

# Transcriptomic responses to ocean acidification in larval sea urchins from a naturally variable pH environment

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## Abstract

Some marine ecosystems already experience natural declines in pH approximating those predicted with future anthropogenic ocean acidification (OA), the decline in seawater pH caused by the absorption of atmospheric CO<sub>2</sub>. The molecular mechanisms that allow organisms to inhabit these low pH environments, particularly those building calcium carbonate skeletons, are unknown. Also uncertain is whether an enhanced capacity to cope with present day pH variation will confer resistance to future OA. To address these issues, we monitored natural pH dynamics within an intertidal habitat in the Northeast Pacific, demonstrating that upwelling exposes resident species to pH regimes not predicted to occur elsewhere until 2100. Next, we cultured the progeny of adult purple sea urchins (*Strongylocentrotus purpuratus*) collected from this region in CO<sub>2</sub>-acidified seawater representing present day and near future ocean scenarios and monitored gene expression using transcriptomics. We hypothesized that persistent exposure to upwelling during evolutionary history will have selected for increased pH tolerance in this population and that their transcriptomic response to low pH seawater would provide insight into mechanisms underlying pH tolerance in a calcifying species. Resulting expression patterns revealed two important trends. Firstly, *S. purpuratus* larvae may alter the bioavailability of calcium and adjust skeletogenic pathways to sustain calcification in a low pH ocean. Secondly, larvae use different strategies for coping with different magnitudes of pH stress: initiating a robust transcriptional response to present day pH regimes but a muted response to near future conditions. Thus, an enhanced capacity to cope with present day pH variation may not translate into success in future oceans.

**Keywords:** climate change, gene expression, ocean acidification, transcriptomics, upwelling, urchin

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## Introduction

Ocean acidification (OA) is a direct consequence of human activity and results from the dissolution of atmospheric carbon dioxide (CO<sub>2</sub>) into the global ocean (Doney *et al.* 2009). The progressive uptake of anthropogenic CO<sub>2</sub> drives chemical reactions that alter seawater

chemistry, increasing the concentration of hydrogen ion (H<sup>+</sup>) and decreasing the abundance of carbonate ion (CO<sub>3</sub><sup>2-</sup>) (Caldeira & Wickett 2003). Although OA manifests at a global-scale, its impact on marine organisms is not expected to be homogeneous (Turley *et al.* 2010). Previous research has shown that marine organisms vary considerably in their response to OA (Ries *et al.* 2009; Kroeker *et al.* 2010) and several hypotheses have been proposed providing mechanistic explanations for this trend. For example calcifying species, organisms that manufacture shells, spines and skeletons, are considered, especially vulnerable because shifts in seawater

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chemistry triggered by elevated  $p\text{CO}_2$  which reduces the saturation state of calcium carbonate building blocks needed to form these 'hard parts' (Hofmann *et al.* 2010). Conversely, highly mobile species, such as teleost fish and cephalopods, are considered comparatively resistant because these species possess powerful ion regulatory systems that promote acid-base homeostasis in a low pH environment (Melzner *et al.* 2009). However, knowledge of the role played by present day pH variation in shaping responses to OA remains limited (Kelly & Hofmann 2012).

A detailed perspective of pH variability within the global ocean has recently emerged that highlights asymmetry between habitats (Hofmann *et al.* 2011). These data demonstrate that several marine ecosystems already encounter naturally low pH regimes that are not predicted to occur in the open ocean via anthropogenic OA until next century. Some of these naturally low pH environments have been exploited as analogues for OA (e.g., geothermal  $\text{CO}_2$  seeps), affording a window into the structure and function of future marine communities (Hall-Spencer *et al.* 2008; Kroeker *et al.* 2011). However, this research (and OA research in general) has tended to emphasize species vulnerability. In contrast, comparatively little attention has been directed towards the suite of marine organisms seemingly capable of tolerating high  $p\text{CO}_2$  environments. As a result, our understanding of low pH tolerance is restricted and traits that may be required for success in future oceans remain largely unidentified. Also unclear is how organisms already living in naturally acidic environments will respond to additional acidification as anthropogenic activities drive pH within these habitats even lower.

An ideal environment in which to study organisms and their response to natural pH variation are coastal areas of the Northeast Pacific Ocean that experience episodic upwelling. Upwelling is caused by seasonal shifts in wind direction that push surface waters offshore causing subsurface waters rich in dissolved  $\text{CO}_2$  and low in pH and carbonate ion to flow to the surface (i.e., be upwelled) (Feely *et al.* 2008; Hauri *et al.* 2009; Fassbender *et al.* 2011). Thus, the changes in ocean chemistry that accompany upwelling approximate those occurring from anthropogenic OA. Upwelling-driven declines in pH can be severe. Offshore measurements indicate coastal Oregon as an area of high pH variability, where water with pH as low as 7.8 can be transported onto the continental shelf and expose benthic calcifying organisms to corrosive conditions (Feely *et al.* 2008; Andersson & Mackenzie 2012). Upwelling events in the Northeast Pacific have already increased in severity since pre-industrial times due to anthropogenic inputs of  $\text{CO}_2$  (Hauri *et al.* 2009) and

oceanographic models forecast a rapid progression of OA within the California Current System that will decrease the minimum pH experienced during upwelling to 7.6 in only a few decades (Hauri *et al.* 2009; Gruber *et al.* 2012). While similar magnitude declines in seawater, pH have been shown problematic for a variety of marine species (Kroeker *et al.* 2010), upwelling has been a prominent oceanographic process in the Northeast Pacific for thousands of years (Pisias *et al.* 2001) and we hypothesized that persistent exposure to low pH seawater over these evolutionary timescales will have selected for increased pH tolerance in marine populations inhabiting areas strongly influenced by upwelling. Consequently, these same populations may be better equipped to cope with future acidification expected as a consequence of human  $\text{CO}_2$  emissions.

In this study we target two questions critical to forecasting the effects of OA in marine ecosystems: (i) what are the molecular mechanisms that may allow calcifying organisms to persist in a low pH ocean? and (ii) does evolving in a naturally low pH environment confer resistance to future anthropogenic OA? We address these questions by characterizing the transcriptomic response to current and future pH regimes in a calcifying benthic invertebrate that inhabits a naturally variable pH environment. Firstly, we used high-frequency measurements of environmental variables to develop a 104-day time series of pH, temperature, wind and sea level pressure within an intertidal habitat in coastal Oregon, demonstrating that offshore upwelling dramatically alters pH in this near-shore environment. Secondly, we used these environmental data to frame exposures for experimental cultures of larval purple sea urchins (*Strongylocentrotus purpuratus*) spawned from adults collected within this same intertidal habitat that is strongly affected by upwelling and characterized by variable  $p\text{CO}_2$  exposure. We assumed that urchins recruiting and surviving in this area possess enhanced mechanisms to cope with seawater acidification and we hoped to identify underlying mechanisms that might emerge in a species tolerant of natural variation in seawater chemistry. *Strongylocentrotus purpuratus* is a keystone ecosystem engineer across the Northeast Pacific Ocean whose abundance has a dominant effect on community structure (Pearse 2006; Rogers-Bennet 2007). Larval stages spend extensive periods in the plankton (Strathmann 1978; Rogers-Bennet 2007) where they are likely to be exposed to upwelling-driven declines in pH while developing a calcium carbonate skeleton required for swimming and feeding (Pennington & Strathmann 1990; Strathmann & Grunbaum 2006). These early life stages comprise a critical period in echinoderm life cycles, as recruitment success is primarily determined

by survival at these stages (Rowley 1989), yet larvae are considered much more vulnerable to OA than adults (Byrne 2011). Third, we used microarray-based transcriptomics to provide insight into the molecular mechanisms that may promote function during low pH exposure in this urchin population. Gene expression profiling has emerged as a powerful discovery tool in ocean change biology (Evans & Hofmann 2012) and has increased mechanistic understanding of ecologically significant processes such as coral bleaching (DeSalvo *et al.* 2008, 2010) and responses to acidification (Moya *et al.* 2012) and species invasions (Lockwood *et al.* 2010). Previous research involving OA-induced changes in gene expression in *S. purpuratus* have been implemented using candidate gene approaches that target a defined set of genes chosen *a priori* by the investigator (Todgham & Hofmann 2009; O'Donnell *et al.* 2010; Stumpp *et al.* 2011b; Hammond & Hofmann 2012). For example, Todgham & Hofmann (2009) used a micro array platform to track responses to elevated pCO<sub>2</sub> across a set of ~1000 genes functioning within ten major physiological pathways and Stumpp *et al.* (2011b) targeted a set of 27 genes expected to be involved in the adaptive response to pCO<sub>2</sub>, such as those involved in calcification and acid-base homeostasis. While informative, these approaches place restrictions on the identification of novel mechanisms that may promote survival in future oceans. Here, we expand on previous efforts by tracking gene expression using a microarray platform with complete coverage of the *S. purpuratus* transcriptome (28 036 genes), which provides a comprehensive and unbiased screen of the adaptive response to OA after generations of exposure to upwelled seawater. By comprehensively tracking gene expression, the ability to uncover the molecular basis of sustaining function in a high pCO<sub>2</sub> ocean is greatly enhanced (Wong *et al.* 2011; Moya *et al.* 2012).

