completeness with a complete BUSCO score of 92.2% (Table 2). Therefore, this transcriptome should offer a suitable foundation for transcriptomic analyses of *M. franciscanus*.

The gene discovery and functional annotation analyses identified 35,632 contigs that blasted to known proteins in the public databases (Table S1). From these, 24,900 contigs were linked to GO classifications. Hypothetical or predicted proteins in these databases were excluded by discarding matches associated to “hypothetical”, “predicted”, “unknown” and

“putative” categories. Over 95% of the annotated contigs hit against the genomes of the purple sea urchin, *Strongylocentrotus purpuratus*, followed by the sea star, *Acanthaster planci*, and the sea cucumber, *Apostichopus japonicus*. The functional annotation analysis retrieved 48,990 GO terms, with 23,053 linked to molecular function (mainly protein binding), 15,754 linked to biological process (mainly G protein-coupled receptor signaling pathway, oxidation-reduction process and transmembrane transport), and 10,183 linked to cellular component (mainly integral component of membrane) (Table S2). Finally, the enzyme code annotation with KEGG mapping identified 1,948 transcripts, which represented 433 enzymes in 122 unique pathways (Table 3, S3). KEGG pathways included those related to purine metabolism, biosynthesis of antibiotics, T cell receptor signaling pathway, Th1 and Th2 cell differentiation, and ether lipid metabolism.

Table 2. Statistics of *de novo* transcriptome assembly.

Assembly statistic	Value
No. contigs	115,719
No. contigs > 1kb	19,511
Assembly size (Mb)	96.74
Mean contig length (bp)	836
Median contig length (bp)	341
Max contig length (bp)	132,566
GC content	42.58
N ₅₀ (bp)	3,292
L ₅₀ (bp)	6,526
BUSCO completeness (%)	92.2
BUSCO fragmented (%)	0.8
BUSCO missing (%)	7.0

Table 3. Top 10 KEGG pathways in the transcriptome.

Pathway	Pathway ID	No. transcripts	No. enzymes
Purine metabolism	map00230	825	47
Thiamine metabolism	map00730	727	6
Drug metabolism - other enzymes	map00983	233	16
Biosynthesis of antibiotics	map01130	185	100
T cell receptor signaling pathway	map04660	132	2
Th1 and Th2 cell differentiation	map04658	129	1
Glutathione metabolism	map00480	76	13
Ether lipid metabolism	map00565	69	7
Cysteine and methionine metabolism	map00270	60	23
Sphingolipid metabolism	map00600	58	14

Gene expression patterns in eggs and throughout early development

A total of 35,126 sequences remained after filtering to those with more than 0.5 counts per million mapped reads across at least three of the 21 samples. A PCA of sample-to-sample distances showed that gene expression profiles were more similar within stages (i.e., among sample replicates) than across developmental stages (Figure 2). The first and second dimensions captured 62.94% and 13.09% of the variation, respectively, and revealed a clear separation between egg/early embryonic stages and later developmental stages. Moreover, hierarchical clustering revealed two primary clusters (Figure 2): cluster 1 included cluster 1a, which contained eggs (EG), and cluster 1b, which included the 8- to 16-cell (CL) and morula (MO) stages; cluster 2 included cluster 2a, which contained blastula (BL) and gastrula (GA) stages, and cluster 2b, which contained prism (PR) and pluteus stages (PL). In general, gene expression profiles followed the progression of development during which time major developmental processes and alterations in morphology occurred. Similar separations in transcriptomic patterns between earlier (e.g., egg and cleavage) and later (e.g., blastula and onwards) developmental stages have been observed in several other urchin species, and have been attributed to the transition between maternal and zygotic transcription (i.e., the MZT) (Gildor et al. 2016; Israel et al. 2016). The timing of the MZT in *M. franciscanus* will be discussed in further detail later (section 3.4).

WGCNA was used to highlight groups of genes that were co-expressed in eggs and each developmental stage. After filtering, normalizing and voom-transforming the data, the remaining 35,126 genes were assigned into module eigengenes containing similarly expressed genes. Only 86 genes remained unclustered and unassigned, and were grouped into the grey module (Figure 3). All other genes were assigned into 15 different modules that

were designated by color, and hierarchical clustering of the module eigengenes revealed three main clusters (Figure 3). Each module was related to developmental stage to generate eigengene networks with positive or negative correlation values ranging from 1 to -1 (Figure 3).

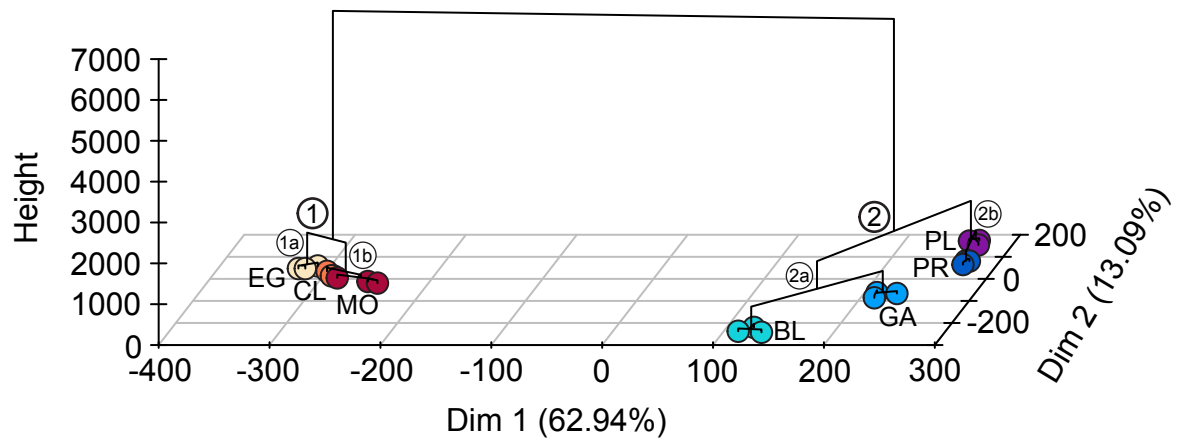


Figure 2. PCA of *Mesocentrotus franciscanus* eggs and early developmental stages showing the first two dimensions and hierarchical clustering of the samples. Sample colors denote the different stages, which include: egg (EG), 8- to 16-cell (CL), morula (MO), blastula (BL), gastrula (GA), prism (PR), and pluteus (PL). Hierarchical clustering show two main clusters (1 and 2) that each contain two clusters (a and b).

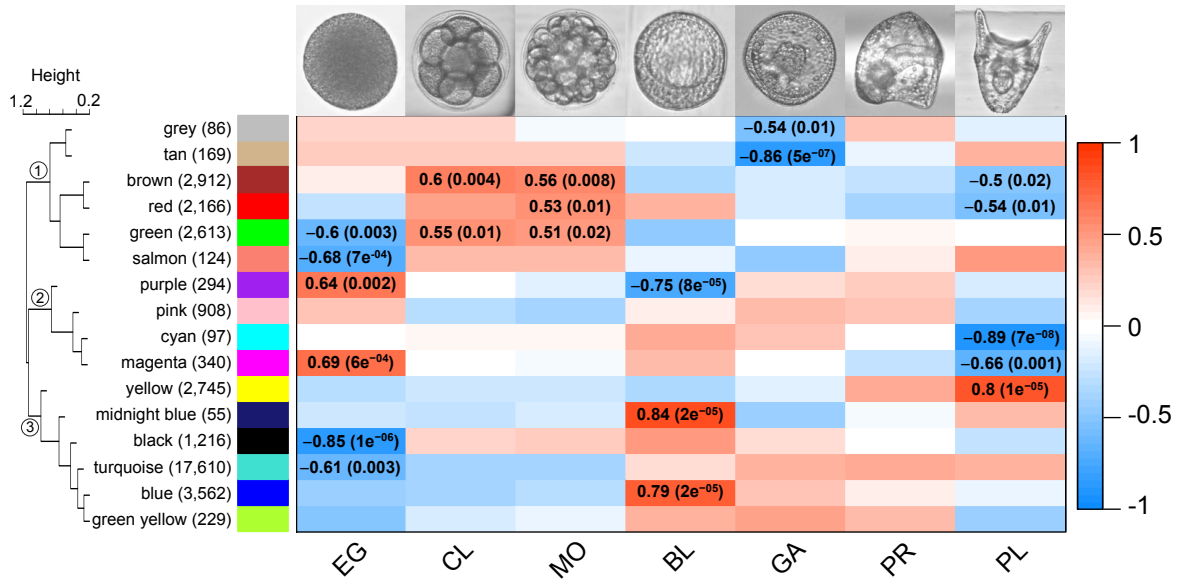


Figure 3. WGCNA identified significant correlations between module eigengenes (rows) and stages (columns). The stages are: egg (EG), 8- to 16-cell (CL), morula (MO), blastula (BL), gastrula (GA), prism (PR), and pluteus (PL). The number of genes within each module eigengene is noted in parentheses following each color name. The red-blue color scale represents the strength of the correlation (1 to -1). Each correlation value (r^2) is followed by a p -value in parentheses. Hierarchical clustering of the module eigengenes revealed three primary clusters of gene expression (1-3).

Overall, WGCNA showed a mixed result for the 15 modules that were identified. Of the 15 module eigengenes, pink (908 genes) and green yellow (229 genes) were not significantly correlated to any stage (r^2 correlation ≤ 0.50 , p -value ≥ 0.05). In addition, functional enrichment analyses did not identify any GO terms within the module eigengenes purple (294 genes), cyan (97 genes), magenta (340 genes), or midnight blue (55 genes). However, there were nine remaining module eigengenes that were significantly correlated to at least one stage (r^2 correlation ≥ 0.50 and p -value ≤ 0.05), and in which functional enrichment analyses successfully identified GO terms. These module eigengenes were tan (169 genes), brown (2,912 genes), red (2,166 genes), green (2,613 genes), salmon (124 genes), yellow (2,745 genes), black (1,216 genes), turquoise (17,610 genes), and blue (3,562 genes).

The WGCNA analysis revealed that the unfertilized eggs (EG) were significantly correlated to six module eigengenes, the greatest number of any developmental stage (Figure 3). A significant negative correlation of EG with module eigengenes green, salmon, black, and turquoise revealed that, relative to the measured developmental stages post-fertilization, the egg transcriptome was characterized by a down-regulation of genes related to metabolic processes and catalytic activity (Tables 4, S4). Unfortunately, while EG had a significant positive correlation with module eigengenes purple and magenta, functional enrichment analyses failed to reveal any GO terms within these modules. However, in other studies, there is evidence that active transcription and translation occurs at low levels in unfertilized sea urchin eggs (Chassé et al. 2018; Ruderman and Schmidt 1981). In the purple urchin, *Strongylocentrotus purpuratus*, eggs possess transcripts of genes related to the cell cycle and DNA replication (Tu et al. 2014). In the Mediterranean sea urchin, *Paracentrotus lividus*,

genes related to the cell cycle and DNA replication are translated upon fertilization (i.e., at the one-cell embryo stage) (Chassé et al. 2018).

Unlike the egg analyses, functional enrichment analyses were able to identify GO terms within module eigengenes positively correlated with early embryo stages. In this study, early embryo cell divisions are represented by the 8- to 16-cell stage (CL) and the morula stage (MO), whose transcriptomes are highly similar to one another (Figure 2). CL and MO were both positively correlated to module eigengenes brown and green (module eigengene cluster 1, Figure 3). The MO stage also had a significant correlation with module eigengene red (module eigengene cluster 1, Figure 3). Enrichment analyses of these modules revealed that during these early cell divisions, the embryos contained transcripts encoding proteins related to metabolic processes, catalytic activity, and organelle and membrane formation (Tables 4, S4), which likely reflect various processes involved in cell proliferation. This finding is consistent with proteomic data from early embryos of *S. purpuratus* (Guo et al. 2015). Additionally, GO terms related to signal transduction, G protein-coupled receptor signaling pathway, and transmembrane transport were identified. This result is similar to observations made in *P. lividus*, in which there was an enrichment of GO terms related to signaling pathways and cellular transport in the early embryological stages (Gildor et al. 2016). Lastly, GO terms related to the cell cycle were identified in each of the brown, green, and red modules. This is comparable to *S. purpuratus*, in which genes encoding proteins related to the cell cycle are prominent in cleavage stage embryos (Tu et al. 2014).

Upon progression to the blastula stage, extensive cell differentiation occurs in which the embryo forms a blastocoel, cilia, and enzymes required to digest the fertilization membrane during the hatching process (Barrett and Edwards 1976; Lepage and Gache 1989).

With regard to stage-specific gene expression, the blastula stage (BL) was negatively correlated with module eigengene purple and positively correlated with module eigengenes midnight blue and blue (Figure 3). Although functional enrichment analyses failed to reveal any GO terms within module eigengenes purple or midnight blue, module eigengene blue contained GO terms related to RNA-directed DNA polymerase activity, protein binding, DNA integration, G protein-coupled receptor activity, and transmembrane transport (Tables 4, S4). The enrichment of these genes is in alignment with other studies in which genes related to DNA replication and energy production were expressed during the blastula stage in *S. purpuratus* (Gildor et al. 2016; Tadros and Lipshitz 2009).

Gastrulation is a major and fundamental process of metazoan development (Wolpert 1992) that begins by invagination at the vegetal plate and the formation of the archenteron (Dan and Okazaki 1956; Etensohn 1984). Somewhat surprisingly, there were few correlations between the gastrula stage (GA) and module eigengenes identified by WGCNA. GA was negatively correlated with only the module eigengene tan (module eigengene cluster 1, Figure 3). GA was also negatively correlated with the grey module, which contained the unclustered and unassigned genes. Functional enrichment analysis of module eigengene tan revealed 39 GO terms, including oxidation-reduction process, integral component of membrane, ion transmembrane transport, and ATPase activity (Tables 4, S4). Unfortunately, the WGCNA did not identify any modules with a significant positive correlation with GA. Previous investigations of the *M. franciscanus* gastrula transcriptome, however, reported GO terms related to cell differentiation and signal transduction involved in cell cycle checkpoints (Gaitán-Espitia and Hofmann 2017). In *S. purpuratus*, there is an increase in expression of

genes related to biomineralization, the nervous system, immunity and the defense once gastrulation begins (Tu et al. 2014).

The digestive tract and supporting skeletal rods are formed during the prism and early pluteus stages, which are necessary for the planktotrophic feeding strategy of the urchin larvae (Burke 1980; Ettensohn and Malinda 1993). The prism stage (PR) was not significantly correlated to any module eigengene (Figure 3). However, its expression patterns were similar to that of the pluteus stage (PL) (Figures 2, 3). PL was negatively correlated to module eigengenes brown and red (module eigengene cluster 1, Figure 3), which include GO terms related to ATP binding, integral component of membrane, transmembrane transport, signal transduction and the cell cycle (Tables 4, S4). PL was also negatively correlated with module eigengenes cyan and magenta, although functional enrichment analyses were unable to identify GO terms within these modules. Lastly, PL was positively correlated with one module eigengene, yellow, which was within module eigengene cluster 3 (Figure 3). Enrichment analysis identified GO terms within yellow that included those related to metalloproteinase activity, metabolism, adhesion, the cytoskeleton, and the immune system (Tables 4, S4). This observation was in agreement with our earlier work (Gaitán-Espitia and Hofmann 2017), in which genes related to these processes and structures were up-regulated in *M. franciscanus* pluteus larvae relative to gastrula stage embryos. The yellow module also included GO terms related to ATP binding, oxidation-reduction process, calcium ion binding, acetylcholine-gated cation-selective channel activity, and ion transmembrane transport (Tables 4, S4). This expression pattern likely reflects the energy production and biomineralization processes necessary to support gut and skeletal formation in the developing pluteus larvae.

Table 4. Select GO term results from functional enrichment analyses of WGCNA module eigengenes.

GO ID	GO term name	GO category	FDR value	No. transcripts (% of ref)
tan: GA ($r^2 = -0.86$)				
GO:0016021	integral component of membrane	Cellular Component	6.67E-06	26 (0.6)
GO:0055114	oxidation-reduction process	Biological Process	2.27E-05	12 (1.2)
GO:0016491	oxidoreductase activity	Molecular Function	2.82E-04	11 (1.1)
GO:0034220	ion transmembrane transport	Biological Process	3.13E-03	6 (2)
GO:0015267	channel activity	Molecular Function	5.97E-03	6 (1.7)
GO:0042623	ATPase activity, coupled	Molecular Function	5.97E-03	5 (2.5)
brown: CL ($r^2 = 0.6$), MO ($r^2 = 0.56$), PL ($r^2 = -0.5$)				
GO:0016021	integral component of membrane	Cellular Component	4.81E-12	207 (4.6)
GO:0005524	ATP binding	Molecular Function	1.63E-10	79 (6.5)
GO:0055085	transmembrane transport	Biological Process	1.12E-05	57 (5.7)
GO:0007049	cell cycle	Biological Process	5.60E-04	20 (8.5)
GO:0035556	signal transduction	Biological Process	6.36E-04	24 (7.4)
GO:0140098	catalytic activity, acting on RNA	Molecular Function	3.75E-03	20 (7.2)
red: MO ($r^2 = 0.53$), PL ($r^2 = -0.54$)				
GO:0016021	integral component of membrane	Cellular Component	9.33E-14	172 (3.8)
GO:0005524	ATP binding	Molecular Function	1.18E-11	68 (5.6)
GO:0022402	cell cycle process	Biological Process	2.01E-05	16 (10.1)
GO:0007165	signal transduction	Biological Process	3.78E-05	80 (3.5)
GO:0055114	oxidation-reduction process	Biological Process	8.93E-04	39 (4.1)
GO:0055085	transmembrane transport	Biological Process	2.53E-02	36 (3.5)
green: EG ($r^2 = -0.6$), CL ($r^2 = 0.55$), MO ($r^2 = 0.51$)				
GO:0005524	ATP binding	Molecular Function	2.79E-34	117 (10)
GO:0035556	intracellular signal transduction	Biological Process	2.35E-21	48 (16.1)
GO:0140098	catalytic activity, acting on RNA	Molecular Function	1.48E-10	31 (11.7)
GO:0016021	integral component of membrane	Cellular Component	4.84E-09	177 (3.9)
GO:0007049	cell cycle	Biological Process	1.24E-08	26 (11.4)
GO:0055114	oxidation-reduction process	Biological Process	5.06E-07	53 (5.7)
salmon: EG ($r^2 = -0.68$)				
GO:0003824	catalytic activity	Molecular Function	1.81E-07	34 (0.4)
GO:0005515	protein binding	Molecular Function	9.29E-04	20 (0.4)
GO:0008152	metabolic process	Biological Process	5.40E-03	26 (0.3)
GO:0008168	methyltransferase activity	Molecular Function	5.40E-03	6 (1.7)
GO:0005543	phospholipid binding	Molecular Function	5.40E-03	4 (4.7)
GO:0016020	membrane	Cellular Component	9.21E-03	21 (0.3)
yellow: PL ($r^2 = 0.8$)				
GO:0055114	oxidation-reduction process	Biological Process	8.55E-23	87 (9.7)
GO:0005509	calcium ion binding	Molecular Function	1.44E-16	77 (8.3)
GO:0022848	acetylcholine-gated cation-selective channel activity	Molecular Function	1.78E-07	9 (60)
GO:0034220	ion transmembrane transport	Biological Process	4.70E-07	27 (9.8)
GO:0005524	ATP binding	Molecular Function	3.95E-04	57 (4.6)
GO:0002376	immune system process	Biological Process	6.06E-04	18 (8.4)
black: EG ($r^2 = -0.85$)				
GO:0005515	protein binding	Molecular Function	1.18E-11	111 (2.4)

GO:0003677	DNA binding	Molecular Function	8.07E-07	39 (3.3)
GO:0006464	cellular protein modification process	Biological Process	3.49E-05	38 (2.8)
GO:0046872	metal ion binding	Molecular Function	4.01E-05	69 (2.1)
GO:0006396	RNA processing	Biological Process	1.46E-04	16 (4.9)
GO:0016021	integral component of membrane	Cellular Component	1.59E-04	86 (1.8)
turquoise: EG ($r^2 = -0.61$)				
GO:0005524	ATP binding	Molecular Function	1.50E-92	499 (63.2)
GO:0015074	DNA integration	Biological Process	5.19E-40	271 (53.3)
GO:0005509	calcium ion binding	Molecular Function	3.64E-36	315 (45.5)
GO:0003964	RNA-directed DNA polymerase activity	Molecular Function	3.09E-33	415 (38)
GO:0006278	RNA-dependent DNA biosynthetic process	Biological Process	4.22E-33	415 (37.9)
GO:0005525	GTP binding	Molecular Function	2.54E-19	136 (51.7)
blue: BL ($r^2 = 0.79$)				
GO:0016021	integral component of membrane	Cellular Component	7.53E-28	296 (6.7)
GO:0003964	RNA-directed DNA polymerase activity	Molecular Function	5.84E-24	132 (9.6)
GO:0005515	protein binding	Molecular Function	5.01E-21	277 (6.1)
GO:0015074	DNA integration	Biological Process	1.28E-10	65 (9.1)
GO:0004672	protein kinase activity	Molecular Function	2.26E-09	48 (10.3)
GO:0055085	transmembrane transport	Biological Process	4.85E-06	67 (6.7)

The maternal-to-zygotic transition

To examine the timing of the MZT, we assessed: 1) the decline of maternally-derived transcripts, and 2) the increase of zygotic transcription. Upon targeting genes that play a role in the degradation of maternal RNAs, one *DGCR8*-like gene (*dgcr8*), one *dicer* gene (*dicer*), one *smaug* homolog (*smaug1*) and three putative *smg* genes (*smg7*, *smg8*, and *smg9*) were identified within the *M. franciscanus* developmental transcriptome (Table 1). The expression levels of *dgcr8*, *dicer*, *smg7*, *smg8*, and *smg9* all peaked during the 8- to 16-cell (CL) and morula (MO) stages (Figure 4A). The *dgcr8* gene plays a role in processing microRNAs that are required for degrading mRNAs in mammals (Marlow 2010; Wang et al. 2007). The Mediterranean sea urchin, *P. lividus*, exhibited a similar pattern of expression of *dgcr8* as reported here, in which there was a peak in expression within 8- and 16-cell embryos (Gildor et al. 2016). The authors attributed this observation to the role of *dgcr8* in degrading maternal mRNAs (Gildor et al. 2016). *Dicer* is involved in clearing maternal messages in zebrafish and mice (Giraldez et al. 2005; Marlow 2010), and mutations in the *dicer* gene are known to alter and arrest embryonic development in some species (Murchison et al. 2007).

The *smg* genes code for proteins that function in nonsense-mediated mRNA decay (NMD) in a variety of organisms (Okada-Katsuhata et al. 2012; Pulak and Anderson 1993; Yamashita et al. 2009). The NMD pathway detects and degrades mRNAs, and is often described as a surveillance pathway that serves as a quality-control mechanism to remove mRNAs with premature termination codons (Chang et al. 2007; Hentze and Kulozik 1999). However, the NMD pathway also serves functional roles that shape gene expression and are important for differentiation and development (Lykke-Andersen and Jensen 2015). Additionally, the NMD pathway has been shown to selectively degrade mRNA transcripts

with longer 3' UTRs, causing a relative enrichment of shorter 3' UTR transcripts (Bao et al. 2016). In zebrafish embryos, 3' UTR length affects the stability of maternal mRNAs because longer 3' UTRs confer resistance to codon-mediated deadenylation, the first step required for mRNA decay (Mishima and Tomari 2016). Therefore, the removal of long 3' UTR transcripts via the NMD pathway may increase the relative proportion of short 3' UTR transcripts available for deadenylation and decay during the CL and MO stages. Overall, the peak in expression of *dgcr8*, *dicer*, *smg7*, *smg8*, and *smg9* during the CL and MO stages supports that maternal mRNAs are degraded during this period of embryonic development.

In contrast to the expression of *dgcr8*, *dicer*, and *smg* genes, *smaug1* was not expressed until the blastula stage (Figure 4A). The *smaug* gene is a transcriptional regulator known to bind to and target maternal RNAs for degradation in *Drosophila melanogaster* and is highly conserved across taxa (Tadros et al. 2007). It is therefore possible that degradation of maternal mRNAs is still ongoing at the blastula stage. This differs from observations in *S. purpuratus*, in which maternal degradation appears to end prior to the blastula stage (Tadros and Lipshitz 2009; Wei et al. 2006). With the exception of *smaug1* expression, the degradation of maternal transcripts appears to primarily occur during the 8- to 16-cell (CL) and morula (MO) stages. This is additionally supported by the WGCNA, which revealed genes related to catalytic activity acting on RNA in module eigengenes brown and green, both of which share significant, positive correlations with the CL and MO stages (Table 4).

Evidence of maternal transcript degradation was also reflected by a decrease in levels of maternal transcripts, which were represented by those expressed in unfertilized eggs (EG). A heatmap of the top 500 transcripts expressed in eggs revealed that expression of these transcripts began to decline at the 8- to 16-cell and morula stages (Figure 5). By the blastula

stage, the overall patterns of the maternal transcript levels had completely changed, with moderate retention of some maternal transcripts, although the majority had dramatically decreased. Most of these maternal transcripts continued to show low levels relative to the eggs during the remaining stages of development (i.e., gastrula through pluteus stages). Taken together, the degradation of maternal RNAs and the resulting reduction in expression of maternal transcripts began as early as the 8-cell stage, although it is possible that the process begins even sooner after fertilization at a stage prior to what was examined in this study (e.g., at the 2-cell or 4-cell stage). This result is similar to the timing of maternal mRNA degradation in *S. purpuratus*, in which maternal transcripts are destabilized by early cleavage stages (Tadros and Lipshitz 2009; Tu et al. 2014; Wei et al. 2006).

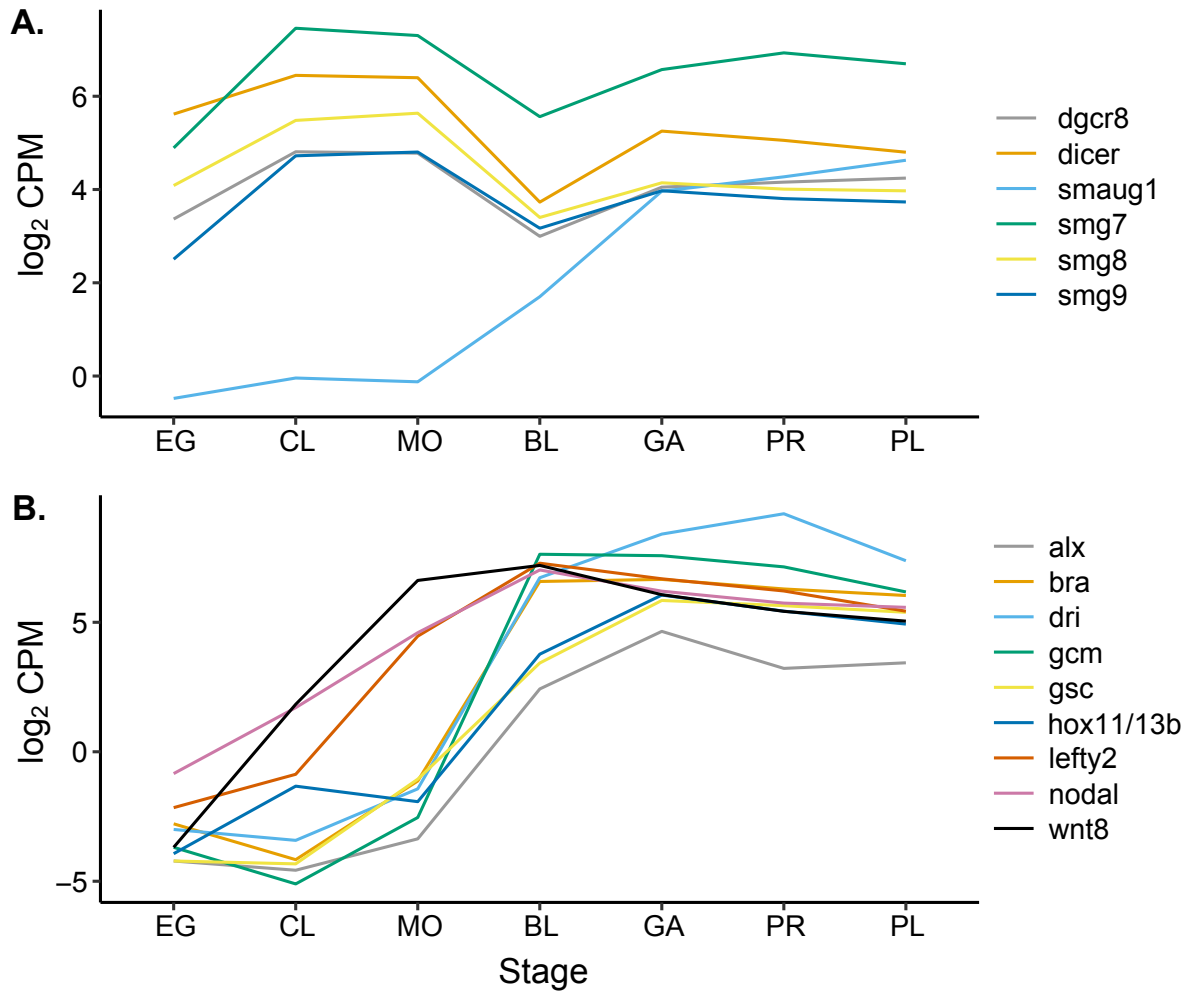


Figure 4. The expression of putative genes that play a functional role during the MZT. These genes **A.** regulate the removal of mRNA, and **B.** regulate zygotic development. The data are in \log_2 counts per million reads (\log_2 CPM) expressed at each stage of development: egg (EG), 8- to 16-cell (CL), morula (MO), blastula (BL), gastrula (GA), prism (PR), and pluteus (PL).

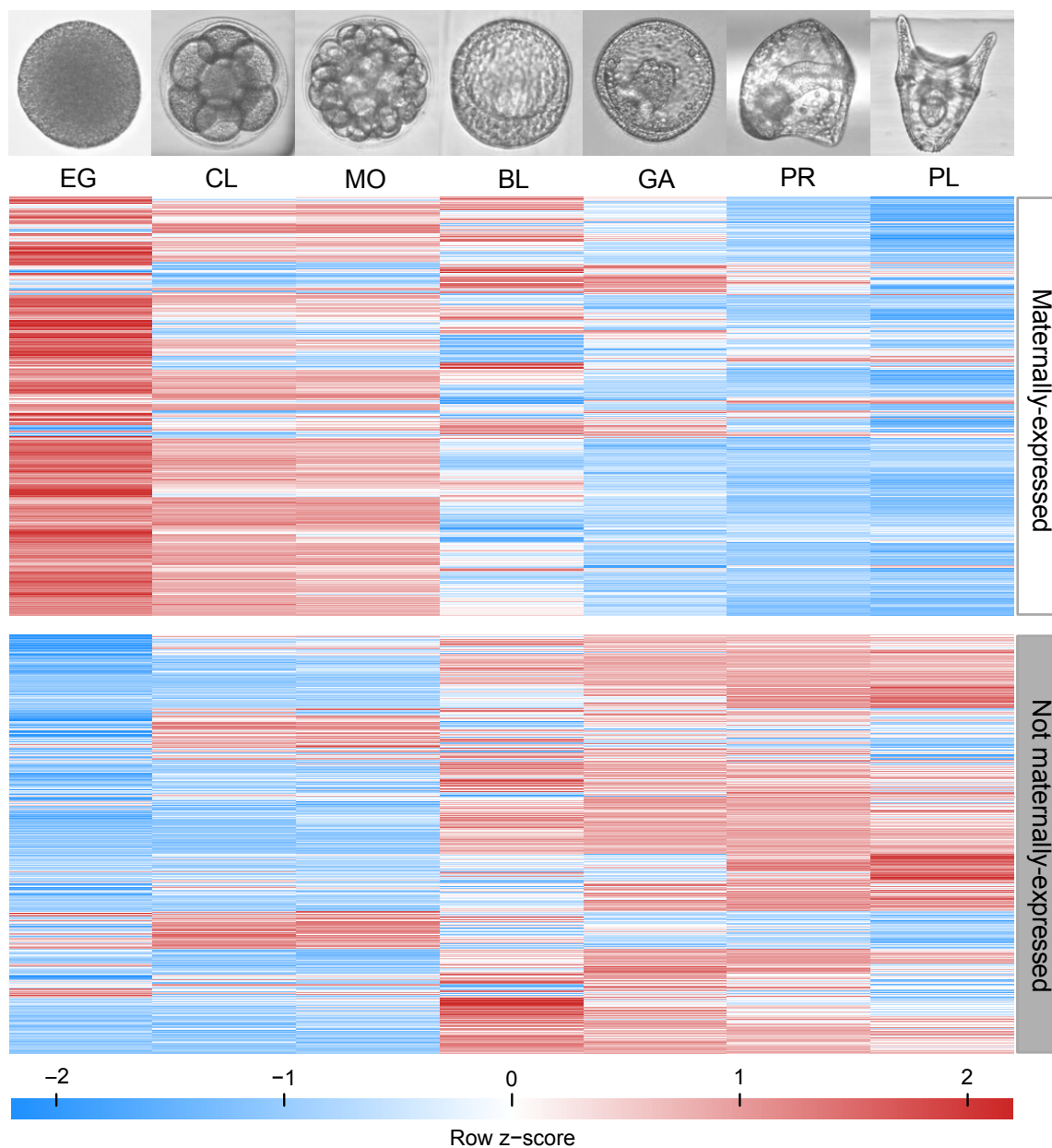


Figure 5. Heatmaps of relative expression profiles for transcripts that are or are not maternally expressed. The rows are transcripts and the columns are in order of developmental stage: egg (EG), 8- to 16-cell (CL), morula (MO), blastula (BL), gastrula (GA), prism (PR), and pluteus (PL). Transcript expression data are in \log_2 counts per million reads (\log_2 CPM), and the data are scaled by row. The top heatmap contains the top 500 maternal transcripts expressed at EG (i.e., transcripts that are maternally expressed). The bottom heatmap contains 19,044 transcripts with a negative expression value of \log_2 CPM (\log_2 CPM < 0) at EG (i.e., transcripts that are not maternally expressed).

To examine the timing of zygotic genome activation, nine putative genes important for zygotic development were identified within the *M. franciscanus* transcriptome (Table 1). Of these, *hox11/13b*, *lefty2*, *nodal*, and *wnt8* increased in expression between egg and the earliest measured developmental stage, the 8- to 16-cell stage (Figure 4B). The expression levels of *wnt8*, *nodal*, and *lefty2* increased further during the morula stage before they plateaued and maintained relatively consistent levels of expression from the blastula stage through the remainder of development. This is somewhat similar to the expression patterns observed in *S. purpuratus*, in which many of the zygotic genes increased during cleavage stages and reached peak expression levels at the blastula stage (Tadros and Lipshitz 2009; Tu et al. 2014; Wei et al. 2006). The *homeobox 11/13b* (*hox11/13b*) gene is one of the earliest transcription factors necessary for endoderm cell specification in echinoderms (Peter and Davidson 2010). Similar transcriptional mechanisms may underlie both *left-right determination factor-2* (*lefty2*) and *nodal* expression, which function together to establish the oral-aboral embryonic axis (Adachi et al. 1999; Duboc et al. 2008; Duboc et al. 2004). Lastly, *wnt8* is required for endomesoderm development in sea urchins, including cell differentiation and gastrulation processes (Minokawa et al. 2005; Wikramanayake et al. 2004). In agreement with our results, in *S. purpuratus* expression of a *wnt8* homolog has been observed beginning at the 16-cell stage (Wikramanayake et al. 2004). The expression of these transcripts during the CL and MO stages support that the activation of zygotic transcription in *M. franciscanus* may occur as early as third cleavage.

The remaining five zygotic transcripts, *alx*, *bra*, *dri*, *gcm*, and *gsc* remained at low levels of expression until the blastula stage (BL), at which time most reached their peak levels of expression (Figure 4B). The expression of these transcripts remained fairly

consistent for the remainder of development. *Aristaless-like homeobox (alx)* expression has a role in primary mesenchyme cell formation, and acts as an early regulatory gene for skeletogenesis (Ettensohn 2009; Ettensohn et al. 2003). *Brachyury (bra)* functions in gastrulation and endoderm development (Peterson et al. 1999; Rast et al. 2002). The *dead ringer (dri)* gene is required for normal embryological development and is highly conserved across taxa (Shandala et al. 1999), functioning in skeletogenesis and oral ectoderm formation in sea urchins (Amore et al. 2003). The *glial cells missing (gcm)* gene functions in endomesoderm specification, particularly those of pigment cells (Ransick and Davidson 2006; Ransick et al. 2002). Lastly, in sea urchin embryos, *gooseoid (gsc)* plays a role in regulating cell specification along the animal-vegetal and oral-aboral axes (Angerer et al. 2001).

The expression patterns of *hox11/13b*, *lefty2*, *nodal*, and *wnt8* may represent a first wave of zygotic activation during CL and MO stages, while the expression patterns of *alx*, *bra*, *dri*, *gcm*, and *gsc* may represent a second wave of zygotic genome activation that occurs at the BL stage. This pattern of zygotic genome activation is very similar to that of *S. purpuratus*, in which there is a minor wave of zygotic transcription during early cell divisions of the embryo, followed by a major wave of zygotic transcription at the blastula stage (Tadros and Lipshitz 2009; Wei et al. 2006). Further evidence of the timing of zygotic genome activation was supported by the appearance of transcripts not associated with the maternal transcriptome. A heatmap of transcripts that were not expressed in unfertilized eggs (i.e., transcripts that were not maternally-derived) showed that a moderate number of these transcripts began to be expressed by the 8- to 16-cell and morula stages (Figure 5). This event may represent the first, minor wave of zygotic activation. The majority of the

transcripts that were not present in eggs, however, were expressed by the blastula, gastrula, prism, and pluteus stages. Thus, the appearance of these transcripts at the blastula stage appear to represent the major wave of zygotic activation in *M. franciscanus* development.

Overall, the timing of the MZT in *M. franciscanus* appears to span from early cleavage through the blastula stage, in which 1) maternal degradation begins at or before the 8- to 16-cell stage and persists to the blastula stage, and 2) zygotic activation occurs as a minor wave at the 8- to 16-cell and morula stages and manifests as a major wave by the blastula stage. The occurrence of the MZT may partially explain the clear separation between egg/early embryonic stages (i.e., EG, CL, and MO) and later developmental stages (i.e., BL, GA, PR, and PL) evident in Figure 2.

