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Parentage influence on gene expression under acidification revealed through single-embryo sequencing

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Abstract

The dissolution of anthropogenic carbon dioxide (CO_2) in seawater has altered its carbonate chemistry in the process of ocean acidification (OA). OA affects the viability of marine species. In particular, calcifying organisms and their early planktonic larval stages are considered vulnerable. These organisms often utilize energy reserves for metabolism rather than growth and calcification as supported by bulk RNA-sequencing (RNA-seq) experiments. Yet, transcriptomic profiling of a bulk sample reflects the average gene expression of the population, neglecting the variations between individuals, which forms the basis for natural selection. Here, we used single-embryo RNA-seq on larval sea urchin *Heliocidaris crassispina*, which is a commercially and ecologically valuable species in East Asia, to document gene expression changes to OA at an individual and family level. Three paternal half-sibs groups were fertilized and exposed to 3 pH conditions (ambient pH 8.0, 7.7 and 7.4) for 12 h prior to sequencing and oxygen consumption assay. The resulting transcriptomic profile of all embryos can be distinguished into four clusters, with differences in gene expressions that govern biomineralization, cell differentiation and patterning, as well as metabolism. While these responses were influenced by pH conditions, the male identities also had an effect. Specifically, a regression model and goodness of fit tests indicated a significant interaction between sire and pH on the probability of embryo membership in different clusters of gene expression.

Cheuk Wang Fung and Kin Yung Chau are equally contribution.

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The single-embryo RNA-seq approach is promising in climate stressor research because not only does it highlight potential impacts before phenotypic changes were observed, but it also highlights variations between individuals and lineages, thus enabling a better determination of evolutionary potential.

KEYWORDS
climate change, development, echinoderm, gene expression, pluteus, RNA-sequencing, sea urchin, single-embryo

1 | INTRODUCTION

Ocean acidification (OA), the change in seawater carbonate chemistry due to increased uptake of anthropogenic carbon dioxide (CO_2), imposes significant stress on marine species (Doney et al., 2020; Gattuso et al., 2015; IPCC, 2014). This change alone has resulted in multiple interacting stressors, including acidosis, hypercapnia and reduced saturation of calcium carbonate (Albright et al., 2016; Gattuso et al., 2015; IPCC, 2014). Calcifying organisms, such as coccolithophores, corals and echinoderms, are deemed more sensitive and exhibit negative outcomes when exposed to OA conditions (Leung et al., 2022; Siegel et al., 2022). These detrimental impacts include the elevated metabolic cost to maintain physiological processes (e.g. acid–base homeostasis) (Liu et al., 2020; Pan et al., 2015) and the subsequent reduction in growth and calcification (Byrne & Hernández, 2020; Melzner et al., 2020).

Various transcriptomic studies have been set to identify the changes in gene expression patterns that underline the phenotypic changes associated with OA conditions (Strader et al., 2020). With several fully sequenced and annotated sea urchin genomes reported (Sodergren, Shen, et al., 2006; Sodergren, Weinstock, et al., 2006; Tu et al., 2012) and their role as ecosystem engineers (Grande et al., 2020; Jones et al., 1997; Rogers-Bennett & Catton, 2019), sea urchins have been the target of such sequencing studies (Strader et al., 2020). Broadly speaking, the responses observed include elevated expression of metabolic genes related to ATP production and the tricarboxylic acid cycle (Evans et al., 2017; Stumpp et al., 2011; Todgham & Hofmann, 2009), stress proteins (Hsp70) and immune response (O'Donnell et al., 2009; Wong & Hofmann, 2021; Zhan et al., 2020); and, a downregulation in genes associated with skeletogenesis and calcification (Devens et al., 2020; Martin et al., 2011; Padilla-Gamiño et al., 2013; Runcie et al., 2016). These gene expression patterns are consistent with whole organism responses observed—reduced digestion rate (Lee et al., 2019; Stumpp et al., 2013), elevated oxygen consumption rate (Lo et al., 2021; Stumpp et al., 2011) and reduced growth and development (Byrne et al., 2013; Dorey et al., 2013; Foo et al., 2020).

Rapid evolutionary responses to near-future acidification have been reported for multiple marine invertebrates, including sea urchins (Bitter et al., 2019; Kang et al., 2022; Pespeni et al., 2013). Standing genetic variations both between populations and within a single population could enhance an organism's capacity to persist in future, rapid environmental change through such evolutionary responses (Bell, 2017; Sunday et al., 2014). One way to assess evolutionary potential is to estimate the broad sense heritability (h^2) of specific traits through parent–offspring comparison (Jury et al., 2019; Sunday et al., 2011). Alternatively, controlled breeding designs together with a mixed linear model can be used to estimate dam and sire contribution towards gene expression patterns (Devens et al., 2020; Martin et al., 2011; Padilla-Gamiño et al., 2013; Runcie et al., 2016). Here, we present single-embryo level sequencing, as an alternative to bulk RNA-seq methods that pool thousands of individuals, to quantify diversity in genetic response to environmental stress. Unlike typical single-cell RNA-seq in which the individual transcriptomes of captured cells are sequenced, single-embryo level sequencing enables gene expression profiling of each embryo's global response to OA and makes possible analysis on paternal-specific, maternal-specific, or individual variations, thus providing an alternative to estimate evolutionary potential.

The urchin *Heliocidaris crassispina* inhabits the low-intertidal and subtidal areas along the coastal waters of the western Pacific from southern Japan to southern China (Agatsuma, 2013; Freeman, 2003). The species is commercially cultured and harvested (Ding et al., 2007), and it is a key grazer (Wai & Williams, 2006). Urchins in our study site are exposed to variable coastal pH conditions (Maboloc & Chan, 2021; Pecquet et al., 2017). Consistent with the prediction of the climatic variability hypothesis that organisms exposed to environmental fluctuations process larger plasticity, that is, ability to withstand stress (Gaitán-Espitia et al., 2017; Vargas et al., 2022). Dorey et al. (2018) showed that larval *H. crassispina* survived 3-day exposure to low pH (pH 7.7) and copper addition (≤0.19 μM), but experienced a reduction in growth and elevated frequencies of abnormality. Given the phenotypic resilience, we hypothesize that there is a reservoir of genetic variation in the local *H. crassispina* that can respond to acidification during larval development and buffer some of the negative consequences associated with pH changes.