## Materials and methods

### *Environmental monitoring*

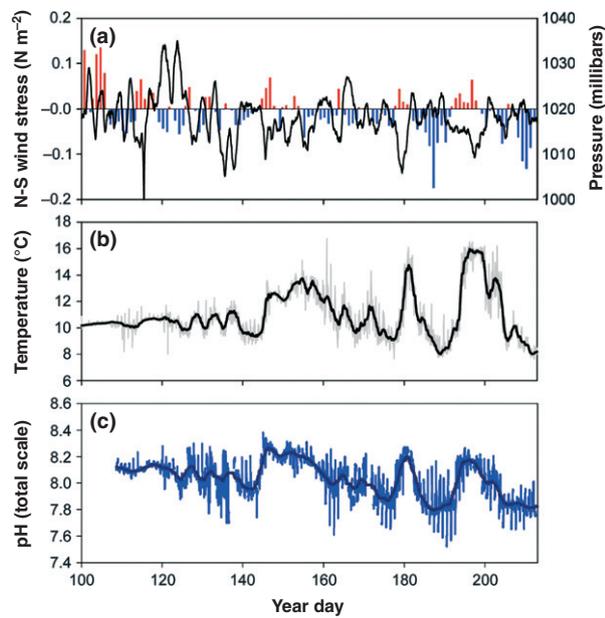
We tracked the natural dynamics of four environmental variables known to shift during upwelling within an intertidal habitat at Fogarty Creek, Oregon, USA (latitude: 44°51'N; longitude 124°00'W): wind stress, sea level pressure, temperature and pH (Huyer 1983; Feely *et al.* 2008). Upwelling is highly seasonal in coastal Oregon (Barth *et al.* 2007) and we monitored these environmental variables during peak upwelling months [April 19, 2011 (year day 108) to July 31, 2011 (year day 211)] to capture the pH minimum occurring in our study site. Importantly, this same period corresponds to when *S. purpuratus* have recently spawned and larvae from

this area are entering pelagic phases and potentially exposed to upwelling events (Miller & Emlet 1997).

North-south wind stress and sea level pressure were calculated using measurements from the National Oceanic and Atmospheric Administration National Data Buoy Center station NWPO3 (44°36'48"N 124°4'0"W) located 25 km south of the study site. Temperature was measured at 5 min intervals via TidbiT Temperature Loggers (Onset Computer Corporation, Pocasset, MA, USA) placed intertidally at Fogarty Creek. pH was obtained via an autonomous sensor deployed intertidally at Fogarty Creek and placed adjacent to the adult urchins used in this study to maximize the likelihood that recorded pH represents that experienced by adult urchins in the wild. The pH sensor was based on a modified version of the Honeywell DuraFET<sup>®</sup>, an ion-sensitive field-effect transistor, with an integrated data logger and power supply (Martz *et al.* 2010). The sensor was calibrated pre- and post-deployment against TRIS-based certified reference materials and pH is reported on the total hydrogen ion concentration scale (Dickson 2010).

### *Adult urchin collection*

Adult *S. purpuratus* were collected from the intertidal site at Fogarty Creek on year day 121 (May 2, 2011). Because our pH time series began on year day 108 (April 19, 2011), we were able to determine the pH conditions experienced by adults in the field prior to collection. As illustrated in Fig. 1, pH during year day 108–121 was more stable than that observed during the remainder of the time series and adults did not encounter pH below 7.97 in the field for at least 13 days prior to collection. Upon collection, adult *S. purpuratus* were transferred to flow-through-seawater tables at the University of California Santa Barbara and maintained for 2 weeks in seawater pumped from the Santa Barbara Channel at ambient pCO<sub>2</sub> at seawater pumped the Santa Barbara Channel. The pH of this incoming seawater was not monitored during the 2-week holding period and it was possible that adults might have been exposed to some variation in seawater pH as a function of natural processes within the Santa Barbara Channel. However, pH dynamics are weaker along this area of the coast relative to Oregon and intertidal regions of the Santa Barbara Channel have a much lower frequency exposure to pH <7.8 than the Fogarty Creek study site used here (Francis Chan, Department of Zoology, Oregon State University, personal communication). Thus, we do not anticipate that adult urchins were exposed to declines in pH typical of their native environment during this very brief holding period.



**Fig. 1** Variability in environmental conditions at Fogarty Creek, OR, USA. Data were collected from April 19, 2011 (year day 108) to July 31, 2011 (year day 211). Record of north (red bars) and south (blue bars) wind stress ( $\text{N/m}^2$ ) and sea level pressure (millibars; black line) plotted as 3-day running means (a). Daily minimum and maximum temperatures ( $^{\circ}\text{C}$ ; gray lines) and 3-day running mean temperature (black line) (b). Daily minimum and maximum pH (total scale; blue lines) and 3-day running mean pH (dark blue line) (c). Temperature and pH are high resolution with measurements recorded every 5 and 10 min, respectively, during sensor immersion.

#### Culturing of larval urchins in $\text{CO}_2$ -acidified seawater

*Strongylocentrotus purpuratus* embryos were cultured in seawater bubbled with three different concentrations of  $\text{CO}_2$  as described in Fangue *et al.* (2010). These three treatment levels were selected based on data obtained by environmental monitoring at Fogarty Creek, OR (Fig. 1) and by future  $\text{pCO}_2$  scenarios predicted for the California current system by Hauri *et al.* (2009). Culture mesocosms were aerated continuously with commercially manufactured air premixed at ( $\pm$  standard deviation, SD):  $435 \pm 16 \mu\text{atm}$  (near ambient conditions at the study site),  $813 \pm 39 \mu\text{atm}$  (representative of increases in  $\text{pCO}_2$  incurred during present day upwelling events) and  $1255 \pm 61 \mu\text{atm}$  [approximating  $\text{pCO}_2$  levels predicted to occur during upwelling events within the next few decades (Hauri *et al.* (2009)]. These  $\text{pCO}_2$  regimes resulted in seawater with the following pH ( $\pm$  standard deviation):  $8.01 \pm 0.01$ ,  $7.77 \pm 0.02$  and  $7.59 \pm 0.007$ , respectively. Water chemistry (salinity, pH and  $\text{pCO}_2$ ) and temperature were monitored daily in each mesocosm according to best practice procedures outlined in Dickson *et al.* (2007) and as described in Fangue *et al.* (2010). A total alkalinity (TA) of

$2243 \pm 3.04 \mu\text{mol/kg}$  of seawater was used in the calculation of pH and  $\text{pCO}_2$  (Robbins *et al.* 2010), which was obtained via titration and represented the average TA in water samples taken from reservoirs at the onset and completion of the exposures ( $n = 6$ ).

Each of the three  $\text{pCO}_2$  treatments contained four replicates, giving a total of 12 experimental cultures. To populate these twelve cultures, spawning was first induced by injecting the coelomic cavity of adults with  $0.5 \text{ M KCl}$  following standard methods (Strathmann 1987). Eggs were fertilized by slowly adding small volumes of diluted sperm. Fertilization rates were continuously monitored in subsamples of eggs using compound microscopy and the addition of sperm halted once fertilization reached 95%. Eggs collected from four females were fertilized separately by sperm from a single male, resulting in four embryo families with shared paternity but dissimilar maternity. A subsample of approximately 125 000 fertilized embryos from a single family was then distributed into one replicate culture (10 embryos/mL) at each  $\text{pCO}_2$  treatment. This process was repeated until all 12 cultures were populated. Each  $\text{pCO}_2$  treatment then contained four replicate cultures with each replicate containing embryos from one of the four embryo families. This experimental design followed that of Todgham & Hofmann (2009) and was chosen because of the limited number of adult urchins that could be collected from our study site. Despite this limitation, our design ensured that some genetic variation was present within  $\text{pCO}_2$  treatments, but that genetic variation between treatments was similar.