Conclusions

The transcriptome presented here is a useful molecular resource for studying *M. franciscanus*, a non-model organism and an important fishery species. We observed distinct patterns of gene expression across the eggs and early development of this sea urchin species, and identified periods of maternal RNA degradation and zygotic transcription. This genomic resource will support future investigations into the early development of *M. franciscanus*, and its response to environmental stress. In terms of developmental studies, the examination of the timing of the MZT will inform future gene expression studies that aim to target stages in which the zygotic transcriptome is fully activated. Lastly, these studies will facilitate our understanding of a marine species that has a significant ecological role in kelp forest ecosystems as a grazer, and is an economically valuable fishery species.

Data deposition

The raw sequence data and the *M. franciscanus* developmental transcriptome are available under NCBI Bioproject PRJNA531463 and Sequence Read Archive accession numbers SRR8866315 – SRR8866335. The assembled transcriptome can be found in the NCBI Transcriptome Shotgun Assembly (TSA) database under accession GHJZ00000000. Additional data files including the transcriptome annotation can be found at <http://doi.org/10.5281/zenodo.2649269>.

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Supplementary material

Table S1 BLAST results for the developmental transcriptome assembly.

Table S2 List of GO terms identified in the developmental transcriptome assembly.

Table S3 KEGG results for the developmental transcriptome assembly.

Table S4 Gene ontology annotation following functional enrichment of genes within module eigengenes generated by WGCNA.

III. The effects of temperature and $p\text{CO}_2$ on the size, thermal tolerance and metabolic rate of the red sea urchin (*Mesocentrotus franciscanus*) during early development

Abstract

Abiotic stressors associated with ocean warming and ocean acidification can adversely impact marine organisms, particularly during their early development. Unfortunately, little is known about how the red sea urchin, *Mesocentrotus franciscanus*, responds to temperature or $p\text{CO}_2$ stress during its early development. Here, embryos of *M. franciscanus* were raised under combinations of two temperatures (13 °C and 17 °C) and two $p\text{CO}_2$ levels (475 μatm and 1050 μatm). Elevated $p\text{CO}_2$ levels led to a decrease in body size of gastrula stage embryos while temperature had no effect. At the prism stage, both temperature and $p\text{CO}_2$ affected body size. The warmer temperature increased the body size of prism stage embryos, counteracting the stunting effect of elevated $p\text{CO}_2$ on growth. Warmer temperature during development also increased thermal tolerance at the prism stage, although this effect was small. Neither temperature nor $p\text{CO}_2$ affected embryo metabolic rate. This study provides important insight into a species of high ecological and economic value and will facilitate our predictive capacity of how climate change will impact future populations.

Introduction

Carbon dioxide (CO_2) concentrations in Earth's atmosphere have been on the rise since the Industrial Revolution, and this upward trajectory is predicted to continue (Godbold and Calosi 2013; IPCC 2013). Ocean warming and ocean acidification are major

consequences of the increase in atmospheric CO₂, and produce considerable alterations in oceanic conditions worldwide. By the end of the 21st century, surface ocean temperatures are predicted to rise by 0.6 – 2.0 °C, while pH levels are expected to drop by 0.1 – 0.4 units (IPCC 2013). Many studies have focused on how a single environmental stressor impacts marine organisms. However, because factors such as ocean temperature and pH are predicted to change, often simultaneously, this approach may not be adequate to accurately predict organismal responses to continuing ocean warming and acidification (Bockmon et al. 2013; Byrne and Przeslawski 2013). It is therefore highly valuable to incorporate multiple stressors into experiments that reflect realistic and ecologically relevant current and future ocean conditions (Frieder et al. 2014; Przeslawski et al. 2015). Such studies are also critical for determining optimal or detrimental conditions for economically valuable species (Munari et al. 2011).

Early developmental stages may be the most vulnerable times during the life history of many marine organisms (Byrne 2011; Dupont and Thorndyke 2009; Gosselin and Qian 1997; Kurihara 2008), and with continuing alterations in the marine environment due to climate change, these stages may act as a bottleneck that determines if a species will be successful in the future (Byrne 2012; Byrne and Przeslawski 2013; Kurihara 2008). Thermal stress and elevated *p*CO₂ levels are expected to affect different life stages to varying degrees, and negative impacts have the potential to carry over into later stages, altering population structure and distribution (Beckerman et al. 2002). In this chapter, I have focused on the early developmental stages of the red sea urchin, *Mesocentrotus franciscanus*, and assessed how they responded to stressors associated with ocean warming and acidification.

Given the large ecological role of *M. franciscanus* within kelp forest ecosystems (Leighton et al. 1966; Rogers-Bennett 2007) and the economic value of the *M. franciscanus* fishery (Kalvass 2000; Rogers-Bennett 2013), surprisingly few studies have been conducted to assess how this species responds to environmental stressors. Elevated $p\text{CO}_2$ levels have been shown to increase sperm limitation and the potential for polyspermy, and decrease fertilization success in *M. franciscanus* (Frieder 2014; Reuter et al. 2011). While multistressor studies have focused on some sea urchin species, examination of *M. franciscanus* development under combined $p\text{CO}_2$ and temperature stressors has remained almost entirely absent from climate change biology. In one study, O'Donnell et al. (2009) found that *M. franciscanus* larvae raised under elevated $p\text{CO}_2$ levels exhibited a decreased ability to respond to acute elevated temperatures, measured by a decrease in expression levels of *hsp70*. Gaps of knowledge remain regarding how this species responds to elevated temperatures and $p\text{CO}_2$ levels, both as singular and as combined stressors. These gaps limit our understanding of how current populations are responding to stress, and restrict our predictive capacity of how future populations will fare under continued ocean warming and acidification.

Here, I investigated the response of *M. franciscanus*, a species that has been overlooked in climate change biology despite its sizeable ecological and economic importance (Pearse 2006; Quinn et al. 1993; Rogers-Bennett 2013), to different temperature and $p\text{CO}_2$ conditions during its early development. Embryos of *M. franciscanus* were raised under different combinations of temperature (13 °C and 17 °C) and $p\text{CO}_2$ level (475 μatm and 1050 μatm) that represented ecologically relevant present and future ocean conditions. I examined how these stressors affected embryo body size at both the gastrula and prism stages

of early development. I also measured thermal tolerance and metabolic rate at the prism stage to examine how developmental temperature and $p\text{CO}_2$ conditions affected embryo physiology.

Materials and methods

Animal collection and culturing

Adult *M. franciscanus* were hand-collected by SCUBA on February 21, 2018 from a site in the Santa Barbara Channel near Ellwood Mesa, Goleta, CA (34° 25.065'N, 119° 54.092'W) at a depth of 14 meters under CA Scientific Collection permit SC-1223. The sea urchins were immediately transported to the Marine Science Institute at the University of California Santa Barbara (UCSB), where they were maintained in flow-through seawater tanks for approximately one week prior to spawning. Adults were spawned via an intracoelomic injection of 0.53 M KCl (Strathmann 1987). Sperm from a single adult male was collected dry, and was activated by dilution in 0.35 μm filtered, UV-sterilized seawater (FSW) immediately prior to performing crosses. Eggs were collected in FSW from five adult females. Eggs were visually inspected for quality and maturity (i.e., approximately symmetrical in shape and lack large, visible germinal vesicles). Subsamples of eggs were fertilized to ensure high male-female compatibility within each cross. An approximately equal number of eggs from each of the five females were gently pooled together. Dilute, activated sperm from the single male was added to the pooled eggs until approximately 98% fertilization success was reached.

The culture vessels and CO_2 system used were as previously described in Chapter II, with minor modifications to reach the desired temperature and $p\text{CO}_2$ conditions of each

treatment. The temperature was modified using two Delta Star ® heat pumps with Nema 4x digital temperature controllers (AquaLogic, San Diego, CA, USA) to maintain the culturing temperatures at either ~13 °C or ~17 °C. A flow-through CO₂-mixing system modified from (Fangue et al. 2010) was used to establish *p*CO₂ levels of either ~475 µatm or ~1050 µatm. Four 5-gallon reservoir tanks were used to establish the target *p*CO₂ levels for each temperature, resulting in four total treatments. These temperature and *p*CO₂ conditions were selected as representative of conditions currently measured in the Santa Barbara Channel and projected conditions given continuing ocean warming and acidification (Chan et al. 2017; IPCC 2013; Kapsenberg and Hofmann 2016; Rivest et al. 2016). Treated water was transported to each culture vessel at a rate of 6 L/hr. Three replicate cultures were used for each experimental condition for a total of 12 culture vessels. Approximately 120,000 embryos were placed into each culture vessels at a concentration of no more than 10 embryos per mL of FSW.

Temperature, pH, salinity, and total alkalinity (TA) were recorded daily throughout embryo development. Temperature was measured using a wire thermocouple (Thermolyne PM 20700 / Series 1218). Salinity was measured using a conductivity meter (YSI 3100). The pH was measured following the standard operating procedure (SOP) 6b (Dickson et al. 2007b), using a *m*-cresol purple (Sigma-Aldrich) indicator dye and a spectrophotometer (Bio Spec-1601, Shimadzu). Water samples for measuring TA were preserved by poisoning FSW with saturated 0.02% mercuric chloride and storing the samples at 4 °C until analyzed. The TA was estimated using SOP 3b (Dickson et al. 2007a). Using the measured temperature, spectrophotometric pH, salinity, and TA, parameters of *p*CO₂ and aragonite saturation state

(Ω_{ara}) were calculated using the carbonic acid dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) using CO₂calc software (Robbins et al. 2010).

Sampling

The early gastrula stage (~23.5 hours post-fertilization (hpf) at ~17 °C and ~32.5 hpf at ~13 °C) and the prism stage (~44 hpf at 17 °C and ~55.5 hpf at 13 °C) were sampled from each culture bucket. The early gastrula stage was identified by the extension of the archenteron to approximately one-half the body length of the embryo and by the formation of secondary mesenchyme cells. The prism stage was designated by the archenteron becoming tripartite, the development of skeletal rods, and the formation of a pyramid-like body shape. Sampling was accomplished by gently siphoning embryos from the culture vessels onto a submerged, mesh filter (35 μm). Embryos were carefully concentrated onto the mesh filter and transferred to a 15 mL falcon tube using a plastic transfer pipette. The total number of embryos collected from each culture vessel at each stage was estimated by counting three small aliquots of embryos so that a coefficient of variance (CV) of less than 10% was reached. The average of the counts was used to calculate the average number of embryos per mL of FSW.

Morphometrics

Gastrula-stage embryos were preserved for body size analyses using 4% formalin in FSW. The fixative for the prism stage was 4% formalin in 0.01 M phosphate buffered saline (PBS) that was buffered with 100 mM sodium perborate to a pH of 8.7. This buffered formalin was used for the prism stage to prevent dissolution of the skeletal rods. The 4%

fixatives were added to an equal volume of seawater with embryos, fixing the samples in a final concentration of 2% formalin in seawater. These samples were stored at 4 °C for no longer than three weeks prior to imaging.

At both the gastrula and prism stages, individual embryos ($n = 35$) from each of the 12 culture vessels (i.e., 4 treatments with 3 replicate vessels each) were photographed. Embryos were digitally photographed under bright field DIC illumination using a compound microscope (Olympus BX50) with an attached digital camera (Infinity Lite) and Motic Images Plus software (version 3.0). The gastrula embryos were oriented so that the side profile of their archenteron was visible and aligned with the center of the vegetal plate. For the gastrula stage, the length (i.e., the linear distance from the anterior to posterior end when measured across the center of the archenteron) and 2-dimensional area were measured (Figure 6). The prism stage was oriented in a ventral view and each individual was measured by the length of the skeletal rod, from the tip of the body rod to the tip of the post-oral rod (Figure 6). The digital images were calibrated for the 20X objective using ImageJ (National Institutes of Health, USA). Differences in body size measured at the gastrula and prism stages were tested using a two-way ANOVA with temperature and $p\text{CO}_2$ set as fixed factors and culture vessel identity set as a random factor. These statistical analyses were performed using JMP Pro software (version 11.2.0).

Thermal tolerance

Thermal tolerance was only measured for the prism stage. For each treatment, embryos across replicate culture vessels were pooled together to obtain approximately 7,000 embryos per treatment. For each treatment, the embryos were divided equally among seven

20-mL vials so that each vial contained approximately 1,000 embryos in 5 mL of FSW (200 embryos/mL). Using two water baths to create a temperature gradient across an aluminum heat block, the embryos were exposed to a range of temperatures spanning from 16.1 to 32.2 °C for one hour. Temperatures were recorded using a wire thermocouple (Thermolyne PM 20700 / Series 1218). All vials were placed randomly within the heat block while ensuring that each temperature contained a vial from each treatment. Following the 1-hour exposure, the vials were removed and 100 embryos from each vial were scored as either alive or dead based on the presence or absence of ciliary movement viewed under a light microscope. Hammond and Hofmann (2010) tested the effects of recovery periods of different lengths following a 1-hour temperature treatment of urchin larvae, and found that recovery periods did not significantly affect mortality. Therefore, it was not deemed necessary to incorporate a recovery period into this experiment. Due to the differences in timing of developmental progression, this procedure was performed separately for embryos raised under the 13 °C treatment and embryos raised under the 17 °C.

Using the binary mortality data (alive or dead) for prism embryos exposed to each temperature, a generalized linear mixed-effects model was used to test for differences in thermal tolerance across treatments. The model included temperature treatment and $p\text{CO}_2$ treatment as fixed factors and vial identity as a random factor. Lethal temperature (LT) values were calculated via a logistic regression for each treatment. While LT_{50} values are the standard metric used when assessing temperature sensitivity (de Vries et al. 2008), LT_{10} and LT_{25} values were also calculated because these smaller reductions in survival may still have biologically meaningful consequences (Collin and Chan 2016). The statistical analyses for

thermal tolerance were performed using the lme4 (Bates et al. 2015), MASS (Venables and Ripley 2002), and base packages in R (version 3.4.4).

Respirometry

To examine metabolic differences in embryos raised under different temperature and $p\text{CO}_2$ conditions, oxygen consumption rates were measured in embryos from each treatment according to Marsh and Manahan (1999), with some modifications. Metabolic rate was only measured for the prism stage. Oxygen concentrations were measured following a 5- to 6-hour incubation of different densities of embryos ($n = 34$ to 534) placed in glass respirometry chamber vials (684 to $795 \mu\text{L}$). Embryos were placed in FSW that matched their respective $p\text{CO}_2$ treatment, and all vials were incubated at a single, intermediate temperature, 15°C . A single incubation temperature was chosen in an attempt to mediate differences in development and growth that would occur across treatments during the incubation period if two different incubation temperatures were used. One vial for each of the three culture replicates per treatment was filled with only FSW and used as a control to account for any possible background respiration. At the end of each incubation, the seawater was transferred using a gas-tight syringe (Hamilton Company, USA) to an optode containing a fiber-optic oxygen meter (Micro TX3; PreSens, Germany) that had been calibrated using sodium sulfite (Na_2SO_3) and FSW. A standard curve was generated from which the rate of oxygen consumption per individual (in $\text{pmol O}_2/\text{hr}/\text{individual}$) was estimated. Differences in respiration rates across treatments were tested using a two-way ANOVA using JMP Pro software (version 11.2.0), with temperature and $p\text{CO}_2$ set as fixed factors and culture vessel identity set as a random factor.

Results

Culturing of embryos

The early development of the *M. franciscanus* was normal, with few observed morphological abnormalities and low mortality rates. In addition, seawater conditions remained largely stable throughout the culturing period; here, target temperatures of ~13 °C or ~17 °C were maintained via chillers, and $p\text{CO}_2$ levels (~475 μatm or ~1050 μatm) were maintained by a CO_2 mixing system described previously in Chapter II (Table 5). The average salinity was 33.3 ± 0.05 and the average TA was $2222 \pm 3 \mu\text{mol kgSW}^{-1}$.

Body size

All morphometric data were tested to verify that they met the assumption of an ANOVA (i.e., approximate normality and homogeneity of variance). For the gastrula stage, there was no significant effect of the temperature treatment on either gastrula length ($F_{1,9} = 4.7359$, $p = 0.0575$) or 2D area ($F_{1,9} = 2.3969$, $p = 0.1560$) (Figure 6A, B). There was, however, a significant effect of the $p\text{CO}_2$ treatment on both gastrula length ($F_{1,9} = 44.6841$, $p < 0.0001$) and 2D area ($F_{1,9} = 43.6387$, $p < 0.0001$). Gastrula raised under low $p\text{CO}_2$ conditions (475 μatm) were on average 4.83% longer and 8.62% greater in area than those raised under high $p\text{CO}_2$ conditions (1050 μatm). There was no significant interaction between temperature and $p\text{CO}_2$ treatment conditions for the gastrula stage ($p > 0.05$).

Table 5. Seawater conditions throughout embryological development. All values are given as mean \pm standard deviation.

Treatment	Temperature ($^{\circ}\text{C}$)	pH	$p\text{CO}_2$	Ω_{ara}
17 $^{\circ}\text{C}$, 1050 μatm	17.2 \pm 0.14	7.67 \pm 0.004	1047 \pm 11	1.15 \pm 0.01
17 $^{\circ}\text{C}$, 475 μatm	17.2 \pm 0.13	7.96 \pm 0.006	491 \pm 6	2.10 \pm 0.03
13 $^{\circ}\text{C}$, 1050 μatm	13.1 \pm 0.10	7.66 \pm 0.005	1039 \pm 13	0.97 \pm 0.01
13 $^{\circ}\text{C}$, 475 μatm	13.2 \pm 0.04	7.98 \pm 0.003	465 \pm 4	1.88 \pm 0.01

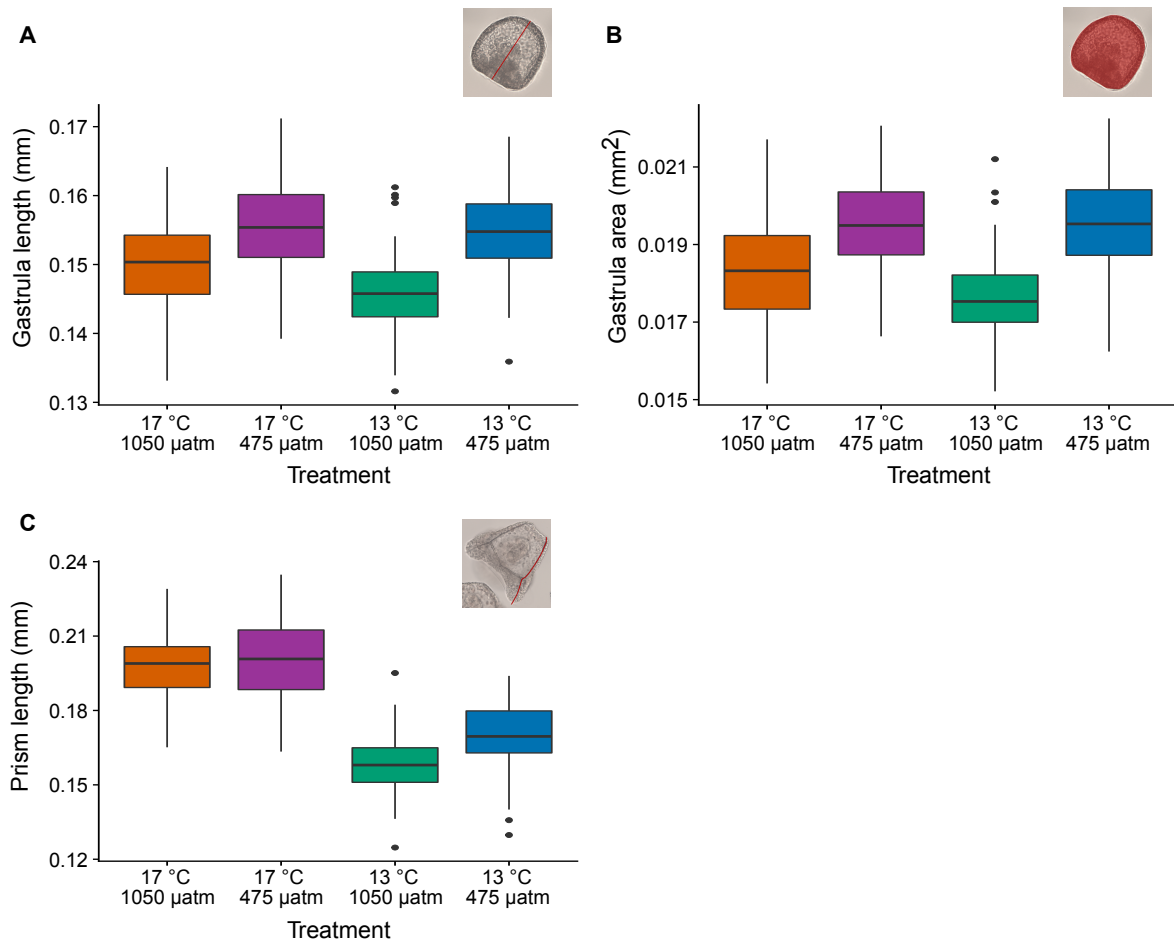


Figure 6. Body size measurements of embryos under different temperature and $p\text{CO}_2$ treatment conditions, including **A.** the length of the gastrula stage, **B.** the 2D area of the gastrula stage, and **C.** the length of the prism stage. Bars show standard error.

Unlike the gastrula stage, at the prism stage there were treatment effects for both temperature and $p\text{CO}_2$. There was a significant effect of temperature treatment on prism length ($F_{1,9} = 160.5712$, $p < 0.0001$), in which embryos raised under high temperature conditions (17 °C) were on average 20.81% greater in length than those raised under low temperature conditions (13 °C) (Figure 6C). There was also a significant effect of $p\text{CO}_2$ treatment on prism length ($F_{1,9} = 7.2205$, $p = 0.0249$), in which embryos raised under low $p\text{CO}_2$ conditions (475 μatm) were on average 4.08% greater in length than those raised under high $p\text{CO}_2$ conditions (1050 μatm). There was no significant interaction between temperature and $p\text{CO}_2$ treatment conditions for the prism stage ($p > 0.05$).

Thermal tolerance

Unfortunately, due to the loss of embryos during the sampling process, I was unable to include embryos from two culture vessels (one replicate of 17 °C, 1050 μatm and one replicate of 17 °C, 475 μatm). Therefore, for these two treatments, only representatives from two of the three replicate culture vessels were pooled together for the thermal tolerance assay. The survivorship of prism stage embryos was assessed across a range of temperatures (16.1 to 32.2 °C) to generate performance curves for each treatment (Figure 7). A generalized linear mixed-effects model found that there was an effect of temperature treatment on the survivorship of embryos in response to temperature ($p = 0.0389$). However, $p\text{CO}_2$ treatment had no such effect ($p = 0.8431$). The calculated LT_{10} , LT_{25} , and LT_{50} values of embryos raised under the high temperature treatment (17 °C) were greater than those of embryos raised under the low temperature treatment (13 °C) (Table 6). Although the effect of temperature treatment on thermal tolerance was statistically significant, the difference in

LT₅₀ values was relatively small. Specifically, the average LT₅₀ of embryos raised at 17 °C was only 0.3 °C higher than the average LT₅₀ of embryos raised at 13 °C.

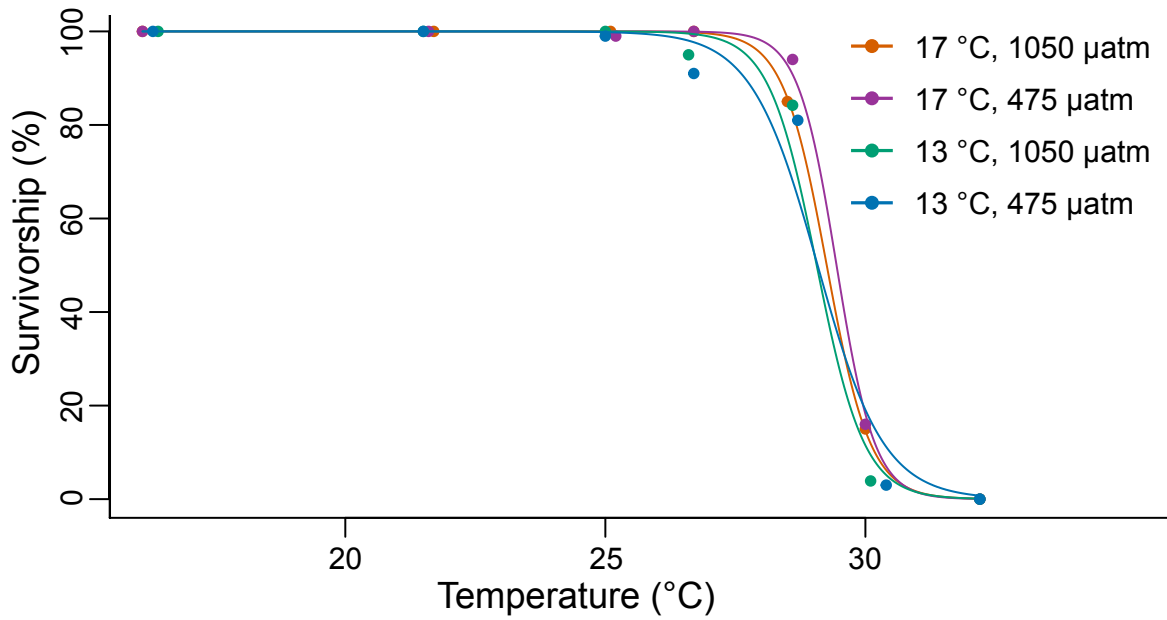


Figure 7. Thermal tolerance of prism stage embryos following 1-hour temperature exposures. Points are plotted as percent survivorship with the lines showing the logistic regression for each treatment.

Table 6. LT₁₀, LT₂₅, and LT₅₀ values for each treatment. All values are given as mean \pm standard error.

Treatment	LT ₁₀	LT ₂₅	LT ₅₀
17 °C, 1050 µatm	28.3 \pm 0.1	28.8 \pm 0.1	29.3 \pm 0.1
17 °C, 475 µatm	28.6 \pm 0.1	29.0 \pm 0.1	29.4 \pm 0.1
13 °C, 1050 µatm	28.1 \pm 0.1	28.6 \pm 0.1	29.1 \pm 0.1
13 °C, 475 µatm	27.7 \pm 0.2	28.4 \pm 0.1	29.1 \pm 0.1

Respiration rate

The respirometry data was tested to verify it met the assumption of an ANOVA (i.e., approximate normality and homogeneity of variance). There was no effect of temperature treatment ($F_{1,9} = 1.9819$, $p = 0.1927$) or $p\text{CO}_2$ treatment ($F_{1,9} = 0.9042$, $p = 0.3665$) on the respiration rate of prism embryos (Figure 8). Additionally, there was no evident relationship between the rate of oxygen consumption and embryo body size (i.e., the average length of prism embryos from each culture vessel) ($p > 0.056$).

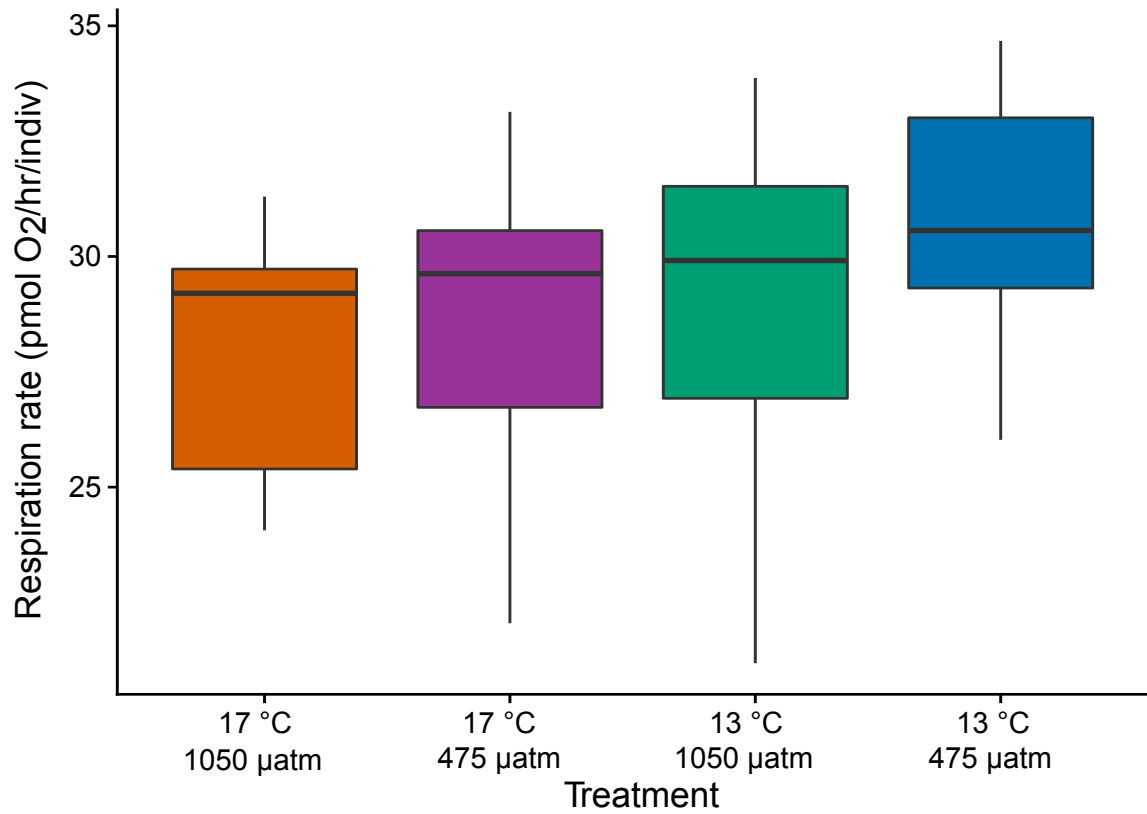


Figure 8. Respiration rates (expressed as pmol O₂ per hour per individual) for prism embryos raised under different temperature and pCO₂ treatment conditions. Error bars show standard error.

Discussion

Increasing temperatures and $p\text{CO}_2$ levels are anticipated to impact the early developmental stages of many marine organisms (Byrne 2011; Byrne and Przeslawski 2013; Kroeker et al. 2013). However, there remains limited knowledge regarding how *M. franciscanus* will be impacted by these environmental stressors despite its considerable economic and ecological value as a fisheries species that inhabits kelp forest ecosystems. In this study, I investigated how elevated temperature and $p\text{CO}_2$ conditions affected the morphometry and physiology of developing *M. franciscanus* embryos. There were four salient findings: 1) at the gastrula stage, only $p\text{CO}_2$ had an effect on embryo body size, 2) at the prism stage, temperature and $p\text{CO}_2$ had an antagonistic effect on body size, although temperature appeared to be the dominant factor, 3) temperature had a modest effect on thermal tolerance of the prism stage, and 4) neither $p\text{CO}_2$ or temperature affected prism metabolic rate.

Only $p\text{CO}_2$ impacts gastrula size

The effects of temperature and $p\text{CO}_2$ on embryo body size differed by developmental stage. Temperature had no effect on body size of gastrula embryos, while elevated $p\text{CO}_2$ conditions decreased both gastrula length and 2D area. Some studies have shown that elevated $p\text{CO}_2$ levels can decrease gastrulation success and survival in some echinoderm species (Ericson et al. 2010; Foo et al. 2014; Martin et al. 2011; Nguyen et al. 2012). In the sea urchins *Strongylocentrotus purpuratus* and *Heliocidaris erythrogramma*, however, high $p\text{CO}_2$ was found to have no effect on developmental progression or morphological abnormality in gastrula embryos (Byrne et al. 2009; Padilla-Gamiño et al. 2013). In this

study, while abnormality and mortality was not directly measured, visual inspections of the cultures supported largely normal gastrula development, with less than 5% abnormality estimated across all culture replicates. Thus, while an effect of $p\text{CO}_2$ on mortality or abnormality was not observed, there was an effect on morphometry.

Most ocean acidification studies have not examined the effects of $p\text{CO}_2$ on embryo size, instead focusing primarily on the body size of later, calcifying stages (e.g., pluteus larvae). However, elevated $p\text{CO}_2$ levels can impact processes other than calcification via alterations in acid-base regulation physiology (Fabry et al. 2008). Similar to what was observed in this study, low pH levels have been shown to decrease gastrula embryo length in the Antarctic urchin, *Sterechinus neumayeri* (Ericson et al. 2010). This decrease in size may have significant biological consequences, as larger body sizes during early development have often been linked to higher fitness (Smith and Fretwell 1974). Organisms that are larger during early development may exhibit greater performance (i.e. greater growth, survivorship, and reproductive output) at later life stages (Marshall et al. 2003; Moran and Emlet 2001). Thus, a decrease in body size, even at a single stage during early development, may have adverse long-term effects on an organism's fitness, particularly when the stressor occurs during an essential developmental process such as gastrulation.

Temperature and $p\text{CO}_2$ impact prism size

Unlike at the gastrula stage, there were effects of both temperature and $p\text{CO}_2$ on body size at the prism stage. Prism development is immediately prior to the formation of the early pluteus larval stage and includes initial calcification processes for skeletal rod formation.

Here, temperature appears to be the dominant factor, though an effect of $p\text{CO}_2$ is still evident. Although a statistically significant interaction between stressors was not detected, temperature and $p\text{CO}_2$ appear to have an antagonistic effect on prism body size, in which elevated temperature appears to increase and offset the decrease in body size due to elevated $p\text{CO}_2$ levels. Similar patterns have been observed in sea urchins species from tropical, temperate, and polar regions in which temperature can mitigate the negative effects of low pH on early developmental stages undergoing calcification (Byrne et al. 2013a; Byrne et al. 2013b; Byrne et al. 2013c; Sheppard Brennan et al. 2010). Therefore, as ocean warming and ocean acidification progress, moderate warming may provide a compensatory effect as ocean pH levels continue to decline.

In the absence of increased temperatures, elevated $p\text{CO}_2$ levels stunt prism growth. This was observed in prism embryos raised under the 13 °C, 1050 μatm $p\text{CO}_2$ treatment (Figure 6). The temperature and $p\text{CO}_2$ level of this treatment are characteristic of water conditions that occur during upwelling events in the Santa Barbara Channel (Kapsenberg and Hofmann 2016; Rivest et al. 2016). Upwelling events off the West Coast of North America are caused by a strengthening of northwesterly winds that typically begin in the early spring season (Fabry et al. 2008; Pennington and Chavez 2000). The timing of these events are highly variable, but they can coincide with the peak spawning period of *M. franciscanus*, which typically occurs in the spring and early summer (Bennett and Giese 1995; Kato and Schroeter 1985; Strathmann 1987). It is therefore possible that *M. franciscanus* embryos experience these combined low temperature and low pH conditions in nature when spawning occurs during or immediately prior to an upwelling event. Additionally, the intensity and duration of upwelling events are expected to increase in the future (Diffenbaugh et al. 2004).

Lastly, lower temperatures decrease the rate of development. Here, embryos reached the prism stage after ~55.5 hours post-fertilization (hpf) when raised at 13 °C in comparison to ~44 hpf at 17 °C. Therefore, lower temperatures may also increase the duration this vulnerable developmental stage is exposed to stressful pH conditions.

Temperature impacts thermal tolerance

In addition to body size, I also investigated how temperature and $p\text{CO}_2$ impacted the physiological performance of *M. franciscanus* during the prism stage. Temperature, but not $p\text{CO}_2$, affected thermal tolerance. These results do not agree with observations by O'Donnell et al. (2009), in which elevated $p\text{CO}_2$ conditions appeared to affect the thermal stress response of *M. franciscanus* larvae raised at 15 °C. Specifically, *M. franciscanus* raised under elevated $p\text{CO}_2$ levels exhibited lower levels of expression of the molecular chaperone *hsp70*, a gene affiliated with thermal defense, which the authors suggested meant larvae exposed to low pH conditions were more vulnerable to heat stress. Additionally, O'Donnell et al. (2009) found that following a 1-hour temperature exposure to 31 °C, cultures displayed over 90% survivorship regardless of $p\text{CO}_2$ treatment. In contrast, during this study, less than 16% survivorship was observed across all cultures following a 1-hour temperature exposure to 30 °C, and the calculated LT_{50} values (i.e., the temperature at which 50% mortality occurs (de Vries et al. 2008)) across all treatments were around 29.1 to 29.4 °C. The observed disparities between this study and O'Donnell et al. (2009) could be due to the slight difference in developmental stage (i.e., prism versus pluteus larvae), alterations in culturing temperatures (i.e. 15 °C versus 13 or 17 °C), genetic variance, or transgenerational effects caused by dissimilarities in adult environmental history and quality.

The effect of temperature on thermal tolerance was relatively modest. Nonetheless, LT_{10} , LT_{25} , and LT_{50} values were consistently higher for embryos raised under the higher temperature of 17 °C. Even small differences in temperature can have biologically meaningful consequences. The results of this study differs from observations made in *S. purpuratus*, in which embryos were raised under different temperatures that matched sites where the adults were collected (i.e., sites that varied across the species biogeographic range) were found not to differ in their thermotolerance (Hammond and Hofmann 2010). Other sea urchin species have exhibited a potential for thermal acclimation and adaptation, with offspring of adults from warmer regions displaying increased thermal tolerance during early development (Byrne et al. 2011; Pecorino et al. 2013). In this study, all of the embryos across treatments were from the same parents collected together from the same site. Therefore, I do not anticipate that variation in parental experience affected the differences in thermal tolerance observed here (i.e., transgenerational effects). Rather, the difference in thermal tolerance, while relatively small, is likely due to rapid acclimation and developmental plasticity within embryos raised under the warmer temperature.

Metabolic rate is unaffected by experiment treatments

I also examined the influence of temperature and pCO_2 conditions on the metabolic rate of *M. franciscanus* embryos. In general, metabolic rates have been shown to increase with temperature (Arnberg et al. 2013; McElroy et al. 2012; Pimentel et al. 2012). Low pH levels, however, can have varying effects on metabolism. For instance, low pH may decrease metabolism via extracellular acid-base imbalances and direct hypercapnic suppression, or it may increase metabolism due to higher costs associated with maintaining calcification and

cellular homeostasis (Hoshijima et al. 2017; Pörtner 2008; Stumpp et al. 2011b; Widdicombe and Spicer 2008). In this study, I found no effect of either stressor on the rate of oxygen consumption at the prism stage. This differs from observations by Padilla-Gamiño et al. (2013), in which simultaneous exposure to a warmer temperature and higher $p\text{CO}_2$ level depressed respiration rates of *S. purpuratus* pluteus larvae.