To test the above-mentioned hypothesis, we reared embryos of *H. crassispina* under three pH conditions (ambient pH 8.0, 7.7 and 7.4). Twelve hours post-fertilization, the oxygen consumption rate was measured as a proxy of metabolic rate and single-embryo RNA-seq was performed. We revealed the transcriptional states of urchin embryos shifted with acidification, but such changes were not mirrored by the physiological measurements taken at the same time. Under acidification, genes essential to embryogenesis were downregulated, while expressions related to metabolism were upregulated. More importantly, paternal lineage likely plays a role in influencing

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offspring's resilience to acidification—when reared at pH 7.7, one of the three parental lineages had a significantly different proportion of descendants with the expression profile associated with enrichment of metabolic genes than the others did. These results provide novel insights into individual-level responses of urchins to future ocean conditions. They also illustrate single-embryo RNA-seq as a viable means to study standing genetic variations, and hence, the evolutionary potential of marine organisms in the face of climate change.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Adult *Heliocidaris crassispina* were collected by snorkelling along the shoreline of HKUST, Clear Water Bay, Hong Kong SAR, China (22.39°N, 114.11°E, Figure 1b). After collection, the sea urchins were transported immediately to the adjacent coastal marine laboratory for gamete extraction. Adult urchins were held in a sea table with running seawater (~33–35 psu, pH 8.01).

2.2 | Seawater chemistry manipulation

The experimental pH levels were chosen to represent the present-day open ocean condition (pH 8.0), the predicted average open ocean pH at 2100 (pH 7.7) and an extremely low pH (pH 7.4), which encompass the pH values *H. crassispina* has experienced at the collection location (Maboloc & Chan, 2021; Pecquet et al., 2017). Seawater pH was altered through CO_2 addition through a mass flow controller. The temperature, voltage and pH_{NBS} were measured with a Metrohm 913 pH meter and unitrode with Pt 1000 (Herisau). Salinity was measured with a handheld refractometer. The measured pH was converted to total scale pH (pH_T) with the use of Tris/HCl buffer solution (salinity = 33) provided by the Dickson Lab at the Scripps Institution of Oceanography (San Diego). Total alkalinity (TA) was measured through Gran titration (905 Titrando, Metrohm). Calibration was performed using the standard seawater provided by the Dickson Lab (Batch 151). The resulting total scale pHs are 7.37, 7.71 and 8.02 respectively. The average TA between duplicates were 2039.4, 2048.5 and 2036.2 μmol kg^{-1} respectively. Using the R package seacarb (Gattuso et al., 2021), the partial carbon dioxide pressure in these treatments was computed as 2067, 901 and 399 μatm.

2.3 | Fertilization and embryo respirometry

Gametes from one female and three males were extracted to generate three groups of paternal half-sibs. A single female was used to avoid the confounding effect of differences in egg quality and focus the investigation on the genetic effect (Lynch & Walsh, 1998). Adults were induced to spawn through the injection of 0.35 M KCl. Eggs were rinsed with filtered seawater (0.45 μm) (FSW) at the three experimental pHs and sperm were collected dry. Eggs were fertilized with sperm at a concentration of approximately 10^4 sperm mL^{-1}, in their respective pH treatments. Fertilization success (>95%) was confirmed by the presence of a fertilization envelope 5 min post-fertilization. The fertilized embryos were incubated in sealed containers at the respective pHs in a growth chamber (Jeio Tech) held at 23°C (Figure 1a). Three replicate containers were used for each male and pH combinations ($N = 27$).

To investigate how gene expression pattern aligns with organismal performance, the oxygen consumption rate of the embryo was also quantified 12 h post-fertilization following the methods outlined in Lo et al. (2021). Five individuals were randomly chosen and introduced to a 200 μL well on a respiration plate and each well was considered a replicate. Four replicates were used for each paternal lineage at each pH. Procedural control that contains only FSW was included to account for bacterial respiration. Fluorescence microplate readers (SDR SensorDish, Loligo PreSens) were used to detect the oxygen concentration in each well for 18 h.

2.4 | Single-embryo RNA sequencing and data preprocessing

Twelve hours post-fertilization, embryos, independent from those used in the oxygen consumption measurement, were individually pipetted into a 96-well plate with lysis mix containing 1 U RNase inhibitor (RNaseOUT Recombinant Ribonuclease Inhibitor; Invitrogen), 0.1% (vol/vol) Triton X-100 solution (Sigma-Aldrich), 2.5 mM each dNTP mix (Thermo Scientific) and 2.5 μM oligo-dT_{30}VN (Integrated DNA Technologies). Samples were then prepared for sequencing library construction using the Smart-seq2 protocol (Picelli et al., 2014). In brief, reverse transcription (RT), followed by PCR was performed on lysate to synthesize cDNA. Qualities and quantities of cDNA libraries were inspected using Qubit 3.0 and Fragment Analyzer (Agilent). Libraries were then tagmented with Nextera XT DNA sample preparation kit (Illumina). After tagmentation, library PCR amplification was done to amplify sequencing libraries with Illumina index primers. Finally, samples were purified using AMPure XP beads and pooled together for sequencing in Illumina NextSeq 500 platform. The result FASTQ files were preprocessed for trimming and ribosomal RNA removal. Trimming was done using fastp (Chen et al., 2018). The order of sequence in read1 and read2 was remapped using Bbmap (Bushnell, 2022). RNAmmer (Lagesen et al., 2007) was employed to predict ribosomal RNA, followed by removal using BBDuk (Bushnell, 2022).