Samples from each culture were collected at two developmental stages: advanced gastrula and early pluteus. Key events in the calcification of the larval skeleton occur at these two stages, including the formation of spicules during gastrulation (Wilt 1999) and the elongation of skeletal rods at pluteus stages (Guss & Etensohn 1997). Gastrulae were collected when the archenteron contacted the overlying ectoderm and plutei when the surface between the dorsal arms became concave and the apical tips of the larvae became pointed (Fig. S1, Supporting information). Development was monitored closely to ensure that increases in  $\text{pCO}_2$  did not affect developmental rate [as reported elsewhere, e.g., Dupont *et al.* (2010); Stumpp *et al.* (2011a)] and that samples were collected at identical developmental stages across  $\text{pCO}_2$  treatments. As larvae approached gastrula or early pluteus stages, subsamples of approximately 50 embryos were taken every 2 h from each culture, viewed under compound microscopy, and scored as either complete or incomplete for having reached developmental landmarks. Sampling was initiated when all embryos within each subsample reached the desired stage. Using this

method, both gastrulae and plutei reached sampling landmarks synchronously across the three pCO<sub>2</sub> exposures, as reported elsewhere (Todgham & Hofmann 2009; O'Donnell *et al.* 2010; Yu *et al.* 2011; Hammond & Hofmann 2012; Place & Smith 2012; Padilla-Gamiño *et al. in revision*; Pespeni *et al. in revision*). Gastrulae were collected at 44 h past fertilization (hpf) by removing half of the volume of seawater from each culture (6 L or approximately 60 000 embryos). Our flow-through system meant the original volume of seawater was quickly restored in each culture and remaining embryos (now at 5 embryos/mL) were allowed to develop until early pluteus stage (92 hpf) at which point the entire culture volume was sampled (12 L and the remaining 60 000 larvae). Both gastrulae and plutei were concentrated into a small volume of seawater using reverse filtration, transferred to 1.5 mL Eppendorf tubes and quickly pelleted by centrifugation. Excess water was then removed, embryos were flash-frozen in liquid nitrogen and 1 mL of TRIzol<sup>®</sup> Reagent (Invitrogen, Carlsbad, CA, USA) was added. Following the addition of TRIzol<sup>®</sup>, embryos were vortexed vigorously and stored at -80 °C until further processing. Larvae were not fed because of the short duration of the experiment.

#### *RNA extraction, amplification and labeling*

A total of 24 samples were used for transcriptional profiling, corresponding to samples collected from four replicate cultures at the three pCO<sub>2</sub> treatments across two developmental stages. Each sample, containing approximately 60 000 larvae, was thawed and further homogenized by repeatedly passing the homogenate through a 21-gauge needle. Total RNA was extracted using the guanidine isothiocyanate method (Chomczynski & Sacchi 1987). Following extraction, RNA was processed to remove tRNA and degraded fragments using a RNeasy<sup>®</sup> Mini Kit according to manufacturer's instructions (Cat. No. 74104, Qiagen, Valencia, CA, USA). RNA yield and purity were assessed by measuring A260 and A260/A280 ratio, respectively, with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Gene expression levels were determined by comparing the amount of RNA transcript in experimental samples relative to a common reference sample, whereby experimental samples were fluorescently tagged with Cy5 (red) dye and the reference with Cy3 (green) dye. The reference sample was acquired by mixing equal proportions of RNA from all samples used in this study. Two hundred nanograms of clean, total RNA from either experimental or reference samples were amplified and labeled with Cy5 or Cy3, respectively, using Agilent's Low Input Quick Amp Two-Color

Labeling Kit (#5190-2306, Agilent Technologies Inc., Santa Clara, CA, USA). Each amplification and labeling reaction also contained 'spike-in' RNA (Agilent's Two-Color RNA Spike-In Kit, # 5188 5279, Agilent Technologies Inc.), which generated expected fluorescent signal intensities used in quality control. Yield and specific activity of the resulting amplified and labeled RNA was determined using a NanoDrop spectrophotometer (NanoDrop Technologies). In accordance with suggested standards (Agilent Technologies Inc.), only amplified and labeled RNA wherein yield and specific activity exceeded 1.875 µg and 6 pmol of fluorescent dye incorporation per µg RNA, respectively, were used in subsequent hybridizations.

#### *Gene expression profiling with microarrays*

Microarray analysis was performed using custom-designed 105 000 feature oligonucleotide microarrays (Agilent Technologies Inc.). Nucleotide sequences of predicted mRNAs from *S. purpuratus* genome assembly version 3.1 were downloaded from SpBase (<http://www.spbase.org/SpBase/>) and annotated as described in Oliver *et al.* (2010). Putative gene sequences were used to design transcript-specific long oligonucleotide probes (60-mer) using Agilent's probe design software (<http://earray.chem.agilent.com/earray/>). Three unique probes were designed against each of the 28 036 putative genes in the *S. purpuratus* genome. Each of these probes was spotted once on the microarray, accounting for 84 108 total features. An additional 17 141 probes from this set were randomly chosen to occupy the remainder of the user-defined features. The remaining 3823 features were occupied by a set of 10 random probes each spotted 15 times and Agilent's set of positive, negative and 'spike-in' control probes, all of which were used for quality control.

Each experimental sample was hybridized to a single array, yielding a dataset of 24 microarrays. Microarray hybridizations were performed according to recommended protocols (Two-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol, Agilent Technologies Inc.). An Axon GenePix 4000B microarray scanner (Axon Instruments, Sunnyvale, CA, USA) was used to capture fluorescent images, adjusting the PMT gain for optimized visualization of each slide. Fluorescent signal intensities for each feature on the microarray were extracted and LOESS normalized within each array using Feature Extraction Software (version 9.5.3.1; Agilent Technologies Inc.). Only features which had signal intensities 2.6 times the background level but below the saturation threshold on all 24 arrays were included for further analyses ( $n = 8821$ ). Because each gene was represented by multiple probes,

expression was summarized by computing the geometric mean of the normalized signal intensities across multiple probes targeting the same gene (Lockwood *et al.* 2010). Resulting signal intensities were then converted to  $\log_2$ -ratios of the experimental channel (Cy5) divided by the reference channel (Cy3).  $\log_2$ -ratio expression data for this set of 3677 genes was then used in downstream statistical analyses.

#### *Statistical analyses of gene expression*

Gene expression data were analyzed using singular-value decomposition (SVD), a statistical approach able to detect genome-wide trends in the dynamics of gene expression (Alter *et al.* 2000). SVD is a data-reduction technique in which the complete gene expression dataset is reduced to a series of 'eigengenes,' each corresponding to a major expression pattern. The first eigengene represents the pattern accounting for the largest proportion of total variation in the dataset, the second eigengene describes the next largest proportion and so on. This statistical approach has been found highly effective in resolving biologically meaningful patterns of gene expression from large transcriptomic datasets (e.g., Gracey *et al.* 2008; Miller *et al.* 2011). Here we used SVD to elucidate dominant transcriptional responses to elevated  $p\text{CO}_2$  in early stage *S. purpuratus* using the National Institute on Aging's Array Analysis tool (Sharov *et al.* 2005). Next, to identify genes wherein expression matched the dominant patterns detected by SVD, we calculated Pearson's correlation coefficients for each gene relative to the first two eigengenes. We only considered genes strongly correlated ( $>0.6$ ) or anticorrelated ( $<-0.6$ ) to either of the first two eigengenes and with mean expression changes  $>1.5$ -fold between exposures to be differentially expressed. This approach allowed us to isolate a set of up- and down-regulated genes responsible for the largest proportions of variation in gene expression and therefore responsive to  $p\text{CO}_2$ . The 1.5-fold-change threshold was imposed to increase the likelihood that the detected transcriptional change influenced organism physiology and fold-change filters were commonly applied in microarray datasets (McCarthy & Smyth 2009). SVD and correlation calculations were performed separately on gastrula and pluteus stage samples, as including expression data from both stages in a single analysis would highlight variation due to developmental stage rather than  $p\text{CO}_2$ .