These discordant results between studies could be due to species-level variances or differences in respirometry methods. Here, I measured respiration rates at a single, intermediate temperature (15 °C) for the purpose of removing the confounding factor of increased developmental rate under warmer temperatures. However, if the temperature treatment was maintained throughout respiration measurements, I may have detected a similar effect of simultaneous high temperature and $p\text{CO}_2$ exposure on metabolic rate that was observed in *S. purpuratus* (Padilla-Gamiño et al. 2013). The lack of metabolic differences in this study could also be due to the developmental stage that was examined. For instance, Stumpp et al. (2011b) found that there was an effect of $p\text{CO}_2$ on metabolic rate only once pluteus larvae began feeding. At earlier embryonic stages, metabolic rate did not vary by $p\text{CO}_2$ treatment. Therefore, the effects of temperature and $p\text{CO}_2$ on metabolic rates in *M. franciscanus* may only be visible later in development.

Conclusions

The goal of this study was to provide much needed information regarding a species of significant ecological and economic value by examining its capacity to respond to stressors related to climate change under an ecologically relevant context. Here, I did not observe a decline in successful gastrulation as has been recorded in other urchin species but did observe

a decrease in body size due to high $p\text{CO}_2$ levels. This leads to two points for consideration regarding studies of this nature. First, even though they may not be actively undergoing calcification, earlier embryological stages should not be overlooked in ocean acidification studies. Low pH can influence important processes other than calcification, and negative impacts during early embryological development can have carry-over effects and/or act as a major bottleneck for populations (Byrne 2011). Second, while most studies report the percent success of gastrulation, few report how stressors impact gastrula body size. Sublethal effects, however, may still have significant biological consequences (Przeslawski et al. 2015).

Moderate ocean warming may be beneficial to *M. franciscanus* during early development, helping to offset the negative effects of ocean acidification or acute warming events. This will not occur, however, during periods of upwelling, in which organisms will experience elevated $p\text{CO}_2$ levels without simultaneous exposure to elevated temperatures. It is also important to note that while elevated temperatures can increase embryo growth and thermal tolerance, there may be unanticipated biological consequences to ocean warming. Future studies would benefit from examining the effects of prolonged exposure to high temperatures, particularly as *M. franciscanus* begin feeding as pluteus larvae and metamorphose into juveniles. There may be negative carry-over effects of elevated temperatures, and what may be beneficial at the prism stage could be detrimental at other life history stages. For instance, warming can decrease gonad quality and increase disease susceptibility in adult sea urchins (Tajima et al. 1997; Uthicke et al. 2014). Additionally, quicker development rates under elevated temperatures may shorten the planktonic larval duration (i.e., the length of time embryos and larvae are in the plankton prior to metamorphosis and settlement), which can affect dispersal, recruitment and gene flow

dynamics (Byrne et al. 2011). Overall, more work is required to understand how positive or negative effects of ocean warming will interact with ocean acidification, and to accurately predict how they will impact populations of *M. franciscanus*.

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IV. Gene expression patterns of red sea urchins (*Mesocentrotus franciscanus*) exposed to different combinations of temperature and $p\text{CO}_2$ during early development

Abstract

Gene expression analyses allow for a molecular-level examination of how organisms respond to their environment. Here, differences in gene expression patterns were observed in embryos of the red sea urchin, *Mesocentrotus franciscanus*, raised under combinations of two temperatures (13 °C and 17 °C) and two $p\text{CO}_2$ levels (475 μatm and 1050 μatm).

Transcriptomic patterns primarily varied by developmental stage (gastrula versus prism), but there were also differences in gene expression as a result of the temperature treatment. Under the warmer temperature, genes related to the cellular stress response were up-regulated in gastrula and prism embryos. Both stages also displayed an up-regulation of epigenetic genes related to histone modifications in embryos raised under the 13 °C temperature. At the prism stage, warmer temperatures also led to an up-regulation of genes related to ciliary activity and cellular metabolic processes. Conversely, $p\text{CO}_2$ had a comparatively minimal impact on gene expression, with neither stage exhibiting a robust transcriptomic response to elevated $p\text{CO}_2$ conditions.

Overview

This chapter utilizes samples generated from the same experiment detailed in Chapter III. While Chapter III focused on *M. franciscanus* physiology (e.g., body size, thermal tolerance, and metabolic rate), this chapter examines the molecular response of *M.*

franciscanus embryos raised under different temperature and $p\text{CO}_2$ conditions. This work uses the *M. franciscanus* developmental transcriptome that was assembled and described in Chapter II to examine differences in gene expression patterns across experiment treatment conditions at two stages of development, gastrula and prism.

Introduction

Transcriptomics is a powerful tool that enables molecular-level examinations of how organisms are affected by environmental change. As next-generation sequencing technologies have advanced and become increasingly affordable, so has the ability to use transcriptomics to answer important ecological questions in non-model organisms (Ekblom and Galindo 2011; Stapley et al. 2010). When used in combination with phenotypic observations, gene expression analyses may illuminate the mechanistic underpinnings of how organisms are responding to stress or an environmental stimulus. In this chapter, I investigated the transcriptomic responses of the red sea urchin, *Mesocentrotus franciscanus*, to different temperature and $p\text{CO}_2$ conditions during their early development.

In Chapter III, elevated temperature and $p\text{CO}_2$ were found to have different effects at different developmental stages (i.e., gastrula versus prism). For instance, there was no effect of temperature at the gastrula stage while warmer temperatures increased body size at the prism stage. Development at the warmer temperature also slightly increased thermal tolerance in prism embryos. Elevated $p\text{CO}_2$ levels had a negative effect on body size at both the gastrula and prism stages. Using samples collected during the same experiment as detailed in Chapter III, here, I examined the embryological transcriptome as a trait, and analyzed how the gene expression patterns of gastrula and prism embryos varied as a result

of their laboratory exposure conditions during development. I used both differential expression (DE) analysis as well as a weighted gene co-expression network analysis (WGCNA) to examine how temperature and $p\text{CO}_2$ influenced the expression of genes in *M. franciscanus* embryos. I then paired my observations of gene expression patterns with the morphometric and physiological observations made in Chapter III to probe the means by which *M. franciscanus* responds to stressors associated with ocean warming and acidification.

Materials and methods

Sample generation

Embryo cultures of *M. franciscanus* were generated following the materials and methods described in Chapter III. Briefly, embryos were raised under a combination of two temperatures (17 °C or 13 °C) and two $p\text{CO}_2$ levels (1,050 μatm or 475 μatm) within triplicate culture vessels for each treatment combination (12 culture vessels total). The early gastrula stage (~23.5 hours post-fertilization (hpf) at ~17 °C and ~32.5 hpf at ~13 °C) and the prism stage (~44 hpf at 17 °C and ~55.5 hpf at 13 °C) were sampled from each culture bucket by gently concentrating embryos onto a 35 μm mesh filter. The concentration of embryos/mL of seawater was calculated by counting three aliquots of embryos so that a coefficient of variance (CV) of less than 10% was reached. At both the gastrula and prism stages, 5,000 embryos from each culture vessel were transferred into a 1.5 mL microcentrifuge tube. Embryos were quickly pelleted by centrifugation, excess seawater was removed, and the samples were flash frozen in liquid nitrogen. The samples were stored at -80 °C until RNA extractions were performed.

Extractions and sequencing

RNA extractions were performed using the same methods described in Chapter II. Extractions were performed on samples from each culture vessel at both the gastrula and prism stages for a total of 24 RNA extractions. RNA purity, quantity, and quality were verified using a NanoDrop[®] ND100, a Qubit[®] fluorometer, and a TapeStation 2200 system (Agilent Technologies). The RNA samples were submitted to the Genome Center at the University of California, Davis where 24 libraries were generated using poly(A) enrichment. The 24 libraries were pooled and sequenced across two lanes on an Illumina HiSeq 4000 with 100 base-pair (bp) single reads.

Data processing and DE analyses

Adapter sequence contamination and base pairs with quality scores below 30 were removed from the raw sequence data using Trim Galore! (version 0.4.1) (Krueger 2015). FastQC (version 0.11.5) (Andrews 2010) was used to verify sequence quality. The trimmed sequence data were mapped onto the *de novo* developmental transcriptome for *M. franciscanus* that was assembled in Chapter II and expression values were calculated using RSEM (version 1.3.0) (Li and Dewey 2011) and bowtie2 (version 2.3.2) (Langmead and Salzberg 2012). The LIMMA package (Ritchie et al. 2015) in R (version 3.4.4) was used to filter the sequence data to those that had at least 0.5 counts per million mapped reads across at least six of the samples. A scale normalization was applied to the read counts using a trimmed mean of M-values (TMM) normalization method (Robinson and Oshlack 2010). LIMMA was used to voom-transform the data, converting the read counts to log-counts per

million while accounting for sample-specific quality weights and the blocking design of the experiment (i.e., biological replicates). A principal component analysis (PCA) was performed on the filtered, normalized, and voom-transformed data to examine distances between all samples using the `prcomp` function in R. Additional separate PCAs were performed for each stage (i.e., gastrula and prism).

Differential expression (DE) analyses were performed in LIMMA by making pairwise comparisons between temperature treatment (17 °C versus 13 °C) and $p\text{CO}_2$ treatment (1,050 μatm versus 475 μatm) for both the gastrula and prism stages. This resulted in four comparisons of interest: (1) gastrula embryos raised under 17 °C versus gastrula embryos raised under 13 °C, (2) gastrula embryos raised under 1,050 μatm versus gastrula embryos raised under 475 μatm , (3) prism embryos raised under 17 °C versus prism embryos raised under 13 °C, and (4) prism embryos raised under 1,050 μatm versus prism embryos raised under 475 μatm . Moderated t -statistics and p -values adjusted by the Benjamini and Hochberg's method were used to control the false discovery rate (Benjamini and Hochberg 1995). Differentially expressed genes (genes relatively down- or up-regulated) were determined for each pairwise comparison of interest (adjusted p -value < 0.05).

A Weighted Gene Co-Expression Network Analysis (WGCNA) (Langfelder and Horvath 2008) was performed in R to identify clusters of similarly expressed genes which were sorted into modules (minimum of 30 genes per module). Modules with highly correlated eigengenes were merged using a threshold of 0.26. Eigengene expression was correlated with each trait. Because I was interested in examining the effects of temperature and $p\text{CO}_2$ at different developmental stages, and not in comparing the two stages, the traits were defined as gastrula temperature, gastrula $p\text{CO}_2$, prism temperature, and prism $p\text{CO}_2$. A

heatmap was generated to visualize significant correlations (p -value ≤ 0.05) between each trait and module eigengene.

Using Blast2GO (version 5.2.5) and the annotated developmental transcriptome from Chapter II, functional enrichment analyses were performed on lists of differentially expressed genes (relatively down- or up-regulated) and on lists of genes within each WGCNA module eigengene that was significantly correlated to at least one trait. Enrichment analyses were performed using a Fisher's Exact Test with an FDR filter value of 0.05 to identify gene ontology (GO) terms within three GO categories: biological process, molecular function, and cellular component.

Results

Summary statistics and overview of RNA-seq

Sequencing of the 24 libraries yielded a total of 728,782,735 100-bp single reads. After quality trimming, an average of 30.3 ± 1.3 million reads per library remained. FASTQC reports of trimmed sequences showed high sequence quality (>30) with limited adapter contamination or presence of overrepresented sequences. Per-library mapping efficiency to the developmental transcriptome using RSEM was at an average of 52.6%. After filtering the data to those with more than 0.5 counts per million mapped reads across at least six samples, 34,190 sequences remained.

Expression differences driven primarily by developmental stage

A PCA of sample-to-sample distances showed that differences in gene expression profiles were primarily between the two developmental stages, gastrula and prism (Figure 9).

Principal Component 1 (PC1) captured the majority of the variance (64.2%) and revealed a clear separation between gastrula and prism stage embryos. Samples that represented the gastrula stage were somewhat separated along the axis of Principal Component 2 (PC2), although PC2 only captured 4.5% of the variance. Samples that represented the prism stage were grouped relatively close to one another.

Differences in gene expression patterns across *M. franciscanus* early development were previously explored in Chapter II, in which a clear separation between gastrula and prism transcriptomes was evident. Therefore, to more closely examine the effects of temperature and $p\text{CO}_2$ rearing conditions on the embryos, the samples were analyzed separately by stage. A PCA of only the gastrula stage showed that replicate samples grouped together (Figure 10A). PC1 captured 26.6% of the variance, while PC2 captured 11.1% of the variance. There was a modest separation of samples by experiment treatment, both by temperature and $p\text{CO}_2$. In contrast, the PCA of only the prism stage had a much clearer separation of samples by experiment treatment (Figure 10B). Along the PC1 axis, which captured 27.1% of the variance, there was a clear separation of samples by treatment temperature (17 °C versus 13 °C). Along the PC2 axis, which only captured 10.3% of the variance, there appeared to be a separation of samples by treatment $p\text{CO}_2$ level (1,050 versus 475 μatm).

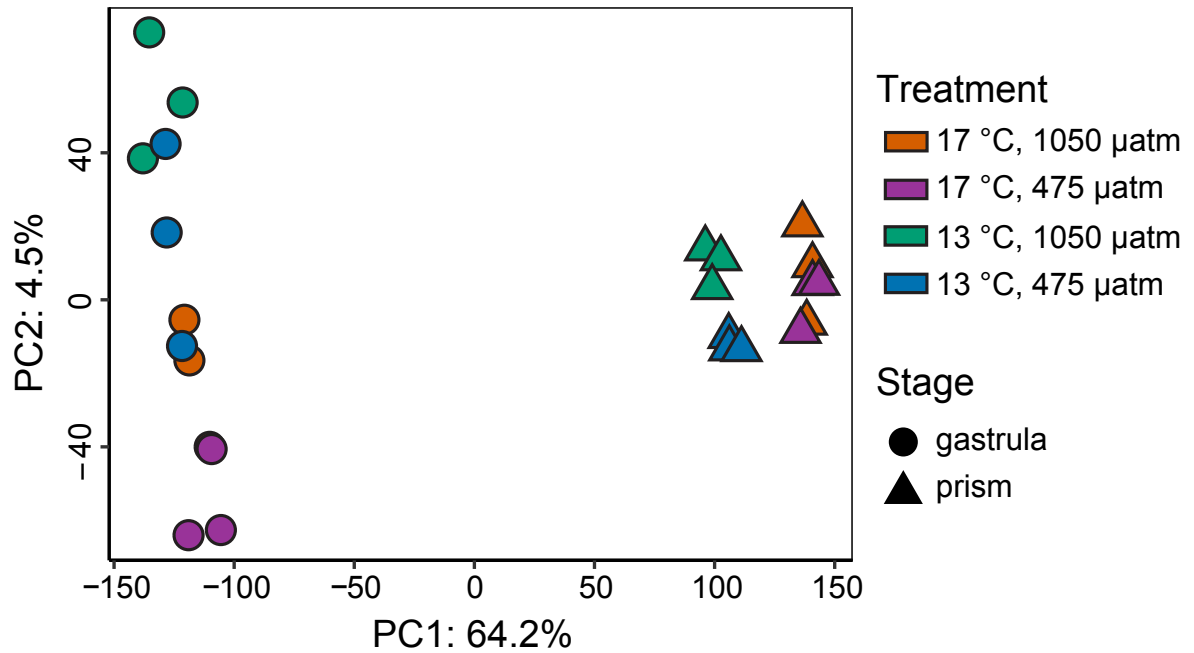


Figure 9. Principal component analysis (PCA) plot showing distances between all samples.

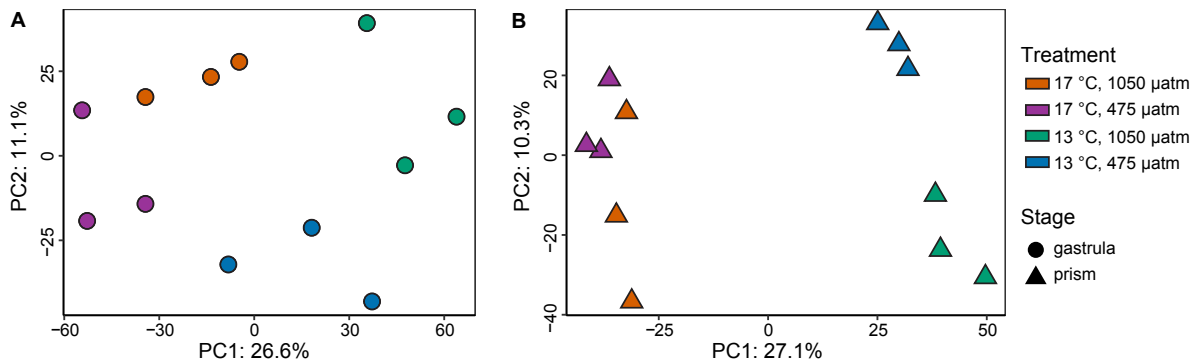


Figure 10. Principal component analysis (PCA) plots showing sample-to-sample distances at the **A.** gastrula and **B.** prism stages.

Differential expression and WGCNA results

Pairwise differential expression analyses conducted in LIMMA identified differentially expressed genes (relatively down- and up-regulated) between different experiment treatments. For each developmental stage, there were many more differential expressed genes between different temperature treatments than there were between different $p\text{CO}_2$ treatments (Figure 11). The WGCNA identified genes that were similarly expressed and their correlation to each trait of interest (i.e., gastrula temperature, gastrula $p\text{CO}_2$, prism temperature, and prism $p\text{CO}_2$). The filtered, normalized and voom-transformed data consisting of 34,190 genes were assigned into seven module eigengenes (Figure 12). There were 124 genes that remained unclustered and unassigned. These were grouped together into the gray module. Each module eigengene was related to the traits of interest to generate eigengene networks with positive or negative correlation values ranging from 1 to -1 (Figure 12). All seven module eigengenes were significantly correlated to at least one trait of interest (Figure 12). Enrichment analyses were used to identify GO terms within all possible lists of differentially expressed genes from pairwise comparisons, and within lists of genes within modules eigengenes.

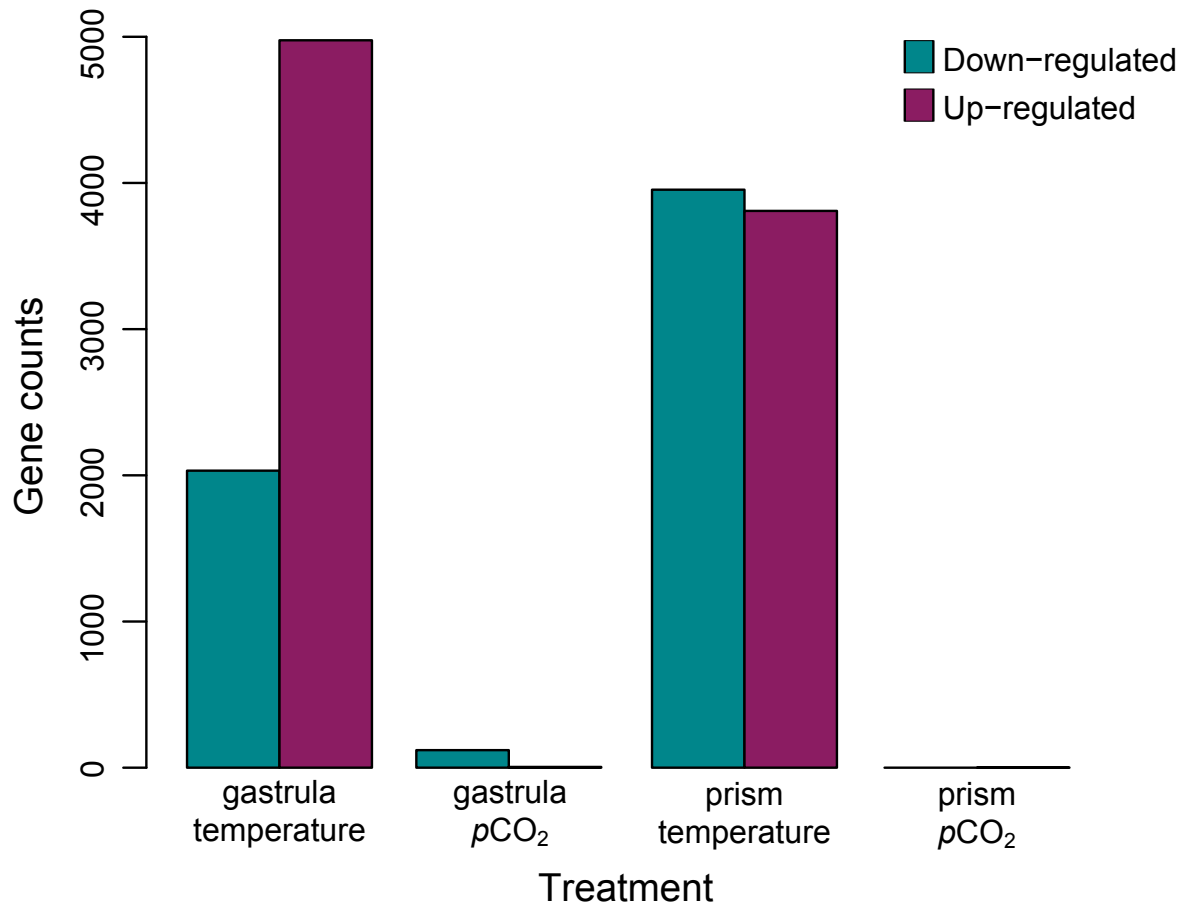


Figure 11. Histogram displaying the number of differentially expressed genes (gene counts) for each pairwise comparison of interest determined using LIMMA (FDR = 0.05).

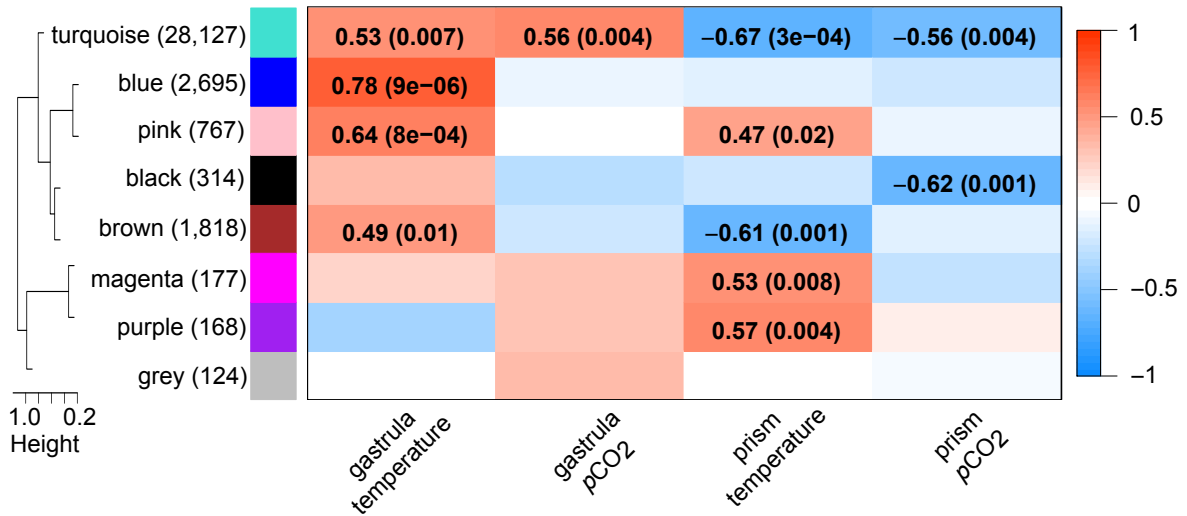


Figure 12. The WGCNA identified significant correlations between module eigengenes (rows) and traits (columns). The traits of interest included temperature and $p\text{CO}_2$ treatments for each of the two developmental stages. The red-blue color scale represents the strength of the correlation (1 to -1). Each correlation value (r^2) is followed by a p -value in parentheses. For the temperature traits, correlations >0 are positively correlated to 17 °C and negatively correlated to 13 °C (and vice versa for correlations <0). For the $p\text{CO}_2$ traits, correlations >0 are positively correlated to 1,050 μatm and negatively correlated to 475 μatm (and vice versa for correlations <0). The number of genes within each module eigengene is noted in parentheses following each color name.

Temperature affected gastrula gene expression patterns

Temperature significantly affected gene expression patterns at the gastrula stage. Although the WGCNA did not identify any module eigengenes negatively correlated to 17 °C conditions (i.e., positively correlated to 13 °C conditions) at the gastrula stage (Figure 12), there were 2,032 genes down-regulated in gastrula raised under 17 °C compared to gastrula raised under 13 °C (Figure 11). Functional enrichment analyses revealed that these genes included those related to protein folding, histone binding, histone acetyltransferase activity, and Swr1 complex (Tables 7, S5).

A total of 4,976 genes were up-regulated in gastrula raised under 17 °C compared to gastrula raised under 13 °C (Figure 11). Also, module eigengenes turquoise (28,127 genes), blue (2,695 genes), pink (767 genes), black (314), and brown (1,818 genes) were positively correlated to 17 °C conditions (i.e., negatively correlated to 13 °C conditions) at the gastrula stage (Figure 12). Functional enrichment analyses of the genes up-regulated in 17 °C gastrula embryos relative to 13 °C gastrula embryos revealed those related to nucleic acid binding, catalytic activity, ATP binding, and DNA metabolic processes (Tables 7, S5). Additionally, genes related to DNA polymerase activity and response to oxidative stress were observed to be relatively up-regulated in gastrula embryos raised under 17 °C.

Temperature affected prism gene expression patterns

Temperature also significantly affected gene expression patterns at the prism stage. A total of 3,954 genes were down-regulated in prism raised under 17 °C compared to gastrula raised under 13 °C (Figure 11), 814 of which were the same genes that were down-regulated at the gastrula stage. Additionally, module eigengenes turquoise (28,127 genes) and brown

(1,818 genes) were negatively correlated to 17 °C conditions (i.e., positively correlated to 13 °C conditions) at the prism stage (Figure 12). Functional enrichment analyses of these genes identified those related to anion binding, ATP binding, and cell cycle process (Tables 7, S5). Genes related to histone acetylation, methylation and modification were also observed to be relatively down-regulated in prism embryos raised under 17 °C.

A total of 3,809 genes were up-regulated in prism raised under 17 °C compared to gastrula raised under 13 °C (Figure 11), 830 of which were the same genes that were up-regulated at the gastrula stage. Also, module eigengenes pink (767 genes), magenta (177 genes), and purple (168 genes) were positively correlated to 17 °C conditions (i.e., negatively correlated to 13 °C conditions) at the prism stage (Figure 12). Functional enrichment analyses of the genes up-regulated in 17 °C prism embryos relative to 13 °C prism embryos revealed those related to catalytic activity, protein binding, ion binding, and Hsp70 protein binding (Tables 7, S5). Genes related to cilium formation and movement, as well as cellular metabolic processes were also observed to be relatively up-regulated in gastrula embryos raised under 17 °C.

pCO₂ had limited effects on gene expression patterns

Unlike the temperature treatment, there were limited effects of the *p*CO₂ treatment on gene expression patterns of the gastrula embryos. Differential expression analysis only found 120 genes that were down-regulated and 5 genes that were up-regulated in gastrula embryos raised under *p*CO₂ conditions of 1,050 µatm relative to those raised under 475 µatm (Figure 11). Unfortunately, a functional enrichment analysis was not able to identify any enriched GO terms across these genes. Furthermore, there were no WGCNA module eigengenes

negatively correlated to 1,050 μatm conditions (i.e., positively correlated to 475 μatm conditions) at the gastrula stage (Figure 12). Only module eigengene turquoise (28,127 genes) was positively correlated to 1,050 μatm conditions (i.e., negatively correlated to 475 μatm conditions) at the gastrula stage (Figure 12). Functional enrichment analysis identified genes within module eigengene turquoise that were related to ATP binding, histone modification, anion binding, and transmembrane transport (Tables 7, S5).

The gene expression patterns of prism embryos also appeared to be minimally impacted by differential $p\text{CO}_2$ conditions. Differential expression analysis found no down-regulated genes and only 1 up-regulated gene in prism embryos raised under $p\text{CO}_2$ conditions of 1,050 μatm relative to those raised under 475 μatm (Figure 11). The WGCNA analysis only identified two module eigengenes with a significant correlation to prism $p\text{CO}_2$ conditions, turquoise (28,127 genes) and black (314), which were negatively correlated to 1,050 μatm conditions (i.e., positively correlated to 475 μatm conditions) at the prism stage (Figure 12). Unfortunately, functional enrichment analyses were unable to identify significant GO terms within module eigengene black. Interestingly, while module eigengene turquoise was positively correlated with 1,050 μatm conditions at the gastrula stage, it was negatively correlated to 1,050 μatm conditions at the prism stage, and included genes related to ATP binding, histone modification, anion binding, and transmembrane transport (Tables 7, S5).

Table 7. Select GO term results from functional enrichment analyses of genes that were differentially expressed in each pairwise comparison of interest and in each WGCNA module eigengene.

Gene list	GO ID	GO term name	GO category	FDR value	No. transcripts (% of ref)
gastrula 17C vs. 13C (Down)	GO:0042393	histone binding	Molecular Function	7.85E-07	7 (53.8)
gastrula 17C vs. 13C (Down)	GO:0006457	protein folding	Biological Process	3.37E-06	12 (14.1)
gastrula 17C vs. 13C (Down)	GO:0004402	histone acetyltransferase activity	Molecular Function	1.83E-05	6 (42.9)
gastrula 17C vs. 13C (Down)	GO:0000812	Swr1 complex	Cellular Component	9.34E-05	3 (NA)
gastrula 17C vs. 13C (Up)	GO:0003824	catalytic activity	Molecular Function	6.00E-207	1039 (13.6)
gastrula 17C vs. 13C (Up)	GO:0003676	nucleic acid binding	Molecular Function	8.35E-77	438 (13.1)
gastrula 17C vs. 13C (Up)	GO:0006259	DNA metabolic process	Biological Process	5.34E-69	337 (14.7)
gastrula 17C vs. 13C (Up)	GO:0005524	ATP binding	Molecular Function	7.02E-35	168 (15)
gastrula 17C vs. 13C (Up)	GO:0034061	DNA polymerase activity	Molecular Function	6.90E-34	189 (13.5)
gastrula 17C vs. 13C (Up)	GO:0005509	calcium ion binding	Molecular Function	2.15E-09	91 (9.9)
prism 17C vs. 13C (Down)	GO:0043168	anion binding	Molecular Function	4.92E-73	261 (14.7)
prism 17C vs. 13C (Down)	GO:0005524	ATP binding	Molecular Function	4.44E-61	188 (17.1)
prism 17C vs. 13C (Down)	GO:0016570	histone modification	Biological Process	1.37E-19	29 (50.9)
prism 17C vs. 13C (Down)	GO:0016573	histone acetylation	Biological Process	3.74E-07	10 (55.6)
prism 17C vs. 13C (Down)	GO:0022402	cell cycle process	Biological Process	1.24E-05	21 (13.7)
prism 17C vs. 13C (Down)	GO:0016571	histone methylation	Biological Process	4.16E-05	8 (42.1)
prism 17C vs. 13C (Up)	GO:0003824	catalytic activity	Molecular Function	0.00E+00	1039 (13.6)
prism 17C vs. 13C (Up)	GO:0005515	protein binding	Molecular Function	2.15E-197	629 (15.1)
prism 17C vs. 13C (Up)	GO:0043167	ion binding	Molecular Function	8.19E-163	609 (13.1)
prism 17C vs. 13C (Up)	GO:0044237	cellular metabolic process	Biological Process	7.73E-44	465 (7)
prism 17C vs. 13C (Up)	GO:0005929	cilium	Cellular Component	2.64E-30	44 (51.2)
prism 17C vs. 13C (Up)	GO:0003341	cilium movement	Biological Process	4.45E-14	14 (156)
turquoise	GO:0003824	catalytic activity	Molecular Function	0.00E+00	4685 (117.7)
turquoise	GO:0043168	anion binding	Molecular Function	2.77E-253	1214 (147.2)

turquoise	GO:0005524	ATP binding	Molecular Function	2.16E-159	768 (147.4)
turquoise	GO:0055085	transmembrane transport	Biological Process	4.44E-139	645 (154.3)
turquoise	GO:0006259	DNA metabolic process	Biological Process	4.47E-46	970 (58.3)
turquoise	GO:0022402	cell cycle process	Biological Process	3.28E-28	113 (185.2)
turquoise	GO:0034061	DNA polymerase activity	Molecular Function	7.59E-13	518 (48.3)
turquoise	GO:0006979	response to oxidative stress	Biological Process	1.20E-05	27 (135)
blue	GO:0006259	DNA metabolic process	Biological Process	9.18E-27	167 (6.8)
blue	GO:0003824	catalytic activity	Molecular Function	8.95E-21	349 (4.2)
blue	GO:0034061	DNA polymerase activity	Molecular Function	8.37E-14	95 (6.4)
blue	GO:0003676	nucleic acid binding	Molecular Function	1.60E-07	149 (4.1)
blue	GO:0005524	ATP binding	Molecular Function	8.33E-04	57 (4.6)
pink	GO:0003824	catalytic activity	Molecular Function	1.14E-21	145 (1.7)
pink	GO:0044237	cellular metabolic process	Biological Process	7.89E-15	113 (1.6)
pink	GO:0005515	protein binding	Molecular Function	8.11E-12	82 (1.7)
pink	GO:0043167	ion binding	Molecular Function	5.02E-11	85 (1.6)
pink	GO:0003676	nucleic acid binding	Molecular Function	6.43E-06	56 (1.5)
pink	GO:0005524	ATP binding	Molecular Function	3.16E-04	25 (2)
pink	GO:0006259	DNA metabolic process	Biological Process	7.51E-03	35 (1.3)
pink	GO:0005509	calcium ion binding	Molecular Function	1.19E-02	18 (1.8)
brown	GO:0003824	catalytic activity	Molecular Function	3.56E-13	237 (2.8)
brown	GO:0043168	anion binding	Molecular Function	1.54E-07	72 (3.7)
brown	GO:0005524	ATP binding	Molecular Function	6.30E-05	46 (3.7)
brown	GO:0006259	DNA metabolic process	Biological Process	2.56E-03	70 (2.7)
brown	GO:0003676	nucleic acid binding	Molecular Function	2.59E-03	93 (2.5)
brown	GO:0022402	cell cycle process	Biological Process	1.10E-02	11 (6.7)
magenta	GO:0005515	protein binding	Molecular Function	3.42E-10	34 (0.7)
magenta	GO:0005929	cilium	Cellular Component	1.01E-03	5 (4)
magenta	GO:0044237	cellular metabolic process	Biological Process	1.75E-03	28 (0.4)

magenta	GO:0003824	catalytic activity	Molecular Function	1.26E-02	29 (0.3)
magenta	GO:0030544	Hsp70 protein binding	Molecular Function	1.40E-02	2 (33.3)
magenta	GO:0043167	ion binding	Molecular Function	2.90E-02	20 (0.4)
purple	GO:0005929	cilium	Cellular Component	3.24E-15	12 (10.2)
purple	GO:0060271	cilium assembly	Biological Process	6.09E-12	9 (12.5)
purple	GO:0005515	protein binding	Molecular Function	1.49E-09	31 (0.7)
purple	GO:0003341	cilium movement	Biological Process	5.72E-08	5 (27.8)
purple	GO:0043167	ion binding	Molecular Function	2.98E-06	27 (0.5)
purple	GO:0003824	catalytic activity	Molecular Function	6.21E-03	28 (0.3)

Discussion

In Chapter III, I investigated how elevated temperature and $p\text{CO}_2$ conditions affected developing *M. franciscanus* embryo morphometry and physiology. Here, I examined how the gene expression patterns of gastrula and prism embryos varied by the developmental temperature and $p\text{CO}_2$ conditions under which they were raised. Additionally, I assessed whether the transcriptomic results reported here align with the morphometric and physiological results observed in Chapter III.

Transcriptomic patterns varied by developmental stage

Developmental stage (gastrula vs prism) appeared to be the primary factor driving differences in gene expression patterns across samples (Figure 1). This was an expected result as transcriptomic differences between these stages were observed and detailed in Chapter II. Because comparing and contrasting gastrula versus prism gene expression patterns was not a goal of this chapter, no direct comparative transcriptomic analyses were performed between these stages.

Interestingly, embryos at different developmental stages also had different transcriptomic responses to temperature and $p\text{CO}_2$ treatments. For instance, at the gastrula stage, many more genes were up-regulated than down-regulated in 17 °C relative to 13 °C, whereas at the prism stage, a similar number of genes were relatively up- and down regulated between the two temperature treatments. This difference in response by developmental stage was also evident in the WGCNA, in which the gastrula and prism stages were significantly correlated to different module eigengenes for the temperature and $p\text{CO}_2$ treatments. Similarly, in Chapter III the morphometric response to different temperature and $p\text{CO}_2$

treatments varied by stage, in which only $p\text{CO}_2$ affected gastrula body size, while both temperature and $p\text{CO}_2$ affected body size at the prism stage.

Different life stages are predicted to have different sensitivities to stress (Byrne 2011). The observed variability between gastrula and prism responses to temperature and/or $p\text{CO}_2$ stress may be explained by a difference in stage-specific vulnerability. The observed variability could also be due to the timing and duration of exposure. The effects of a stressor may become increasingly deleterious as the length of exposure continues. Organisms not permitted time to recover may exhibit increasingly poor performance. Furthermore, during development there may be negative carry-over effects that persist into later life stages (Beckerman et al. 2002; Hettlinger et al. 2012). Alternatively, organisms may acclimate to stressful conditions over time, and are therefore less adversely affected by a stressor following the initial exposure. For example, in the coral *Acropora hyacinthus* the immediate transcriptomic response to heat stress was much higher than the transcriptomic response following 20 hours of exposure to warmed conditions (Seneca and Palumbi 2015). Thus, it remains important to acknowledge that organisms may respond differently to various environmental stressors depending on when during their life history they are exposed as well as the timing and duration of the exposure.