2.5 | RNA-seq analysis

The preprocessed FASTQ data were aligned to the *H. crassispina* genome assembled in-house using STAR (Dobin et al., 2013). Transcript quantification was performed using RSEM (Li & Dewey, 2011). Sequencing data were analysed in R (R Core Team, 2022). Data processing was done with the Seurat package (Butler et al., 2018;

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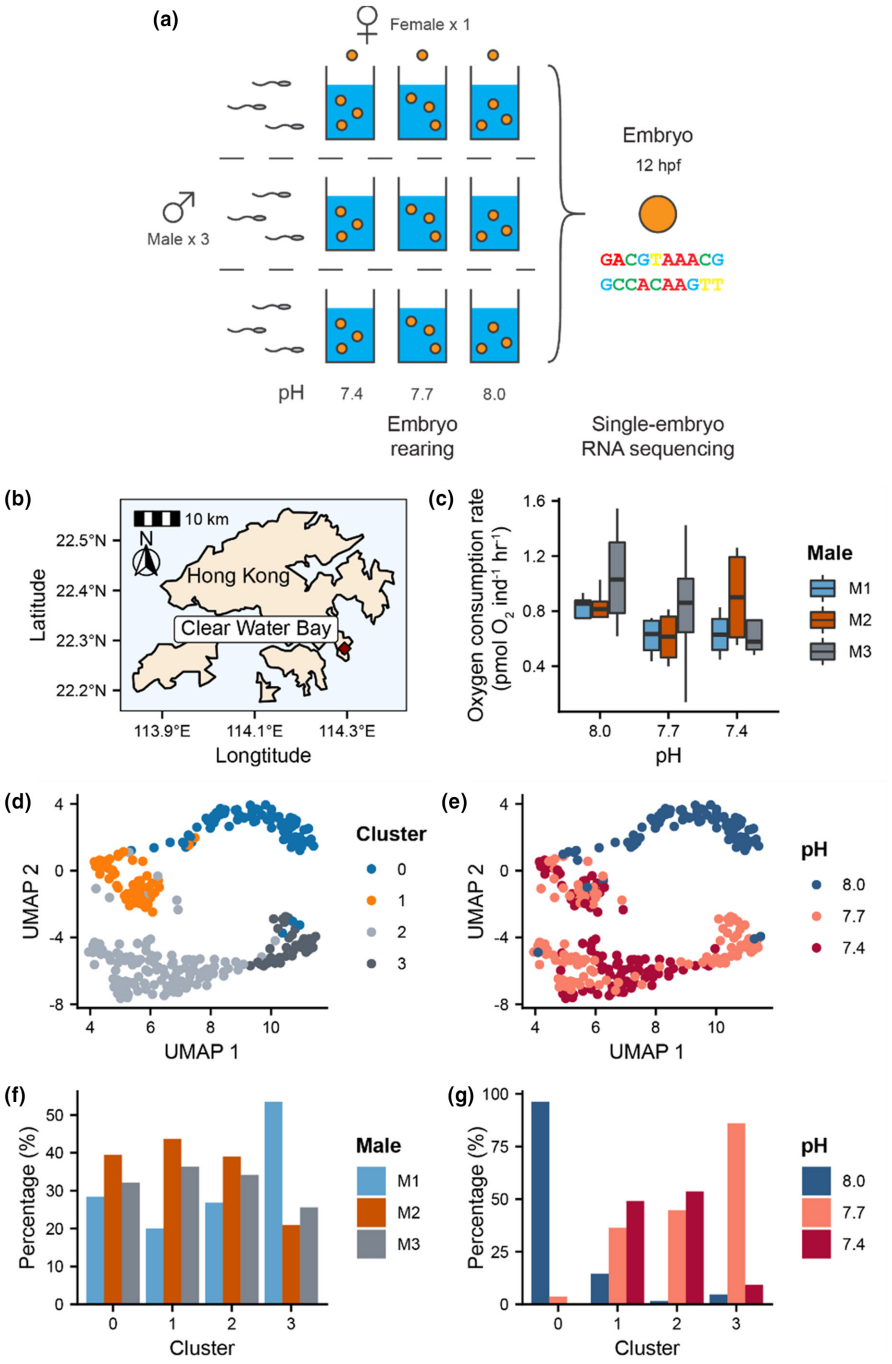

FIGURE 1 (a) Experimental schematic of single-embryo RNA-seq. (b) Collection site of *Heliocidaris crassispina* in Clear Water Bay, Hong Kong S.A.R. (c) Oxygen consumption rate of the urchin embryos. (d–e) Visualization of *H. crassispina* embryos transcriptome with UMAP after dataset integration using FIRM. Each dot represents an individual embryo. (d) Labelled by cluster from unsupervised clustering; (e) Labelled by pH of seawater in which the embryos were exposed. (f, g) Contribution of embryos in different clusters. (d) From males; (e) From different pH environments.

Hao et al., 2021; Satija et al., 2015; Stuart et al., 2019). To eliminate batch effects, FIRM integration was performed by stratifying datasets by Embryos with raw counts higher than 400,000 and a total number of genes lower than 200 or higher than 3000 were discarded. Genes expressed in fewer than three embryos were also removed. The final expression matrix contains 302 embryos and 20,076 genes (90 embryos for pH 8.0, 115 embryos for pH 7.7 and 97 embryos for pH 7.4). After log normalization, FIRM integration (Ming et al., 2022) with male stratification was applied to obtain the scaling matrix and eliminate the batch effect in later visualization.

Our goals are similar to single-cell transcriptomic profiling experiments, which are to identify groups of individuals that form subpopulations of interest, while also highlighting the heterogeneities and trends within and between subpopulations. Hence, we deployed an analysis pipeline similar to previous single-cell RNA-sequencing atlas and publications (Almanzar et al., 2020; Jones et al., 2022; Travaglini et al., 2020; Yu et al., 2023). To visualize our dataset, we applied Uniform Manifold Approximation and Projection (UMAP), a non-linear, non-deterministic dimensionality reduction method that captures the global data structure and proximity of data points to each other, presenting the best 2D approximation of that connectivity between data points (McInnes et al., 2018). Separately, we use a shared nearest neighbour-based clustering algorithm to group individuals with similar gene expression

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patterns into clusters. Specifically, we used the R package Seurat, functions FindNeighbors() and FindClusters(), which uses the Louvain method of clustering (Butler et al., 2018; Hao et al., 2021; Satija et al., 2015; Stuart et al., 2019). Differential gene expression analysis was performed using MAST (Finak et al., 2015). Gene orthologues between *S. purpuratus* and *H. crassispina* were mapped using reciprocal blast best hit of protein sequence by BLASTp (Altschul et al., 1990).