#### *Hierarchical clustering*

Hierarchical clustering was used to compare gene expression patterns across  $p\text{CO}_2$  treatments. Clustering was based upon normalized  $\log_2$ -ratios using the

Spearman correlation dissimilarity matrix and the Ward agglomerative linkage method. Analyses were conducted using the *gplots* package in R (Warnes *et al.* 2009).

#### *Gene set enrichment analyses*

Gene set enrichment analysis (GSEA) was performed to better resolve the biological significance of genes responding to  $p\text{CO}_2$ . GSEA is a statistical tool that uses functional information to identify categories of genes (i.e., ontologies) significantly over- or under-represented within a user-defined list. Significance was determined by the binomial statistic i.e. the probability that the number of genes observed from a given ontology occurred by chance relative to a background list. GSEA was performed within the PANTHER database (Thomas *et al.* 2003, 2006; Mi & Thomas 2009) using four different ontology databases: (i) Gene Ontology: Biological Process, (ii) Gene Ontology: Molecular Function, (iii) PANTHER Protein Class and (iv) PANTHER Pathway. Integrating information across multiple databases provided a method to summarize large-scale physiological processes influenced by elevated  $p\text{CO}_2$ . Following PANTHER recommendations, we considered ontologies with Bonferroni false-discovery rate corrected *P*-values  $< 0.05$  to be significant (Thomas *et al.* 2006).

## **Results**

#### *Environmental data from the Oregon intertidal site*

We monitored the natural dynamics of four environmental variables to describe variability in abiotic factors at our study site and determine whether offshore upwelling influences pH regimes in our near-shore study site. Data collected during the first half of the upwelling season on the Oregon coast (from mid-April to end July) showed that the intertidal habitat at Fogarty Creek exhibited large fluctuations in all four measured parameters with N-S wind stress ranging from 0.3 to  $-0.2 \text{ N m}^{-2}$ , sea level pressure from 999 to 1035 millibars (Fig. 1A), temperature from 8 to 17 °C (Fig. 1B) and pH from 8.38 to 7.47 (Fig. 1C). Temperature and pH fluctuated on a consistent daily scale, reflecting day-night cycles that drive alternating periods of respiration and photosynthesis in the intertidal (Wootton *et al.* 2008). Daily pH averaged over the entire 104 day-time series was  $7.95 \pm 0.139$  ( $\pm\text{SD}$ ;  $n = 104$ ). The lowest observed daily pH was  $7.79 \pm 0.124$  ( $\pm\text{SD}$ ;  $n = 101$ ) and was detected during a 5-day period when mean daily pH remained low for several consecutive days (year day 185–190) and was therefore not a function of diurnal respiration/photosynthesis cycles. This

sustained decrease in pH was temporally associated with a sharp decline in water temperature and an increase in pressure and southerly winds; concurrent shifts in these environmental variables are a signature of upwelling. Raw environmental data are provided in Fig. S2 (Supporting information).

These environmental pH data were subsequently used to choose pCO<sub>2</sub> levels used in laboratory cultures of sea urchins (Fig. 2). The daily pH averaged across the entire time series (pH = 7.95) was used to derive pCO<sub>2</sub> for cultures at the lower range of natural variability, referred to hereafter as the Low pCO<sub>2</sub> treatment. We used the lowest daily pH observed during the upwelling event that occurred between year day 185–190 (pH = 7.79) to set pCO<sub>2</sub> in cultures representing upwelling in this region, which we refer to hereafter as the Mid pCO<sub>2</sub> treatment. Finally, because very high pCO<sub>2</sub> levels can on occasion be reached during upwelling and anthropogenic inputs of CO<sub>2</sub> are expected to further decrease the minimum pH experienced during upwelling (Hauri *et al.* 2009, 2012), we included a high pCO<sub>2</sub> treatment to investigate the effects of extreme upwelling and to simulate future upwelling events. Hauri *et al.* (2009) predicted that surface pH within the California current would decrease to 7.6 during upwelling in the next few decades, a conservative near future scenario (Melzner *et al.* 2012). We used this predicted value to derive pCO<sub>2</sub> in cultures hereafter referred to as the High pCO<sub>2</sub> treatment. The CO<sub>2</sub> mixing system approximated these chosen pCO<sub>2</sub> levels very closely during the laboratory experiment. Resulting mean seawater pH within the Low treatment cultures was  $8.01 \pm 0.01$ , very near the target value of 7.95.

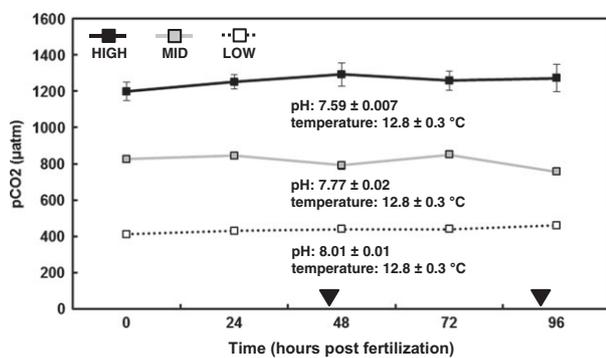


Fig. 2 Time-course of seawater pCO<sub>2</sub> in experimental cultures. Each point represents the mean daily pCO<sub>2</sub> across four replicate mesocosms at each of the three treatment levels: Low (dashed line), Mid (gray line) and High (black line). Data points are shown  $\pm$  standard deviation. Some error bars are obstructed by data points. Inverted triangles along the x-axis denote sampling times for gastrula (44 hpf) and pluteus (92 hpf) stages.

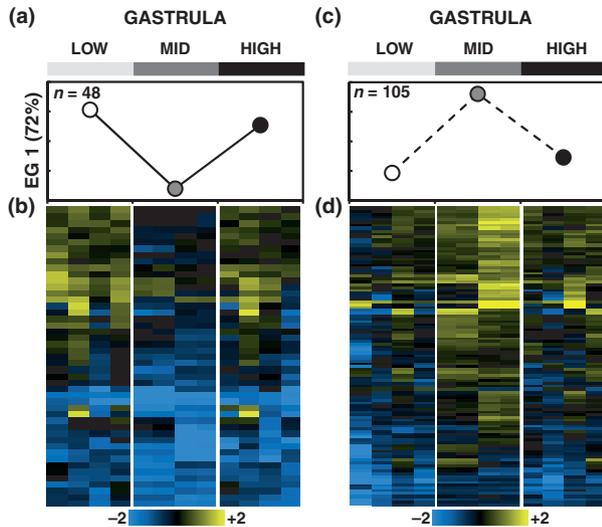
Our target pH for the mid cultures was 7.79 and mean seawater pH within these cultures was  $7.77 \pm 0.02$ . Mean seawater pH within the high treatment intended to simulate a near future scenario was  $7.59 \pm 0.007$ , again very close to our target of 7.6.

#### Transcriptional responses to acidified seawater

As an overview, transcriptomic analysis revealed that gene expression did vary between pCO<sub>2</sub> treatments in early stage urchins with genes in physiologically significant classes showing changes during development and revealing several important trends. Firstly, there was a distinct difference between the developmental stages, as gastrulae showed much greater change in their transcriptome in response to elevated pCO<sub>2</sub> when compared with the later stage plutei. Secondly, most of the transcriptomic change in gastrulae occurred in response to the Mid pCO<sub>2</sub> treatment. Third, both gastrulae and plutei largely failed to respond transcriptionally to the High pCO<sub>2</sub> treatment. Finally, when examined with regard to cellular function, we found that four processes were influenced by elevated pCO<sub>2</sub> in gastrulae: calcium homeostasis, ion transport, cell signaling and transcription. These results are described below in greater detail.