Temperature influences cellular stress response

Examining the cellular stress response can be a useful metric in climate change biology (Evans and Hofmann 2012). This response includes the removal of damaged macromolecules as well as nucleic acid and chromatin stabilization and repair (Kültz 2003). Here, temperature, but not $p\text{CO}_2$, appeared to be the dominant factor affecting differences in

gastrula and prism transcriptomic patterns. Elevated temperatures led to an up-regulation of genes associated with a cellular stress response in gastrula and prism embryos. Genes associated with the 17 °C compared to the 13 °C treatment included those related to catalytic activity. A similar overexpression of catalytic gene ontologies was observed in the intertidal copepod, *Tigriopus californicus*, exposed to heat stress (Kelly et al. 2017). The authors suggested that the increase in expression of catalytic genes was indicative of the removal of misfolded proteins caused by heat shock (Kelly et al. 2017). Thus, an increase in catalytic activity in *M. franciscanus* embryos may be a means of removing macromolecules damaged from the high temperature treatment.

In the gastrula stage embryos, the warmer treatment also elicited a relative up-regulation of genes related to DNA replication and repair (i.e., nucleic acid binding, DNA metabolic processes and DNA polymerase activity). Similarly, observations of heat stress in *Acropora* corals have reported an up-regulation of DNA replication and repair genes (DeSalvo et al. 2010; Seneca and Palumbi 2015). Furthermore, there was a relative up-regulation of genes related to an oxidative stress response in gastrula raised at 17 °C. Heat stress increases the expression of oxidative stress genes in other marine organisms including coral and blue mussels (DeSalvo et al. 2008; Lockwood et al. 2010; Voolstra et al. 2009). As detailed in Chapter III, temperature had no visible effect on gastrula abnormality, mortality, or body size. Thus the considerable transcriptomic alterations due to temperature suggests that the transcriptomic response observed was sufficient to buffer gastrula embryos from high temperature stress.

The cellular stress response is also associated with the increased synthesis of molecular chaperones (Kültz 2003), including heat shock proteins (HSPs) that facilitate

protein folding and repair (Lindquist 1986). In this study, genes associated with Hsp70 protein binding were relatively up-regulated in prism embryos raised under the higher temperature conditions. 70-kDa heat shock proteins (Hsp70s), the most extensively studied and highly conserved HSP family, are associated with the development of thermotolerance (Lindquist 1986; Whitley et al. 1999). The up-regulation of genes related to Hsp70 in prism embryos raised at 17 °C may have contributed to the slight increase in thermotolerance that was described in Chapter III. In other studies of sea urchin early development, Hsp70 was generally not observed under moderate warming scenarios. An examination of Hsp70 expression in *M. franciscanus* found that Hsp70 was not transcriptionally up-regulated until larvae were exposed to temperatures at or above 20 °C (O'Donnell et al. 2009). Similarly, induction of Hsp70 in the purple sea urchin *Strongylocentrotus purpuratus* was only found to occur at temperatures above 21 °C (Bédard and Brandhorst 1986; Hammond and Hofmann 2010). However, these studies implemented sudden temperature shocks on embryos raised under control temperature conditions. Runcie et al. (2012) demonstrated that more subtle increases in temperature could elicit increased expression of Hsp70 given chronic exposure to warmer conditions. Here, embryos were exposed to an ecologically relevant temperature of 17 °C immediately following fertilization. It is possible that, given the moderate warming treatment used in this study, genes related to Hsp70 were only up-regulated later in development at the prism stage as a result of a longer period of exposure.

Temperature influences epigenetic processes

In both gastrula and prism embryos, genes associated with the 13 °C compared to the 17 °C treatment included those related to epigenetic processes connected with histone

modifications (e.g., histone binding, histone methylation, histone acetyltransferase activity, and Swr1 complex). This is consistent with observations made in *S. purpuratus* larvae exposed to simultaneous elevated temperatures and $p\text{CO}_2$ conditions, in which there was a downregulation of histone encoding genes (Padilla-Gamiño et al. 2013). Epigenetic modifications, primarily consisting of DNA methylation, histone modifications, and small RNAs, are a mechanisms of nongenetic variation by which the phenotypes of organisms can be altered faster than, and without the need of, changes in genotype (Bondurianski and Day 2009; Deans and Maggert 2015). The dynamic changes in different epigenetic marks can act to regulate gene function without altering the DNA sequence, promoting phenotypic plasticity and potentially modulating the response to different environmental conditions (Eirin-Lopez and Putnam 2019; Hofmann 2017; Verhoeven et al. 2016).

One epigenetic regulatory mechanism of gene expression is the availability of different chromatin regions for transcription. Histone variants and post-translational modifications of histones may activate or repress transcription processes by modifying chromatin structures (Berger 2007). Histone modifications have been shown to mediate responses to changing environmental conditions in marine organisms (Gonzalez-Romero et al. 2017; Rodriguez-Casariego et al. 2018). Therefore, a relative up-regulation of genes related to histone modifications in embryos raised under conditions 13 °C compared to those raised under 17 °C, suggests greater epigenetic regulation of transcription in the cooler temperature. This suggests that there is a greater amount of potential plasticity in embryos raised under 13 °C, whereas embryos in the 17 °C treatment have a lower capacity for epigenetic regulation in response to changing environmental conditions. Comparative epigenetic analyses between treatments, including differences in DNA methylation, histone

variants, and small RNAs, would help elucidate the role of epigenetic processes in the response of *M. franciscanus* to different environmental stressors.

Temperature influences prism metabolism and motility

Prism stage embryos raised under the warmer temperature exhibited a relative up-regulation of genes associated with cilium formation and movement as well as cellular metabolic processes. This may indicate that under elevated temperatures, prism embryos increase energy expenditure towards locomotion for the purposes of feeding and/or swimming. As was observed in this study, temperature has a direct effect on sea urchin species' rates of embryonic development, with embryos developing faster at warmer temperatures (Strathmann 1987). Generally, this negative relationship between temperature and development rate has been attributed to increased metabolic rates at higher temperatures. This conflicts with the observed down-regulation of genes related to cell cycle processes in embryos raised under 17 °C. Although genes cell cycle processes were underrepresented, there was nevertheless an increase in metabolic genes and a faster rate of development at warmer temperatures.

Given the quicker development in embryos raised under 17 °C, these embryos may have been faster to expend their finite amount of maternally-derived provisions, and have concentrated their energy towards developing feeding structures. At the prism stage, embryos have developed a tripartite archenteron and are nearly at their first larval feeding stage (i.e., pluteus larvae) (Strathmann 1987). The observed up-regulation of genes related to cilium assembly may indicate preparation for increased feeding requirements at higher

temperatures. Indeed, warmer temperatures have been observed to increase feeding activity in a variety of marine invertebrates (Newell and Branch 1980).

Due to the effects of temperature on development rate, the planktonic larval duration (PLD), the period of time at which larvae remain in the plankton prior to metamorphosis, is predicted to decrease with increasing temperatures, affecting the potential dispersal distance of many marine species (O'Connor et al. 2007). However, given the observed increase in genes related to metabolism and ciliary movement, it is possible that embryos in warmer temperatures are also capable of swimming faster. In larvae of the sand dollar *Dendraster excentricus*, there is a positive relationship between swimming speed and temperature (Chan and Grünbaum 2010), and although *M. franciscanus* embryos and larvae are planktonic, they are not entirely passive drifters. Therefore, it is possible that *M. franciscanus* embryos and larvae in warmer temperature conditions are able to swim greater horizontal distances in a shorter period of time, thereby helping to offset a reduction in PLD.

Limited transcriptomic response to pCO₂ conditions

In other studies, echinoderms raised under elevated pCO₂ conditions exhibited altered expression of genes related to skeletogenic pathways, spicule matrix proteins, cellular stress response, ion regulation and transport, apoptosis, metabolism and ATP production (Evans et al. 2013; Evans and Watson-Wynn 2014; Padilla-Gamiño et al. 2013; Stumpp et al. 2012; Todgham and Hofmann 2009; Wong et al. 2018). Here, the pCO₂ treatment had a relatively small effect on gene expression patterns at both the gastrula and prism stage. This conflicts with observations made at the organism level, in which elevated pCO₂ resulted in decreased gastrula and prism body size. One possible explanation for this is that the *M. franciscanus*

developmental transcriptome used to map the sequencing reads failed to capture genes that are important in the response of the embryos to elevated $p\text{CO}_2$ levels. However, this seems unlikely as the developmental transcriptome was assembled using sequence data from *M. franciscanus* embryos and larvae that had been raised under low and high pH levels (Gaitán-Espitia and Hofmann 2017).

It is also possible that while there is a clear phenotypic difference in embryos raised under high versus low $p\text{CO}_2$ conditions, the transcriptomic changes underlying this difference are too subtle to be identified statistically. Alternatively, the embryos may simply lack a robust transcriptional response to the 1,050 μatm $p\text{CO}_2$ treatment. Evans et al. (2013) found that *S. purpuratus* gastrulae had different transcriptomic responses depending on the magnitude of the pH stress exposure. The transcriptomic response of gastrulae raised under a high $p\text{CO}_2$ treatment designed to reflect near-future levels was relatively muted relative to those raised under a more moderate $p\text{CO}_2$ treatment designed to reflect present-day low pH conditions (Evans et al. 2013). Evans et al. (2013) speculated that the transcriptional response required for acclimating to a more extreme $p\text{CO}_2$ level might have been too metabolically expensive, and instead, gastrulae conserved their limited maternally-derived provisions to successfully survive the lecithotrophic phase of their embryological development. While a failure of embryos to respond at the transcriptomic level may allow for continued successful development under high $p\text{CO}_2$ conditions, there may be important physiological consequences such as the reduction in growth (i.e., body size) that was observed in Chapter III. Thus, the lack of a transcriptomic response to high $p\text{CO}_2$ may have important fitness consequences for *M. franciscanus*.

Additionally, the parental generation can influence the transcriptomic response of their offspring to environmental stressors. In the green sea urchin *Strongylocentrotus droebachiensis*, a quantitative genetic breeding design implemented by Runcie and colleagues demonstrated that changes in gene expression as a result of differences in pH exposure were minor relative to gene expression differences as a result of parentage (Runcie et al. 2016). Thus, minimal changes in gene expression as a result of the $p\text{CO}_2$ treatment may be due to the genetic variation within *M. franciscanus*. While all embryo cultures for this experiment were composed of the same mixture of progeny from a cross between one male and five females, it is possible that the sea urchins collected for this experiment may be from a population with a relatively muted transcriptomic response to high $p\text{CO}_2$ conditions.

The genetic structure of the sea urchins used in this experiment may have contributed to the lack of an expected response to the $p\text{CO}_2$ treatment. It has been proposed that selection and local adaptation acts on populations that are regularly exposed to high $p\text{CO}_2$ conditions, such as populations that often experience upwelling conditions within the California Current System (CCS) (Chan et al. 2017), and that these populations may harbor genotypes that are resistant to low pH conditions (Evans et al. 2017; Kapsenberg et al. 2017; Kelly et al. 2013; Padilla-Gamiño et al. 2013; Pespeni et al. 2013a; Pespeni et al. 2013b). In *S. purpuratus*, transcriptomic responses to high $p\text{CO}_2$ levels can vary by the frequency in which the sea urchin populations are exposed to upwelling conditions (Evans et al. 2017). In particular, urchins from populations frequently exposed to low pH have greater transcriptomic responses to high $p\text{CO}_2$ than those that experience low pH less often, up-regulating genes related to ATP production. The adult sea urchins used in this study were collected from a site in the Santa Barbara Channel near Ellwood Mesa, Goleta, CA ($34^\circ 25.065'N$, 119°

54.092°W). While this area does experience periods of low pH due to upwelling (Kapsenberg and Hofmann 2016; Rivest et al. 2016), it experiences low pH less often than more northern sites within the CCS (Chan et al. 2017; Hofmann et al. 2014; Kroeker et al. 2016). Therefore, the urchins used in this study may be comparatively less adapted towards mounting a transcriptomic response to high $p\text{CO}_2$.

Additionally, the high $p\text{CO}_2$ treatment used in this experiment was designed to represent an ecologically-relevant $p\text{CO}_2$ level. *In situ* measurements have recorded $p\text{CO}_2$ levels at or around 1,050 μatm within the Santa Barbara Channel during the spring season, particularly between the months of March to May (Hofmann and Washburn 2018; Hoshijima and Hofmann 2019). Furthermore, this period of time coincides with the spawning season of *M. franciscanus* (Bernard 1977). Thus, it is likely that sea urchin populations within the Santa Barbara Channel already experience these $p\text{CO}_2$ conditions in nature, as adults and as developing embryos and larvae. It is therefore possible that the $p\text{CO}_2$ level of 1,050 μatm used in this experiment was not stressful enough to elicit a transcriptomic response in *M. franciscanus*. Differential expression under high $p\text{CO}_2$ conditions may have been observed given a more extreme $p\text{CO}_2$ treatment that represented future ocean conditions.

In addition to genetic contributions, transgenerational plasticity may have also influenced the lack of a transcriptomic response to high $p\text{CO}_2$. In Chapter VI (Wong et al. 2018), I observed that transcriptomic responses to elevated $p\text{CO}_2$ levels can vary greatly by the environmental exposure history of the adults. For instance, in adult female *S. purpuratus* that experienced simulated non-upwelling conditions (400 μatm $p\text{CO}_2$ and 17 °C) during gametogenesis produced progeny that had a relatively minimal transcriptomic response to high $p\text{CO}_2$ when compared to the progeny of females that experience simulated upwelling

conditions (1,100 $\mu\text{atm } p\text{CO}_2$ and 14 °C) (Wong et al. 2018). Here, adult *M. franciscanus* were not acclimated to any particular conditions and were spawned shortly after being collected from the field. While details of the conditions that the adults used in this study experienced during gametogenesis is unknown, it is possible that they led to the production of progeny with relatively low transcriptomic responses to high $p\text{CO}_2$ levels.

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Supplementary material

Table S5 GO term results from functional enrichment analyses of differentially expressed genes from each pairwise comparison of interest and in each WGCNA module eigengene.

V. Transgenerational effects in an ecological context: Conditioning of adult sea urchins to upwelling conditions alters maternal provisioning and progeny phenotype

Abstract

Transgenerational plasticity occurs when the conditions experienced by the parental generation influences the phenotype of their progeny. This may in turn affect progeny performance and physiological tolerance, providing a means by which organisms cope with rapid environmental change. We conditioned adult purple sea urchins, *Strongylocentrotus purpuratus*, to combined $p\text{CO}_2$ and temperature conditions reflective of *in situ* conditions of their natural habitat, the benthos in kelp forests of nearshore California, and then assessed the performance of their progeny raised under different $p\text{CO}_2$ levels. Adults were conditioned during gametogenesis to treatments that reflected static non-upwelling ($\sim 650 \mu\text{atm } p\text{CO}_2$, $\sim 17^\circ\text{C}$) and upwelling ($\sim 1,300 \mu\text{atm } p\text{CO}_2$, $\sim 13^\circ\text{C}$) conditions. Following approximately 4 months of conditioning, the adults were spawned and embryos were raised under low $p\text{CO}_2$ ($\sim 450 \mu\text{atm } p\text{CO}_2$) or high $p\text{CO}_2$ ($\sim 1,050 \mu\text{atm } p\text{CO}_2$) treatments to determine if differential maternal conditioning impacted the progeny response to a single abiotic stressor: $p\text{CO}_2$. We examined the size, protein content, and lipid content of eggs from both sets of conditioned female urchins. Offspring were sampled at four stages of early development: hatched blastula, gastrula, prism, and echinopluteus. This resulted in four sets of offspring: (1) progeny from non-upwelling-conditioned mothers raised under low $p\text{CO}_2$, (2) progeny from non-upwelling-conditioned mothers raised under high $p\text{CO}_2$, (3) progeny from upwelling-conditioned mothers raised under low $p\text{CO}_2$, and (4) progeny from upwelling-conditioned

mothers raised under high $p\text{CO}_2$. We then assessed the effects of maternal conditioning along with the effects of developmental $p\text{CO}_2$ levels on body size of the progeny. Our results showed that differential maternal conditioning had no impact on average egg size, although non-upwelling females produced eggs that were more variable in size. Maternal conditioning did not affect protein content but did have a modest impact on egg lipid content. Developing embryos whose mothers were conditioned to simulated upwelling conditions ($\sim 1,300 \mu\text{atm } p\text{CO}_2$, $\sim 13 \text{ }^\circ\text{C}$) were greater in body size, although this effect was no longer evident at the echinopluteus larval stage. Although maternal conditioning affected offspring body size, the $p\text{CO}_2$ levels under which the embryos were raised did not. Overall, this laboratory study provides insight into how transgenerational effects may function in nature. The impacts of parental environmental history on progeny phenotype during early development have important implications regarding recruitment success and population-level effects.

Overview

This chapter describes the results of experiments in which I acclimated adult purple sea urchins to combined temperature and $p\text{CO}_2$ conditions to examine differences in maternal provisioning and offspring phenotype. This work was supported by funds from the UC Climate Champion award from the University of California to my advisor, Dr. Gretchen Hofmann, as well as by resources from Santa Barbara Coastal Long Term Ecological Research program (NSF award OCE-1232779). The research presented in this chapter was conducted in collaboration with my advisor, Dr. Gretchen Hofmann, EEMB doctoral students in the Hofmann Lab, Ms. Logan Kozal and Mr. Terence Leach, and a former member of the

Hofmann Lab, Dr. Umihiko Hoshijima. This chapter is currently in published in the *Journal of Experimental Marine Biology and Ecology*.

Introduction

Mechanisms that alter phenotypic plasticity are recognized as potential biological responses to climate change (Buckley and Kingsolver 2012; Hoffmann and Sgro 2011; Munday et al. 2013b; Reusch 2014; Somero 2012), and importantly, can drive a rapid shift in physiological performance and the capacity to resist abiotic stress, a shift that occurs on ecological rather than evolutionary timescales. Recently, mechanisms involved in transgenerational plasticity (TGP) in marine metazoans have attracted interest within the research community (Chirgwin et al. 2018; De Wit et al. 2016; Hofmann 2017; Marshall et al. 2016; Munday 2014; Putnam and Gates 2015; Ross et al. 2016). Here, the research goal has been to explore how and whether the environmental history of the parents, driven by maternal provisioning or epigenetic mechanisms, has a role in bolstering the resistance of progeny to future anthropogenic changes in marine ecosystems. Alternatively, TGP may be disadvantageous if environmental stress causes a reduction in offspring quality (Putnam and Gates 2015; Shama and Wegner 2014), or if a significant mismatch between parental and offspring environments leads to progeny that are poorly prepared for their environment (Bondurianski et al. 2012; Putnam and Gates 2015; Reed et al. 2010). Numerous recent studies indicate that conditioning of the parental generation does alter characteristics of the progeny, and in some cases, confers resistance and tolerance to abiotic stress in a global change context (Munday 2014; Ross et al. 2016; Shama et al. 2014). Investigating TGP in a global change context has expanded our understanding of the role of phenotypic plasticity as

a first response to anthropogenic global change, and importantly, the potential of application in conservation efforts (Chakravarti et al. 2016). The goal of this study was to examine TGP, in the form of maternal effects, in early stage purple sea urchins, *Strongylocentrotus purpuratus* (Stimpson, 1857), and to contribute to insights on the ‘plasticity first’ hypothesis (Levis and Pfennig 2016; Schwander and Leimar 2011) in marine organisms.

Examples of TGP in marine metazoans have been described in the literature, with most studies conducted in the laboratory (Donelson et al. 2017; Munday 2014; Ross et al. 2016). Examples of environmental conditioning of adults *in situ* have also been reported. Murray and colleagues demonstrated TGP in a wild population of Atlantic silverside, *Menidia menidia*, whose natural habitat was characterized by an annual pH decline throughout their spawning season (Murray et al. 2014). Offspring produced later in the spawning season exhibited greater tolerance to high CO₂, suggesting an effect of *in situ* parental exposure to seasonal acidification. Similarly, in the European squid, *Loligo vulgaris*, the phenology of egg laying (i.e., summer versus winter) influenced the response of early developmental stages to ocean acidification and warming (Rosa et al. 2014). Overall, TGP may fulfill the role of a fast-acting response mechanism to rapid environmental change, providing time for and potentially assisting adaptive evolution processes. Here, the basic concept is that the variant forms of early life stages have differential performance in a dynamic environment and, if that phenotype is heritable, it could create the grist for future adaptation, and possible evolutionary rescue (Levis and Pfennig 2016; Marshall et al. 2016). In an eco-evolutionary context, evolution of traits is a process that occurs in response to environmental change, and thus, shifts in progeny traits via TGP is a likely mechanism of rapid response to climate change (Hendry 2016).

An inherent challenge in understanding the significance of TGP in a global change context is that, in many cases, we often know relatively little about what early stages experience *in situ*. Increasingly, investigators are realizing that natural variability in the marine environment is selecting for tolerant genotypes, and that local adaptation likely plays a key role on a biogeographic scale (Hofmann et al. 2014; Kelly et al. 2017; Pereira et al. 2017; Sanford and Kelly 2011; Vargas et al. 2017). Such observations have been made for tropical corals (Barshis et al. 2013), sea urchins (Evans et al. 2017; Kelly et al. 2013), abalone (De Wit and Palumbi 2013) and other marine snails (Calosi et al. 2017; Gleason and Burton 2013), and intertidal copepods (Kelly et al. 2017). Recent research has indicated that local adaptation will likely influence organismal responses to future ocean change (Calosi et al. 2017; Pereira et al. 2017).

Importantly, field observations of pH and temperature conditions are increasingly available, an advance that supports studies aimed at coupling natural conditions with experimental approaches. For example, in the California Current System (CCS), episodic upwelling dominates the region (Feely et al. 2008), and the progression of ocean acidification is expected to result in dramatic changes to ocean chemistry in this nearshore region (Gruber et al. 2012). Recently, Chan and colleagues reported that waters corrosive to aragonite, a more soluble form of calcium carbonate, currently cover large coastal areas of the CCS, and that upwelled waters already display the effects of increased anthropogenic CO₂ contributions following the Industrial Revolution (Chan et al. 2017). Oceanographic observations from the kelp forest show that pH and temperature conditions are highly variable, likely driven by biological processes such as photosynthesis and respiration, interacting with episodic upwelling (Frieder et al. 2012; Hoshijima 2018; Hoshijima and

Hofmann 2019; Kapsenberg and Hofmann 2016; Koweek et al. 2017). Overall, the present-day physicochemical environment of the kelp forest creates a highly variable mosaic of pH and temperature conditions that likely influences the history of adults, with gradients existing in relation to the proximity to kelp (e.g., inside versus outside kelp bed differences) (Hoshijima and Hofmann 2019). Here, we used a simplified set of conditions as a representation of two distinct conditions that can be observed in kelp forest environments *in situ*. We did not incorporate the full extent of variability that is observed, but instead focused on the question: does differential conditioning of the parents result in progeny with different properties? Thus, this study represents the first steps to examine whether adult conditioning of a benthic marine invertebrate in the natural physicochemical environment of a kelp forest might induce variable performance in the progeny.

Here, we conditioned adult *S. purpuratus* to two divergent temperatures and $p\text{CO}_2$ levels during gametogenesis that represent end-member conditions in the kelp forest during upwelling and non-upwelling (i.e., relaxed) conditions (Kapsenberg and Hofmann 2016; Koweek et al. 2017). To examine if maternal conditioning to different $p\text{CO}_2$ levels and temperatures elicited a transgenerational effect, the offspring of differentially conditioned mothers were tested using two $p\text{CO}_2$ levels at one temperature representative of conditions at the time of year the embryos and larvae would be in the water column, in this case, 15 °C. Thus, offspring of the females conditioned under the upwelling and non-upwelling treatments were raised at the same temperature, but under either a low or high $p\text{CO}_2$ level. Sea urchins are vulnerable to high $p\text{CO}_2$ (i.e., low pH) conditions, particularly during their early development (Byrne 2011; Byrne et al. 2013c; Byrne and Przeslawski 2013; Clark et al. 2009). Exposure to high $p\text{CO}_2$ levels can affect body size (Byrne et al. 2013b; Kurihara and

Shirayama 2004; O'Donnell et al. 2009; Sheppard Brennan et al. 2010), internal acid-base balance (Miles et al. 2007), metabolic processes (Padilla-Gamiño et al. 2013; Stumpp et al. 2011b), and gene expression (Evans et al. 2013; Pespeni et al. 2013b; Stumpp et al. 2011a; Wong et al. 2018). Therefore, investigating how maternal conditioning influences the response of the progeny to high $p\text{CO}_2$ conditions represents a valuable and ecologically relevant opportunity to explore TGP in *S. purpuratus*.

In a previous study using a similar experimental design and laboratory conditions, we found maternal conditioning affected the transcriptome of the progeny, and gene expression patterns suggested that the offspring of upwelling-conditioned mothers may be primed to respond to high $p\text{CO}_2$ levels (Wong et al. 2018). In this study, we report the size, protein content, and lipid content of the eggs from differently conditioned females to evaluate maternal provisioning and egg quality. We also report a response variable in the form of body size that was assessed across four stages of early development to evaluate changes in performance of the progeny relative to the conditioning of the adults. In a companion study, DNA methylation was examined as an epigenetic mechanism that potentially mediates transgenerational and intragenerational plasticity in *S. purpuratus* (Strader et al. 2019). Although transgenerational effects can be maternal, paternal, or a combination of both, only maternal contributions to TGP were examined. Specifically, we examined an average maternal effect across a pool of genotypes, using an experimental approach that mimics female mass spawning events that occur in nature. Our results indicated that there is a transgenerational effect that links environmental conditioning of the mothers to changes in phenotype of the early life stages. In nature, such an outcome could drive differences in progeny dispersal and fitness.

Material and methods

Animal collection and conditioning

Approximately 90 adult purple sea urchins, *S. purpuratus*, were hand-collected on SCUBA on October 5, 2016 from a site in the Santa Barbara Channel near Goleta Beach, Goleta, CA (34° 24.840' N, 119° 49.742' W). Urchins were transported to the Hofmann Lab at UC Santa Barbara, where they were held in flow-through seawater tanks for approximately 2.5 weeks prior to adult conditioning. To begin the adult conditioning trial, urchins were randomly sorted into 20-gallon glass treatment tanks (8-13 urchins per tank). There were three replicate tanks for each of two conditioning treatments, for a total of 6 treatment tanks. Adults were held under two combined $p\text{CO}_2$ and temperature experimental conditions: (1) a non-upwelling treatment (N: $\sim 650 \mu\text{atm } p\text{CO}_2$, $\sim 17 \text{ }^\circ\text{C}$) and an upwelling treatment (U: $\sim 1,300 \mu\text{atm } p\text{CO}_2$, $\sim 13 \text{ }^\circ\text{C}$). These two static conditions represent ecologically relevant values observed using ocean sensors deployed in the Santa Barbara Channel (Kapsenberg and Hofmann 2016; Rivest et al. 2016). Adult urchins were maintained at these two conditioning treatments for approximately four months and were fed an excess of kelp blades (*Macrocystis pyrifera*) three days per week.

Conditioning treatments were maintained using a flow-through CO_2 mixing system modified from Fanguie et al. (2010) to reach the target $p\text{CO}_2$ levels, and two Delta Star[®] heat pumps with Nema 4x digital temperature controllers (AquaLogic, San Diego, CA, USA) to control the temperature. Treated seawater flowed to each adult conditioning tank at a rate of 20 L/hr. Aquaria were fitted with a small fountain pump (Total Pond, 70gph) to aid in water mixing. Every one to two days, temperature (Omega HH81A), salinity (YSI-3100) and

spectrophotometric pH (Shimadzu UV-1800 and m-cresol dye) were analyzed according to best-practice procedures outlined by Dickson et al. (2007b). Total alkalinity was measured every three to five days (Mettler-Toledo T50) according to best-practice procedures outlined by Dickson et al. (2007a).

Measured spectrophotometric pH, total alkalinity, salinity, and temperature were used to calculate *in-situ* carbonate chemistry parameters using CO₂calc (Robbins et al. 2010) with equilibrium constants from Mehrbach and colleagues (Mehrbach et al. 1973) refit by Dickson and Millero (1987). As total alkalinity was taken less periodically than the other parameters, calculations were conducted using the last recorded alkalinity sample.

Spawning and egg assessment

At the conclusion of adult conditioning, spawning was induced via injection of 0.53 M KCl into the coelom. Sperm was collected dry and stored on ice until activation. Eggs from each female were collected in filtered seawater (FSW) (i.e., UV-sterilized seawater filtered to 0.35 μ m). A subset of eggs ($n > 100$) from each individual female were visually inspected for maturity (i.e., ova that lack large, visible germinal vesicles) and tested for male-female compatibility to ensure the use of only high quality, viable eggs. Nine females from each maternal treatment were selected to contribute eggs for egg analyses and subsequent embryo culturing. Prior to conducting fertilizations, eggs from each individual female were sampled, resulting in eggs from 18 separate females for analysis (i.e., $n = 9$ females per maternal treatment). Size, total protein, and lipid content were measured for the eggs.

Egg morphometric analyses

Samples for morphometric analyses were preserved by adding 4% formalin in 0.01 M phosphate buffered saline (PBS) to an equal volume of seawater with eggs, fixing the samples in a final concentration of 2% formalin in seawater. Formalin preservation has been shown to cause eggs to shrink slightly in some echinoderm species (Lessios 1987). However, all eggs were preserved using the same formalin and methods, so any formalin effects should have affected all eggs equally. Samples were stored at 4 °C until imaged, for no longer than two months. Eggs were digitally photographed under bright field DIC illumination on a compound microscope (Olympus BX50) with an attached digital camera (Infinity Lite) using Infinity Capture software (version 6.2.0). Digital images were calibrated using a stage micrometer for the 20X objective using ImageJ (National Institutes of Health, USA). Eggs ($n \geq 20$) from each of 18 separate females (i.e., eggs from $n = 9$ females from each maternal treatment) were photographed for morphometric analyses. All digital images were analyzed using ImageJ. Eggs were measured by their average diameter and total 2-dimensional (2D) area (Figure 13 A,B). The average diameter of the eggs was determined by taking the average of three independent diameter measurements for each egg.

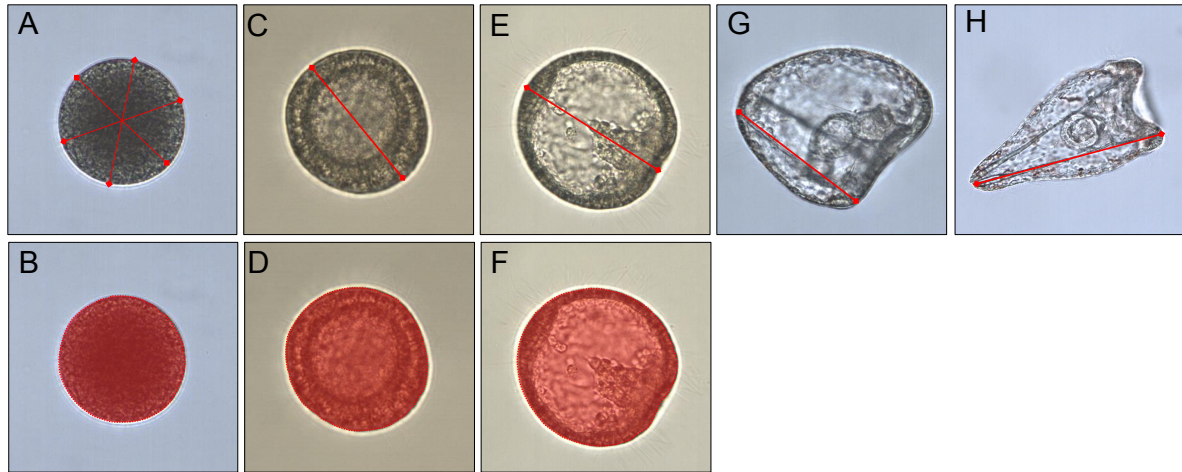


Figure 13. Body size was measured using ImageJ (National Institutes of Health, USA). Eggs size was determined by **A.** taking the average of three independent diameter measurements, and **B.** measuring the 2-dimensional (2D) area. The average diameter was subsequently used to calculate egg volume by assuming the eggs were spherical in shape. Body size of the hatched blastula stage was determined by **C.** measuring the maximum length, from the animal to vegetal end, and **D.** measuring the 2D area. Body size of the gastrula stage was determined by **E.** measuring the maximum length, from the anterior to posterior end, when measured across the center of the archenteron, and **F.** measuring the 2D area. Body size of the prism stage was determined by **G.** measuring the tip of the body rod to the tip of the postoral rod. Body size of the echinopluteus stage was determined by **H.** measuring the length of the left postoral arm, from the spicule tip of the postoral arm to the spicule tip of the aboral point.

Protein quantification

Egg quantities were estimated by counting small aliquots of eggs and calculating their concentration. To preserve samples for protein quantification, approximately 5,000 eggs were placed in a 1.5 mL Eppendorf tube. The samples were pelleted by centrifugation, excess seawater was carefully removed using a pipette, and the tube was immediately frozen in liquid nitrogen and stored at -80 °C. Three tubes of eggs from each of the 18 separate females (i.e., eggs from n = 9 females from each maternal treatment) were used to quantify protein content. Protein samples were extracted using methods modified from (Byrne et al. 2008) and Prowse et al. (2008). Samples were sonicated on ice for 20 seconds in 100 μ L

homogenization buffer (20 mM Tris-HCl (pH 7.6); 130 mM NaCl, 5 mM EDTA) containing 1% Triton-X and 1% Protease Inhibitor Cocktail (Sigma-Aldrich) using a Sonic Dismembrator 550 (Fisher Scientific). Samples were shaken on ice for 15 minutes and centrifuged for 20 minutes at 14,000 rpm. The retained supernatant was diluted 1:2 to 1:4 in deionized water and total soluble protein was quantified at 562 nm on a microplate reader (Bio-Rad) using a BCA protein assay kit following manufacturer's instructions (Catalog number 23225, Pierce Biotechnology, Rockford, IL, USA).). Following quantification of total protein (ng) content per egg, protein density (ng/nl) was calculated using the average volume of the eggs produced by each adult female urchin. Equivalents of energy content (mJ) and density (mJ/nl) from protein were estimated using the combustion enthalpy coefficient (24.0 kJ/g protein) from Gnaiger (1983).

Lipid content

Eggs for lipid extractions were preserved using the same methods described for protein quantification (5,000 eggs per tube). Three tubes of eggs from each of the 18 separate females (i.e., eggs from $n = 9$ females from each maternal treatment) were analyzed for lipid content. Lipids were extracted from frozen egg samples following the methods described by Sewell (2005), with some modifications. Each sample was sonicated on ice for three 25-second bursts. Samples were transferred to 1 mL glass V-vials (Wheaton) and combined with 250 μ l of methanol, 75 μ l of chloroform, and 50 μ l of ketone internal standard (1 μ g/ μ l in chloroform) (Parrish 1987). After vigorous shaking, V-vials were centrifuged for 5 minutes at 4 °C. Using a pulled Pasteur pipette, both the aqueous and chloroform layers were transferred to a clean V-vial; chloroform and water were added to a final volume ratio of

4:3:2 (water:chloroform:methanol). After centrifuging under the same conditions, only the bottom chloroform layer was transferred and stored under nitrogen at -20 °C.

Immediately prior to loading the samples, the extract was dried down using nitrogen gas and re-suspended in 50 µl of chloroform. The lipid classes were separated and quantified using an Iatroscan MK-6/6s thin layer chromatography/flame ionization system and silica gel S-4 Chromarods (Parrish 1987; Parrish 1999). For the chromatographic separation, 10 µl of sample was added to each of two duplicate rods. The rods were developed under a double development system modified from Parrish (1999). Following sample application, the rods were first developed in hexane:diethyl ether:acetic acid (98.95:1.0:0.05 by volume) for 25 minutes, dried for 5 minutes at 60 °C in a drying oven, and partially scanned through the ketone peak. The rods were then developed in hexane:diethyl ether:acetic acid (79:20:1 by volume) for 40 minutes, dried in the same manner, and fully scanned. Chromatographs were collected using the software Peak Simple (version 4.54; SRI Inc.). Raw values were calibrated using a multilevel calibration curve as outlined by (Matson et al. 2012). Free fatty acid (palmitic acid) was excluded from our calibration solutions as it is not detected in unfed larvae and eggs of *S. purpuratus* (Meyer et al. 2007).

Although ketone (KET; 3-hexadecanone) was included as an internal standard as a means of calculating percent yield, it was ultimately not used to adjust the final lipid concentrations because we found that the concentration of ketone in our lipid extractions was not a reliable indicator of total yield. Specifically, there was a weak relationship between the amount of ketone and the unstandardized amount of total lipid in each sample ($r^2 = 0.0001$) and coefficients of variation (CVs) between technical replicate samples were routinely higher after standardizing the lipid concentrations to the ketone standard (unstandardized CVs =

2.16 to 18.42; standardized CVs = 2.64 to 41.52). We processed both sets of eggs (non-upwelling and upwelling) simultaneously, so we do not anticipate a treatment bias occurred during the extraction process. Therefore, we have assumed that any minor differences in percent yield are randomly distributed between the two maternal treatments. Furthermore, we are only interested in the relative concentration of lipids between the eggs of the two treatments (non-upwelling versus upwelling), and not the absolute quantity of lipids.

Aliphatic hydrocarbon (Nonadecane) was consistently at or below the level of detection for this method (Matson et al. 2012), and was not used in our quantification of lipids. Therefore, total lipid content was represented by one energy storage lipid class: triacylglycerols (TAG; Tripalmitin), and two structural lipid classes: sterol (ST; cholesterol) and phospholipids (PL; L- α -phosphatidylcholine). Lipid density (ng/nl) was subsequently calculated using the average egg volume of the eggs produced by each adult female urchin. Equivalent energy content (mJ) and density (mJ/nl) from lipids were estimated using the combustion enthalpy coefficient (39.5 kJ/g lipid) from Gnaiger (1983).

Principal component analysis

A principal component analysis (PCA) biplot was used to visualize the data from all three analyses in tandem to provide an overall assessment of the eggs. The PCA biplot was created in R (version 3.3.3) using a combination of data from eggs of each female urchin. The data used to generate the biplot included: 1) morphometric data (i.e., average egg diameter), 2) protein data (ng of protein per egg), and 3) lipid data (ng of total lipid per egg). The average of each data set was combined by individual female identity (n = 9 females from

each maternal treatment). Groupings (i.e., eggs from either non-upwelling (N) or upwelling (U) females) were tested using a PERMANOVA technique.