2.6 | Gene ontology enrichment analysis

Gene ontology terms were enriched with gene list overrepresentation enrichment analysis in PANTHER (Mi et al., 2017). After differential gene expression analysis, genes with adjusted *p*-value <.01 and average log fold-change >0 were used to enrich GO terms in GO-Slim biological process annotation set using *S. purpuratus* whole genome as the reference. GO terms with FDR < 0.05 were reported in this paper. Only terms with fold enrichment >10 (i.e. $\log_{10}$(fold enrichment) > 1) in at least one cluster were shown in the heatmap visualization.

2.7 | Statistical analysis

The respiration rates of embryos were computed by applying a linear regression model of measured oxygen concentrations against time after Lo et al. (2021). The relationship between embryo cluster classification and the interactive effect of male and pH was interpreted using multinomial logistic regression in R with the package nnet (Venables & Ripley, 2002). A follow-up likelihood ratio test was performed between the two regression models: a full model with the main and interaction effect of male and pH, and a reduced model with only the main effect, under the null hypothesis that the cluster data fits equally well in both, meaning the interactive effect is not significant in the gene expression diversity (cluster classification) of the embryos.

3 | RESULTS

3.1 | Single-embryo RNA-sequencing, but not respiratory, reveals OA impact

The average individual oxygen consumption rate of *Heliocidaris crassispina* embryos was affected neither by pH, male identities nor their interactions (Figure 1c, $F \leq 1.38$, $p \geq .27$). In contrast, single-embryo sequencing and analysis utilizing the transcriptome assembly of *H. crassispina* showed both pH and male-dependent patterns. Gene expression data were filtered to remove poor-quality embryos which resulted in 302 embryos for analysis. Based on the transcriptional profile of each embryo, unsupervised clustering after dimension reduction categorized embryos at similar transcriptional states into four clusters (Figure 1d).

As expected, embryos exposed to different pHs had different transcriptomic profiles (Figure 1e). The distribution of embryos from the males in each cluster was similar between male two (M2) and male three (M3). Male one (M1) had a higher percentage of embryos in cluster 3, up to 32.6% more than the other males (Figure 1f, see section on parental effect). Meanwhile, 96.3% of embryos in cluster 0 were from ambient pH 8.0. As pH decreased, more embryos were clustered into clusters 1 (pH 7.7: 36.4%, pH 7.4: 49.1%), 2 (pH 7.7: 44.7%, pH 7.4: 53.7%), and 3 with embryos reared at pH 7.7 contributed more significantly to cluster 3 (pH 7.7: 86.0%, pH 7.4: 9.3%) (Figure 1g).

Gene ontology (GO) enrichment analysis of the differentially expressed genes pointed to specific biological processes for each cluster (Figure 2; Tables S1 and S2). Notably of GO terms with fold enrichment higher than 10 [i.e. log(fold enrichment) > 1], embryos from cluster 0 expressed genes enriching terms related to cilia movement (Figure 2; Table S1), and the genes corresponding to this term included *Tekt1/2/3* and *Cfap20* (Figure S1a–d). While these genes were upregulated in the pH 8.0 embryos which exclusively belong to cluster 0, moderate expression levels could still be observed in other clusters of embryos from pH 7.7 and 7.4 (Figure S1a–d).

Gene expression of embryos from pH 7.7 and 7.4 constituting cluster 1 had enriched terms related to both endoplasmic reticulum tubular network organization and MAP kinase activity (Figure 2; Table S1). Meanwhile, cluster 2 which had an equal mix of embryos from the pH 7.7 and 7.4 treatment had a lower level of expression of MAP kinase-related genes (Figure S1e–k).

While the differentially expressed genes from clusters 2 and 3 both resulted in GO terms related to respiration and ATP synthesis (Figure 2; Table S1), further focused differential expression analysis segregated the two clusters (Table S3). Differentially expressed genes from cluster 2 were enriched for cell cycle-related terms (Table S3), specifically G1/S phase transition and cellular response to TGF-β stimulus. In contrast, cluster 3 continued to show enriched terms related to ATP synthesis and mitochondrial electron transport (Figure 3a,c; Table S3). Further investigation on the expression level of corresponding genes revealed that the SMAD family genes, including *Smad1/2/4/6*, were upregulated in cluster 2, with an average $\log_2$ fold-change ($\log_2$FC) in gene expression of 0.52, 0.46, 0.36 and 0.63 respectively (Figures 3b and S2a–d; Table S3). As the members of TGF-β superfamily, these genes play a pivotal role in cell proliferation, differentiation and migration (Lapraz et al., 2006). Additionally, G1/S phase regulatory genes *CycD*, *Psme3* and *Rbl1* were also upregulated, with average $\log_2$FC of 0.75, 0.79 and 0.25 respectively (Figures 3b and S2e–g; Table S3). On the other hand, expressions of metabolic genes *Cox2*, *Cox5a* and *Cox6a1* were the highest in cluster 3 among all other clusters, with respective average $\log_2$FC of 0.94, 0.80 and 1.26 with respect to cluster 2 (Figures 3b and S2h–j; Table S3).