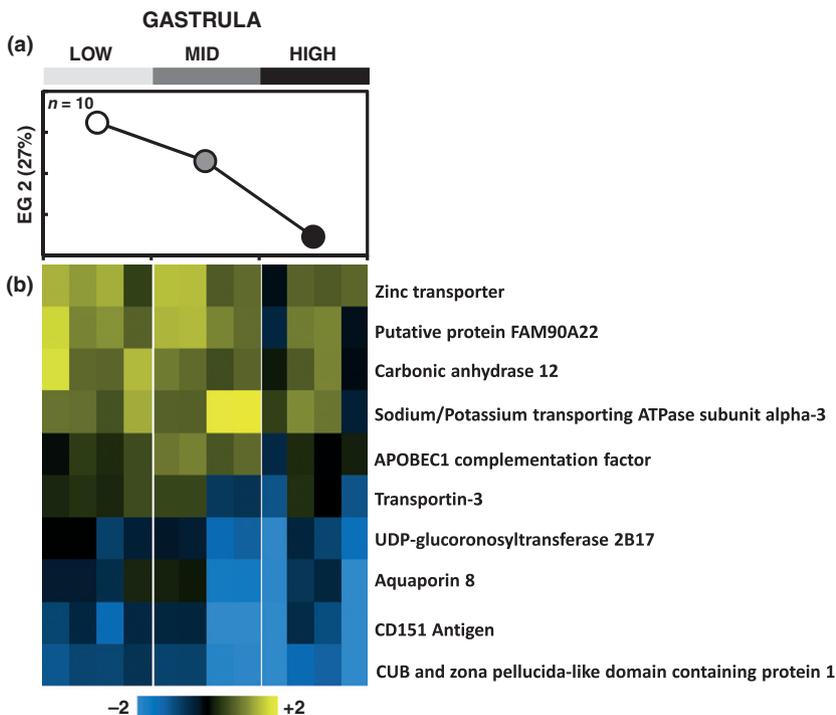
*Gastrulae.* SVD revealed a dominant trend in the response of early stage *S. purpuratus* to pCO<sub>2</sub>: the vast majority of differential gene expression occurred exclusively in the earlier stage gastrulae at the Mid pCO<sub>2</sub> treatment (i.e., pCO<sub>2</sub> = 813 µatm, pH = 7.77). One hundred and 53 genes were differentially expressed in gastrulae at the Mid pCO<sub>2</sub>, 48 of which were down-regulated (Fig. 3A,B) and 105 up-regulated (Fig. 3C,D) relative to the low cultures. Names of each differentially expressed gene and their order in each heatmap are provided in Fig. S3 (Supporting information). This dominant expression pattern (i.e., eigengene 1) explained 72% of the observed variation in gastrulae. In comparison, differential expression in the High pCO<sub>2</sub> treatment was unexpectedly small (i.e., eigengene 2), accounting for only 27% of the variation and only ten genes were considered differentially expressed (Fig. 4A,B). All 10 of these genes were down-regulated relative to low cultures. Only transportin-3 was differentially expressed under both Mid and High pCO<sub>2</sub> treatments in gastrulae.

A large number of genes with important functions in skeletogenesis were among those 163 responding to Mid and High pCO<sub>2</sub> in gastrulae. At least 36 genes differentially expressed here were also expressed or had isoforms expressed in the primary mesenchyme, a population of cells exclusively involved in skeletogenesis (Fig. S3, Supporting information) (Zhu *et al.* 2001).



**Fig. 3** Genes differentially expressed in response to the Mid pCO<sub>2</sub> treatment in gastrulae. Genes are listed to correspond to the order presented in the heatmaps shown in Fig 3B and 3D from top to bottom.

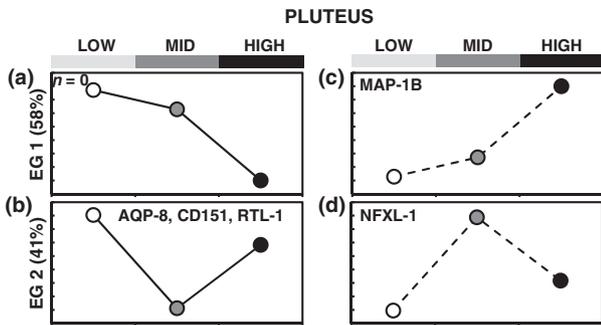
An additional 13 are expressed or had isoforms expressed in the test and spine of *S. purpuratus* (Fig. S3, Supporting information) (Mann *et al.* 2008). Notable were C-type lectin domain family 19 member A, carbonic anhydrase and collagen alpha-1(XV) chain. C-type lectin domains are a conserved feature of a subfamily of spicule matrix proteins (Illies *et al.* 2002), carbonic anhydrase has an important function in the mineralization



**Fig. 4** Genes down-regulated at the High pCO<sub>2</sub> treatment in gastrulae. The expression pattern explaining the second largest proportion of variation in gene expression [i.e., eigengene 2 (27%)] (a) and genes strongly correlated (b;  $n = 10$ ) with this expression pattern are illustrated as a hierarchical cluster heatmap that displays the normalized log<sub>2</sub>-ratio for each gene (rows) across four replicates at each pCO<sub>2</sub> (columns). Sequence identifiers: UDP-glucuronosyl transferase 2B17 (SPU\_000692), Zinc transporter ZIP11 (SPU\_016878), APOBEC1 complementation factor (SPU\_022391), CD151 antigen (SPU\_002137), Sodium/potassium-transporting ATPase subunit alpha-3 (SPU\_025815), Aquaporin-8 (SPU\_027252), Transportin-3 (SPU\_000250), Carbonic anhydrase 12 (SPU\_024809), CUB and zona pellucida-like domain-containing protein 1 (SPU\_016301), putative protein FAM90A22 (SPU\_007031).

of calcite used in urchin skeletons (Killian & Wilt 2008) and collagen provides an important substrate for skeletogenesis (Benson *et al.* 1990).

*Plutei*. In stark contrast to the large number of genes responding to elevated pCO<sub>2</sub> in gastrulae, only five genes met identical criteria in the more advanced pluteus stage larvae across all three pCO<sub>2</sub> exposures (Fig. 5). Four of these five genes were differentially expressed at the Mid pCO<sub>2</sub>: aquaporin-8, CD151 antigen, retrotransposon-like protein 1 and NFX1-type zinc finger protein (Fig. 5B,D). Microtubule-associated protein 1B was induced at the High pCO<sub>2</sub> (Fig. 5C). The vastly reduced number of differentially expressed genes in pluteus stage samples is a function of genes changing less than 1.5-fold between treatments and therefore not meeting the minimal fold-change requirement for being differentially expressed. This trend was illustrated by the fact that eigengene 1 explained 58% of the variation, but only a single gene wherein expression changed greater than 1.5-fold was strongly correlated or anticorrelated with this expression pattern (MAP-1B; Fig. 5C). Based on this information, we hypothesized that plutei did not respond robustly to elevated pCO<sub>2</sub> at the level of the transcriptome. To address this hypothesis, we plotted expression (mean log<sub>2</sub> ratio) for each gene at Low pCO<sub>2</sub> versus expression at the Mid and High pCO<sub>2</sub> (overlaid on the same graph) for both gastrulae and plutei. The resulting graphs illustrated global differences in expression as a function of pCO<sub>2</sub> at each stage, whereby genes



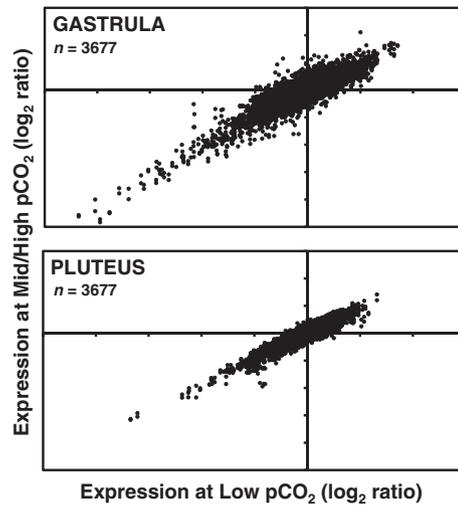
**Fig. 5** Genes differentially expressed in response to elevated pCO<sub>2</sub> in plutei. The two most dominant expression patterns, eigengene 1 (58%) (a) and eigengene 2 (41%) (b), and the names of genes strongly correlated (solid line) or anticorrelated (dashed line; c,d) with these expression patterns are listed above each graph. AQP-8 = Aquaporin-8 (SPU\_027252); CD151 = CD151 antigen (SPU\_002137); MAP-1B = microtubule-associated protein 1B (SPU\_000133); RTL-1 = retrotransposon-like protein 1 (SPU\_005430); NFXL-1 = NFX1-type zinc finger protein (SPU\_016237).

with similar expression levels under Low and Mid/High pCO<sub>2</sub>, and therefore unresponsive to pCO<sub>2</sub>, aligned along a clear linear axis. Conversely, genes affected by pCO<sub>2</sub> would deviate from this axis and yield a more dispersed plot. Comparison of plots between gastrulae and plutei confirmed that changes in gene expression due to elevated pCO<sub>2</sub> were markedly reduced in older pluteus stage larvae (Fig. 6).