Embryo and larval culturing

An equal number of eggs from each female were collected and gently pooled together by treatment. This was meant to simulate the mixture of genotypes that occurs during mass spawning events in nature, while also ensuring that each female contributed equally to the pools of eggs. This resulted in two pools of eggs, in which one pool was composed of eggs from 9 females acclimated to non-upwelling conditions and one pool was composed of eggs from 9 females acclimated to upwelling conditions. Because this study is focused on only the maternal contributions to TGP, and to somewhat limit genotypic diversity that would otherwise confound analyses, only a single male conditioned to non-upwelling conditions was crossed with both groups of females, resulting in only half- and full-sibling progeny (Figure 14). Sperm was collected dry and activated with FSW. Dilute, activated sperm was added to each pool of eggs until at least 95% fertilization success was reached.

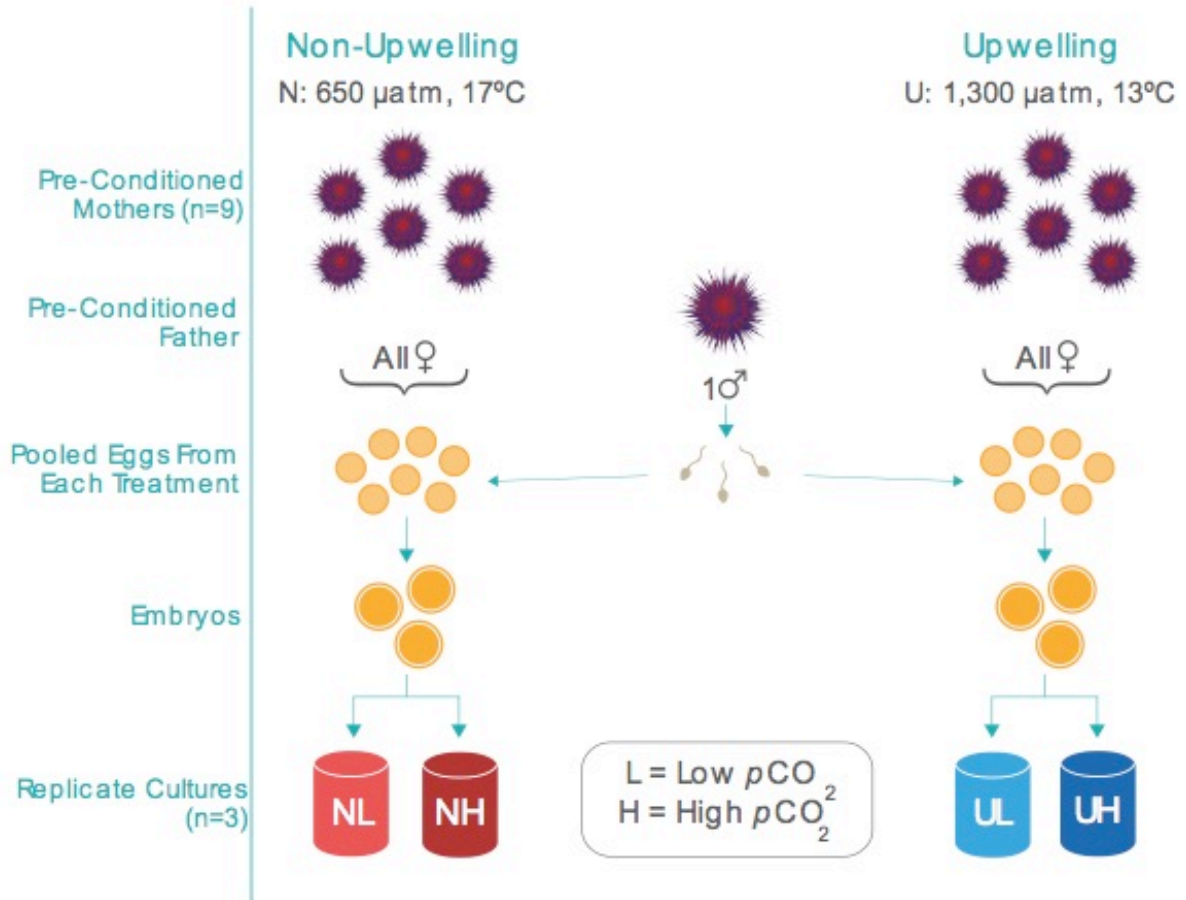


Figure 14. Cross design of adult urchins conditioned under treatments that mimic non-upwelling (N: $\sim 650 \mu\text{atm } p\text{CO}_2$, $\sim 17^\circ\text{C}$) or upwelling (U: $\sim 1,300 \mu\text{atm } p\text{CO}_2$, $\sim 13^\circ\text{C}$) conditions observed in the kelp forest ecosystem (Kapsenberg and Hofmann 2016; Rivest et al. 2016). Embryos were raised at the same temperature ($\sim 15^\circ\text{C}$) under low $p\text{CO}_2$ (L: $\sim 450 \mu\text{atm}$) or high $p\text{CO}_2$ (H: $\sim 1,050 \mu\text{atm}$) conditions. This resulted in four embryo treatments: 1) progeny of females that experienced non-upwelling conditions raised under low $p\text{CO}_2$ levels (NL), 2) progeny of females that experienced non-upwelling conditions raised under high $p\text{CO}_2$ levels (NH), 3) progeny of females that experienced upwelling conditions raised under low $p\text{CO}_2$ levels (UL), and 4) progeny of females that experienced upwelling conditions raised under high $p\text{CO}_2$ levels (UH).

Embryos from each cross were divided and transplanted into either low pCO₂ (L: ~450 μatm) or high pCO₂ (H: ~1,050 μatm) conditions. All embryos were raised at the same temperature, ~15 °C, to isolate the observed response to one factor (i.e., different pCO₂ levels) as well as to ensure synchronous development and consistent sampling across all stages. This experimental design resulted in four sets of treatments: (1) progeny of non-upwelling mothers raised in low pCO₂ conditions (NL), (2) progeny of non-upwelling mothers raised in high pCO₂ conditions (NH), (3) progeny of upwelling mothers raised in low pCO₂ conditions (UL), and (4) progeny of upwelling mothers raised in high pCO₂ conditions (UH) (Figure 14). Approximately 150,000 embryos were added to each culture vessel immediately after verification that fertilization success was ≥ 95%. The embryos cultures for each treatment (i.e., NL, NH, UL and UH) were cultured in triplicate (i.e., 12 total culture vessels). Each culture vessel was composed of two, nested 5-gallon buckets with a flow-through rate of 6 L/hr. Each vessel was fitted with a small paddle attached to a 12-V motor to aid in gentle mixing of the cultures. Temperature, salinity, and pH measurements were taken daily for the cultures, following the same procedure implemented during adult conditioning.

Once culturing commenced, embryos and larvae were sampled from each culture vessel at four discrete stages of early development: hatched blastula, gastrula, prism, and early echinopluteus. To maintain consistent pCO₂ and temperature conditions, seawater was filtered for embryos or larvae and immediately replenished to the culture vessels during sampling. Additionally, due to the flow-through nature of our culturing system, treated seawater was continuously added to the culture vessels at 6 L/hr during all sampling processes. As a result, it was not feasible to accurately determine the concentration of

embryos and larvae within each culture vessel at each developmental stage, and therefore, mortality rates could not be accurately assessed throughout development.

Generally, the developmental schedule adhered to what might be expected for *S. purpuratus* (Strathmann 1987). At 15 °C, blastulae were sampled at ~18 hours post-fertilization (hpf). The blastula stage was designated by the development of cilia and the occurrence of hatching and swimming. At ~30 hpf, the gastrula stage was characterized by the formation and extension of the archenteron to 1/2 of the total body length. At ~45 hpf, the archenteron had grown towards one side of the body and become tripartite. At this time, the prism stage was designated by the first development of skeletal rods and the formation of a pyramid-like body shape. Finally, at ~70 hpf, the early echinopluteus stage was sampled, by which time the larvae had developed defined internal structures, including the mouth, esophagus, stomach, anus, anterolateral and postoral skeletal body rods, as well as the early formation of feeding arms. While echinopluteus larvae are capable of feeding, larvae were sampled immediately upon reaching this stage. As such, it was not necessary to feed the larvae and potential starvation did not impact the findings of this study.

Morphometric analyses of embryos and larvae

Samples for morphometric analyses were preserved by the same methods used to preserve eggs. Digital images were calibrated using a stage micrometer for the 10X objective using ImageJ (National Institutes of Health, USA). Embryos and larvae from each treatment replicate were photographed for morphometric analyses ($n \geq 30$ per culture vessel) at each developmental stage.

All digital images were analyzed using ImageJ to determine various size metrics for each developmental stage (Figure 13C-F). The hatched blastula stage was oriented such that a full lateral view was visible, evident by uneven thickness of walls on the animal versus vegetal poles of the embryo. The maximum length of each hatched blastula, measured from the animal to vegetal end, was determined as well as the 2D area. When photographing the gastrula stage, larvae were oriented such that the full side profile of the archenteron was visible and aligned with the center of the vegetal plate. The gastrula stage was measured by length (i.e., the maximum distance from the anterior to posterior end, when measured across the center of the archenteron) and by 2D area. Prism stage larvae were photographed in a lateral view, and length was determined by measuring the tip of the body rod to the tip of the postoral rod. When photographing the echinopluteus stage, larvae were oriented such that the dorsal side was down against the microscope slide, with the postoral arms facing upwards. The length of the left postoral arm of each echinoplutei was measured, from the spicule tip of the postoral arm to the spicule tip of the aboral point (following Yu et al. 2011).

Statistical analyses

Statistical analyses were performed using JMP Pro (version 11.2.0) and R (v. 3.4.1). The morphometric, protein, and lipid data were tested to verify approximately normal distributions. A Levene test was used to test for unequal variance between treatments. A one-way analysis of variance (one-way ANOVA) was used to compare the means between egg samples with experimental treatment (i.e., non-upwelling or upwelling) set as a fixed factor and female identity set as a random effect. A Best Linear Unbiased Predictor (BLUP) (Robinson 1991) was used to evaluate the random factor effect. A two-way analysis of

variance (two-way ANOVA) was used to examine body size in embryos and larvae by setting maternal treatment (i.e., non-upwelling or upwelling) and offspring treatment (i.e., low or high $p\text{CO}_2$) as fixed factors and testing for an interaction between maternal and offspring treatment. For all developmental stages (i.e., blastula, gastrula, prism, pluteus), culture vessel replicate identity was set as a random effect in the model.

Results

Outcome of the adult conditioning phase and raising cultures of offspring

Adult urchins were successfully conditioned to the two experimental treatments (i.e., either simulated non-upwelling or upwelling treatment conditions) for a 4-month acclimation period. During this period, under controlled $p\text{CO}_2$ and temperature conditions (Table 8), there was no mortality and adults remained healthy and fecund. Although the seawater chemistry was somewhat variable due to respiration processes by the adults, a clear separation between the non-upwelling and upwelling treatment remained throughout the conditioning period (Figure 15). In addition, the embryo culturing experiment was successful with $p\text{CO}_2$ conditions remaining stable throughout the 3-day culturing period (Table 8, Figure 15). All culture buckets displayed synchronous development with each batch of embryos reaching key stages on the appropriate development schedule for *S. purpuratus* raised at 15 °C (Strathmann 1987).

Table 8. Average (\pm standard deviation) carbonate chemistry parameters and temperatures for adult conditioning (N or U) and offspring culturing (L or H). The $p\text{CO}_2$ and Ω_{Arag} were calculated from measured pH, salinity (33.1 ± 0.1), and total alkalinity values ($2211.25 \pm 7.10 \mu\text{mol kg}^{-1}$).

	pH_{total}	$p\text{CO}_2$ (μatm)	Ω_{Arag}	Temperature ($^{\circ}\text{C}$)
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 $p\text{CO}_2$ (L)	7.99 ± 0.01	446 ± 12.4	2.03 ± 0.05	15.0 ± 0.11
High $p\text{CO}_2$ (H)	7.66 ± 0.01	1050 ± 37.7	1.02 ± 0.03	14.9 ± 0.05

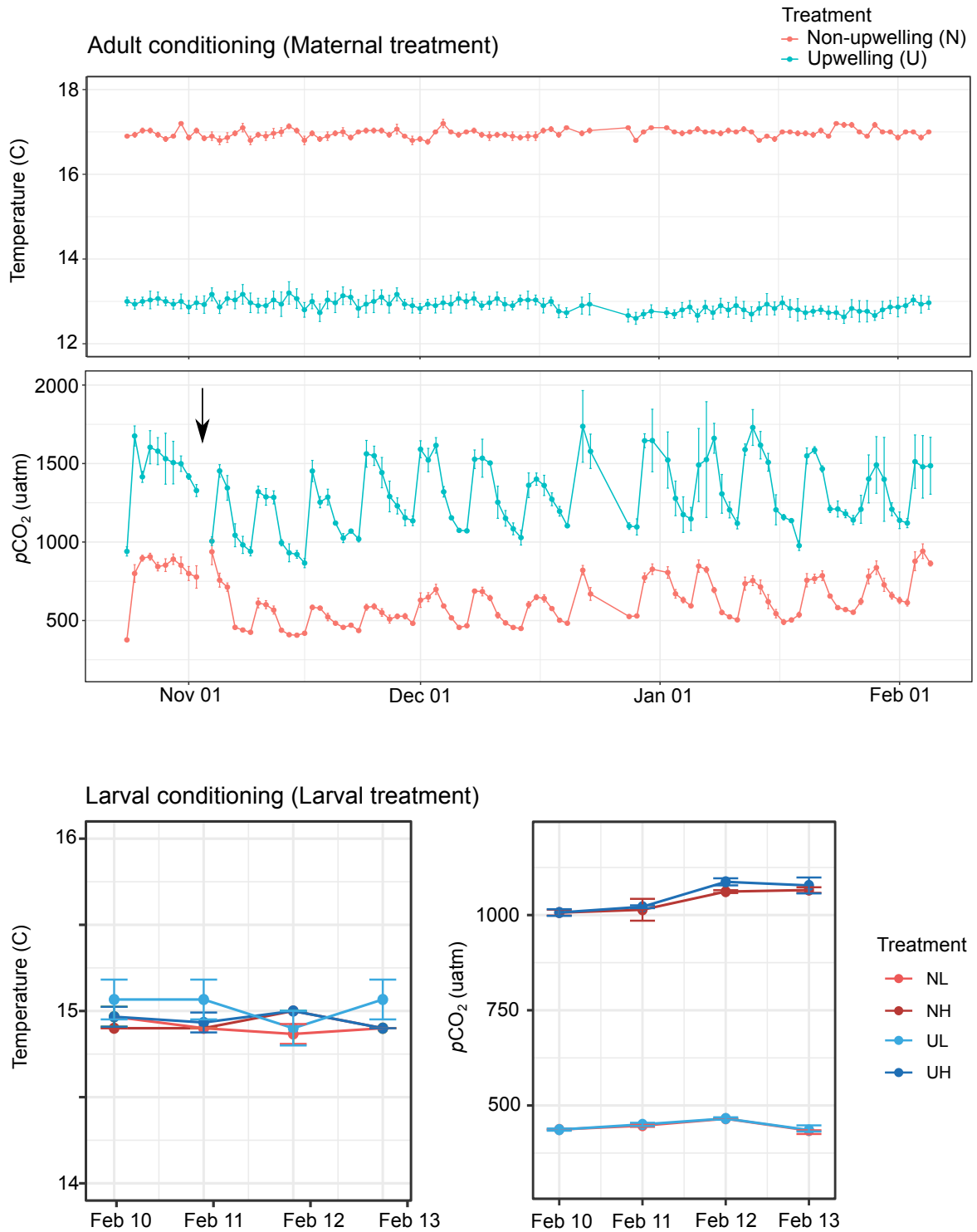


Figure 15. Temperature and $p\text{CO}_2$ conditions for the duration of the adult and embryo/larval conditioning periods. The arrow denotes an anomaly due to an unexpected campus-wide seawater pump failure during system maintenance, in which all conditions experienced $>3,000 \mu\text{atm } p\text{CO}_2$ for less than 24 hours. Error bars represent \pm the standard deviation across the three replicate tanks (maternal conditioning) or cultures (larval conditioning).

Assessment of the eggs

Egg morphometric analyses

There was no significant effect of parental history on the average egg size. Here, average egg diameter and 2D area were highly correlated ($r^2 = 0.924$). The average diameters of the eggs produced by the 9 non-upwelling females differed by which female produced them ($F_8 = 58.5399$, $p < 0.0001$). The 9 upwelling females also produced eggs that differed in size between females ($F_8 = 8.1425$, $p < 0.0001$). After accounting for size differences between the eggs of different females by treating female identity as a random effect, there was no apparent effect of maternal treatment (i.e., conditioning to non-upwelling versus upwelling) on average egg diameter ($F_{1,16} = 0.1658$, $p = 0.6892$) (Figure 16A). On average, eggs from non-upwelling females (N) were 0.09103 ± 0.002535 mm in diameter while eggs from upwelling females (U) were 0.09138 ± 0.002196 mm in diameter (Table 9). In examining variance across all eggs produced by both sets of females, the eggs produced by non-upwelling females exhibited more variance in size ($F_{1,651} = 7.9524$, $p = 0.0049$).

The 2D areas of the eggs produced by the 9 non-upwelling females differed by which female produced them ($F_8 = 56.2295$, $p < 0.0001$). The 9 upwelling females also produced eggs that differed in 2D area between females ($F_8 = 11.5792$, $p < 0.0001$) (Figure 17A). When examining variance of all eggs cumulatively across both maternal treatments, the eggs produced by non-upwelling females exhibited more variance in size ($F_{1,651} = 6.6736$, $p = 0.0100$). There was no significant maternal treatment effect on average egg 2D area ($F_{1,16} = 0.0563$, $p = 0.8155$) (Figure 17A) when incorporating individual female identity as a random effect into our model. Eggs from females that experienced non-upwelling conditions (N) during gametogenesis had an average area of 0.006673 ± 0.000363 mm² and eggs from

females that experienced upwelling conditions (U) during gametogenesis had an average area of $0.006705 \pm 0.000316 \text{ mm}^2$ (Table 9).

As expected, because average egg diameter was used to calculate egg volume, there was also no significant maternal treatment effect on egg volume ($F_{1,16} = 0.1555, p = 0.6985$) when incorporating female identity as a random effect into our model. On average, eggs from non-upwelling females (N) were $0.396 \pm 0.033 \text{ nl}$ in volume while eggs from upwelling females (U) were $0.400 \pm 0.029 \text{ nl}$ in volume (Table 9).

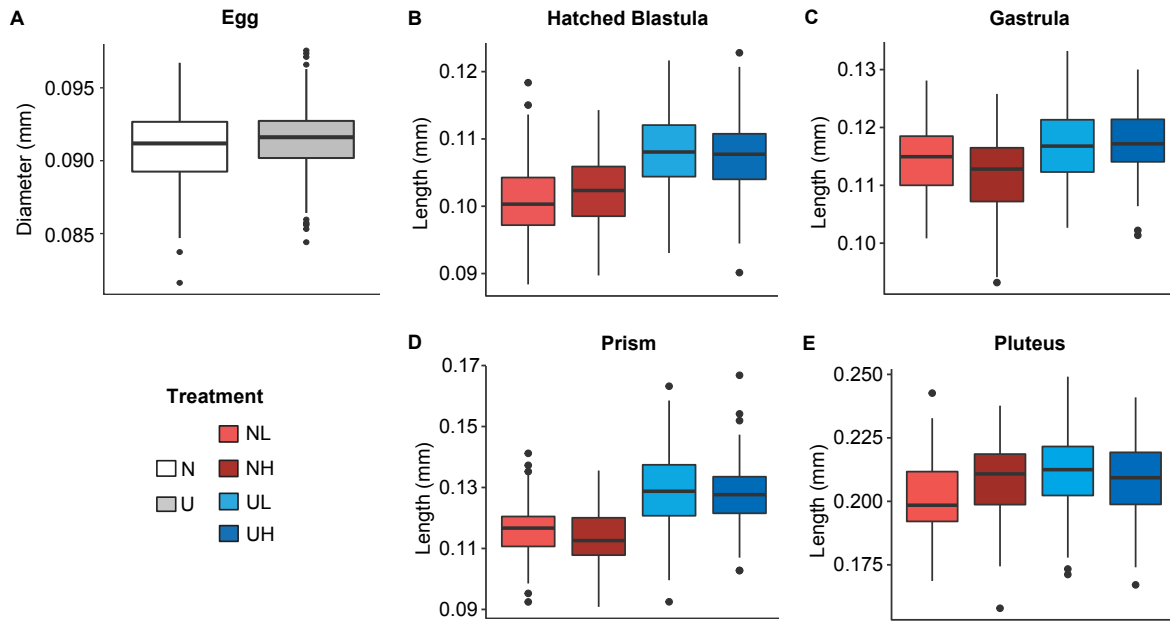


Figure 16. Size differences across developmental stages of offspring, with error bars showing standard error. **A.** The average diameter of eggs from female urchins conditioned under either non-upwelling (N) or upwelling (U) treatments. The average length of developmental stages, including **B.** hatched blastula, **C.** gastrula, **D.** prism, and **E.** echinopluteus larvae stages of each of the four treatment types (i.e., NL, NH, UL, and UH).

Table 9. Average (\pm standard deviation) sizes, protein contents and densities, lipid contents and densities, and energy equivalents and densities of eggs produced by females conditioned to the Non-upwelling treatment (N) and females conditioned to the Upwelling treatment (U).

	Non-upwelling (N)	Upwelling (U)
Diameter (mm)	0.09103 \pm 0.002535	0.09138 \pm 0.002196
Volume (nl)	0.396 \pm 0.033	0.400 \pm 0.029
Total protein (ng/egg)	36.10 \pm 10.95	42.88 \pm 8.79
Protein density (ng/nl)	91.05 \pm 26.44	107.66 \pm 23.86
Total energy from protein (mJ/egg)	0.87 \pm 0.26	1.03 \pm 0.21
Energy density from protein (mJ/nl)	2.19 \pm 0.63	2.58 \pm 0.57
Total lipid (ng/egg)	17.01 \pm 5.90	20.69 \pm 4.08
Lipid density (ng/nl)	43.10 \pm 14.50	51.82 \pm 10.91
Total energy from lipid (mJ/egg)	0.67 \pm 0.23	0.82 \pm 0.16
Energy density from lipid (mJ/nl)	1.70 \pm 0.57	2.05 \pm 0.43

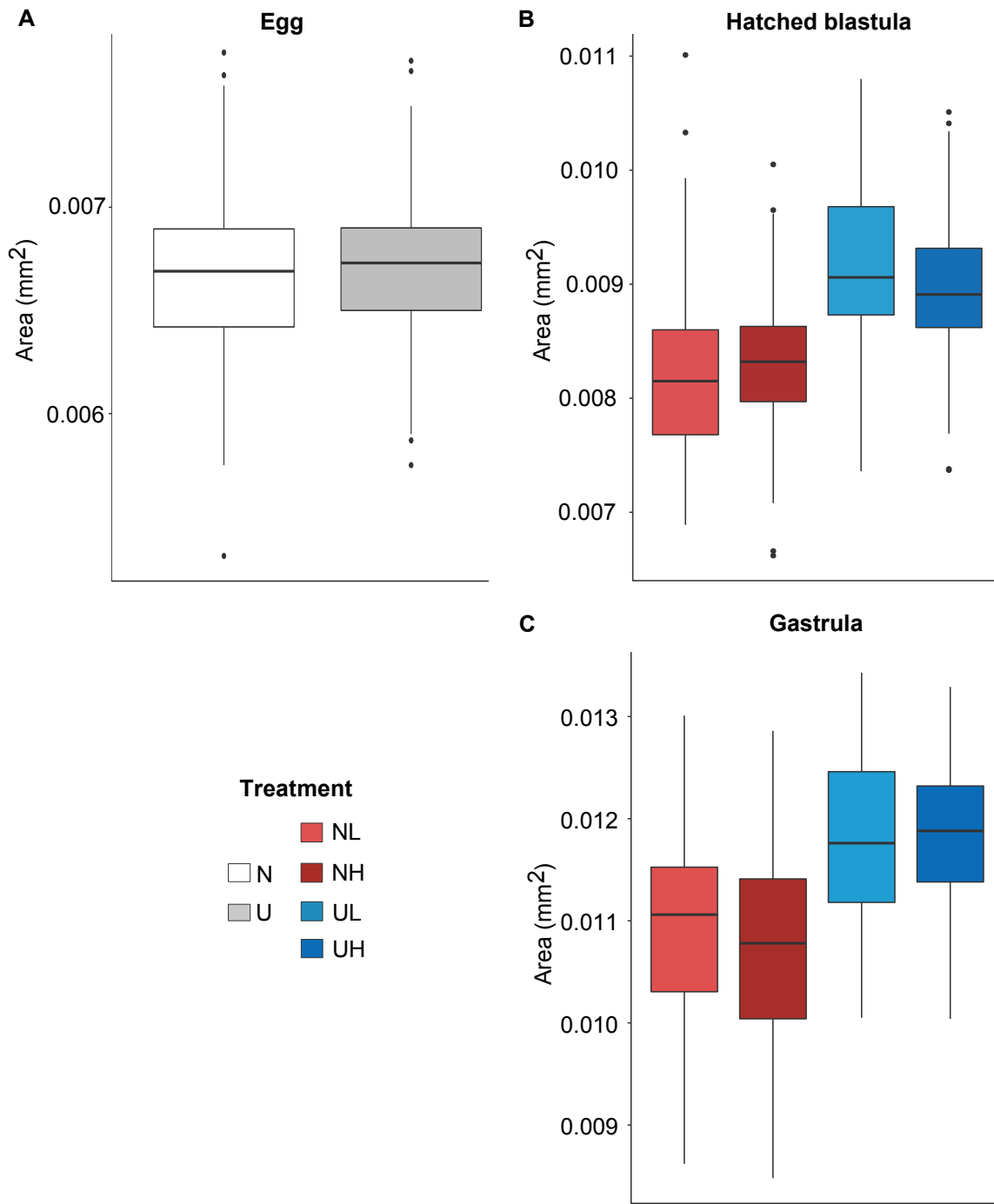


Figure 17. Size differences across eggs and developmental stages of offspring, with error bars showing standard error. **A.** The average 2D area of eggs from female urchins conditioned under either non-upwelling (N) or upwelling (U) treatments, and the average 2D area of **B.** hatched blastula and **C.** gastrula stages of each of the four treatment types (i.e., NL, NH, UL, and UH).

Protein quantification

There was no significant effect of maternal treatment on total protein content in the eggs ($F_{1,16} = 2.2991$, $p = 0.1490$), although on average, eggs from upwelling-conditioned females had more total protein (average of 42.88 ± 8.79 ng/egg) than eggs from non-upwelling-conditioned females (average of 36.10 ± 10.95 ng/egg) (Table 9). The variability of protein content did not differ between eggs from non-upwelling females and eggs from upwelling females ($F_{1,52} = 1.2793$, $p = 0.2632$).

When examining egg protein content, female identity was incorporated into all models as a random effect. There was no significant difference in egg protein density (ng/nl) between maternal treatments ($F_{1,16} = 2.1320$, $p = 0.1636$) (Table 9). The total energy available from protein (mJ) per egg also did not significantly differ by maternal treatment ($F_{1,16} = 2.2991$, $p = 0.1490$). Additionally, there was no significant difference in energy density (mJ/nl) from protein between the maternal treatments ($F_{1,16} = 2.1320$, $p = 0.1636$). Lastly, there was no difference in variance of protein density, energy available from protein, and energy density from protein between the eggs produced by non-upwelling and upwelling females ($p > 0.05$).

Lipid content

The lipid content (ng per egg) was determined by measuring the concentration of three lipid classes: triacylglycerols (TAG), sterol (ST), and phospholipids (PL), in which the total lipid content was the combination of the three classes measured. After accounting for variability between eggs from different females of the same conditioning treatment, there were modest differences in lipid content between the eggs from urchins conditioned to

different maternal treatments. Specifically, there was a significant difference in the concentration of phospholipids (PL), in which eggs from females conditioned to the upwelling treatment had on average a higher concentration of PL (7.89 ± 1.58 ng/egg) than those from females conditioned to the non-upwelling treatment (6.41 ± 1.96 ng/egg) ($F_{1,16} = 4.6935$, $p = 0.0454$) (Figure 18). There was no significant treatment effect on triacylglycerol content (TAG) ($F_{1,17} = 2.3072$, $p = 0.1474$), sterol content (ST) ($F_{1,16} = 3.0881$, $p = 0.0978$), or total lipid content (TAG + PL + ST) ($F_{1,16} = 0.0762$). However, there was a clear trend overall in which eggs from females that experienced upwelling conditions had a higher lipid content of all lipid classes (Figure 18). On average, eggs produced by upwelling-conditioned females had a total lipid content of 20.69 ± 4.08 ng/egg, while eggs produced by non-upwelling-conditioned females had a total lipid content of 17.01 ± 5.90 ng/egg (Table 9). There was equal variance of total lipid content between the eggs from the two sets of females for every lipid class ($p > 0.05$).

When examining egg lipid density, female identity was incorporated into all models as a random effect. There was no significant maternal treatment effect on triacylglycerol density (TAG) (ng/nl) ($F_{1,17} = 2.1372$, $p = 0.1624$), sterol density (ST) (ng/nl) ($F_{1,16} = 2.8707$, $p = 0.1095$), phospholipid density (PL) (ng/nl) ($F_{1,16} = 4.0213$, $p = 0.0619$), or total lipid density (TAG + ST + PL) (ng/nl) ($F_{1,16} = 3.2333$, $p = 0.0908$). These results for lipid densities (ng/nl) differed slightly from the results for lipid content (ng/egg). Specifically, whereas total phospholipid content (ng/egg) was significantly different between maternal treatments, phospholipid density (ng/nl) was not, although it was nearly significant ($p = 0.0619$). Nonetheless, the pattern of lipid densities was highly alike to the pattern observed

when examining lipid content, in which eggs from upwelling-conditioned females consistently possessed a higher density of lipids across all lipid classes (Figure 19).

The total energy available from lipids (mJ) per egg also did not significantly differ by maternal treatment ($F_{1,16} = 3.5886, p = 0.0761$). There was also no significant maternal treatment effect on energy density (mJ/nl) from lipids ($F_{1,16} = 3.2333, p = 0.0908$). Finally, there was no difference in variance of lipid density, energy available from lipids, and energy density from lipids between the eggs produced by non-upwelling and upwelling females ($p > 0.05$).

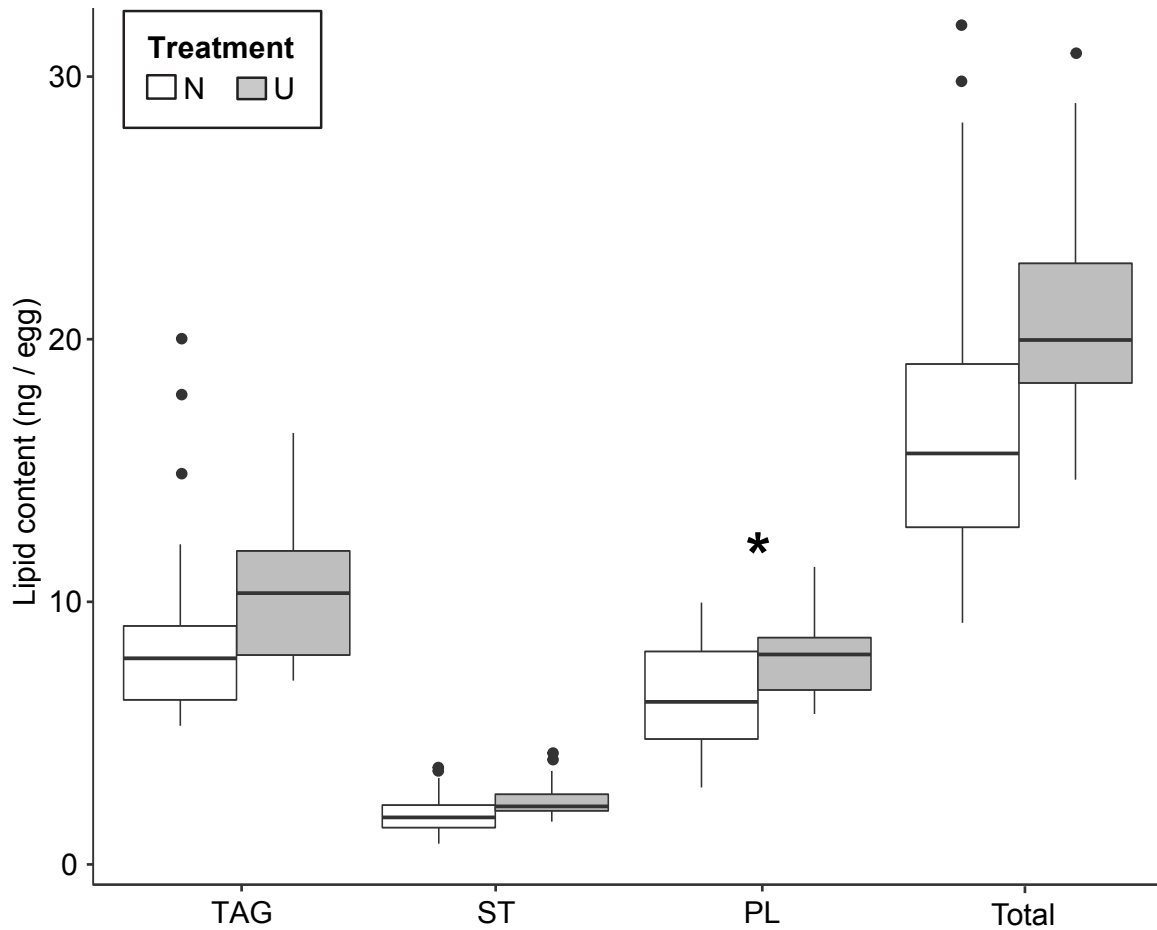


Figure 18. Lipid content (ng/egg) of eggs from females conditioned to the non-upwelling treatment (N) and females conditioned to the upwelling treatment (U), with error bars showing standard error. Three lipid classes were measured including triacylglycerols (TAG; Tripalmitin), sterol (ST; cholesterol) and phospholipids (PL; L- α -phosphatidylcholine). Total is the total of all three lipid classes. Any significant difference between treatments is marked with an asterisk (*).

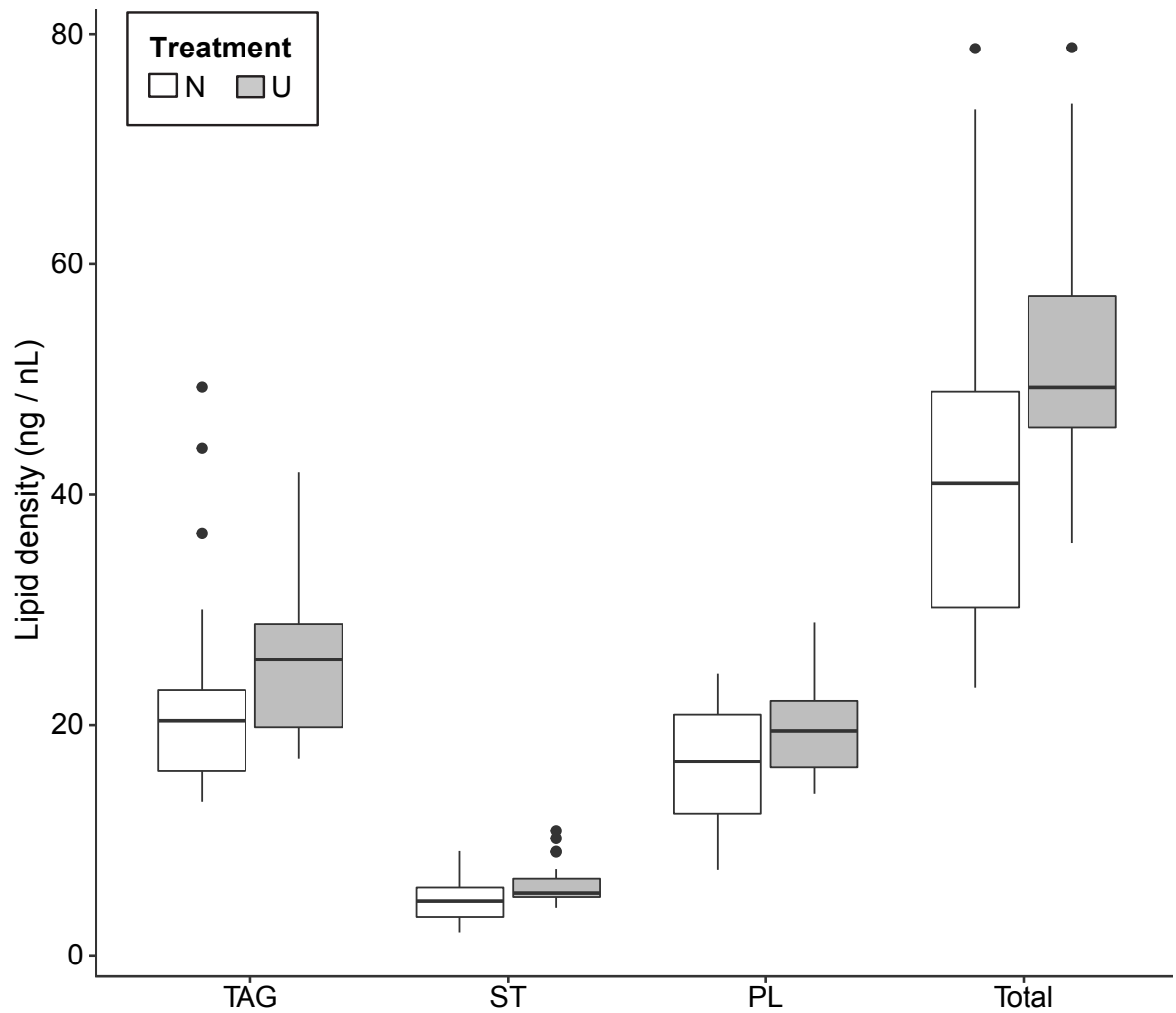


Figure 19. Per egg lipid density (ng/nl) of eggs produced by females conditioned to the non-upwelling treatment (N) and females conditioned to the upwelling treatment (U), with error bars showing standard error. Three lipid classes were measured including triacylglycerols (TAG; Tripalmitin), sterol (ST; cholesterol) and phospholipids (PL; L- α -phosphatidylcholine). Total is the total of all three lipid classes (TAG + ST + PL).