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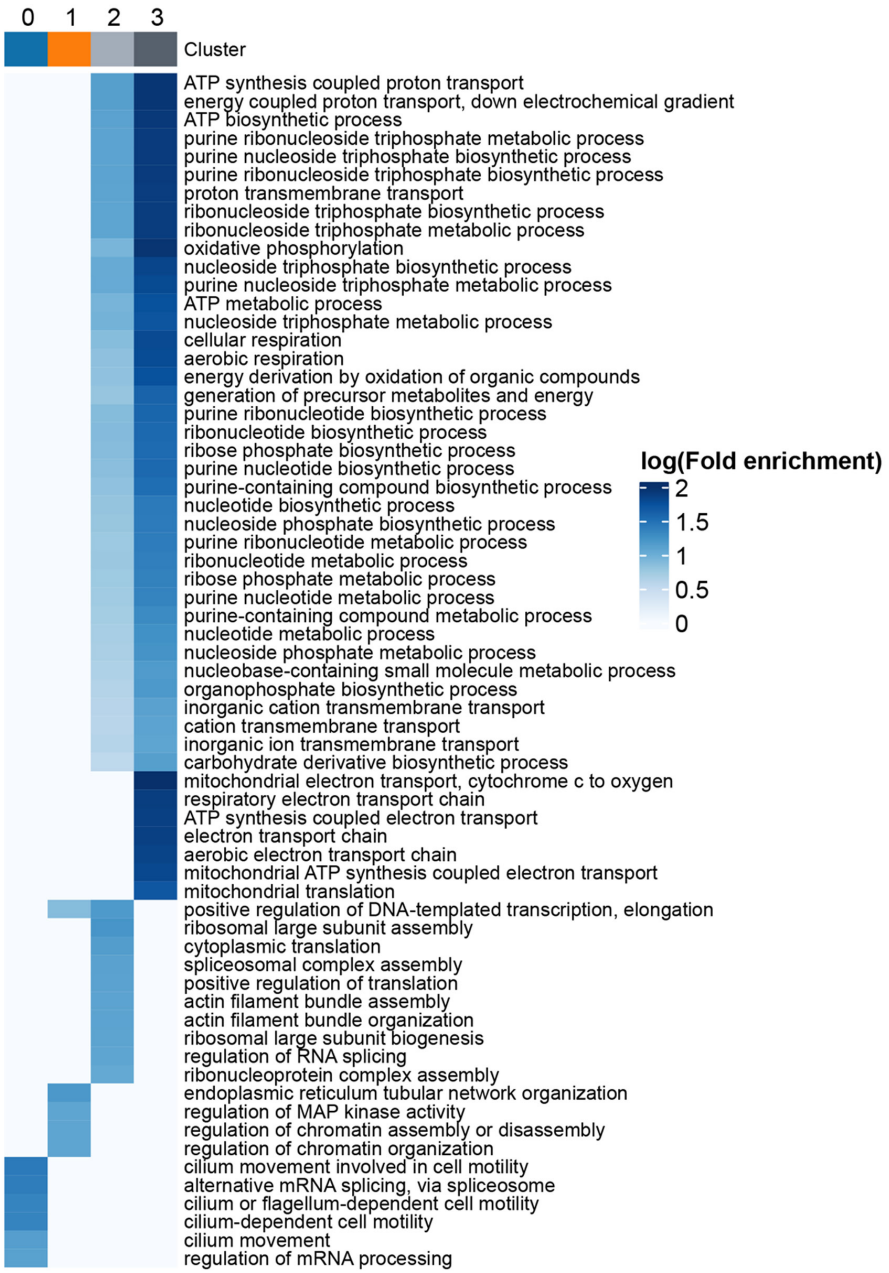

FIGURE 2 Logarithmic fold enrichment of GO terms after gene list overrepresentation enrichment analysis with genes differentially expressed in the four clusters (Table S2, FDR < 0.05). Higher fold enrichment of a term indicates the embryos in that cluster were more involved in the process. Only showing terms with fold enrichment higher than 10 [i.e. log(fold enrichment) > 1] in at least one cluster.

3.2 | Differential gene expression between pH treatments

To investigate population-level gene expression changes when exposed to acidification conditions, differential gene expression analysis between pH groups was performed by pooling across all paternal lineages (see Table S4 for average $\log_2$FC). Of note among the downregulated genes in embryos exposed to reduced pH were the TGF-β superfamily genes *dvr1*, *Univin*, *Nodal* and *Lefty* (average $\log_2$FC: 0.91, 0.45, 0.89, 0.56, see Table S4) with reduced expression level (Figure 4a–d) that are linked to cilia motility and subsequent developmental patterning (Duboc et al., 2004, 2005; Luo & Su, 2012; Molina et al., 2013). Similarly, *Ets1* controls the expression of *Alx1* which determines the skeletogenic fate of the primary mesenchymal cells (PMCs) (Ettensohn et al., 2003; Sharma & Ettensohn, 2010) were also downregulated (average $\log_2$FC: 2.84, see Table S4). While acidified embryos contributed towards clusters 1, 2 and 3, those in cluster 1 had a significantly higher *Ets1* expression (average $\log_2$FC: 2.79, see Table S4) than the clusters 2 and 3 (Figure 4e), highlighting individual variations in skeletogenesis response when exposed to the same pH condition.

Other noteworthy metabolism-related genes were upregulated among acidified embryos. (Figure 4f–h). *Gfpt1* (GFAT) expression level was elevated (Figure 4f) by an average $\log_2$FC of 1.09 (see Table S4). This gene encodes the first and rate-limiting enzyme in the hexosamine biosynthetic pathway, a glucose metabolism pathway that results in the synthesis of a nucleotide sugar UDP-GlcNAc. This sugar is subsequently used for the post-translational modification of intracellular proteins that regulate nutrient sensing and stress response (Yamazaki, 2014; Yi et al., 2019). Ribosomal genes were also