*Physiological responses to acidified seawater in gastrulae*

To isolate large-scale physiological processes influenced by elevated pCO<sub>2</sub> in gastrulae, we performed GSEA to identify functional categories significantly over- or under-represented among differentially expressed genes. Four gene sets from gastrula stage embryos were analyzed: genes down-regulated (*n* = 48) or up-regulated (*n* = 105) at the Mid pCO<sub>2</sub>, genes down-regulated at the High pCO<sub>2</sub> (*n* = 10) and the union of these three gene sets (*n* = 163). We did not perform GSEA on pluteus stage samples due to the very low number of input genes (*n* = 5). All genes in the urchin genome were used as the background list (*n* = 28 036). We summarized results by grouping similar ontologies, subsequently resolving a set of four physiological processes most strongly influenced by pCO<sub>2</sub> in gastrulae: calcium homeostasis, ion transport, cell signaling and transcription (Fig. S4, Supporting information).

Ontologies associated with calcium homeostasis occurred most frequently in genes up-regulated at the Mid pCO<sub>2</sub> treatment and had the lowest *P*-values (Table 1). Three calcium transporters were induced at



**Fig. 6** Gene expression (mean log<sub>2</sub> ratio) at the Low treatment (*x*-axis) versus expression at Mid and High pCO<sub>2</sub> treatments (*y*-axis) in gastrulae and plutei for each gene used in statistical tests (*n* = 3677). This plot allows comparison of global differences in expression as a function of pCO<sub>2</sub> at each stage.

this pCO<sub>2</sub>: voltage-dependent T-type calcium channel subunit alpha-1H, the sodium/calcium exchanger 2 and LETM1 and EF-hand domain-containing protein 1 [a Ca<sup>+2</sup>/H<sup>+</sup> antiporter (Waldeck-Weiermair *et al.* 2011)]. Enkurin, which encodes a protein involved in the regulation of calcium channels, was also induced, as were the calcium-binding proteins calmodulin-like protein 5,

**Table 1** Over-represented gene ontologies relating to calcium homeostasis

Over-Represented Ontology	Database	<i>P</i> -value	Rank
Calcium channel	PANTHER Protein Class	8.82E-07	2 of 29
Calcium-binding protein	PANTHER Protein Class	2.15E-05	4 of 29
Intracellular calcium-sensing protein	PANTHER Protein Class	4.16E-03	12 of 29
Calmodulin	PANTHER Protein Class	4.16E-03	13 of 29
Voltage-gated calcium channel activity	Gene Ontology: Molecular Function	8.82E-07	1 of 25
Calcium ion binding	Gene Ontology: Molecular Function	6.53E-06	2 of 25
Calmodulin binding	Gene Ontology: Molecular Function	4.60E-02	24 of 25

Ontologies were ranked by *P*-values from lowest to highest. Complete results from the gene set enrichment analysis can be found in Fig. S4 (Supporting information).

fillagrin-2 and sarcoplasmic reticulum histidine-rich calcium-binding protein (www.uniprot.org).

Ontologies relating to ion transport were significantly enriched in all four gene sets and included 'transporter', 'transmembrane transporter activity', 'voltage-gated ion channel activity' and 'cation transmembrane transporter activity' (Fig. S4, Supporting information). While the differential expression of calcium transporters contributed to the enrichment of these ontologies, three genes that transport substrates in a sodium-dependent manner were also differentially expressed: the Na<sup>+</sup>/K<sup>+</sup>-ATPase subunit alpha 3, solute carrier family 10 member 6 and solute carrier family 23 member 2.

Significantly enriched ontologies associated with cell signaling and transcription were abundant. Given the difficulty in linking ambiguous ontologies such as 'signal transduction' or 'transcription factor activity' (Fig. S4, Supporting information) to specific physiological outcomes, we used the PANTHER Pathways database (Mi & Thomas 2009) to identify specific pathways modulated by pCO<sub>2</sub> and driving the enrichment of these ontologies. Cellular pathways with diverse functions in urchin development, but established links to skeletogenesis, were most numerous. These included FGF, Wnt and TGF-beta (Table 2; Morales *et al.* 1993; Zhu *et al.* 2001; Zito *et al.* 2003; Kiyomoto *et al.* 2004; Croce *et al.* 2006; Röttinger *et al.* 2008; Byrum *et al.* 2009; Croce & McClay 2010). No pathways were significantly enriched among the ten genes down-regulated at the High pCO<sub>2</sub>. Complete results of the GSEA are provided in Fig. S4 (Supporting information).

**Table 2** Over-represented pathways relating to urchin skeletogenesis

Over-Represented Pathway	Database	P-value	Rank
Phenylethylamine degradation	PANTHER Pathways	8.92E-07	1 of 11
FGF signaling pathway	PANTHER Pathways	3.51E-05	2 of 11
Axon guidance mediated by Slit/Robo	PANTHER Pathways	8.82E-04	4 of 11
Wnt signaling pathway	PANTHER Pathways	3.90E-03	6 of 11
TGF-beta signaling pathway	PANTHER Pathways	4.98E-03	7 of 11

Ontologies were ranked by *P*-values from lowest to highest. Complete results from the PANTHER Pathways analysis can be found in Fig. S4 (Supporting information).

## Discussion

The over-arching goal of this study was to explore whether populations are adapted to local pH regimes and tolerant of the seasonal changes in seawater chemistry associated with strong upwelling on the Oregon coast. Local adaptation, the fine-tuning of physiological responses to local environmental conditions (Sanford & Kelly 2011), has been explored in the intertidal with regard to temperature (e.g., Kuo & Sanford 2009; Kelly *et al.* 2012), but rarely with regard to OA. In the context of the unique pH conditions encountered at Fogarty Creek, we hypothesized that persistent exposure to low pH seawater during evolutionary history will have selected for traits that increase pH tolerance among resident populations, and further, that organisms recruiting and surviving in this region may possess resistance to future anthropogenic acidification. The first step in addressing this larger hypothesis was to characterize the response to acidic seawater in a potentially tolerant population. Therefore, we profiled the transcriptomic response to elevated pCO<sub>2</sub> in a population of purple sea urchins that experience intense variation in seawater chemistry in its natural habitat on the Oregon coast. The study had two elements: (i) the collection of environmental data to characterize ecologically significant variation in environmental parameters, in this case, pH, temperature, wind stress and sea level pressure and (ii) a transcriptomic analysis of gene expression in urchin larvae spawned from adults that as a population are likely acclimatized to upwelling regimes.

### Upwelling and pH variability in the intertidal

Seasonal upwelling is a dominant oceanographic process in the Northeastern Pacific Ocean that mixes deep, CO<sub>2</sub>-rich waters with surface layers causing significant declines in seawater pH (Hauri *et al.* 2009). Using high-frequency pH sensors, we demonstrate that upwelling events off the coast of Oregon strongly influence pH dynamics in a nearshore intertidal habitat. Our pH time series revealed that upwelling-driven declines within the intertidal are considerable, exposing these biodiverse areas to pH that can average 7.8 over a 24-h period. Similar declines in seawater pH are not expected to occur in other marine habitats via anthropogenic ocean acidification until next century (Intergovernmental Panel on Climate Change 2007). Because seasonal upwelling overlaps with pelagic phases of marine invertebrates, including *S. purpuratus* (Strathmann 1987), we speculate that planktonic stages in this region are also exposed to low pH seawater (Miller & Emlet 1997). While upwelling could sweep plankton offshore (Morgan *et al.* 2009), paired pH sensors placed both in

the intertidal and in nearby pelagic habitats demonstrate that pH dynamics driven by upwelling cause similar pH changes in both habitats (Francis Chan, Department of Zoology, Oregon State University, personal communication). Consequently, both larval and adult life stages are likely to require some means of coping with these dramatic changes in ocean chemistry.