Principal component analysis

A PCA biplot of the combined size (average diameter), protein (ng/egg of total protein), and lipid data (ng/egg of total lipid) for the eggs showed that Principal Components 1 and 2 (PC1 and PC2) cumulatively explain a majority of the variance in the data (96.5%) (Figure 20). A PERMANOVA showed that the grouping identity of the eggs (i.e., eggs from females conditioned to non-upwelling versus upwelling) were not significantly associated with PC1 ($F_{1,16} = 4.11$, $p = 0.05962$) or PC2 ($F_{1,16} = 0.04294$, $p = 0.8385$). However, the eggs from the females conditioned to upwelling are generally clustered more closely to one another than are the eggs from females conditioned to non-upwelling (Figure 20). As noted earlier, only morphometric analyses showed a significant difference in variance between the two groups of eggs, in which eggs from non-upwelling females were significantly more variable in size.

In terms of our egg metrics, total protein content had a high positive correlation with total lipid content ($r = 0.9151$). Additionally, protein content and lipid content both have a positive correlation with PC1 ($r = 0.7073$ and 0.7027 , respectively), which represents the most variability in the data (63.1%). Protein and lipid content show nearly no relationship to the size of the eggs ($r = 0.08445$ and 0.004050 , respectively). Size has a high, positive correlation with PC2 ($r = 0.9935$), which represents 33.4% of the variability in the data. In general, eggs from females conditioned to upwelling (U) appeared to have more protein and lipids per egg (Figure 20). In contrast, egg size does not appear to differ between the upwelling and non-upwelling eggs, other than by tighter clustering of the upwelling eggs.

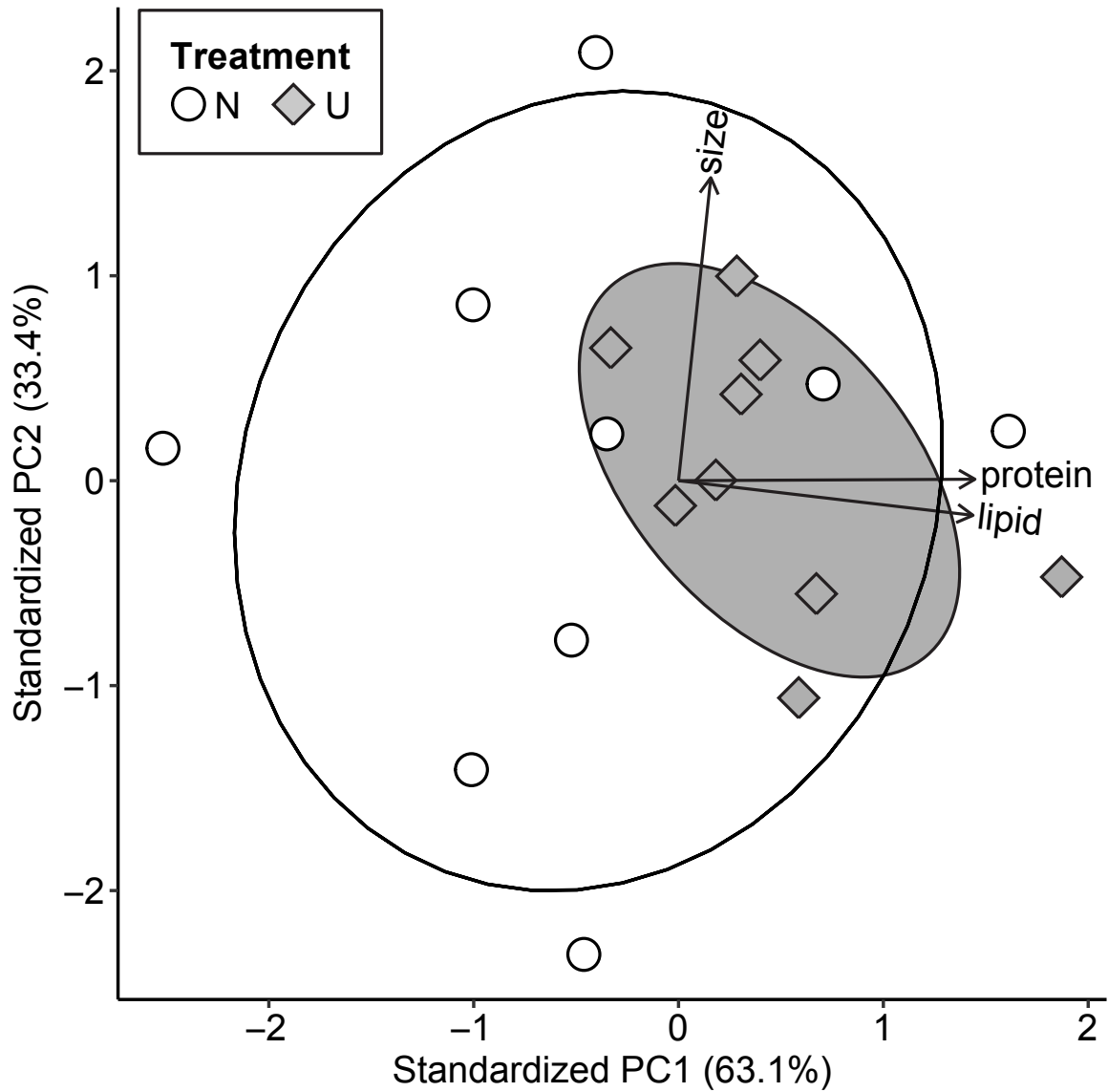


Figure 20. A PCA biplot of the eggs from females conditioned to the non-upwelling treatment (N) and from females conditioned to upwelling treatment (U), using a combination of the egg data, including: 1) size (average egg diameter), 2) protein (the average ng of protein per egg), and 3) lipid (the average ng of total lipid per egg).

Morphometric analyses of embryos and larvae

Although the average size of the eggs was not significantly affected by maternal conditioning, treatment effects became evident during early development upon examining average maternal effects across a pool of embryo genotypes. There was a significant maternal treatment effect on the lengths of hatched blastulae ($F_{1,8} = 53.7797, p < 0.0001$) (Figure 16B). The progeny of upwelling-conditioned mothers (UL and UH) were, on average, 6.5% longer than the progeny of non-upwelling-conditioned mothers (NL and NH) at the hatched blastula stage. While there was an effect of maternal treatment, there was no significant effect of offspring treatment (i.e., raised under high versus low $p\text{CO}_2$ at $\sim 15^\circ\text{C}$) on blastula body size ($F_{1,8} = 0.3008, p = 0.5987$). There was also no significant interaction between maternal and offspring treatment at the blastula stage ($F_{1,8} = 0.6219, p = 0.4536$).

There was also a significant maternal treatment effect on the 2D area of the hatched blastulae ($F_{1,8} = 20.3612, p = 0.0020$) (Figure 17B). Specifically, the progeny of upwelling-conditioned mothers was, on average, 9.7% greater in area than the progeny of non-upwelling-conditioned mothers. There was no significant effect of offspring $p\text{CO}_2$ treatment ($F_{1,8} = 0.0972, p = 0.7632$) or a significant interaction between maternal and larval treatment ($F_{1,8} = 0.3235, p = 0.5853$) on blastula area. This pattern is highly similar to that observed for blastula length, in which the progeny of upwelling-conditioned mothers was larger than the progeny of non-upwelling-conditioned mothers.

The effect of differential maternal conditioning on embryo size was also evident at the gastrula stage (Figure 16C). We found a significant effect of maternal treatment (non-upwelling versus upwelling) on the length of the gastrulae ($F_{1,8} = 18.0220, p = 0.0025$). Like the hatched blastula stage, progeny at the gastrula stage were greater in length if their

mothers experienced upwelling conditions (U: $\sim 1,300 \mu\text{atm } p\text{CO}_2$, $\sim 13 \text{ }^\circ\text{C}$) during gametogenesis (i.e., UL and UH), and were, on average, 3.9% longer than the progeny of mothers that experienced non-upwelling conditions (N: $\sim 650 \mu\text{atm } p\text{CO}_2$, $\sim 17 \text{ }^\circ\text{C}$) (i.e., NL and NH). There was no effect of offspring treatment (i.e., raised under high versus low $p\text{CO}_2$ at $\sim 15 \text{ }^\circ\text{C}$) on gastrula length ($F_{1,8} = 0.1064$, $p = 0.7522$). There was not a significant interaction between maternal and offspring treatment (i.e., an interaction between maternal conditioning and the response of the offspring to their environment) ($F_{1,8} = 4.6172$, $p = 0.0621$).

There was also a significant maternal treatment effect on the 2D area at the gastrula stage ($F_{1,8} = 52.5014$, $p < 0.0001$) (Figure 17C). The progeny of upwelling-conditioned mothers (UL and UH) were, on average, 9.9% greater in area than the progeny of non-upwelling-conditioned mothers (NL and NH). There was no significant effect of offspring $p\text{CO}_2$ treatment ($F_{1,8} = 0.3670$, $p = 0.5604$) or a significant interaction between maternal and larval treatment ($F_{1,8} = 0.7796$, $p = 0.4015$).

The same morphometric patterns observed at the gastrula stage remained evident at the prism stage (Figure 16D). There was a significant maternal treatment effect on prism skeletal rod length ($F_{1,8} = 44.6129$, $p = 0.0002$). The skeletal rods in offspring of upwelling-conditioned mothers were, on average, 11.6% longer than the progeny of non-upwelling-conditioned mothers. There was no effect of offspring treatment (i.e., raised under high versus low $p\text{CO}_2$ at $15 \text{ }^\circ\text{C}$) on prism rod length ($F_{1,8} = 0.06152$, $p = 0.4563$). Lastly there was no significant interaction between maternal and offspring treatment ($F_{1,8} = 0.7419$, $p = 0.4152$).

While maternal conditioning to upwelling versus non-upwelling conditions during gametogenesis impacted body size at the hatched blastula, gastrula, and prism stages, this effect was no longer evident later in development. There was no significant effect of maternal treatment ($F_{1,8} = 1.2122$, $p = 0.3023$), offspring treatment ($F_{1,8} = 0.0363$, $p = 0.8535$), or significant interaction between maternal and offspring treatment ($F_{1,8} = 1.0591$, $p = 0.3329$) on the arm length of the echinopluteus stage larvae (Figure 16E).

Discussion

Transgenerational effects have been demonstrated to influence the performance of marine invertebrate embryos and larvae in an environmental change context (Donelson et al. 2017; Ross et al. 2016). In this study, we investigated whether adult sea urchins that experience different conditions during gametogenesis might produce offspring with varying responses to different $p\text{CO}_2$ conditions during development. One working hypothesis here is that in kelp forest ecosystems, adult urchins likely perform gametogenesis across a range of seawater conditions (see Chan et al. 2017; Hoshijima 2018; Hoshijima and Hofmann 2019), and this could point to the role of kelp, upwelling, and altered physicochemical conditions influencing ecological and evolutionary dynamics via TGP. In a previous study, differential gene expression in gastrula-stage embryos of *S. purpuratus* was shown to vary significantly with the conditioning treatment of the adults (Wong et al. 2018). In the present laboratory experiment, we investigated whether conditioning of the adults to combined $p\text{CO}_2$ and temperatures that are simple representations of upwelling versus non-upwelling conditions in a coastal kelp forest would lead to a change in phenotype of the early life history stages exposed to elevated $p\text{CO}_2$ levels. Here, we were able to detect a maternal effect on offspring

phenotype. However, we were unable to measure a maternal effect on offspring response to stress because the offspring $p\text{CO}_2$ treatment alone failed to elicit an observable change in phenotype. Nonetheless, TGP may provide rapid resilience to environmental stress in the early life stages of echinoderms.

Because the timing of adult conditioning is likely an important factor in TGP (Ross et al. 2016; Uthicke et al. 2013), we conditioned adult *S. purpuratus* at the onset of seasonal gonadal development as a means of capturing the entirety of gametogenesis. The adult conditioning period reported here appears to have been of sufficient duration to elicit observable differences in offspring phenotype. Here, we report four salient findings: 1) maternal conditioning did not affect average egg size, though there was a difference in the variability of egg sizes produced, 2) maternal conditioning affected egg lipid content, 3) there an effect of maternal conditioning on body size during the early development of the embryos (transgenerational effect), but no detectable effect of offspring $p\text{CO}_2$ treatment on body size (intragenerational effect).

Impact of differential maternal conditioning on eggs

In this study, we found that differential maternal conditioning to combined $p\text{CO}_2$ and temperatures representative of upwelling or non-upwelling conditions in the kelp forest environment affected some, but not all, of the response variables used to measure the females' eggs. Egg size has been associated with a variety of important developmental characteristics, including larval duration (Strathmann 1985; Wray and Raff 1991a) and juvenile success (Emlet and Hoegh-Guldberg 1997). Other studies have found that maternal exposure to either high $p\text{CO}_2$ levels or low temperatures can increase egg sizes. In the

Antarctic sea urchin *Sterechinus neumayeri*, adults conditioned to lowered pH conditions for 17 months produced larger eggs than adults conditioned under average pH levels (Suckling et al. 2015). Regarding temperature TGP effects on oocyte size, females exposed to colder temperatures have been observed to produce larger eggs in a variety of organisms (Atkinson et al. 2001; Baldanzi et al. 2015; Blanckenhorn 2000; Feiner et al. 2016; Shama 2015). In contrast, we found that maternal exposure to the high $p\text{CO}_2$ level and low temperature associated with the upwelling treatment had no significant effect on the average size of the eggs, although this observation may be subject to egg size variability between females. In fact, urchins that experienced non-upwelling conditions produced eggs that were more variable in size than those that experienced upwelling conditions.

Intraspecific variability in egg size occurs within and between populations of sea urchins in nature (George et al. 1990; Hagström and Lönning 1967). It is possible that the natural size variability that exists in *S. purpuratus* egg production was evident under relaxed $p\text{CO}_2$ and temperature conditions (i.e., non-upwelling), but the elevated $p\text{CO}_2$ level, lower temperature, or the combination of both factors decreased the variability of the eggs produced by the upwelling-conditioned females. Variability in egg size can also be affected by differences in genetic factors of the adult females that produced the eggs (Moran and McAlister 2009). Although adult urchins were randomly sorted into their conditioning treatments, we cannot rule out that the non-upwelling treatment may have harbored more genetic variability. Thus, the difference in egg size variability between the maternal treatments could also be caused by genetic variability and/or variability in gene-environment interactions.

Biochemical composition (e.g., amount of protein and lipid) are often used to assess egg quality and maternal provisioning (Falkner et al. 2006; George et al. 1990; Jong-Westman et al. 1995; McAlister and Moran 2012). We did not find that egg size was correlated to egg biochemical composition. Although egg energy content generally scales with size across echinoderm species, at finer intraspecific scales, this relationship can be quite weak (Moran and McAlister 2009). Therefore, egg size does not necessarily provide a reliable indication of energetic content and quality (George et al. 1990; Jong-Westman et al. 1995; McAlister and Moran 2012; McEdward and Morgan 2001; Thompson 1983). In this study, there was no observable effect of maternal conditioning on the total protein content of the eggs. However, there was a maternal treatment effect on the lipid content of the eggs, in which upwelling-conditioned females produced eggs with more lipids. This increase in lipid content could be caused by either the low temperature, the high $p\text{CO}_2$ level, or a combination of both factors. Given our experimental design, however, it is not possible to identify which component(s) of the upwelling maternal treatment contributed to the increase in egg lipid content. Nevertheless, in nature, sea urchins are likely exposed to both factors simultaneously due to the cold, low pH conditions characteristic of upwelled waters.

Our results indicate that females that experience upwelling conditions increase maternal provisioning of lipids to their eggs, particularly in the form of phospholipids, a structural lipid class. Phospholipids regulate membrane morphology, are involved in nuclear envelope formation during mitosis, and modulate the activity level of integral membrane proteins, including ion channels of the plasma membrane (Falkenburger et al. 2010; Larijani et al. 2014). Although not statistically significant, we also observed a trend in which upwelling-conditioned females provisioned more triacylglycerols (Figures 18, 19).

Triacylglycerols are energy storage lipids used by echinoderms to fuel pre-feeding development (Falkner et al. 2006; Meyer et al. 2007; Prowse et al. 2008; Sewell 2005; Villinski et al. 2002). Therefore, an increase in provisioning of lipids to the eggs can impact performance, growth, and cellular morphology during embryonic and larval development.

Comparing our data to other studies on *S. purpuratus*, the average size of the eggs reported here (0.091 mm in diameter) are larger than what has previously been reported from other observations (0.078 – 0.084 mm) (Chase 1935; Levitan 1993; Moore 1943). Additionally, because formalin preservation can cause echinoderm eggs to shrink slightly (Lessios 1987), the actual sizes of the eggs we measured may be even larger than reported. Similarly, the amount of total protein measured here was greater than what has been reported in *S. purpuratus* (Matson et al. 2012; Meyer et al. 2007; Shilling and Manahan 1990). However, other studies used the Bradford assay (Sapan et al. 1999), which differs from the methods employed in this study. Furthermore, egg protein content can vary by the abundance and protein content of the adult urchins' diet during gametogenesis (George et al. 1990; Hammer et al. 2006; Jong-Westman et al. 1995). The total lipid content per egg reported here is greater than that reported by Matson et al. (2012), but less than what has been reported by Meyer et al. (2007). However, for this study, the lipid concentrations were not adjusted using an internal ketone standard. Additionally, adults were not acclimated to controlled conditions prior to spawning in Matson et al. (2012) or Meyer et al. (2007).

Importantly, much evidence suggests echinoderm egg size and quality is affected by the food quantity and quality available to the adults during gametogenesis (Bertram and Strathmann 1998; George 1996; George et al. 2001; Jong-Westman et al. 1995; Thompson 1983). It is possible that the relatively large egg size and high biochemical content reported

in this study were due to the feeding design adopted in this experiment. We chose to feed the adult urchins in excess throughout the conditioning period to minimize any potential effects of feeding differences between the treatments and to ensure we could obtain enough high quality gametic material to create viable embryo cultures. The effect of feeding may have dominated and diminished the visible effect of our experimental treatment on the eggs. Greater differences in egg size and biochemistry may have been observed given a more conservative feeding regime. Nevertheless, there was still an observable effect of maternal conditioning on the eggs, in which maternal provisioning of lipids was higher in females conditioned to simulated upwelling (i.e., high $p\text{CO}_2$ and low temperature).

Impact of differential maternal conditioning on early developmental stages

A fundamental tenet of life-history theory is that increased offspring size is linked to increased fitness (Smith and Fretwell 1974). Organisms with larger body sizes during early development can exhibit greater performance at later life stages (e.g., as juveniles and adults) in terms of growth, development, survivorship and reproductive output (Marshall et al. 2003; Moran and Emlet 2001). In marine organisms with planktonic embryos and larvae, body size can be positively correlated with dispersal time and distance (Marshall and Keough 2003) that in turn affects recruitment and species ranges. Here, we examined average maternal effects across a pool of genotypes to mimic mass spawning events that occur in nature. While we cannot rule out whether there was differential mortality between embryos from different mothers, the two progeny groups (pooled offspring of upwelling-conditioned mothers and pooled offspring of non-upwelling-conditioned mothers) exhibited distinct phenotypic differences when raised under different $p\text{CO}_2$ levels (low or high $p\text{CO}_2$) at the same

temperature. We observed maternal treatment effects (i.e., transgenerational) on offspring body size, which have possible implications for progeny fitness and dispersal, but no offspring treatment effects (i.e., intragenerational) that would have allowed for an indication of differential stress response.

Although there was no significant maternal treatment effect on egg size, at the hatched blastula, gastrula and prism stages, the progeny from females that experienced upwelling conditions (U: $\sim 1,300 \mu\text{atm } p\text{CO}_2$, $\sim 13^\circ\text{C}$) had larger body sizes. In general, parents that experience lower temperatures produce larger offspring (Atkinson et al. 2001; Burgess and Marshall 2011). The production of larger offspring by mothers that experience colder environments has been attributed to increased maternal provisioning intended to offset the greater energetic costs of developing at lower temperatures (Pettersen et al. 2019). Parental exposure to low pH, however, may have a limited impact on offspring size, although this can vary somewhat depending on the duration of adult exposure (Lamare et al. 2016; Suckling et al. 2014; Suckling et al. 2015). Thus, although we cannot separate the effects of high $p\text{CO}_2$ from low temperature (i.e., the upwelling maternal treatment), it is possible that maternal temperature was the predominant factor eliciting transgenerational effects that facilitated larger embryo body sizes. Eggs produced by upwelling-conditioned females contained more structural lipids, which are used to construct the bodies of the embryo and larva (Prowse et al. 2008; Sewell 2005). Therefore, an increase in maternal provisioning of lipids may have contributed to larger progeny during the blastula through prism stages.

At the echinopluteus larval stage, there was no longer a detectable maternal treatment effect on body size. In general, initial larval size is positively correlated to egg size (McEdward 1986) and here, neither egg nor larval size was significantly different between

the maternal treatments. The loss of an evident maternal effect could be explained by a difference in timing of energy usage and growth. For example, the progeny of mothers conditioned to the upwelling treatment may have spent more energy towards growth during the earlier stages of development (i.e., hatched blastula through prism stages) and slowed their growth as they reached the first larval stage. On the other hand, the progeny of mothers conditioned to the non-upwelling treatment may have grown less during their early development, ultimately catching up in size to the upwelling progeny by the time they reached the echinopluteus stage. Thus, once maternally-provided energy stores were largely exhausted and the offspring had reached their planktotrophic feeding stage, all larvae were approximately equal sizes. A more detailed analysis of how biochemical constituent quantities changed throughout development would be required to verify how progeny differ in their energy use over time. Although there is no significant maternal effect at the larval stage, the success of embryonic stages and processes, particularly gastrulation, are necessary for success in the pelagic zone, dispersal, and eventual recruitment. As such, even transgenerational effects that impact only select stages of development likely play a role in a population's success under rapid environmental change.

Offspring $p\text{CO}_2$ treatment had no effect on offspring body size for any developmental stage. This was an unexpected result because generally, elevated $p\text{CO}_2$ levels have been shown to negatively affect body size and growth during sea urchin early development (Byrne et al. 2013b; Byrne et al. 2013c; Clark et al. 2009; Kurihara and Shirayama 2004; Sheppard Brennard et al. 2010), particularly in developmental stages that undergo calcification processes (e.g., prism and echinopluteus), although the effects of ocean acidification can vary across stages of early development (Byrne 2011; Ericson et al. 2010; Kurihara 2008).

However, there have been exceptions when high $p\text{CO}_2$ does not always cause reduced growth in during the early development of sea urchins, such as in *Pseudechinus huttoni*, in which low pH conditions have no effect on echinopluteus body size (Clark et al. 2009). In some cases, temperature and $p\text{CO}_2$ effects act antagonistically; temperature can offset a reduction in larval body size due to elevated $p\text{CO}_2$ (Byrne and Przeslawski 2013; Przeslawski et al. 2015). Here, all the offspring were raised at the same temperature. It is possible that during this study, developmental temperature rather than developmental $p\text{CO}_2$ level was the dominant factor dictating intragenerational body size.

We found no significant interaction between maternal and offspring treatments for any developmental stage. In other words, adult conditioning did not impact the effect of offspring $p\text{CO}_2$ treatment (i.e., raised under low or high $p\text{CO}_2$ levels at $\sim 15^\circ\text{C}$) on progeny body size. Thus, because the offspring $p\text{CO}_2$ treatment did not elicit an observable effect on progeny body size, we were not able to measure how maternal conditioning may have affected the progeny's response to stress. While the response to $p\text{CO}_2$ was not detectable by examining body size, a past study suggested that maternal conditioning can affect offspring response to $p\text{CO}_2$ stress at the transcriptomic level (Wong et al. 2018). Future studies may include more extreme $p\text{CO}_2$ conditions or other abiotic stressors that can induce an observable effect on body size. In this study, only the maternal treatment affected offspring phenotype. Although we were unable to test if this maternal effect altered the offspring response to $p\text{CO}_2$ stress, the production of larger offspring by upwelling-conditioned mothers suggests that the fitness and dispersal potential of progeny can be affected by the environmental conditions experienced by the parental generation, thereby providing evidence of transgenerational plasticity.

Mechanisms of transgenerational plasticity

In this study, we explored mechanisms that could control the variation phenotype of sea urchins during early development. Such mechanisms could include maternal effects, a situation where the phenotype of an individual is influenced by the phenotype or environmental experience of the mother. Provided environmental conditions are reliably predictable, the progeny's phenotype may be adjusted accordingly via maternal effects so that they are better suited to their particular environment (Bondurianski et al. 2012; Mousseau and Fox 1998). Differential maternal provisioning by females in different CO₂ and temperature treatments has been observed in sea urchins (Foo et al. 2012; Suckling et al. 2015; Sunday et al. 2011) and copepods (Vehmaa et al. 2012). Eggs of the copepod *Acartia* sp. increased in quality over longer exposure times of the adults, which reduced negative effects of changing pH on hatching success, an observation that was attributed to better maternal provisioning (Vehmaa et al. 2012). Increased maternal provisioning, via an additional supply of nutrients and potential energy reserves, can result in larger body sizes and may functionally act to improve an organism's ability to respond to environmental stressors. Here, lipid content was higher in eggs of females conditioned to upwelling conditions (Figure 18), which potentially contributed to the physiological differences that were observed during the early development of the progeny (Figures 16, 17).

Alternatively, transgenerational plasticity may be facilitated by epigenetic mechanisms that operate to alter offspring phenotype, a process that has been proposed for invertebrates (Roberts and Gavery 2012) and marine fish (Metzger and Schulte 2016), although studies on marine metazoans within a global change context are rare (Hofmann

2017). In a companion paper, we found that the maternal environment experienced by adult *S. purpuratus*, but not the developmental environment experienced by their offspring, affected the DNA methylation patterns of the progeny (Strader et al. 2019). Previous research in our group demonstrated that differential maternal conditioning also influenced the expression of genes related to epigenetic processes, such as methylation, methyltransferase activity, and histone modification (Wong et al. 2018). In many cases, epigenetic mechanisms, such as DNA modifications via methylation, operate to alter gene expression, changing the transcriptome in a manner that creates plasticity in response to new abiotic conditions (Roberts and Gavery 2012). This has been observed in corals, in which lower levels of DNA methylation facilitated flexible gene expression across environments (Dixon et al. 2014). In addition, Putnam and colleagues suggest that environmentally-induced changes in DNA methylation may generate phenotypic plasticity in corals exposed to low pH conditions (Putnam et al. 2016). Epigenetic modifications can provide a rapid response to environmental change (Eirin-Lopez and Putnam 2019; Hofmann 2017), and have the potential to subsist across multiple generations, eventually contributing to long term adaptation in marine systems (Suarez-Ulloa et al. 2015).

Overall, there has been a resurgence of studying TGP and maternal effects in a global change context in marine systems (Donelson et al. 2017; Ross et al. 2016). In predicting how marine systems will be impacted by continuing global change, there are important questions regarding the interactions between TGP and evolution (Chirgwin et al. 2018; Donelson et al. 2017). TGP can alter variation in phenotype at a rate faster than, and without the need of, changes in genotype, influencing the fitness landscape and resulting evolutionary processes (Bondurianski et al. 2012; Bondurianski and Day 2009). There exists the possibility that TGP

confers resistance to progeny that acts on an ecological rather than an evolutionary timescale. This resistance is later the source of adaptation (i.e., ‘plasticity first’) (Levis and Pfennig 2016; Schwander and Leimar 2011), buying time for these organisms to respond to rapid changes in their environment. Alternatively, TGP may decelerate evolutionary processes by weakening selection on genetic variation or by providing maladaptive phenotypes when mismatches between parental and offspring environments occur (Bondurianski et al. 2012; Reed et al. 2010).

The interplay between TGP and adaptation is important for predicting future changes in marine populations, communities and ecosystems. Furthermore, there is interest in management approaches in which TGP may have a role in conservation efforts (Chakravarti et al. 2016; Evans et al. 2014; van Oppen et al. 2015). TGP has also been examined in the context of fisheries species and aquaculture practices, in which conditioning of adult broodstocks may lead to more resilient offspring (Burt et al. 2011; Gavery and Roberts 2017; Utting and Millican 1997). Regardless of the conceptual framework, there is still much to do to fully understand TGP, how it connects to creating phenotypic plasticity in environmentally-induced variants during development, and whether these new phenotypes bolster the adaptive capacity of marine populations in a changing ocean. Future research should continue to examine the precise mechanisms of TGP (Bondurianski and Day 2009; Eirin-Lopez and Putnam 2019; Jablonka and Raz 2009), paternal contributions to TGP (Crean and Bondurianski 2014; Crean et al. 2013; Guillaume et al. 2016), and TGP in variable and unpredictable environments in nature (Burgess and Marshall 2014; Donelson et al. 2017).

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Data accessibility

All morphometric, protein, and lipid data are deposited in the Dryad Digital Repository at <https://doi.org/10.5061/dryad.4nv0nb8>.

VI. Transcriptomics reveal transgenerational effects in purple sea urchins exposed to upwelling conditions, and the response of their progeny to differential $p\text{CO}_2$ levels

Abstract

Understanding the mechanisms with which organisms can respond to a rapidly changing ocean is an important research priority in marine sciences, especially in light of recent predictions regarding the pace of ocean change in the coming decades.

Transgenerational effects, in which the experience of the parental generation can shape the phenotype of their offspring, may serve as such a mechanism. In this study, adult purple sea urchins, *Strongylocentrotus purpuratus*, were conditioned to regionally and ecologically relevant $p\text{CO}_2$ levels and temperatures representative of upwelling (low temperature, high $p\text{CO}_2$) and non-upwelling (average temperature, low $p\text{CO}_2$) conditions typical of coastal upwelling regions in the California Current System. Following 4.5 months of conditioning, adults were spawned, and offspring were raised under either high or low $p\text{CO}_2$ levels, to examine the role of maternal effects. Using RNA-seq and comparative transcriptomics, our results indicate that differential conditioning of the adults had an effect on the gene expression patterns of the progeny during the gastrula stage of early development. For example, maternal conditioning under upwelling conditions intensified the transcriptomic response of the progeny when they were raised under high versus low $p\text{CO}_2$ conditions. Additionally, mothers that experienced upwelling conditions produced larger progeny. The overall findings of this study are complex but do suggest that transgenerational plasticity *in*

situ could act as an important mechanism by which populations might keep pace with rapid environmental change.

Overview

This chapter describes the results of an experiment in which adult purple sea urchins were acclimated to combined temperature and $p\text{CO}_2$ conditions to examine differences in the gene expression patterns of their offspring exposed to different $p\text{CO}_2$ conditions. This work was supported by funds from the UC Climate Champion award from the University of California to my advisor, Dr. Gretchen Hofmann. Additionally, the Santa Barbara Coastal LTER (NSF award OCE-1232779; Director: Dr. Dan Reed) provided boating support and access to longterm datasets. The research presented in this chapter was conducted in collaboration with my advisor, Dr. Gretchen Hofmann, a former member of the Hofmann Lab, Dr. Kevin Johnson, and a collaborating researcher at Louisiana State University, Dr. Morgan Kelly. This chapter has been published in *Molecular Ecology* (Wong et al. 2018).

Introduction

Transgenerational plasticity (TGP), or transgenerational effects, in which the history of the parental generation shapes the phenotype of the offspring (Bondurianski and Day 2009; Marshall 2008), has recently attracted attention in marine systems as a means of rapid response to global climate change (GCC) (Donelson and Munday 2015; Goncalves et al. 2016; Munday 2014; Parker et al. 2015; Ross et al. 2016; Schunter et al. 2018; Schunter et al. 2016; Thor and Dupont 2015). Research in this area has examined how laboratory conditioning of adults (Lane et al. 2015; Parker et al. 2015; Putnam and Gates 2015; Salinas

and Munch 2012; Schunter et al. 2018; Shama et al. 2016; Thor and Dupont 2015) and how adult history *in situ* might alter the tolerances of the progeny to abiotic stressors (Evans et al. 2017; Kelly et al. 2011; Murray et al. 2014). Transgenerational effects do not involve transmission of DNA sequences (Bondurianski and Day 2009), and may be somatic, cytoplasmic, nutritional, or epigenetic (Munday 2014). Epigenetic modulation of the transcriptome has been identified as one mechanism that could operate quickly in a rapidly changing environment. Here, we assess the role of TGP in the transcriptomic response of the progeny and hypothesize that abiotic conditions experienced by adults during gametogenesis may alter the response of the F1 generation to different $p\text{CO}_2$ levels.

Marine organisms face a suite of complex and interacting abiotic factors that will change and intensify in the future (Boyd et al. 2015; Breitburg et al. 2015; Gunderson et al. 2016), and the early developmental stages (EDSs) may be particularly vulnerable (Byrne 2011; Gosselin and Qian 1997; Kurihara 2008). Any means of altering tolerance to abiotic stress may facilitate the continued survival of a species (Somero 2010). TGP may be paramount to conferring such tolerance, buying time for adaptation to take place (Chevin et al. 2013). Here, the ‘plasticity first’ hypothesis (*sensu* Schwander and Leimar 2011; West-Eberhard 2005) proposes that phenotypic plasticity initiated by abiotic conditions during development is an important source of tolerance to environmental stress (Levis and Pfennig 2016). Although TGP may allow species to cope with rapid environmental changes (Bossdorf et al. 2008; Salinas and Munch 2012), it may also be disadvantageous if there is a negative parental response (Putnam and Gates 2015), such as a reduction in gamete quality, or if a rapid environmental change causes the offspring to experience conditions very different than what their parents prepared them for (Marshall 2008; Munday 2014).

Regardless, failing to consider the parents' environmental history, particularly during gametogenesis, may lead to inaccurate predictions regarding how EDSs respond to current and future ocean stressors.

The purple sea urchin, *Strongylocentrotus purpuratus*, is an ecologically important keystone species (Pearse 2006) that is frequently used as a model organism in developmental biology (Jasny and Purnell 2006). Populations of *S. purpuratus* inhabit coastal regions characterized by highly dynamic and variable water conditions, and as such, may be exposed to a range of abiotic stressors. Most notably, seasonal upwelling can dominate areas such as the California Current System (CCS), bringing deep waters characterized by low pH and temperature up to the surface (Feely et al. 2008; Hauri et al. 2009). In coastal areas within the Santa Barbara Channel, low total pH (~7.83) and low temperatures (11.5 – 14 °C) have been observed during upwelling events (Kapsenberg and Hofmann 2016; Rivest et al. 2016). *S. purpuratus* in this area typically spawn annually from January to July, while undergoing gametogenesis in the interim months, primarily during the fall and winter (Strathmann 1987). As a result, adult *S. purpuratus* may experience upwelling conditions during gametogenesis. This can influence their gametes and the eventual phenotype of their progeny, including how their offspring respond to abiotic stressors such as elevated $p\text{CO}_2$ levels. As calcifying organisms, sea urchins are potentially vulnerable to low pH/high $p\text{CO}_2$ conditions due to the lowered availability of carbonate ions, used in skeletogenesis. Low pH conditions have also been shown to disrupt internal acid-base balance in urchins (Miles et al. 2007). Exposure of EDS echinoderms to elevated $p\text{CO}_2$ levels can lead to a reduction in size (Byrne et al. 2013b; Clark et al. 2009; Dupont et al. 2008; Kurihara and Shirayama 2004; O'Donnell et al. 2009; Sheppard Brennan et al. 2010) and scope for growth (Stumpp et al. 2011a; Stumpp et al.

2012; Stumpp et al. 2011b), alter metabolic rates (Padilla-Gamiño et al. 2013; Stumpp et al. 2011b), and affect gene regulation, including skeletogenic pathways, spicule matrix proteins, and ion regulation and transport (Evans et al. 2013; Evans and Watson-Wynn 2014; Padilla-Gamiño et al. 2013; Stumpp et al. 2012). Due to the highly variable ocean conditions experienced by urchin populations and the potential adult exposure to upwelling conditions, which may subsequently impact offspring response to $p\text{CO}_2$ stress, this species provides a valuable opportunity with which to examine TGP.

Some *S. purpuratus* populations may be relatively tolerant to low pH conditions (Byrne et al. 2013c; Gaitán-Espitia et al. 2014), due to generations of frequent low pH exposure within upwelling regimes (Evans et al. 2017; Kelly et al. 2013; Padilla-Gamiño et al. 2013; Pespeni et al. 2013a; Pespeni et al. 2013b). However, future oceans will possess a greater intensity of abiotic stressors, possibly forcing this species beyond its threshold of tolerance. The continuation of ocean acidification (OA) due to rising levels of atmospheric CO_2 (Doney et al. 2009; Hoegh-Guldberg and Bruno 2010; Orr et al. 2005) will magnify the effects of upwelling (Gruber et al. 2012), further decreasing pH conditions in coastal waters, an occurrence that is already being observed today (Feely et al. 2008). Additionally, the intensity and duration of upwelling is expected to rise with future climate change (Difffenbaugh et al. 2004). Mechanisms such as local adaptation require long time scales across multiple generations and may not be sufficient in the future with the rapid progression of GCC and OA. However, TGP can function on much shorter timescales.

Given the dynamic environment of the CCS, exploring patterns of gene expression is an ideal means of assessing organismal response. Transcriptomics allows for a molecular-level examination, providing valuable insight into physiological effects and how organisms

respond to their environment (DeBiasse and Kelly 2016; Evans and Hofmann 2012; Gracey 2007). As a phenotypic trait, the transcriptome can be examined and compared between organisms that experience different environmental conditions (DeBiasse and Kelly 2016; Zhou et al. 2012). Previous studies have successfully used transcriptomics to examine how organisms respond to high $p\text{CO}_2$ environments (Evans et al. 2017; Moya et al. 2012; Padilla-Gamiño et al. 2013; Todgham and Hofmann 2009; Walder et al. 1983), and how transgenerational effects may alter the transcriptional response to stress (De Wit et al. 2016; Goncalves et al. 2016; Schunter et al. 2018; Schunter et al. 2016; Shama et al. 2016). To investigate whether differential conditioning of the adults affected the response of their progeny, RNA-seq and comparative transcriptomics were used to examine gene expression patterns during the gastrula stage of early development. In addition, we measured body size as an organismal trait that is a commonly used proxy for performance in early stage invertebrates. The gastrula stage was selected for this study because gastrulation is considered to be perhaps the most vital and fundamental process during metazoan development (Wolpert 1992; Wray and Raff 1991b). Additionally, the maternal-to-zygotic transition (MZT) in *S. purpuratus* occurs very early in development (Tadros and Lipshitz 2009; Wei et al. 2006), and by the gastrula stage, the zygotic genome is fully activated.