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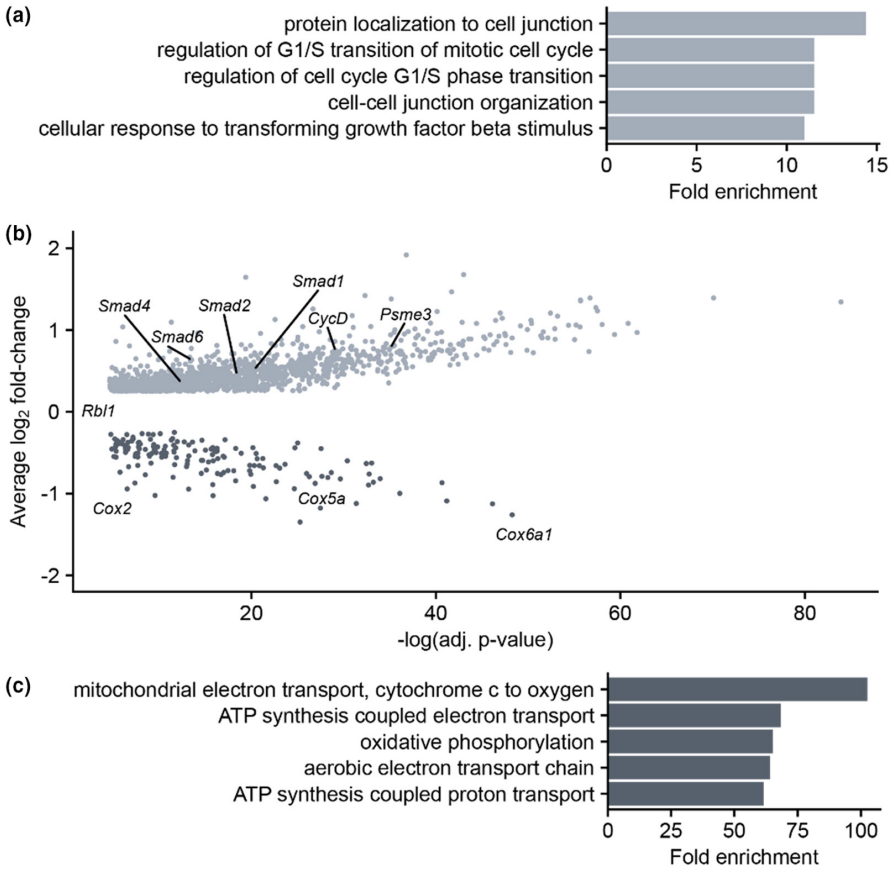

FIGURE 3 (a) Fold enrichment of GO terms with genes differentially expressed in cluster 2 compared to 3 (Table S3, FDR < 0.05). (b) Volcano plot showing differentially expressed genes in clusters 2 and 3. Genes represented in terms are labelled. (c) Fold enrichment of GO terms with genes differentially expressed in cluster 3 compared to 2. Note that enriched GO terms are identified based on genes that are differentially expressed between clusters; the GO enrichment level does not directly reflect the gene expression level of underlying genes.

expressed at a higher level (Figure 4g,h) compared to those exposed to ambient pH, including the genes *Rps8* and *Rps4x* (average $\log_2$FC in order: 2.03, 2.00, see Table S4).

Other cell cycling-related genes also showed pH-dependent expression patterns, including the E2F family genes (*E2f3* and *E2f8*, Figure S3a,b; Table S4), *Cycb3*, *Cdk2/6/9* (Figure S3c–f) and *Cdc20* (Figure S3g, Table S4). While *E2f8* together with *Cycb3*, *Cdk2/6* and *Cdc20* were downregulated (average $\log_2$FC: 3.63, 0.26, 0.48 and 1.76, see Table S4) in the pH 7.4 and 7.7 treatments, *E2f3* and *Cdk9* were downregulated (average $\log_2$FC: 0.53, 0.43, see Table S4) in the pH 8.0 embryos. These genes were linked to progression between different phases of cell division, for example, *E2f8* is associated with G1/S transition (Christensen et al., 2005), *Cdk2/6* is linked to G1 progression and G1/S transition (Johnson, 1998; Meyerson & Harlow, 1994), *Cycb3* and *Cdc20* are involved in G2/M transition and other cell cycle checkpoints (Gallant & Nigg, 1994; Irniger, 2002; Yu, 2002).

3.3 | Paternal difference in cluster distribution

We applied a multinomial logistic regression on the cluster outcome to examine the effect of male identity and pH treatments on gene expression profiles. The likelihood ratio test suggested the interactive effect of male and pH significantly improved the goodness of fit ($\chi^2 = 12$, $p = 1.39 \times 10^{-4}$) that is, the interaction between sire and pH treatment affect cluster classification. The most notable difference between males was that for male 1 (M1) a higher proportion of its embryos were assigned to cluster 3 compared to the other males (M2 and M3, Figure 5a) when reared at pH 7.7. This observation is recapitulated in the predicted probability of embryos being classified into each cluster generated from the full regression model (Figure 5b). Such that cluster classification was similar between all three males at pH 8.0 and 7.4; and embryos of M1 were distinct from the others at pH 7.7, and the proportion of embryos assigned to cluster 3 from M1 was larger than that in M2 and M3.

4 | DISCUSSION

Through profiling single-embryo transcriptomes of the urchin *H. crassispina*, a commercially and economically important species found in the subtropics, this study highlights the transcriptional plasticity of coastal species to cope with acidification stress. While there were changes in gene expression profiles and up/down regulations of genes associated with development, skeletogenesis and metabolism, changes in oxygen consumption were not detected. Our analysis also highlights a subtle, yet significant difference, between parental lineages in gene expression profile—there were shared gene expression profiles across males that shifted with acidification, however, the relative proportion of offspring having a particular profile differed between males. This ability to quantify individual variations and standing genetic variation is crucial for understanding the adaptive potential of organisms in the face of global climate change.