Our environmental data are consistent with previous analyses of natural pH dynamics in the Northeast Pacific. An 8-year time series of intertidal pH at an outer coast site in Washington also highlights the variability of pH in this region (Wootton *et al.* 2008). Importantly, Wootton *et al.* (2008) conclude that the effect of upwelling predominated pH variability on an annual timescale, lending additional support to the hypothesis that populations inhabiting upwelling regions are consistently exposed to low pH seawater and may have evolved an enhanced capacity to cope with low pH stress. pH in the kelp forests of Southern California is also variable and while this environment does not encounter the extreme low pH events that occur in Fogarty Creek, benthic marine communities within this region are predicted to be acclimatized and adapted to fluctuations in pH (Frieder *et al.* 2012). In general, without environmental data regarding the range of pH variability in nature, it is difficult to anticipate biological responses to OA or identify populations capable of tolerating future ocean conditions (Price *et al.* 2012). The study of local adaptation and use of tools within molecular ecology will be increasingly important as the research community works to forecast whether species possess physiological tolerance toward OA (e.g., Moya *et al.* 2012; Stumpp *et al.* 2012a,b) or whether scope for adaptation exists in natural populations (Kelly & Hofmann 2012).

#### *Perspectives on acclimatization and local adaptation to natural variation in pH*

Our study provides evidence that *S. purpuratus* is tolerant of low and variable pH. From a broad perspective, the transcriptomic response to elevated pCO<sub>2</sub> was informative both in terms of genes that were differentially expressed as well as genes that remained unchanged under our experimental conditions. For example, gene expression patterns in some pathways suggest compensation for processes such as calcification (see below), however, expression profiles also indicate *S. purpuratus* embryos and larvae did not protect homeostasis by inducing an overt cellular stress response typical of exposure to acute stress (Kültz 2005; Runice *et al.* 2012). Overall, 153 genes were differentially expressed in gastrula stage larvae at the Mid pCO<sub>2</sub> level, while only four genes were differentially expressed at the same treatment level in older plutei sampled 2 days later

(Fig. 3,5). These trends imply that even early stage embryos and larvae from this area are capable of rapidly adjusting to changes in seawater chemistry.

From a comparative perspective, mechanistic insight into resistance toward OA can be resolved from relating responses to elevated pCO<sub>2</sub> between resistant and vulnerable species. Important similarities and differences emerge when comparing the transcriptomic response to acidified seawater in *S. purpuratus* with that of the coral *Acropora millepora*. Unlike *S. purpuratus*, exposure of adult *A. millepora* to high pCO<sub>2</sub> triggered the differential expression of genes diagnostic of a cellular stress response, including an up-regulation of genes functioning within oxidative stress and apoptotic pathways (Kaniewska *et al.* 2012). Conserved features of the transcriptomic response to OA between early life stages of urchins and corals include the differential expression of carbonic anhydrase and extracellular matrix proteins such as collagen (Moya *et al.* 2012). Finally, transcriptomic responses present in *S. purpuratus* but largely absent in corals may highlight mechanistic bases underlying relative resistance to elevated pCO<sub>2</sub>. Such responses identified in this study included an ability to modify the bioavailability of calcium to promote calcification, transport ions to facilitate pH regulation and manufacture a skeleton without further up-regulation of spicule matrix proteins (Todgham & Hofmann 2009; Martin *et al.* 2011; Stumpp *et al.* 2011b).

#### *Transcriptomic responses to pCO<sub>2</sub>-acidified seawater in early stage *S. purpuratus**

Gene expression profiling identified a set of four physiological processes modified during exposure to elevated pCO<sub>2</sub> in *S. purpuratus* gastrulae: calcium homeostasis, ion transport, cell signaling and transcription. We hypothesize that adaptively modulating gene networks controlling these processes work to promote development of the larval skeleton in a carbonate-limited environment and re-establish pH homeostasis.

#### *Calcification*

Analyses of the *S. purpuratus* transcriptome indicate that modifying the bioavailability of calcium aids in sustaining calcification in a high pCO<sub>2</sub> ocean. Calcium is the primary cation used in the biomineralized structures of urchins and large amounts must be first be transported from seawater intracellularly before moving via exocytosis into the extracellular space for deposition into the larval skeleton (Wilt 2002). Genes up-regulated at the Mid pCO<sub>2</sub> were enriched for ontologies relating to calcium homeostasis and the expressions of three calcium transporters and three calcium-binding proteins

were induced. Calcium channel blockers inhibit biomineralization in *S. purpuratus* larvae (Mitsunaga *et al.* 1986; Hwang & Lennarz 1993), thus increases in the abundances of these proteins may facilitate the transport of calcium from the external environment and promote skeletogenesis in a low pH ocean. In support of this conjecture, the sodium/calcium exchanger up-regulated in this study, which catalyzes the transport of three Na<sup>+</sup> ions for one Ca<sup>2+</sup> (Philipson & Nicoll 2000), has been previously shown responsive to elevated pCO<sub>2</sub> in *S. purpuratus* (Todgham & Hofmann 2009). Similarly, increased expression of voltage-dependent T-type calcium channel subunit alpha-1H may promote the transport of Ca<sup>2+</sup> to the site of calcification. Two T-type calcium channels were differentially expressed in the coral *A. millepora* exposed to low pH seawater during the initiation of calcification (Moya *et al.* 2012), although ontologies relating to calcium transport were not significantly enriched in corals as they were here. Interestingly, T-type calcium channels are involved in the transport of Ca<sup>2+</sup> required for bone development in vertebrates (Bergh *et al.* 2006). LETM1 and EF-hand domain-containing protein 1, a Ca<sup>2+</sup>/H<sup>+</sup> antiporter (Waldeck-Weiermair *et al.* 2011), may perform dual functions in response to seawater acidification in *S. purpuratus* by transporting Ca<sup>2+</sup> for use in calcification and H<sup>+</sup> to regulate intracellular pH. Finally enkurin, whose expression increased in this study, is thought to act as a calcium sensor linking signaling pathways with calcium channels (Sutton *et al.* 2004) and may represent an important intermediary between environmental pH and adaptive responses that promote Ca<sup>2+</sup> transport. More broadly, it may be that the availability of calcium exerts a greater influence on calcification than the induction of spicule matrix proteins, as genes encoding these major components of calcified tissue were largely unaffected in this study, down-regulated in previous studies of early stage *S. purpuratus* exposed to CO<sub>2</sub>-driven acidification (Todgham & Hofmann 2009; Stumpp *et al.* 2011b), but induced in coral (Moya *et al.* 2012) and larvae of the urchin *Paracentrotus lividus* (Martin *et al.* 2011) when exposed to high pCO<sub>2</sub>. While links between calcium transport, calcification and ocean acidification provide a plausible explanation for the induction of these genes, an alternative possibility is that exposure to elevated pCO<sub>2</sub> disrupts normal cell signaling events, of which Ca<sup>2+</sup> is a major contributor. Calmodulin, a major calcium-binding messenger protein was differentially expressed in this study as well as in *A. millepora* exposed to high pCO<sub>2</sub> (Kaniewska *et al.* 2012).

Adjusting pathways that regulate skeletogenesis may also facilitate calcification in a low pH ocean. Pathway analysis revealed that gene networks with diverse functions in development, but established roles in

skeletogenesis, were enriched during exposure to elevated pCO<sub>2</sub> in *S. purpuratus* gastrulae (Table 2). TGF-beta provides an inductive signal necessary for skeleton elongation (Zito *et al.* 2003; Kiyomoto *et al.* 2004), FGF signaling governs skeletal morphogenesis (Röttlinger *et al.* 2008), the calcium dependent noncanonical Wnt pathway is involved in spiculogenesis (Croce *et al.* 2006; Byrum *et al.* 2009) and Notch signaling controls the specification of nonskeletogenic and skeletogenic mesoderm (Croce & McClay 2010). Differential regulation of pathways involving phenylethylamine metabolism and Slit/Robo are also affiliated with urchin skeletons as Slit/Robo is expressed in the primary mesenchyme (Zhu *et al.* 2001) and phenylethylamine has been shown to regulate the movement of spines in adults (Morales *et al.* 1993). Modification of these signaling pathways may ultimately affect downstream effector genes involved in calcification. Despite the differential expression of only a single putative spicule matrix protein (C-type lectin domain family 19 member A), numerous other genes functioning within calcification pathways were differentially expressed in this study (Fig. S3).