Adult *S. purpuratus* were conditioned to regionally and ecologically relevant $p\text{CO}_2$ levels and temperatures representative of conditions measured during non-upwelling ($\sim 400 \mu\text{atm } p\text{CO}_2$, $\sim 17^\circ\text{C}$) and upwelling ($\sim 1,100 \mu\text{atm } p\text{CO}_2$, $\sim 14^\circ\text{C}$) physical conditions that are typically observed in the CCS (Chan et al. 2017; Kapsenberg and Hofmann 2016). Our goal was to determine if differential adult conditioning could elicit a transgenerational effect, so for this study, we acclimated adult urchins to a simplified set of conditions and did not

incorporate $p\text{CO}_2$ and temperature variability that can be observed *in situ* into the acclimation period. In addition, although the mechanisms of TGP can be maternal and/or paternal, here we chose to focus on only maternal contributions. Following 4.5 months of conditioning, adults were spawned and their offspring were raised at a single, ecologically relevant temperature (15 °C), but at two different $p\text{CO}_2$ levels that represent end-members of what the embryos might experience *in situ* in coastal waters: a low $p\text{CO}_2$ level (~400 μatm) or a high $p\text{CO}_2$ level (~1,100 μatm), to examine the role of maternal effects on body size and in the transcriptomic response of the progeny to different $p\text{CO}_2$ conditions. While other studies have investigated how genetic variation affects plasticity (Runcie et al. 2016; Runcie et al. 2012), here, we investigated average maternal effects incurred by each of our treatments, as measured across pooled genotypes, in an experimental setup which mimics *in situ* situations in which female urchins mass spawn, to investigate TGP within a population of *S. purpuratus*. Our goal was to determine if differential abiotic conditioning of females would change the performance of the progeny (Sultan 2017) where the transcriptome itself was the trait to be measured. Using EDS culturing to raise embryos in a “common garden” trial, we found that there were transgenerational (i.e., intergenerational) and within-generational (i.e., intragenerational) differences in gene expression. Lastly, we aimed to characterize the differential response among offspring. This study aims to investigate TGP, a potential mechanism by which populations might keep pace with rapid environmental change, in *S. purpuratus*.

Materials and methods

Animal collection and conditioning

Adult urchins were collected on September 2, 2015 from Goleta Beach, Goleta, CA (34° 24' 49.38'' N, 119° 49' 19.56'' W) and transported to the Marine Science Institute at the University of California Santa Barbara (UCSB) (Santa Barbara, CA, USA). Two treatments were chosen to reflect conditions recorded in the CCS during periods between and during upwelling events (Kapsenberg and Hofmann 2016; Rivest et al. 2016). The non-upwelling treatment (N) was maintained at ~400 μatm and ~17 °C, while the upwelling treatment (U) was maintained at ~1,100 μatm and ~14 °C. Adult conditioning began prior to the onset of seasonal gonadal development in order to capture the entirety of gametogenesis. Adults were conditioned for ~4.5 months, during which time they were fed kelp (*Macrocystis pyrifera*) once every week and the aquaria were regularly cleaned.

A flow-through CO₂-mixing system modified from Fanguie et al. (2010) was used to maintain experimental $p\text{CO}_2$ conditions. Two 20-gallon glass aquaria were established as reservoir tanks for each treatment, which each fed 0.35 μm filtered, UV sterilized seawater (FSW) to three replicate adult tanks per treatment at a rate of 8 L/hr (16 L/hr total). Filtered, CO₂-scrubbed, dry air was mixed with pure CO₂ using SmartTrak® 100L Series Mass Flow Controllers and MicroTrak™ 101 Series Mass Flow Controllers (Sierra Instruments, USA), respectively, and used to establish the target $p\text{CO}_2$ levels within the reservoir tanks. Each adult tank was composed of a 20-gallon glass aquarium fitted with a small fountain pump (Total Pond, 70gph) to aid mixing. Treatment temperatures were controlled using Delta Star® heat pumps with Nema 4x digital temperature controllers (AquaLogic, San Diego, CA, USA).

Temperature, salinity, and pH measurements were taken every 1 to 2 days during adult conditioning. Temperature was measured using a wire thermocouple (Thermolyne PM 20700 / Series 1218) and salinity was measured using a conductivity meter (YSI 3100). Measurements of pH were conducted following the standard operating procedure (SOP) 6b (Dickson et al. 2007b), using a spectrophotometer (Bio Spec-1601, Shimadzu) and *m*-cresol purple (Sigma-Aldrich) indicator dye. A sample for total alkalinity (TA) was taken every 3 to 5 days, and was estimated using SOP 3b (Dickson et al. 2007a). CO₂calc (Robbins et al. 2010) was used to estimate parameters of $p\text{CO}_2$ and $\Omega_{\text{aragonite}}$ using the carbonic acid dissociation constants of Lueker et al. (2000).

Spawning and culturing

Urchin spawning was induced via intracoelomic injection of 1.0 M KCl. Because sea urchins cannot be accurately sexed prior to spawning, a maximum number of urchins were conditioned that could be held within the space constraints of our CO₂ system. Due to uneven sex ratios, eggs of high viability and quality were only obtained from 5 females conditioned to the non-upwelling treatment and 7 females conditioned to the upwelling treatment. Eggs from females of each treatment were pooled together. We prioritized incorporating the maximum number of female contributors because our questions focused on the average maternal effects for each treatment, as measured across a pool of genotypes. Two crosses were performed in which a single male conditioned to the non-upwelling treatment was crossed with each set of females (Figure 21). Because we were not examining paternal effects during this study, only one male was used to ensure all offspring were full or half-siblings, limiting genotypic diversity that might otherwise confound our results. Dilute,

activated sperm and was slowly added to the eggs until at least 95% fertilization success was reached.

Embryos from each cross were divided and raised under either a low $p\text{CO}_2$ (L) level at $\sim 400 \mu\text{atm}$ or a high $p\text{CO}_2$ (H) level at $\sim 1,100 \mu\text{atm}$. We chose to raise the embryos at one temperature to ensure synchronous development and sampling across all cultures. All progeny was raised at $\sim 15^\circ\text{C}$, and since we know that low pH conditions can alter the physiology of EDSs (Kelly et al. 2013; Padilla-Gamiño et al. 2013; Yu et al. 2011), $p\text{CO}_2$ was altered to drive the calcification environment (see Byrne 2011; Ross et al. 2016). These $p\text{CO}_2$ levels and temperature combinations would occur in nature when the early stages are developing *in situ*, as supported by environmental data from field sensors (Hofmann and Washburn 2015; Kapsenberg and Hofmann 2016). The EDS culturing vessels were composed of two, nested 5-gallon buckets (12 L capacity), in which the inner bucket was fitted with 64-micron mesh to prevent the loss of the embryos while allowing for a flow-through of treated seawater (at a rate of 6 L/hr). Embryos were initially placed in each culture vessel at a density of 10 embryos/mL, although sampling processes decreased larval densities throughout the experiment. Each culture vessel contained a paddle driven by a 12-V motor to allow continuous, gentle mixing. Treatment conditions were designated as follows: offspring of mothers conditioned to non-upwelling raised under low $p\text{CO}_2$ conditions (NL), offspring of mothers conditioned to non-upwelling raised under high $p\text{CO}_2$ conditions (NH), offspring of mothers conditioned to upwelling raised under low $p\text{CO}_2$ conditions (UL), and offspring of mothers conditioned to upwelling raised under high $p\text{CO}_2$ conditions (UH). Three replicate culture vessels were used for each of the four treatments (12 vessels total). The

temperature, salinity, and pH of each reservoir and culture vessel were measured daily, implementing the same methods used during adult conditioning.

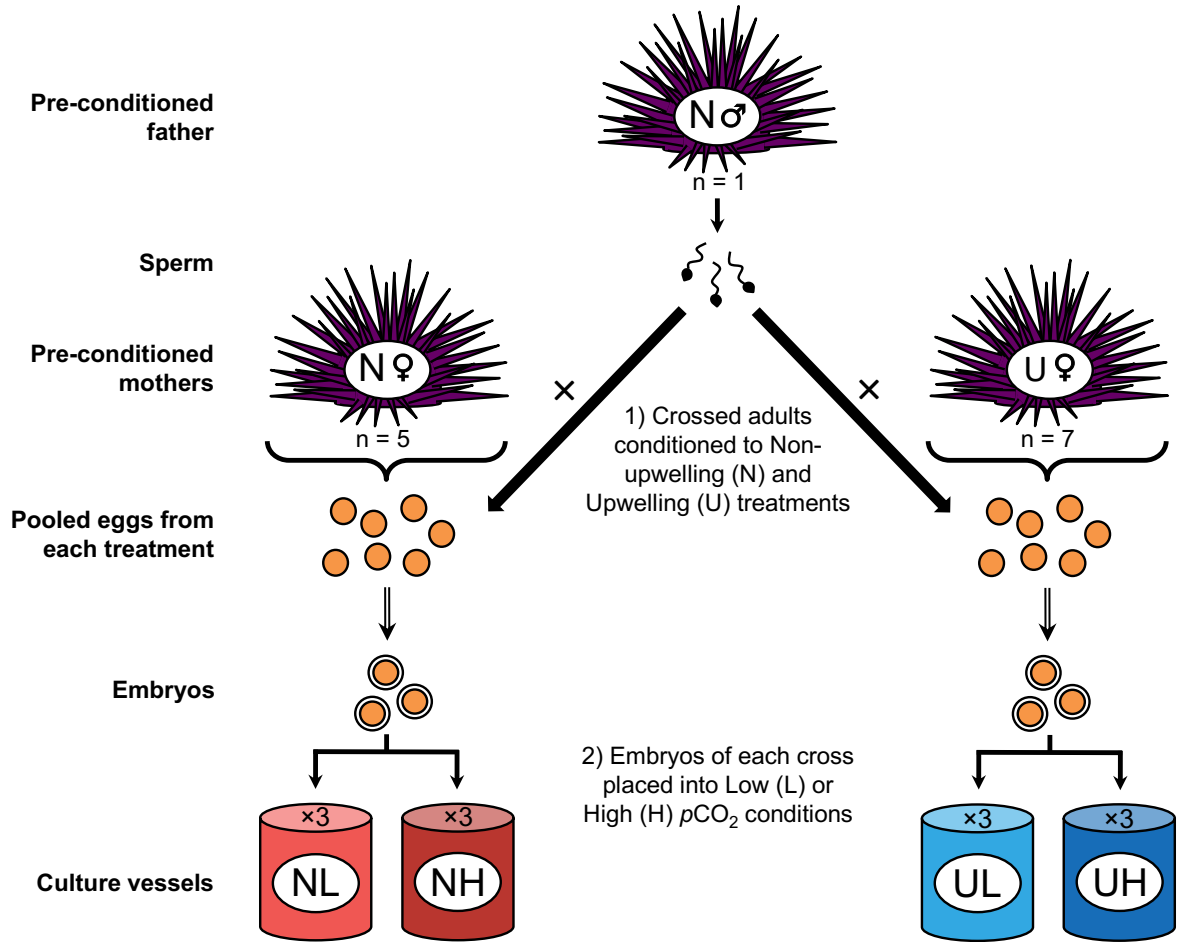


Figure 21. Experimental design showing crosses of non-upwelling- and upwelling-conditioned females with a single non-upwelling-conditioned male. The embryos from each cross were placed into either low or high $p\text{CO}_2$ culturing vessels (with three replicates each), resulting in four treatment types: NL, NH, UL, and UH.

Sample collections

Eggs were sampled from each female for morphometric analyses. The eggs were fixed in 2% formalin in seawater and stored at 4 °C until imaged, for no longer than two months. At approximately 30 hours post-fertilization, the progeny reached the late gastrula stage, at which time the archenteron was invaginated about one-half to four-fifths the body length of the gastrulae. The gastrulae were concentrated using gentle filtration onto a fine mesh screen (35 µm). Gastrula morphometric samples were preserved in the same manner used for eggs. The samples for transcriptomic analyses were collected by transferring approximately 5,000 gastrulae into a 1.5 mL Eppendorf tube. The gastrulae were quickly pelleted by centrifugation, and excess seawater was removed. Samples were then flash frozen in liquid nitrogen, and stored at -80 °C.

Morphometrics

Eggs from each female and gastrulae from each treatment were digitally photographed under bright field DIC illumination using a compound microscope (Olympus BX50) with an attached digital camera (Infinity Lite) and Infinity Capture software (version 6.2.0). ImageJ (National Institutes of Health, USA) was used to analyze the digital photographs and obtain morphometric measurements. Eggs from each female and gastrulae from each treatment replicate were measured ($n \geq 30$). The diameter of each egg was measured three times to calculate the average egg diameter. Gastrulae were oriented such that the full side profile of the archenteron was visible and aligned with the center of the vegetal plate. The maximum distance from the anterior to posterior end, down the center of the archenteron, was measured to obtain the length of each gastrula. A one-way analysis of

variance (one-way ANOVA) was used to compare the means between samples with experimental treatment set as a fixed factor, and mother identity (for eggs) or culture vessel (for gastrulae) set as random effects. The data was tested to verify it met the assumptions of an ANOVA, including normality and homogeneity of variance. If a difference in means was detected, pairwise comparison tests were conducted by a test using false discovery rate (FDR). All statistical analyses were performed using JMP Pro (version 11.2.0).

RNA extractions and sequencing

Total RNA was extracted using 500 μ L of Trizol[®] reagent, following the manufacturer's instructions (Invitrogen). For each sample, 500 μ L of Trizol[®] was added to ~5,000 gastrulae that had been frozen in liquid nitrogen and stored at -80 °C. The sample tissue was homogenized by quickly passing the Trizol/sample mixture three times each through decreasing sizes of sterile needles (21-gauge, followed by 23-gauge, followed by 25-gauge). An addition of chloroform and centrifugation was used to isolate the RNA-containing upper aqueous phase. The RNA was precipitated using isopropyl alcohol. Prior to resuspension, the RNA pellet was washed twice using cold 80% ethanol. The RNA was suspended in DEPC-treated water and kept frozen at -80 °C when not in use. All of the extractions were performed on a single day in two rounds and by the same person, using the same protocol and reagents. RNA purity, quality, and quantity were verified using a NanoDrop[®] ND100 (Thermo Scientific), a TapeStation 220 system (Agilent technologies), and a Qubit[®] fluorometer (Life Technologies) for each sample of total RNA prior to proceeding to library preparation.

Two samples from each culture vessel were used to generate a total of 24 libraries. Libraries were generated from high-quality RNA using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (New England Biolabs). The quality, size, and quantity of each library was verified using a TapeStation 220 system (Agilent) and a Qubit® fluorometer (Life Technologies) at UC Santa Barbara prior to their submission to the Genome Center at the University of California, Davis. The sequencing center at UC Davis also verified library quality and quantity prior to sample pooling and sequencing. Sequencing was performed on an Illumina HiSeq 4000 sequencer, with 150 base-pair paired-end reads on a single lane.

Data processing and DE analyses

Adapter sequences were trimmed from reads using Trim Galore! (version 0.4.1) (Krueger 2015) and base pairs with quality scores below 30 were removed. Sequence quality was verified using FastQC (version 0.11.5) (Andrews 2010). The presence of splice isoforms decreased alignment and mapping efficiency for paired-end reads. Therefore, only single-end reads were mapped to the 21,092 predicted genes of the *S. purpuratus* developmental transcriptome (Tu et al. 2012) and counted to calculate expression values using RSEM (Li and Dewey 2011). Differential gene expression (DE) analyses were performed using the package *limma* (Ritchie et al. 2015) in R (version 3.3.1). The data were filtered to sequences that have more than 0.5 counts per million mapped reads across all 24 samples. Scale normalization was applied to the read counts using a trimmed mean of M-values (TMM) normalization method (Robinson and Oshlack 2010). A voom transformation was applied to the read counts to convert them to log-counts per million while accounting for sample-

specific quality weights and blocking design. Samples were blocked by biological replicate (i.e. the two samples taken from each sample vessel). Principal component analysis (PCA) was used to examine distances between samples. Only the filtered, normalized, and voom transformed data were used for PCA and DE analyses using *limma*.

A contrast matrix was used to make pairwise comparisons between the treatment types, resulting in four comparisons of interest: 1) offspring of upwelling-conditioned mothers versus offspring of non-upwelling-conditioned mothers all raised under low $p\text{CO}_2$ conditions (UL vs. NL), 2) offspring of upwelling-conditioned mothers versus offspring of non-upwelling-conditioned mothers all raised under high $p\text{CO}_2$ conditions (UH vs. NH), 3) offspring of non-upwelling-conditioned mothers raised under high versus low $p\text{CO}_2$ conditions (NH vs. NL), and 4) offspring of upwelling-conditioned mothers raised under high versus low $p\text{CO}_2$ conditions (UH vs. UL). *limma*, which uses moderated t -statistics and p -values adjusted by the Benjamini and Hochberg's method to control the false discovery rate (Benjamini and Hochberg 1995), was used to determine differentially expressed genes (relatively down- or up-regulated) for each comparison (adjusted p -value < 0.05).

WGCNA

While *limma* is useful for conducting pairwise comparisons, the Weighted Gene Co-Expression Network Analysis (WGCNA) (Langfelder and Horvath 2008) package in R allows the user to examine patterns of gene expression across all treatments simultaneously. WGCNA was used to generate a correlation network to identify clusters of similarly expressed genes into modules, with a minimum of 30 genes per module. A module's eigengene summarizes the overall module expression profile. Modules with highly correlated

eigengenes were merged using a threshold value calculated based on the number of samples using the `dynamicMergeCut` WGCNA function in R. Eigengene expression was then correlated with the four experimental treatment types (NL, NH, UL, and UH). A heatmap was used to visualize all significant correlations between modules and treatments.

Functional gene enrichment

Gene ontologies from www.echinobase.org were used to perform functional enrichment analyses in Blast2GO (version 4.0) (Fisher's Exact Test FDR = 0.05). Lists of gene ontology (GO) terms were generated for all differentially expressed genes in each pairwise comparison of interest. GO terms fell within 3 GO categories: (1) Cellular Component, (2) Molecular Function, and (3) Biological Process. These same methods were used to identify GO terms associated with each WGCNA module eigengene.

KEGG analyses

Sequences of interest (i.e. from differential gene expression lists and from module eigengene lists) were paired with their corresponding enzyme codes (downloaded from www.echinobase.org). The Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper (Kanehisa et al. 2012) was used to search and map pathways using enzyme codes. The enzyme codes were searched against all pathways for *S. purpuratus* in the KEGG database, and matches were returned with the number of enzymes that matched to each pathway for each enzyme code list provided.

Results

Parental conditioning and carbonate chemistry

Adult urchins were conditioned for 4.5 months. Urchins that exhibited any indication of poor health were removed from the experiment prior to spawning, but very low mortality was observed. Little to no observable mortality of EDSs was noted in the cultures prior to sampling. Although water chemistry showed some variability during the experiment, $p\text{CO}_2$ treatments remained divergent throughout the course of both adult conditioning and embryo culturing (Table 10). Variation in $p\text{CO}_2$ was likely attributable to fluctuations caused by respiration. TA, salinity, and temperature remained stable throughout the experiment (Table 10).

Table 10. Seawater conditions during the experiment. Adult conditioning included additional temperature differences to mimic non-upwelling and upwelling conditions. Values are given as mean \pm standard deviation.

	pH_{total}	$p\text{CO}_2$ (μatm)	$\Omega_{\text{aragonite}}$	TA ($\mu\text{mol kgSW}^{-1}$)	Salinity	Temp ($^{\circ}\text{C}$)
Adult conditioning						
Non-upwelling (N)	7.98 ± 0.10	485 ± 166	2.18 ± 0.39	2225 ± 5	33.0 ± 0.8	16.8 ± 0.8
Upwelling (U)	7.76 ± 0.10	1208 ± 345	0.91 ± 0.19	2225 ± 5	33.0 ± 0.8	13.6 ± 0.8
Progeny treatment						
low $p\text{CO}_2$ (L)	8.06 ± 0.01	371 ± 12	2.38 ± 0.05	2220 ± 0.7	33.4 ± 0.1	15.4 ± 0.1
high $p\text{CO}_2$ (H)	7.68 ± 0.10	1011 ± 235	1.11 ± 0.25	2220 ± 0.7	33.4 ± 0.1	15.3 ± 0.03

Morphometric analyses

Eggs from females that experienced combined high $p\text{CO}_2$ and low temperature upwelling conditions (U) during gametogenesis were on average 3.2% larger in diameter than eggs from females that experienced combined low $p\text{CO}_2$ and average temperature non-upwelling conditions (N), although this difference was not significant when the variability in egg sizes between individual females of both treatments was accounted for in the analysis (F-ratio = 4.0104, p -value = 0.0731) (Figure 22). There was a maternal treatment effect, but not an offspring treatment effect, on the length of the gastrulae. Specifically, the progeny of mothers that experienced upwelling conditions ($\sim 1,100 p\text{CO}_2$, $\sim 14^\circ\text{C}$) were greater in length than those of mothers that experienced non-upwelling conditions ($\sim 400 p\text{CO}_2$, $\sim 17^\circ\text{C}$) (Figure 23). UL gastrulae were the greatest in length, averaging 1.4% longer than UH, 6.4% longer than NL, and 8.2% longer than NH. Progeny that were raised under the same conditions, but whose mothers experienced different conditions were significantly different from each other (i.e., UL vs. NL (FDR q -value = 0.0294) and UH vs. NH (FDR q -value = 0.0294)). In contrast, there were no significant differences between offspring treatments in which the parental conditions were shared (i.e., NL vs. NH (FDR q -value = 0.4897) and UL vs. UH (FDR q -value = 0.4897)).

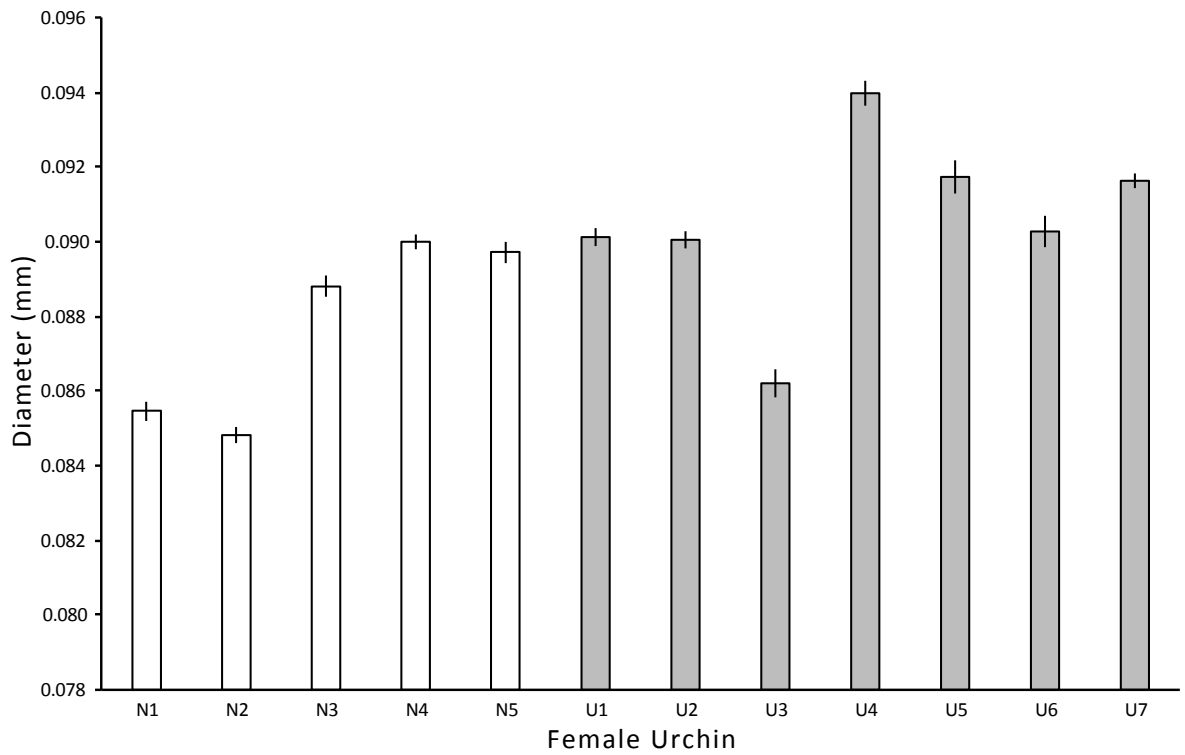


Figure 22. Average diameter of eggs from each female urchin, with error bars showing standard error. Eggs were collected from five females conditioned under the non-upwelling treatment (i.e., N1 – N5) and from seven females conditioned under the upwelling treatment (i.e., U1 – U7).

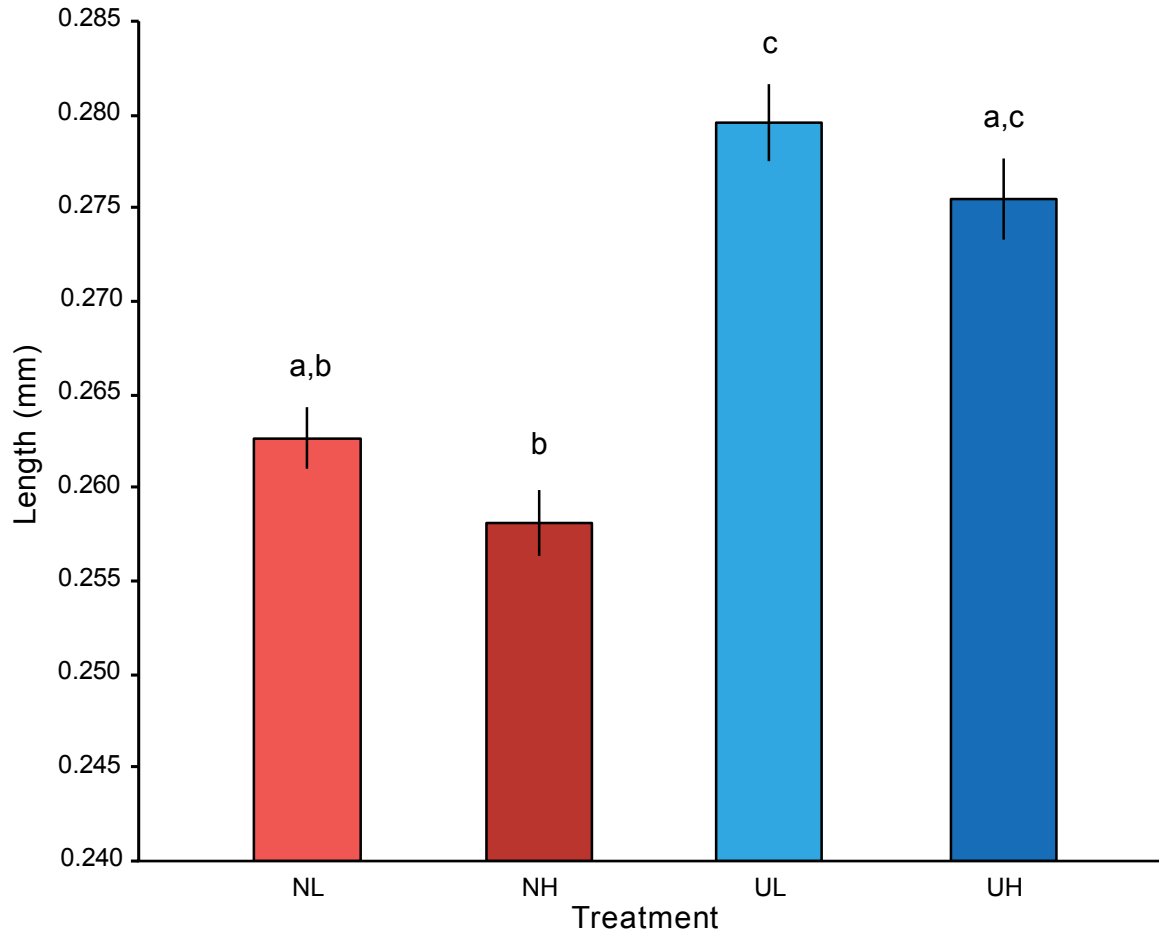


Figure 23. Average length of gastrulae from each of the four treatment types (i.e., NL, NH, UL, and UH), with error bars showing standard error. Any treatments that do not share assigned letters (i.e., a, b, or c) are significantly different from one another.

Summary statistics and overview of RNA-seq

Trimming the sequence reads resulted in 13 to 18 million reads per sample, with an average of 15.7 million reads per sample. FastQC results of trimmed sequences showed high sequence quality (> 30) without adapter contamination or overrepresented sequences. Per-library mapping efficiency using RSEM ranged from 36 to 53%, with an average of 44%. Reads that failed to map to the *S. purpuratus* developmental transcriptome were a combination of mitochondrial rRNA sequences and trace contamination. After using *limma* to filter the data to those with more than 0.5 counts per million mapped reads, 13,967 sequences remained.

Influence of adult conditioning on the gastrula transcriptome

Overall, the transcriptomes of the embryos differed as a function of maternal conditioning, in which one group of adults was conditioned to $\sim 400 \mu\text{atm } p\text{CO}_2$ and $\sim 17^\circ\text{C}$ (i.e., the non-upwelling treatment), and one group of adults was conditioned to $\sim 1,100 \mu\text{atm } p\text{CO}_2$ and $\sim 14^\circ\text{C}$ (i.e., the upwelling treatment). (Figures 24-26). To examine overall similarities in patterns of gene expression across the experiment treatments, a PCA plot was used to examine sample-to-sample distances following voom transformation of the read counts (Figure 24A). In general, the biological and technical replicates within each treatment clustered together. Gene expression patterns of the progeny grouped together depending on what their parents experienced, which resulted in two major clusters: (1) offspring of mothers who experienced non-upwelling conditions (NL and NH), and (2) offspring of mothers who experienced upwelling conditions (UL and UH). Conversely, grouping of progeny was not necessarily dependent on which conditions the offspring experienced during development.

Therefore, the progeny grouped by maternal conditioning (i.e., non-upwelling vs. upwelling) despite the environment in which they developed (i.e., offspring conditions which differed by $p\text{CO}_2$ level). In summary, the PCA plot showed that parental conditions were more predictive of transcriptomic similarity than were offspring conditions.

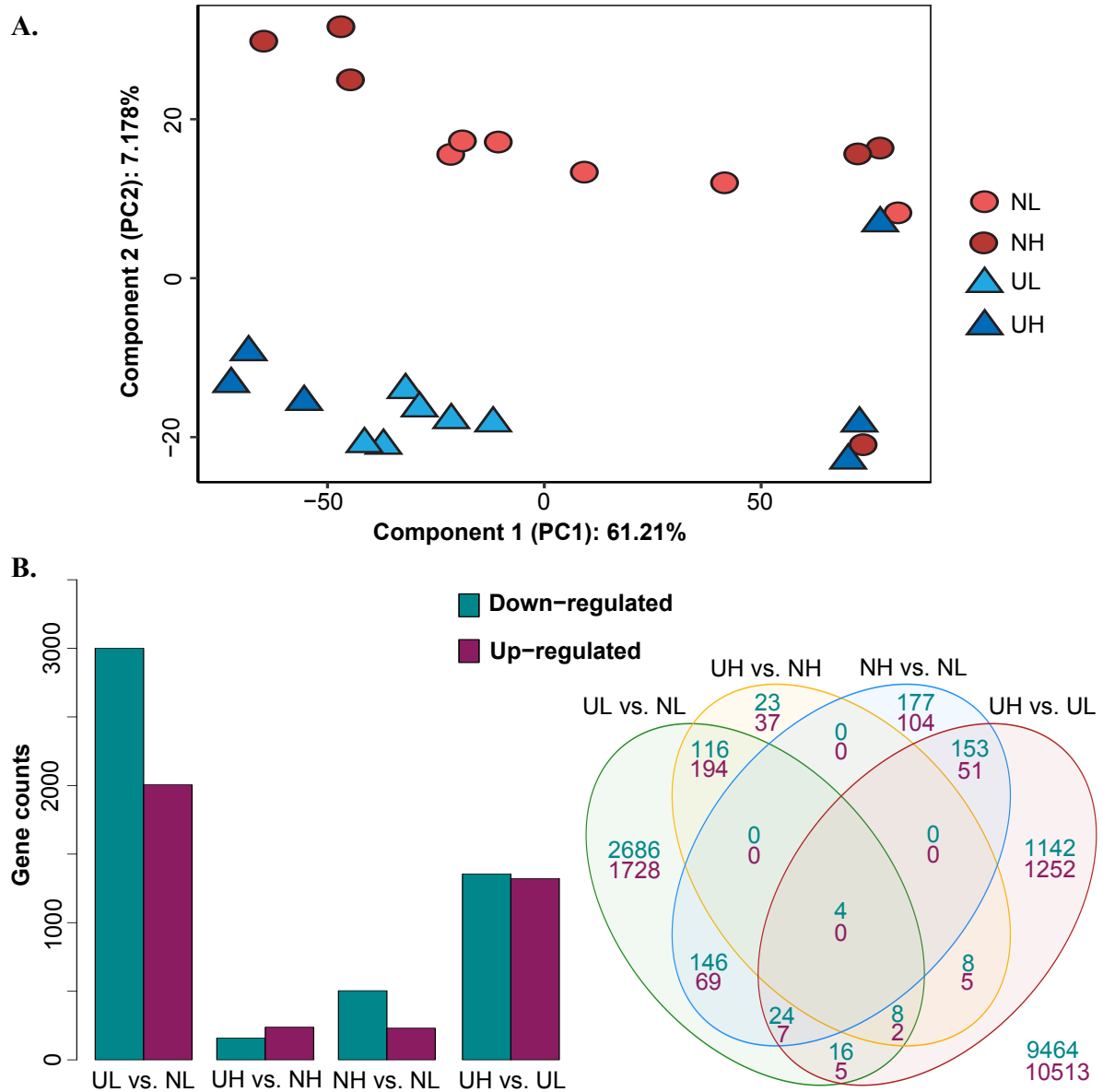


Figure 24. Global RNA-seq patterns of the gastrula stage progeny. **A.** Principal Component Analysis (PCA) plot showing distances between samples. The PCA was performed on gene counts that had undergone filtering and voom transformation using *limma*. **B.** Histogram and Venn diagram displaying the amount of differentially expressed genes (FDR = 0.05) for each treatment pairwise comparison of interest determined using *limma*.

Pairwise comparisons of the gastrula transcriptomes

As noted, the combined $p\text{CO}_2$ and temperature acclimation conditions of the adults influenced the gene expression patterns of the gastrulae, when they were raised under the “common garden” conditions of low or high $p\text{CO}_2$ levels at $\sim 15^\circ\text{C}$. Below, we describe differential gene expression comparisons between each set of treatments. Complete lists of GO terms and KEGG results associated with each comparison are available as supplementary material.

Comparison I: Different maternal conditions + progeny raised under low $p\text{CO}_2$ – UL vs. NL

When the embryos were raised under the low $p\text{CO}_2$ treatment, they exhibited significant differences in gene expression between those whose mothers experienced upwelling conditions (UL) and those whose mothers experienced non-upwelling conditions (NL). The comparison of UL versus NL had more differentially expressed genes than any other pairwise comparison (Figure 24B).

3,000 genes were down-regulated in offspring from mothers who experienced upwelling conditions (UL) compared to those whose mothers experienced non-upwelling conditions (NL) (Figure 24B). Functional enrichment analyses identified down-regulated GO terms including some related to metabolic and epigenetic processes as well as to gene expression (Tables 11, S6). 2,005 genes were up-regulated in offspring from mothers who experienced upwelling conditions (UL) compared to those whose mothers experienced non-upwelling conditions (NL) (Figure 24B). Up-regulated GO terms were identified, including terms related to oxidative phosphorylation, ion transport, and ATP synthesis (Tables 11, S6).

Table 11. Select GO term results from functional enrichment analyses of genes that were differentially expressed in each pairwise comparison of interest.

Down/Up Regulated	GO ID	GO Term Name	GO Category	FDR value	No. Transcripts (% of ref)
UL vs. NL					
Down	GO:0044237	cellular metabolic process	Biological Process	5.84E-37	914 (17.8%)
Down	GO:0010467	gene expression	Biological Process	8.47E-37	455 (23.1%)
Down	GO:0006351	transcription, DNA-templated	Biological Process	1.88E-11	245 (19.5%)
Down	GO:0006417	regulation of translation	Biological Process	4.74E-07	51 (32.1%)
Down	GO:0040029	regulation of gene expression, epigenetic	Biological Process	2.33E-06	22 (61.1%)
Down	GO:0016570	histone modification	Biological Process	4.57E-04	36 (28.6%)
Down	GO:0006304	DNA modification	Biological Process	0.0386	9 (50.0%)
Down	GO:0003676	nucleic acid binding	Molecular Function	5.45E-45	425 (26.4%)
Down	GO:0043167	ion binding	Molecular Function	1.46E-12	590 (15.9%)
Down	GO:0008135	translation factor activity, RNA binding	Molecular Function	1.55E-06	33 (41.8%)
Down	GO:0042393	histone binding	Molecular Function	1.56E-04	19 (50.0%)
Up	GO:0006119	oxidative phosphorylation	Biological Process	2.40E-04	18 (34.0%)
Up	GO:0034220	ion transmembrane transport	Biological Process	4.60E-04	36 (18.8%)
Up	GO:0050801	ion homeostasis	Biological Process	0.00362	28 (18.7%)
Up	GO:0042773	ATP synthesis coupled electron transport	Biological Process	0.00371	12 (36.4%)
Up	GO:0016491	oxidoreductase activity	Molecular Function	1.67E-07	109 (14.9%)
Up	GO:0046933	proton-transporting ATP synthase activity, rotational mechanism	Molecular Function	0.0112	5 (125%)
UH vs. NH					
Up	GO:0032502	developmental process transcription factor activity, RNA	Biological Process	0.0475	39 (1.6%)
Up	GO:0003705	polymerase II distal enhancer sequence-specific binding	Molecular Function	0.0222	5 (21.7%)
Up	GO:0008301	DNA binding, bending	Molecular Function	0.0222	4 (36.4%)
NH vs. NL					
Down	GO:0045862	positive regulation of proteolysis	Biological Process	5.17E-05	14 (12.7%)
Down	GO:0030163	protein catabolic process	Biological Process	4.58E-04	22 (6.3%)
Down	GO:0000502	proteasome complex	Cellular Component	1.67E-11	17 (34.0%)
Down	GO:1905369	endopeptidase complex	Cellular Component	5.08E-11	17 (29.8%)
Down	GO:0004298	threonine-type endopeptidase activity	Molecular Function	6.38E-05	7 (46.7%)
UH vs. UL					

Down	GO:0051603	proteolysis involved in cellular protein catabolic process	Biological Process	1.11E-05	39 (14.4%)
Down	GO:0010498	proteasomal protein catabolic process	Biological Process	0.00190	22 (15.0%)
Down	GO:0006811	ion transport	Biological Process	0.00608	67 (8.3%)
Down	GO:0006119	oxidative phosphorylation	Biological Process	0.00982	12 (20.3%)
Down	GO:0050801	ion homeostasis	Biological Process	0.0214	20 (12.7%)
Down	GO:0070003	threonine-type peptidase activity	Molecular Function	0.03042	6 (37.5%)
Up	GO:0010467	gene expression	Biological Process	1.57E-12	194 (8.7%)
Up	GO:0008152	metabolic process	Biological Process	2.00E-11	460 (6.6%)
Up	GO:0006351	transcription, DNA-templated	Biological Process	6.48E-04	105 (7.5%)
Up	GO:0010608	posttranscriptional regulation of gene expression	Biological Process	0.0128	24 (11.3%)
Up	GO:0006417	regulation of translation	Biological Process	0.0147	22 (11.7%)
Up	GO:0005524	ATP binding	Molecular Function	3.37E-09	119 (9.4%)
Up	GO:0008135	translation factor activity, RNA binding	Molecular Function	0.0107	15 (15.5%)

Comparison II: Different maternal conditions + progeny raised under high $p\text{CO}_2$ – UH vs. NH

In contrast to the previous comparison, there were many fewer genes differentially expressed in embryos raised in a high $p\text{CO}_2$ environment, but who did not share the same parental treatment conditions (Figure 24B). Only 159 genes were down-regulated in gastrulae whose mothers experienced upwelling conditions (UH) compared to those whose mothers experienced non-upwelling conditions (NH). No GO terms were identified for down-regulated genes. 238 genes were up-regulated in UH relative to NH. Up-regulated GO terms were related to transcription factor activity and DNA binding (Tables 11, S6).