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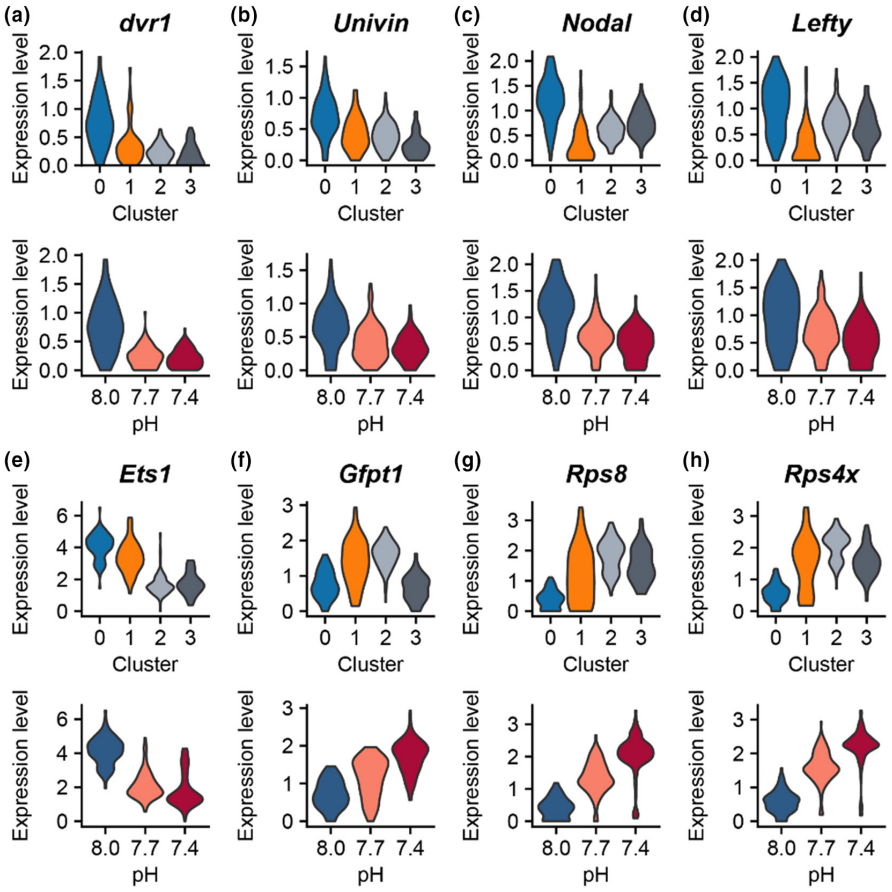

FIGURE 4 Violin plot of gene expressions in the effect of OA grouped by cluster (top) and pH (bottom). (a–d) Expression level of TGFβ superfamily genes *dvr1*, *Univin*, *Nodal* and *Lefty*. (e) Expression of skeletogenic gene *Ets1*. (f) Expression level of hexosamine biosynthesis pathway gene *Gfpt1*. (g, h) Expression level of ribosomal protein gene *Rps8* and *Rps4x*.

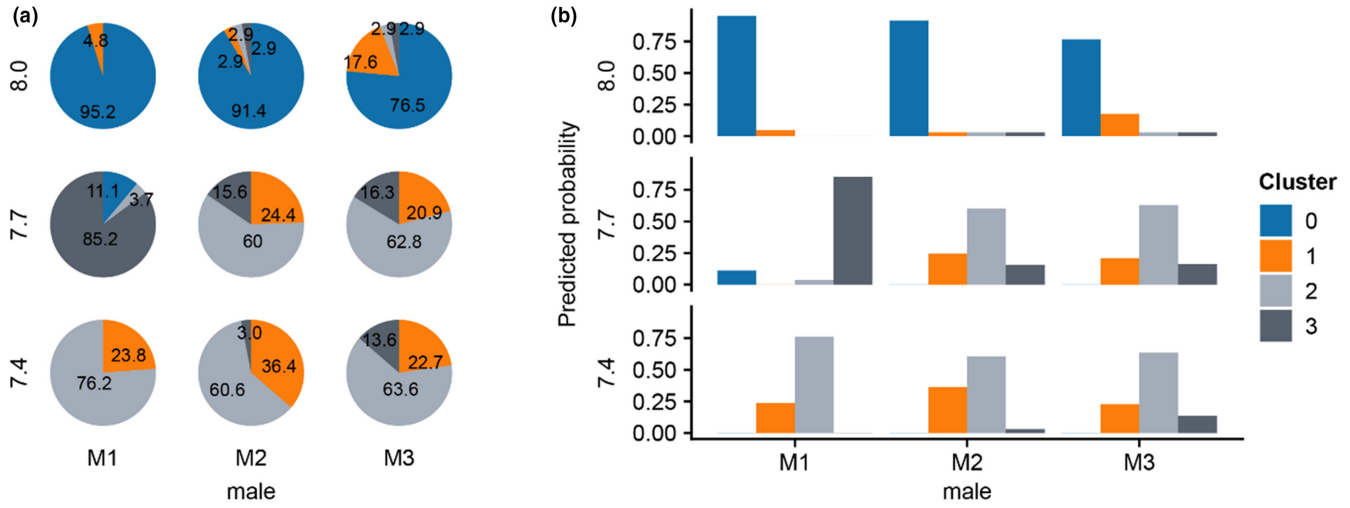

FIGURE 5 (a) Percentage of embryos in each cluster from males and pH conditions. (b) Predicted probability of embryos classified into each cluster.

4.1 | Assessing individual responses with a single-embryo transcriptomic approach

At a single-embryo level, the effect of OA can be profiled at a much higher resolution compared to the conventional bulk sample approach, enabling us to both interrogate individual response and population-level trend. For instance, the expression level of the *E2F* family genes associated with cell-cycle control differed between individuals, as the embryos did not have a synchronous development (see violin plot in Figure S3). However, the downregulation of these genes averaged across pH treatments support the notion that acidification stress led to developmental delay (Byrne & Hernández, 2020).