#### pH regulation

The outer cell layer of echinoderm larvae is highly permeable to ions and small molecules and these characteristics make early life stages vulnerable to changes in ocean chemistry. However, an analysis of the pH regulatory capacity of larval urchins suggested even these early life stages could compensate for disruptions in acid-base homeostasis caused by elevated pCO<sub>2</sub>. While the pH of extracellular spaces conforms to that of the surrounding seawater, measurements of intracellular pH indicated that larvae of the green urchin *S. droebachiensis* could regulate pH within the compartments surrounding the calcifying primary mesenchyme cells during exposure to high seawater pCO<sub>2</sub> (Stumpp *et al.* 2012a,b). The principal method of this compensation is the transmembrane movement of protons (H<sup>+</sup>) and bicarbonate (HCO<sub>3</sub><sup>-</sup>) occurring via Na<sup>+</sup>/H<sup>+</sup>- and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers, respectively (Wheatly & Henry 1992). The expressions of these transport proteins did not change in this study, corroborating previous findings (Todgham & Hofmann 2009; Stumpp *et al.* 2011b; Moya *et al.* 2012) and implying that the activity of existing proteins was sufficient to regulate pH under our experimental conditions. However, intracellular pH compensation in *S. droebachiensis* larvae was also dependent upon the transport of sodium to provide energy needed to export protons (Stumpp *et al.* 2012a,b). In agreement with this prominent role for sodium transport during pH regulation, three genes that transport

substrates in a sodium-dependent manner were differentially expressed here, including sodium/potassium transport ATPase subunit alpha-3 (Fig. 4). Considering the transmembrane movement of ions was metabolically expensive, up to 77% of larval metabolism in sea urchins (Leong & Manahan 1997), these data might illustrate an important trade-off for pH tolerance and increased 'costs of living' in a high pCO<sub>2</sub> ocean (Stumpp *et al.* 2012b).

The down-regulation of carbonic anhydrase 12 might also be a component of acid-base compensation in *S. purpuratus*. Carbonic anhydrases catalyze the interconversion of HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> and their expressions appear sensitive to seawater acidification in diverse marine species: carbonic anhydrase 12 was also down-regulated in prism stage *S. purpuratus* (Todgham & Hofmann 2009), several isoforms decreased expression in *A. millepora* (Moya *et al.* 2012) and the abundance of glycosyl-phosphatidylinositol-linked carbonic anhydrase VII was reduced in the gills of the green crab *Carcinus maenas* (Fehsenfeld *et al.* 2011). These data imply the regulation of these enzymes may be a conserved component of adaptive responses to OA. While some carbonic anhydrases also function in biomineralization, only three of the 19 isoforms encoded in the *S. purpuratus* genome are expressed in the primary mesenchyme (of which carbonic anhydrase 12 is not one; Livingston *et al.* 2006). These data suggest most carbonic anhydrases encoded in *S. purpuratus* genome the function in pH regulation.

#### *Different responses to present day and future acidification*

Our analyses suggest that gastrula stage *S. purpuratus* use different physiological strategies for coping with different magnitudes of seawater acidification. At the High pCO<sub>2</sub> gastrulae modified the expression of only ten genes. Three of these ten genes were transport proteins consistent with previously described roles in pH regulation. However, this transcriptomic response is minimal when compared to the broad-scale shifts in gene expression (153 genes) initiated by gastrulae at the Mid pCO<sub>2</sub>. The apparent lack of transcriptomic change at the High pCO<sub>2</sub> is difficult to reconcile, but being that gastrulae initiated a robust transcriptional response to lower pCO<sub>2</sub>, the possibility that gastrulae need only modify the expression of ten genes to maintain homeostasis at an even higher pCO<sub>2</sub> seems unlikely. A speculative explanation is that acclimatization to this high pCO<sub>2</sub> is metabolically expensive and gastrulae instead limit transcriptional responses to the environment to conserve the fixed supply of maternally deposited yolk needed to complete development. If upwelling is

indeed the selective agent driving responses to pCO<sub>2</sub> in this population of *S. purpuratus*, this strategy may be feasible given upwelling-driven increases in pCO<sub>2</sub> are temporary and larvae could later recover. Regardless, these data suggest gastrulae may be sensitive to OA and raise important questions regarding the ability of *S. purpuratus* to cope with future pH regimes. While this muted response may not negatively affect short-term survival [mortality rates did not increase in *S. purpuratus* embryos cultured for 21-days under similar conditions (Stumpp *et al.* 2011a)], the minimal transcriptional response may be interrelated with non-lethal effects on physiology that ultimately reduce fitness and influence survival over longer temporal scales. Feeding stage *S. purpuratus* larvae exhibit an elevated metabolic rate and a decreased scope for growth at similar seawater pCO<sub>2</sub> (Stumpp *et al.* 2011a), results consistent with an increased 'cost of living'. It may be that gastrulae are already operating near the 'guardrails' of physiological tolerance and, contrary to our hypothesis, an ability to tolerate present day heterogeneity in pH may not necessarily translate into success in future oceans (Langenbuch & Pörtner 2004; Thomsen *et al.* 2010). The ability to apply trends regarding the pH tolerance of *S. purpuratus* to species in other environments is problematic considering the unique pH regimes encountered at Fogarty Creek, yet whether these same principles apply to sympatric species which evolved under similar pH conditions will be an important question to address in the future.

#### *Summary and future directions*

In this study, transcriptomic analyses were used to examine mechanisms of low pH tolerance in embryos and larvae of purple sea urchins inhabiting a naturally low and variable pH environment. Our data indicate that even very early life stage embryos can mount an adaptive response to elevated pCO<sub>2</sub> to sustain calcification and regulate intracellular pH. Importantly, this response occurred in the absence of an overt generalized stress response. Our gene expression data were matched with environmental data showing that the intertidal site on the Oregon coast inhabited by the parents of these larvae experience pulses of low pH water that would act as a selective force to establish local adaptation of *S. purpuratus* at this site. In summation, these data support the hypothesis that this population of urchins possesses an enhanced capacity to copewith pH variation. However, we also reveal broader-scale trends influencing the response to OA in this population, including differences in the response to elevated pCO<sub>2</sub> between developmental stages and between present day and near future pCO<sub>2</sub> scenarios.

One limitation in the current study was the inability to compare the response to acidification in urchins collected at Fogarty Creek to those collected from a less corrosive upwelling site within the Northeast Pacific and we acknowledge that much more conclusive data regarding the link between upwelling and evolved tolerance to low pH will emerge from these comparisons. At the time these experiments were performed, our knowledge surrounding natural pH dynamics in nearshore environments along the California Current was limited, preventing the identification of a site within the range of *S. purpuratus* with more stable pH regimes. Thus, this study represents a 'first cut' analysis of the physiological profile one might expect from organisms that are locally adapted to variable pH. Recently our research group has increased the number of pH sensors deployed within the California Current System and as a result we now know that *S. purpuratus* will experience considerably different pH regimes across its wide biogeographic range. In light of this information, we believe that the California Current System will emerge as a powerful system for future OA research. From a wider perspective, how OA will interact with other climate change variables in the Northeast Pacific (e.g., warming and hypoxia) will be important questions for forecasting the effects of climate change in this valuable and productive marine ecosystem (Costanza *et al.* 1997).

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### Data accessibility

Microarray data were submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under series GSE37522 and platform GPL15481.

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TGE and GEH designed the research; TGE, FC and BAM performed research; TGE, GEH, FC and BAM analyzed data and TGE, GEH, FC and BAM wrote the paper.

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### Supporting information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Representative photographs of gastrulae (A) and pluteus (B) stages sampled in this study.

**Fig. S2** Raw environmental data gathered for the study site at Fogarty Creek, OR.

**Fig. S3** Genes differentially expressed in response to the Mid pCO<sub>2</sub> treatment in gastrulae. Genes are listed to correspond to the order presented in the heatmaps shown in Fig 3B and 3D from top to bottom.

**Fig. S4** Gene set enrichment analyses (GSEA) performed on genes differentially expressed in response to pCO<sub>2</sub> in gastrulae.