Comparison III: Mothers conditioned to non-upwelling + different progeny $p\text{CO}_2$ conditions – NH vs. NL

Embryos exhibited a relatively muted transcriptomic response to high $p\text{CO}_2$ when their mothers were conditioned to non-upwelling conditions (NH vs. NL). In gastrulae whose mothers shared the same non-upwelling conditions, 504 genes were down-regulated in those raised under high $p\text{CO}_2$ (NH) compared to those raised under low $p\text{CO}_2$ (NL) (Figure 24B). Down-regulated GO terms included those related to protein catabolism (Tables 11, S6). Only 231 genes were up-regulated when the offspring were raised under high $p\text{CO}_2$ (NH) compared to those raised under low $p\text{CO}_2$ (NL) (Figure 24B). Functional enrichment analyses of the up-regulated genes did not identify any GO terms.

Comparison IV: Mothers conditioned to upwelling + different progeny pCO₂ conditions – UH vs. UL

Unlike the previous comparison, the transgenerational effect of mothers who experienced upwelling conditions had a large impact on the response of the offspring to different $p\text{CO}_2$ levels (UH vs. UL), and resulted in many more genes that were differentially expressed (Figure 24B). Among the progeny of mothers conditioned under upwelling, 1,355 genes were down-regulated in the offspring raised under high $p\text{CO}_2$ (UH) compared to those raised under low $p\text{CO}_2$ (UL). Down-regulated GO terms included those related to protein catabolism, ion transport, and oxidative phosphorylation (Tables 11, S6). 1,322 genes were up-regulated in those raised under high $p\text{CO}_2$ (UH) compared to those raised under low $p\text{CO}_2$ (UL) (Figure 24B). Within these, GO terms included those related to gene expression, metabolic processes, and ATP binding (Tables 11, S6).

In summary, the transcriptomic response of gastrulae to the culturing conditions was very different depending on the acclimation conditions experienced by their mothers. The offspring of mothers conditioned to upwelling ($\sim 1,100 \mu\text{atm } p\text{CO}_2$, $\sim 14^\circ\text{C}$) differentially expressed many more genes than did the offspring of mothers conditioned to non-upwelling ($\sim 400 \mu\text{atm } p\text{CO}_2$, $\sim 17^\circ\text{C}$) conditions when the progeny was raised under the higher versus lower $p\text{CO}_2$ conditions (UH vs. UL compared to NH vs. NL) (Figures 24B, 25).

Additionally, the log-fold change in expression of these differentially expressed genes was generally greater for the offspring of mothers who experienced upwelling conditions (Figure 25). Relatively few differentially expressed genes were shared between the two comparisons (Figure 24B) and their log-fold change in expression was similar (i.e. clustered near the line with a slope equal to 1) (Figure 25). These shared genes (i.e., genes significantly

differentially expressed in both the UH vs. UL and the NH vs. NL comparisons) included 181 that were down-regulated and 58 that were up-regulated. A functional enrichment analysis of the shared down-regulated genes included GO terms related to protein catabolic processes (Table S6). Enrichment analysis of the shared up-regulated genes only found one GO term, which was related to arginine kinase activity.

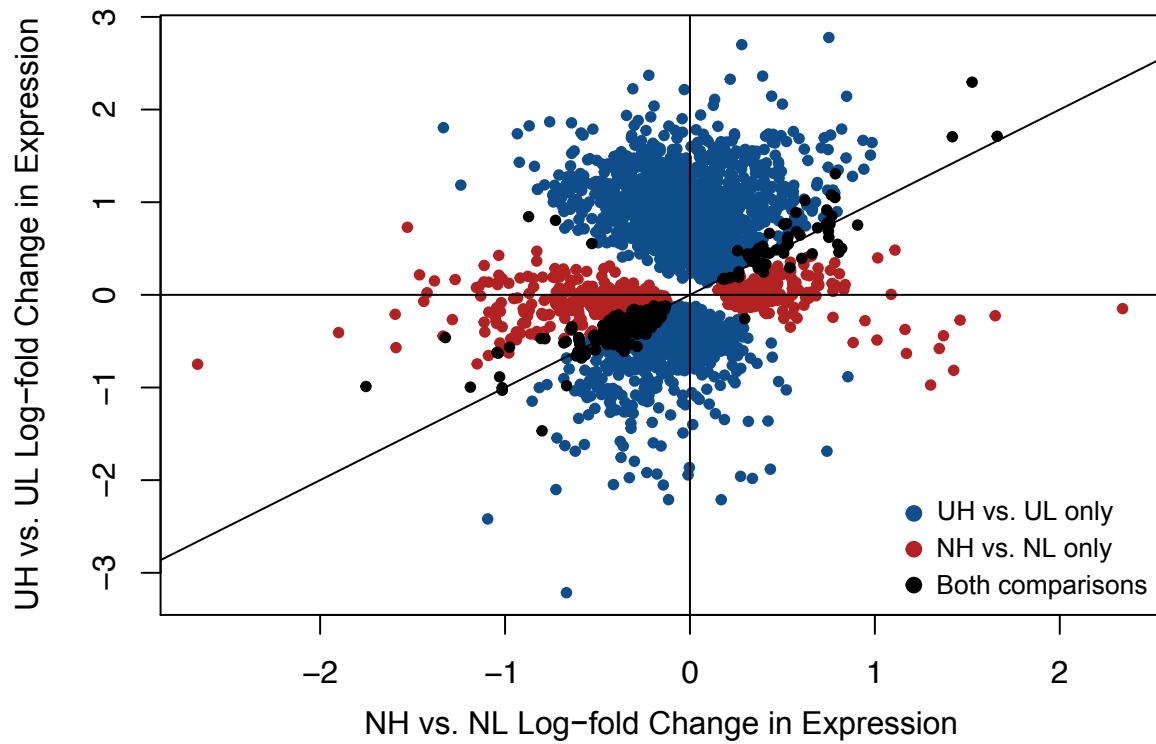


Figure 25. Log-fold change in expression of differentially expressed genes in response to high versus low $p\text{CO}_2$ treatment of the offspring, separated by maternal treatment. A line with slope = 1 is shown.

WGCNA – data summary and module overview

The WGCNA package was used to highlight groups of genes co-expressed within gastrulae of each experimental treatment. This allowed for a broader examination of gene expression patterns across all four treatments, instead of comparing two treatments at a time. WGCNA was used to generate a gene co-expression matrix with modules that represented similarly expressed genes. All 13,967 genes were assigned into 21 modules, designated by color, which merged modules with highly correlated eigengenes (Figure 26A). These 21 modules were then merged using a threshold of 0.26, which corresponded to a height cut of 0.26 and a correlation of 0.74 for merging. Merging resulted in 10 modules. These modules were related to the experiment treatments to generate eigengene networks with assigned correlation values (-1 to 1) (Figure 26B). Of the 10 module eigengenes, “turquoise” (7,831 genes), “green yellow” (243 genes), and “light cyan” (111 genes) did not have any significant correlations (p -value ≤ 0.05) with any of the experiment treatments. Functional enrichment analyses of the module eigengenes, “magenta” (363 genes), “light green” (177 genes), and “grey” (29 genes) did not identify any GO terms. However, the remaining four module eigengenes, “red” (999 genes), “green” (586 genes), “blue” (3,222 genes), and “black” (406 genes) were significantly correlated to at least one treatment, and functional enrichment analyses successfully identified GO terms within each. Analyses of these eigengenes and their significant correlations to each treatment are described below. Complete lists of GO terms and KEGG results associated with each module eigengene are available as supplementary material (Tables S6, S7).

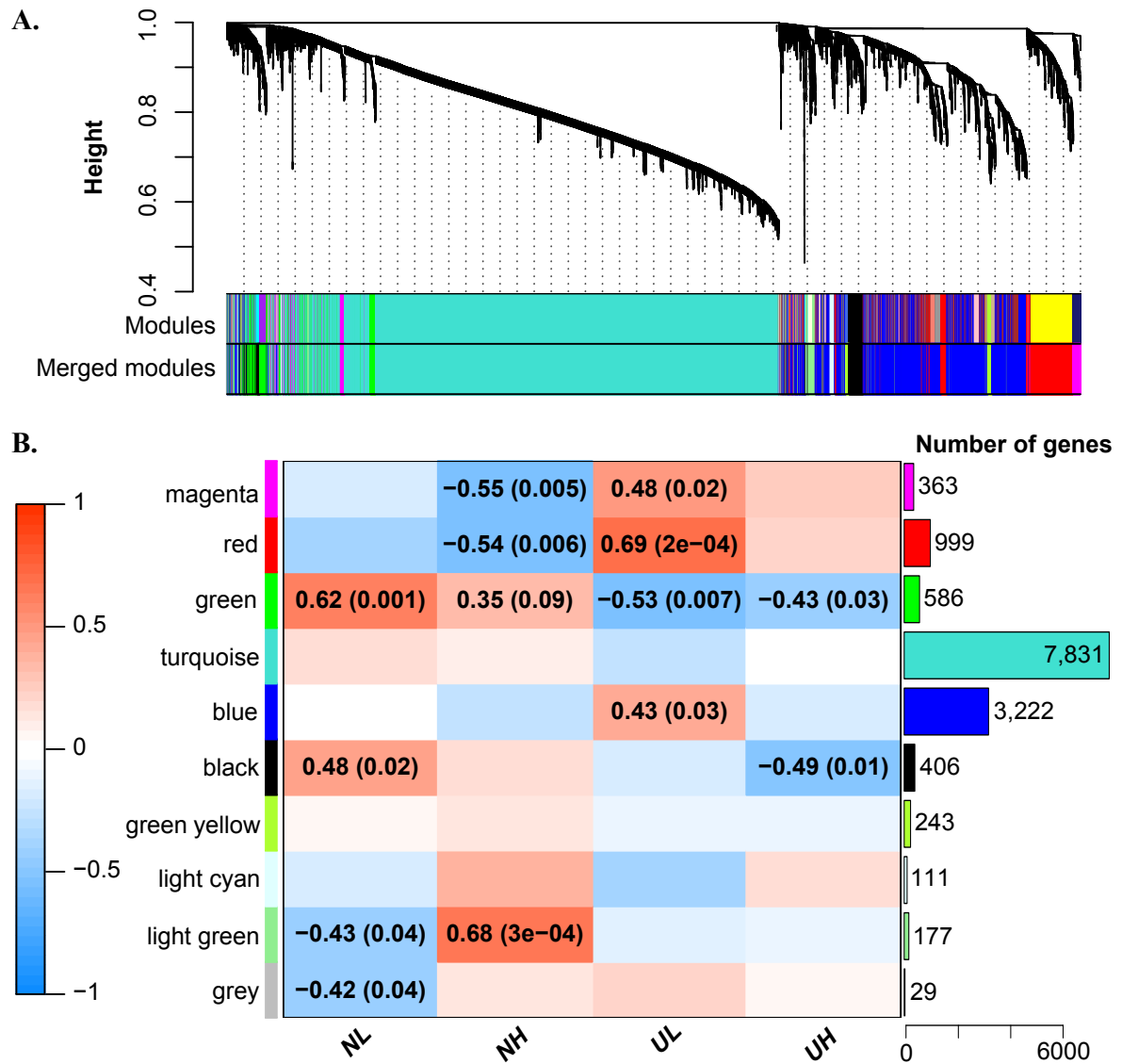


Figure 26. WGCNA, including module generation and their correlation to experiment treatments. **A.** Cluster dendrogram of all 13,967 genes, with dissimilarities based on topological overlap. Modules are assigned below, including modules remaining after merging based on a cut height of 0.26. **B.** Significant relationships between module eigengenes (rows) and treatments (columns) in which the color scale (red-blue) represents the strength of the correlation (1 to -1). Each correlation is represented by a r^2 value and p-value in parenthesis.

Module eigengene, “red”

The module eigengene, “red” had a significant, negative correlation to offspring of mothers that experienced non-upwelling conditions and were raised under high $p\text{CO}_2$ ($r^2_{\text{NH}} = -0.54$, $p\text{-value}_{\text{NH}} = 0.006$), but a significant, positive correlation to offspring of mothers that experienced upwelling conditions and were raised under low $p\text{CO}_2$ ($r^2_{\text{UL}} = 0.69$, $p\text{-value}_{\text{UL}} = 0.0002$) (Figure 26B). GO terms were identified within “red,” which included those related to ion transport and metabolic processes (Tables 12, S6).

Module eigengene, “green”

Module eigengene, “green” had significant correlations to every treatment (Figure 26B). Specifically, “green” had a significant, positive correlation to progeny of non-upwelling mothers raised under low $p\text{CO}_2$ levels ($r^2_{\text{NL}} = 0.62$, $p\text{-value}_{\text{NL}} = 0.001$) and, to a lesser extent, progeny of non-upwelling mothers raised under high $p\text{CO}_2$ levels ($r^2_{\text{NH}} = 0.35$, $p\text{-value}_{\text{NL}} = 0.09$). In contrast, “green” had a significant, negative correlation to both treatments in which the mothers experienced upwelling conditions, regardless of the offspring $p\text{CO}_2$ treatment ($r^2_{\text{UL}} = -0.53$, $p\text{-value}_{\text{UL}} = 0.007$; $r^2_{\text{UH}} = -0.43$, $p\text{-value}_{\text{UH}} = 0.03$). A functional enrichment analysis of “green” identified GO terms that included those related to metabolic processes, gene expression, and methylation (Tables 12, S6).

Module eigengene, “blue”

The module eigengene, “blue” only had a significant, positive correlation to offspring of mothers that experienced upwelling conditions and were raised under low $p\text{CO}_2$ levels ($r^2_{\text{UL}} = 0.43$, $p\text{-value}_{\text{UL}} = 0.03$) (Figure 26B). Within “blue,” GO terms that were identified

included those related to oxidative phosphorylation, ATP metabolic processes, and hydrogen transport (Tables 12, S6).

Module eigengene, “black”

The module eigengene, “black” had a significant, positive correlation to offspring of mothers that experienced non-upwelling conditions and were raised under low $p\text{CO}_2$ levels ($r^2_{\text{NL}} = 0.48$, $p\text{-value}_{\text{NL}} = 0.02$), but a negative correlation to offspring of mothers that experienced upwelling conditions and were raised under high $p\text{CO}_2$ levels ($r^2_{\text{UH}} = -0.49$, $p\text{-value}_{\text{UH}} = 0.01$) (Figure 26B). Within “black,” GO terms were identified that included those related to gene expression regulation and cellular metabolic processes (Tables 12, S6).

Table 12. Select GO term results from functional enrichment analyses of genes within WGCNA module eigengenes.

GO ID	GO Term Name	GO Category	FDR value	No. Transcripts (% of ref)
red: NH ($r^2 = -0.54$), UL ($r^2 = +0.69$)				
GO:0006811	ion transport	Biological Process	5.82E-06	65 (8.0%)
GO:0055085	transmembrane transport	Biological Process	5.82E-06	56 (8.7%)
GO:0006355	regulation of transcription, DNA-templated	Biological Process	0.00386	74 (5.9%)
GO:0008152	metabolic process	Biological Process	0.00696	310 (4.3%)
GO:0005667	transcription factor complex	Cellular Component	0.00386	48 (6.8%)
GO:0015075	ion transmembrane transporter activity	Molecular Function	8.71E-06	50 (9.1%)
GO:0005216	ion channel activity	Molecular Function	0.00945	21 (9.4%)
GO:0009055	electron carrier activity	Molecular Function	0.0218	18 (9.3%)
green: NL ($r^2 = +0.62$), NH ($r^2 = +0.35$), UL ($r^2 = -0.53$), UH ($r^2 = -0.43$)				
GO:0044237	cellular metabolic process	Biological Process	4.73E-08	188 (3.2%)
GO:0010467	gene expression	Biological Process	3.88E-06	90 (3.9%)
GO:0006479	protein methylation	Biological Process	0.0101	8 (13.8%)
GO:0006351	transcription, DNA-templated	Biological Process	0.0102	53 (3.7%)
GO:0032259	methylation	Biological Process	0.0278	10 (8.9%)
GO:0008168	methyltransferase activity	Molecular Function	0.0462	13 (6.6%)
blue: UL ($r^2 = +0.43$)				
GO:0006119	oxidative phosphorylation	Biological Process	3.25E-06	27 (61.4%)
GO:0022900	electron transport chain	Biological Process	1.13E-05	26 (57.8%)
GO:0046034	ATP metabolic process	Biological Process	1.58E-05	49 (33.8%)
GO:0006091	generation of precursor metabolites and energy	Biological Process	4.30E-05	61 (28.6%)
GO:0006818	hydrogen transport	Biological Process	0.00105	26 (40.0%)
GO:0042773	ATP synthesis coupled electron transport	Biological Process	0.00272	16 (55.2%)
GO:0005746	mitochondrial respiratory chain	Cellular Component	0.00641	14 (56.0%)
GO:0008137	NADH dehydrogenase (ubiquinone) activity	Molecular Function	0.0345	10 (58.8%)
GO:0016491	oxidoreductase activity	Molecular Function	0.0462	124 (17.3%)
black: NL ($r^2 = +0.48$), UH ($r^2 = -0.49$)				
GO:0006417	regulation of translation	Biological Process	0.00343	14 (7.1%)
GO:0010608	posttranscriptional regulation of gene expression	Biological Process	0.00343	15 (6.8%)
GO:0010467	gene expression	Biological Process	0.0103	60 (2.5%)
GO:0044237	cellular metabolic process	Biological Process	0.0251	118 (2.0%)
GO:0003723	RNA binding	Molecular Function	0.0173	24 (3.7%)

KEGG analyses

KEGG analyses were performed using enzyme codes affiliated with lists of differentially expressed genes generated from each pairwise comparison of interest. Enzyme codes that matched genes down-regulated in UL compared to NL were used to identify 92 KEGG pathways, while enzyme codes that matched genes up-regulated in UL compared to NL were used to identify 100 KEGG pathways (Table S7). One pathway, identified as inositol phosphate metabolism (spu00562), included the up-regulation of an enzyme related to the production of glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate is used in glycolysis/gluconeogenesis, in which glucose is used to produce ATP. Additionally, 20 enzymes were identified within the oxidative phosphorylation pathway (spu00190), all associated with genes up-regulated in UL compared to NL.

Enzyme codes that matched genes down-regulated in UH compared to NH were used to identify 29 KEGG pathways, while enzyme codes that matched genes up-regulated in UH compared to UL were used to identify 41 KEGG pathways (Table S7). This included the pathway for oxidative phosphorylation (spu00190), in which 17 enzymes related to NADH dehydrogenase complex were up-regulated in UH relative to NH. The NADH dehydrogenase complex transfers electrons from NADH to ubiquinone, helping create the electrochemical potential difference needed to produce ATP.

Enzyme codes that matched genes down-regulated in NH compared to NL were used to identify 52 KEGG pathways, while enzyme codes that matched genes up-regulated in NH compared to NL were used to identify 33 KEGG pathways (Table S7). One such pathway was DNA replication (spu03030), in which eight enzymes related to all three DNA

polymerase complexes were up-regulated in NH relative to NL. Eight enzymes related to RNA polymerase (spu 03020) were also up-regulated in NH compared to NL.

Enzyme codes that matched genes down-regulated in UH compared to UL were used to identify 93 KEGG pathways, while enzyme codes that matched genes up-regulated in UH compared to UL were used to identify 100 KEGG pathways (Table S7). Several of these pathways were related to metabolism, including purine metabolism (spu00230), carbon metabolism (spu01200), pyrimidine metabolism (spu00240), arginine and proline metabolism (spu00330), and fatty acid metabolism (spu01212). All of these pathways contain more up-regulated enzymes than down-regulated enzymes in UH relative to UL.

KEGG analyses were also performed on module eigengenes generated by WGCNA that were significantly correlated with experiment treatments. Genes within the eigengene, “red” (999 genes), which was negatively correlated to NH and positively correlated to UL, were used to identify 77 KEGG pathways (Table S7). This included pathways for oxidative phosphorylation (spu00190), glycolysis / gluconeogenesis (spu00010), fatty acid degradation (spu00071) and inositol phosphate metabolism (spu00562). Genes within the eigengene, “green” (586 genes), which was positively correlated to NL and NH and negatively correlated to UL and UH, were used to identify 55 KEGG pathways. This included pathways for oxidative phosphorylation (spu00190), RNA polymerase (spu03020), and DNA replication (spu03030). Genes within the eigengene, “blue” (3,222 genes), which was positively correlated to UL, were used to identify 106 KEGG pathways. This included pathways for carbon metabolism (spu01200), oxidative phosphorylation (spu00190), glycolysis / gluconeogenesis (spu00010), fatty acid degradation (spu00071), glycerolipid metabolism (spu00561), and citrate cycle (TCA cycle) (spu00020). Lastly, genes within the

eigengene, “black” (406 genes), which was positively correlated with NL and negatively correlated with UH, were used to identify 53 KEGG pathways. This included pathways for glycolysis / gluconeogenesis (spu00010), glycerolipid metabolism (spu00561) and carbon metabolism (spu01200).

Discussion

A growing number of studies have used experimental approaches to demonstrate the role of transgenerational effects in organismal responses to environmental change (Bondurianski and Day 2009; Donelson and Munday 2015; Goncalves et al. 2016; Munday 2014; Parker et al. 2015; Putnam and Gates 2015; Salinas and Munch 2012; Schunter et al. 2018; Thor and Dupont 2015). The study presented here was designed to test for the presence and effects of TGP in *S. purpuratus* by conditioning adults to abiotic conditions in the lab that are ecologically relevant, in this case, combined $p\text{CO}_2$ and temperature conditions typical of non-upwelling ($\sim 400 \mu\text{atm } p\text{CO}_2$, $\sim 17^\circ\text{C}$) and upwelling ($\sim 1,100 \mu\text{atm } p\text{CO}_2$, $\sim 14^\circ\text{C}$) conditions in purple urchin coastal habitat. We then asked how progeny from differently acclimated adults would respond to conditions that embryos would experience in nature, here, a low and a high $p\text{CO}_2$ level ($\sim 400 \mu\text{atm}$ and $\sim 1,100 \mu\text{atm}$) at $\sim 15^\circ\text{C}$. This “common garden” trial approach was designed to explore whether adult experience could alter performance of the offspring, a transgenerational effect. Our study has three salient findings: (1) the history of adult sea urchins in the laboratory affected the gene expression patterns of the progeny, (2) maternal conditions had a greater effect on differential expression (intergenerational effects) than did the response of the gastrulae to developing under high $p\text{CO}_2$ levels while in culture (intragenerational effects), and (3) the results indicated a

“priming” of the embryonic transcriptome to high $p\text{CO}_2$ levels in gastrulae whose mothers experienced upwelling conditions during gametogenesis. While potential prior differences in environmental history between adult urchins cannot be discounted or erased, all urchins used in this study were collected at the same time and from the same location. Therefore, they were likely from the same population, with shared similar environmental histories to one another. As such, the primary difference between the conditions experienced by the adults were the laboratory-controlled exposures imposed on them during this study. Here, we showed that the $p\text{CO}_2$ levels and temperatures that the adults experienced during gametogenesis impacted the phenotype of their offspring, and their transcriptomic response to different $p\text{CO}_2$ levels.

Parental experience possibly played a greater role in the response of their offspring than did the conditions the embryos experienced during development (i.e., inter- versus intragenerational effects). The adults experienced two different experimental conditions, a combination of $p\text{CO}_2$ levels and temperatures, meant to represent conditions observed in nature (Kapsenberg and Hofmann 2016; Rivest et al. 2016). While the experimental design described here cannot separate the impacts of $p\text{CO}_2$ and temperature from one another, the combination of factors elicited an observable transgenerational effect in the offspring. Overall, the gastrula-stage progeny of mothers conditioned to upwelling were larger in size than those whose mothers were conditioned to non-upwelling. Larger offspring may be the result of the lower temperature and/or lower pH level associated with the upwelling treatment. Generally, females that experience colder waters produce larger offspring, which has been observed in a variety of organisms including fish (Feiner et al. 2016), amphipods (Baldanzi et al. 2015), insects (Blanckenhorn 2000; Fischer et al. 2003), and bryozoans

(Burgess and Marshall 2011). In other urchin species, adults that experience decreased pH levels have been shown to produce larger eggs and larvae, although this observation was dependent on the duration of adult exposure (Suckling et al. 2014; Suckling et al. 2015). At the transcriptomic level, an examination of gene expression patterns shows a clear separation of progeny of mothers that experienced non-upwelling conditions from progeny of mothers that experienced upwelling conditions, whereas there was no evident pattern across offspring $p\text{CO}_2$ treatment (Figure 24A). For instance, samples of progeny raised under low $p\text{CO}_2$ levels (UL and NL) did not cluster together and had the greatest number of differentially expressed genes of any pairwise comparison (Figure 24). We acknowledge that the clustering of samples by maternal treatment may be reflective of separate groups of pooled maternal genotypes. While it is not possible to separate maternal genotype effects from treatment effects, the expression patterns and GO terms identified here appear to be indicative of a transcriptomic response to different environmental conditions. These observations are addressed for the remainder of the Discussion.

Maternal influence on epigenetic processes

A notable difference in the transcriptome comparison was the differential expression of genes related to epigenetic processes. Here, genes related to methylation, methyltransferase activity, histone modification, and epigenetic regulation of gene expression were relatively down-regulated in offspring of mothers that experienced upwelling conditions compared to offspring of mothers that experienced non-upwelling conditions (Tables 11, 12). The environmental history of the parents may have impacted certain epigenetic characteristics in their progeny. Epigenetic inheritance is one mechanism by which TGP may

operate, but unfortunately, little is known about epigenetic mechanisms in marine metazoans and how they operate in an environmental change context (Hofmann 2017). Epigenetic modifications can rapidly alter gene expression and may subsist across generations, potentially contributing to long term adaptation in marine systems (Suarez-Ulloa et al. 2015). In this study, gastrulae that exhibited a relative down-regulation of genes related to epigenetic processes also showed a relative down-regulation of genes related to transcription, regulation of transcription, transcription factor activity, and translation factor activity. However, there is much to be understood regarding how epigenetic modifications regulate gene expression in marine metazoans, particularly in echinoderms (Hofmann 2017). It has been proposed that limiting DNA methylation can contribute to an increase in phenotypic plasticity under highly variable conditions by effectively increasing transcriptional opportunities of genes involved in the response to changing environmental conditions (Roberts and Gavery 2012). As such, the reduction of DNA methylation in the progeny may allow gastrulae to be primed and ready to respond to more extreme or variable environmental conditions. Future epigenetic analyses will continue to help elucidate the transcriptomic patterns observed here.

Maternal influence on metabolic processes

The progeny of the two groups of differentially conditioned mothers also differed in their expression of genes related to metabolic processes, which were relatively down-regulated in the progeny of the upwelling-conditioned mothers (Tables 11,12). It is possible that the colder temperature experienced by the upwelling-conditioned urchins during gametogenesis led to a reduced metabolism in their offspring. However, all the embryos were

raised at the same temperature, so the temperature under which development occurred would not be expected to directly affect metabolic processes. In other studies, metabolic depression has been an observed physiological response in marine invertebrates exposed to elevated $p\text{CO}_2$ (Carter et al. 2013; Evans and Watson-Wynn 2014; Nakamura et al. 2011; Todgham and Hofmann 2009). Thus, the findings reported in this study are in-line with what might be expected in organisms raised under high $p\text{CO}_2$ levels or upwelled waters (i.e., combined high $p\text{CO}_2$ and low temperature). The primary departure here is that these characteristics were not observed in offspring raised under high $p\text{CO}_2$ conditions at $\sim 15^\circ\text{C}$ if their mothers were exposed to non-upwelling conditions ($\sim 400 \mu\text{atm } p\text{CO}_2$, $\sim 17^\circ\text{C}$) (i.e., NH), but were instead found in progeny if their mothers were exposed to upwelling conditions ($\sim 1,100 \mu\text{atm } p\text{CO}_2$, $\sim 14^\circ\text{C}$) during gametogenesis, and remained regardless if they were raised under low or high $p\text{CO}_2$ conditions at $\sim 15^\circ\text{C}$ (i.e., UL and UH). Therefore, these characteristics of the embryonic transcriptome may tentatively be attributed to maternal effects from adult urchins that have experienced upwelling conditions, rather than an immediate response of the progeny to their culturing environment. In the future, metabolic rate can be measured to determine if a difference is detectable between embryos at the physiological level.

TGP in the response to high $p\text{CO}_2$

Regardless of parental conditioning, all progeny appeared to down-regulate genes related to protein homeostasis processes as a response to elevated $p\text{CO}_2$ levels, including genes associated with protein catabolic processes, proteolysis, peptidase complex and endopeptidase activator activity (Tables 11, S6). Under elevated $p\text{CO}_2$ levels, *S. purpuratus* has been shown to increase rates of protein synthesis while decreasing protein depositional

efficiency (Pan et al. 2015). The progeny raised under high $p\text{CO}_2$ levels may be down-regulating genes associated to protein breakdown because an increase in protein turnover is likely energetically costly. Despite these similarities, the transcriptomic response of the progeny of upwelling mothers to different $p\text{CO}_2$ levels (i.e., UH versus UL) was largely distinct from the response of the progeny of non-upwelling mothers (i.e., NH versus NL) (Figure 25). Notably, the transcriptomic response to high $p\text{CO}_2$ conditions was comparatively minimal in offspring whose mothers experienced non-upwelling conditions during gametogenesis, both in terms of the number of genes that were differentially expressed as well as their log-fold change in expression.

It may be expected that offspring would actively regulate ion transport and homeostasis in response to high $p\text{CO}_2$ conditions, as urchin larvae have been shown to transport ions across cell membranes to regulate their internal pH (Stumpp et al. 2012). Prior transcriptomic studies have reported an over-representation of genes related to ion transport in urchins exposed to experimentally acidified conditions (Evans and Watson-Wynn 2014). However, this was not observed here as only progeny of upwelling-conditioned mothers raised under low $p\text{CO}_2$ (UL) exhibited these expression patterns (Table 12). The progeny of upwelling-conditioned mothers raised under low $p\text{CO}_2$ levels (i.e., UL) exhibited a relative up-regulation of genes related to ion regulation, including those related to ion binding, ion transport, ion homeostasis, ion transmembrane transporter activity, ion channel activity, and hydrogen transport. Interestingly, ion regulatory genes were up-regulated in UL relative to UH, and no such genes were differentially expressed between the progeny of non-upwelling mothers (i.e., NH versus NL). Therefore, it appears parental conditioning had an impact on the ion regulatory response of their offspring.

Additionally, UL gastrulae exhibited a relative up-regulation of genes related to oxidative phosphorylation, ATP synthesis coupled electron transport, ATP binding and ATP metabolic processes (Tables 11, 12). Furthermore, KEGG analyses of the UL vs. NL differential expression comparison included an up-regulation of enzymes used to produce important compounds used to produce energy. Pan et al. (2015) found that, in *S. purpuratus* raised under elevated $p\text{CO}_2$ conditions, a higher level of ATP was allocated towards ion regulation. The UL gastrulae may be focusing more transcriptomic regulation towards increasing ATP production to provide more energy towards ion regulation and acid-base homeostasis as a means of coping with a change in pH conditions (Pan et al. 2015). Evans et al. (2017) found that *S. purpuratus* from populations that frequently encounter low pH seawaters expressed greater levels of genes related to ATP production than urchins that encounter low pH less frequently. Therefore, females conditioned to high $p\text{CO}_2$ levels associated with the upwelling treatment may have “primed” their offspring to experience similar $p\text{CO}_2$ levels. However, this was not observed in the upwelling-conditioned progeny raised under high $p\text{CO}_2$ levels (UH). It is possible that UH gastrulae were already suitably prepared for high $p\text{CO}_2$ levels and did not need to actively regulate ions to reach homeostasis. We propose the expression patterns observed in UL are due to maternal conditioning in preparation for upwelling-like conditions, acting in combination with the sudden change to low $p\text{CO}_2$ conditions during early development, forcing the embryos to adjust to conditions different to what their mothers experienced.

It has been suggested that existing resistance to low pH conditions in some *S. purpuratus* populations is a result of exposure to upwelling and the resulting natural pH variation over long timescales, allowing for selection and possible local adaptation that

favors tolerance to low or fluctuating pH levels (Evans et al. 2017; Kapsenberg et al. 2017; Kelly et al. 2013; Padilla-Gamiño et al. 2013; Pespeni et al. 2013a; Pespeni et al. 2013b). Due to the natural mosaic of pH variability found in the CCS (Chan et al. 2017; Feely et al. 2008; Gaitán-Espitia et al. 2014; Hauri et al. 2009; Kroeker et al. 2016), some populations may be more tolerant than others (Evans et al. 2017; Kelly et al. 2013; Pespeni et al. 2013a; Pespeni et al. 2013b). For instance, Kelly et al. (2013) found lower sensitivity to low pH experimental treatments in offspring of sires from a site characterized by stronger, more frequent upwelling. Evans et al. (2017) reported that populations exposed more frequently to low pH conditions in nature responded differentially to low pH experimental treatments, particularly in expression patterns of genes related to ATP production. While local adaptation acts over long timescales and across multiple generations, here, we demonstrate that differences in transcriptomic responses to high $p\text{CO}_2$ levels can occur over relatively short timescales; in this case, between parents and their offspring. Furthermore, although it can be difficult to tease apart the effects of local adaptation from transgenerational mechanisms, the transcriptomic differences observed during this study were between the progeny of adults from the same population, collected at the same time, and with presumably similar environmental histories prior to the start of experimental conditioning. We acknowledge that the static treatments the adults experienced during this study do not reflect the observed variability that occurs in nature (Feely et al. 2008; Gaitán-Espitia et al. 2014; Hauri et al. 2009; Kroeker et al. 2016) and recommend that fluctuating experiment treatments consistent with natural variability be incorporated in future studies. Nevertheless, this study provides evidence that parental experience during gametogenesis can impact the F1 generation via

TGP and suggests that urchin populations can adjust to changing ocean conditions faster than adaptation can functionally occur.

While most studies exploring TGP in marine organisms have examined transgenerational effects at the physiological level (Ross et al. 2016), very few have explored the molecular basis of these effects (De Wit et al. 2016; Goncalves et al. 2016; Schunter et al. 2018; Schunter et al. 2016; Veilleux et al. 2015). This study explored how TGP impacts gene expression in echinoderms and examined molecular-level transgenerational effects in marine organisms during their early stages of development. We examined the role of TGP in the response of urchin larvae to an environmental stressor and found that parental experience greatly altered progeny gene expression patterns. When the offspring of mothers that experienced upwelling conditions ($\sim 1,100 \mu\text{atm } p\text{CO}_2$, $\sim 14 \text{ }^\circ\text{C}$) were raised under two different $p\text{CO}_2$ levels at a common temperature ($\sim 400 \mu\text{atm}$ or $\sim 1,100 \mu\text{atm}$ at $\sim 15 \text{ }^\circ\text{C}$), they exhibited expression patterns that might be indicative of the combined $p\text{CO}_2$ and temperature conditions the parental generation experienced, including a decrease in epigenetic and metabolic processes. This expression pattern persisted regardless of which $p\text{CO}_2$ condition the progeny was raised under (i.e., UL and UH). Adult conditioning impacted how the progeny responded to high $p\text{CO}_2$ levels, affecting ATP production, possibly for use in ion transport and ion homeostasis. These results might be indicative of mothers effectively “priming” their offspring to respond to similar conditions that they themselves experienced during gametogenesis. This study highlights the importance of transgenerational effects in the response of *S. purpuratus* EDSs to abiotic stress, and provides support that in nature, TGP may be an important mechanism by which this species copes with rapid changes in the

environment. A better understanding of these processes will facilitate predictions regarding how *S. purpuratus* populations will persist under future ocean conditions.

Data accessibility

Raw sequencing reads are available at NCBI Bioproject PRJNA378799 and Sequence Read Archive accession SRP128972. Analysis scripts and comparative transcriptomic data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.k214j>.

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Supplementary material

Table S6 GO term results from functional enrichment analyses of differentially expressed genes from each pairwise comparison of interest and in each WGCNA module eigengene.

Table S7 KEGG pathways for differentially expressed genes and genes within module eigengenes.

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