Another advantage of this single-embryo transcriptomic approach is that there was no a-priori assumption of differences

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in expressions between pH treatments or males. Instead, an unsupervised clustering approach was applied followed by a comparison of a portion of individuals within a cluster in a particular treatment group. In this particular case, it is likely that a bulk approach could make a distinction between expression profiles of embryos reared under control pH (pH 8.0, cluster 0) and those in acidified condition (pH 7.7 and 7.4, cluster 1–3, Figure 1e), but not between the two lower pHs. Our approach was able to identify at least three different possible transcriptomic states when exposed to reduced pH. Cluster 1 was enriched in endoplasmic reticulum tubular network organization genes that are linked to protein synthesis (Terasaki, 2000) and MAP kinase activity regulation that is related to cell division (Kumano & Foltz, 2003); cluster 2 was enriched in genes linked to G1/S phase transition and cellular response to TGF-β stimulus which regulate skeletal morphogenesis and cluster 3 was enriched in genes associated with ATP synthesis and mitochondrial electron transport (Figures 2 and 3; Table S3). These three different transcriptomic states (clusters) could reflect the difference in developmental schedule as pipetting individual embryos into lysis buffer was time consuming such that lysis did not occur simultaneously for all embryos. More importantly, the probability of embryo membership in each cluster was in turn shaped by both pH treatments and sire identity.

4.2 | Acidification led to differential gene expression

Our single-embryo RNA-seq approach reveals the transcriptional changes in the sea urchin embryo development in response to OA when there was no significant difference in oxygen consumption rate, an organismal metabolism proxy, was detected (Figure 1). It is likely that the observed difference in gene expression pattern would lead to phenotypic changes with increased rearing duration as Dorey et al. (2018) and Lo et al. (2021) both showed acidification reduces the larval size of *H. crassispina*.

Similar to other sea urchins, acidification appeared to cause downregulation of genes associated with ciliogenesis and development as well as skeletogenesis but upregulation in genes associated with metabolism and DNA replication/repair (Figure 4, Chang et al., 2021; Devens et al., 2020; Evans et al., 2013; Strader et al., 2020). Furthermore, the embryos from the two acidified treatments had different expression profiles. The majority of individuals in cluster 3 that is enriched with metabolic genes were reared under pH 7.7, while most individuals reared at pH 7.4 were grouped under clusters 1 and 2, which were enriched in genes associated with cell division, body patterning and skeletogenesis. This trend of first experiencing metabolic stress and then stunned development with decreasing pH supports the notion that the elevated metabolic cost associated with acidification stress and the resulting reallocation of energy caused a developmental delay in larval urchins (Pan et al., 2015).

4.3 | Paternal influence on the diversity of transcriptional responses

Assuming the trait of interest is heritable, paternal variation is crucial in determining the adaptive potential of organisms to future climate (Kelly & Hofmann, 2013; Sunday et al., 2014). From the sequencing data, we observed that instead of a universal gene expression in response to OA within a paternal line, male identity influenced the diversity of the offspring responses. In this case, we observed the number of offspring that expressed genes associated with ATP synthesis and mitochondrial electron transport at pH 7.7 (cluster 3) from one paternal lineage (M1) was significantly different from the others (Figure 5). The likelihood ratio test and predicted probability confirmed the interactive effect between sire and pH constituted the observed difference. The offspring of this male appeared to elevate metabolic rate without depression of cell division or growth. Interestingly, pooled embryos from this male did not express a particularly large variance in oxygen consumption compared to the others (Figure 1e). This difference could be a result of limited biological samples used in the respirometry measurement (4 replicates with 5 individuals in each). Alternatively, the all embryos examined shared the same dam, and hence, the mitochondrial OXPHOS capacities were similar between the half-sibs group. At least, for fish exposed to elevated temperatures, the metabolic response was modulated by the maternal effect (Shama et al., 2014).

Bulk transcriptomic analysis of the conspecific *H. erythogramma* that has lecithotrophic larvae suggested that male identities accounted for ~6% of the variance of the expression profiles observed among the three paternal half-sibs group; the genotype by pH interaction explained an even smaller share (0.3%) of the variation in the gene expression (Devens et al., 2020). This diminished interactive effect is consistent with our observation that the interaction of genotype (sire) and acidification had little effect on the types of expression profiles (the 4 clusters in Figure 1) but the relative abundance in each cluster, highlighting the benefit of surveying individual responses. Indeed, working with a larger set of parents (7 males × 3 females to create 21 families), Runcie et al. (2016) suggested that of all the differentially expressed genes across pH about 20% varied between sires.

The observed differences in gene expressions between males at low pH imply differences in organismal functions. Earlier sperm function analysis suggested that male urchins with sperm that underperform at present-day pH (pH 8.0) had improved fertilization success under acidified conditions (Smith et al., 2019). Sire identity has been shown to affect the developmental success (cleavage) of other echinoderms exposed to acidification conditions (Foo et al., 2012; Sparks et al., 2017). For purple urchins, offspring sired by males from sites experiencing more intense upwelling conditions had larger offspring under low pH (Kelly et al., 2013). Future experiments that include the transcriptome of sperm and later developmental stages with additional biological samples would help better link the genotypic changes to phenotype.

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The interactive effect of sire and pH on gene expression profiles highlights that this population of sea urchins that experience periodic low pH has standing genetic variations to cope with acidification stress. While such variation implies there is potential to cope with future ocean acidification through evolutionary responses, a sufficiently large population is needed to sustain this response and such selective pressure could lead to a reduction in genetic diversity (Pespeni et al., 2013). Nevertheless, our work illustrates the promise of single-embryo sequencing in identifying and quantifying subtle yet important variations, e.g., sire×pH interactive effect, which help inform both molecular, organismal and evolutionary responses to anthropogenic stress.

AUTHOR CONTRIBUTIONS

Kit Yu Karen Chan and Angela Ruohao Wu conceptualized the research. Danson Shek Chun Loi and Ziuwin Leung harvested the sea urchins. Daniel Chun Sang Tong and Claire Knox maintained and crossed the sea urchins. Sindy Sing Ting Tam, Sin Yen Tan, Danson Shek Chun Loi, Ziuwin Leung and Ying Xu prepared sea urchin embryos for experiments. Yi Lan and Pei-Yuan Qian provided analytical tools and insights to the research. Cheuk Wang Fung and Kin Yung Chau analysed the data. Cheuk Wang Fung, Kin Yung Chau, Kit Yu Karen Chan and Angela Ruohao Wu wrote the manuscript. All authors reviewed the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE227051, reference number GSE227051.

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