TITLE:

Underlying impact of elevated carbon dioxide on manila clam (*Ruditapes philippinarum*) larvae revealed through RNA-Seq analysis

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ABSTRACT:
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Introduction

Manila clams (*Ruditapes philippinarum*) are filter feeding bivalve molluscs that have directly impact water quality and are subjected to the affects of alterations in seawater chemistry. Changing environmental conditions can negatively impact survival and as this species is of commercial importance the ramifications would be experienced on ecological and economical fronts. Manila clams are benthic infauna with a planktonic larval period lasting for approximately two weeks before the larvae settle and burrow into the sediment. Studies on manila clam larvae have documented sensitivity to several common environmental stressors such as temperature, salinity, pathogens, and food availability (Inoue et al., 2006; Xiwu et al., 2009; Numaguchi et al., 1998; Paillard et al., 2004). Studies regarding the survival and physiological processes affected by ocean acidification have been documented for other species of larvae however there are no reports concerning the impact of decreased pH and changing carbonate chemistry on *R. philippinarum* larvae.

Ocean acidification is a result of increased carbon emissions equilibrating with the dissolved CO_2 of surface seawater (Caldiera and Wickett 2003). Large scale ocean processes can result in submerged transport of these water masses. This water later resurfaces during upwelling events such as those documented off the west coast of the United States where pCO_2 levels exceeding 1000ppm have been observed (Feely et al. 2008). Ocean acidification is an immediate concern to the health of calcifying organisms and global production of shellfish (Cooley et al. 2009). The aragonite levels of this upwelled water are less than 1, which is the threshold level for which calcifying organisms can properly form carbonate structures (Feely 2008). In addition to the influx of corrosive upwelled waters, changes in the carbonate chemistry of estuaries are exacerbated by geographical and biological landscapes. Processes such as respiration, decomposition of organic matter, and freshwater runoff result in increased levels of dissolved inorganic carbon that exceed those of sea surface level predictions (Feely et al., 2010). Furthermore, carbonate chemistry in the intertidal regions such as tide pools are extremely variable (Morris et al 1982). Clam larvae settle in these environments and are consequently susceptible to changes in carbonate chemistry and potentially tolerable of wide fluctuations.

Understanding the physiological mechanisms underlying the response to increased CO_2 conditions is necessary to better understand the organismal and ecological impacts of ocean acidification. An organism's ability to tolerate changing environmental conditions depends on the ability to maintain homeostasis in a changing environment. Genes and corresponding proteins are primary regulators of cellular homeostasis. Thus, understanding physiological responses at the transcriptomic level can provide valuable insight into processes resulting in adaptation or shifts in population structure. Recent advances in sequencing technology have allowed for comprehensive molecular investigations that were not previously achievable in non-model species.

In this study, growth and mortality of larval manila clams are characterized in elevated CO_2 conditions. These analysis are coupled with the application of high-throughput sequencing to determine the transcriptomic response of elevated CO_2 conditions compared to ambient sea surface conditions. To our knowledge, this represents the first characterization of the manila clam larval transcriptome.

Materials and Methods

Experimental Design

All experimental treatments were conducted at the NOAA Northwest Fisheries Science Center (NWFSC) in Seattle, Washington. Manipulation of dissolved CO_2 concentrations was achieved by the addition of CO_2 into two independent reservoirs with CO_2 levels monitored using a Licor XXX gas analyzer and controlled using Labview Software (XXX). One system maintained near present day (ambient) oceanic sea surface levels of pCO2 (400 µatm) and the second system maintained an elevated pCO_2 concentration (1000 µatm) to represent acidified conditions. Spectrophotomectric pH was measured on each sampling day in larval chambers within each system. Water samples for total alkalinity and dissolved inorganic carbon were taken on days 3, 5, and 12 and analyzed at the NOAA Pacific Marine Environmental Laboratory (PMEL). Seawater (~30 psu) was collected from Elliot Bay, Seattle, Washington, filtered to XX µm, and maintained at 18°C for the duration of the study.

Manila clam larvae were obtained from Taylor Shellfish in Kona, Hawaii. Larvae were distributed in twelve 4.5L CO_2 impermeable chambers at a density of approximately 11 larvae/mL (48,600 larvae/chamber). Larval chambers were placed in the appropriate

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recirculating seawater treatment system (6 chambers/treatment). Average flow rate for the experiment was 3L/hour. Larval clams were fed a mixture of algae (*Nannochloris* sp., *Chaetoceros muelleri*, I*sochrysis galbana*, and *Pavlova lutherii*) twice daily at a final concentration of 50,000-80,000 cells/ml. Chambers were cleaned on a semi-weekly schedule that coincided with sampling.

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Larval mortality and size analysis

On days 1, 4, 7, 11, and 14 three larval chambers from each treatment were sampled for mortality and size analysis. Larvae were isolated on a 50um screen and two replicate samples of ~50 larvae each were transferred into 12 well plates. Consecutive sampling of jars between sampling days was avoided to maintain similar larval densities between replicates. Larval mortality was determined by counting the number of dead larvae using an inverted compound microscope at 20x magnification (manufacturer). Ethanol (75%) was then added to immobilize live larvae in order to quantify total number of larvae per well. Larval size was determined by analyzing photographs taken at 5x magnification. Total surface area for each larva was calculated using ImageJ (Rasband 1997-2011; Abramoff et al., 2004). Data collected from replicate samples within a chamber were averaged. Two way ANOVA statistical analysis was used to determine significant effects of CO2 treatments and time using SPSS statistical software.

Larvae RNA Isolation

Samples for RNA isolation were taken on day 7 from two chambers in each treatment by isolating larvae on a 50 µm screen. Samples for RNA extraction consisted of ~30,000 larvae from each larval chamber. RNA was isolated using Tri-reagent (Molecular Research Center, Inc) following manufacturer protocols. Equal quantities of total RNA (20µg) from each replicate were pooled in equal quantity for construction of two transcriptome libraries.

High-throughput sequencing

Library construction and sequencing was performed at the University of Washington High Throughput Genomics Unit (UWHTGU) on the Illumina Hi-seq platform (Illumina inc., San Diego, CA) using standard protocols. CLC Genomics Workbench version 4.0 (CLC bio) was used for all sequence analysis. Initially, sequences were trimmed based on a quality scores of

0.05 (Phred; Ewing, Green, 1998; Ewing et al., 1998) and the number of ambiguous nucleotides (>2 on ends). Sequences smaller than 25 bp were also removed.

RNA-Seq analysis

RNA-Seq analysis was carried out to determine differential gene expression patterns between the two libraries. For RNA-seq analysis RuphiBase, a transcriptome database (http://compgen.bio.unipd.it/ruphibase/) was used as the reference transcriptome. At the time of analysis this database consisted of 32,606 contiguous sequences (contigs) generated from 454 (Roche) reads (Milan et al., 2011), 5656 Sanger ESTs, and 51 publicly available mRNA sequences. Contigs in RuphiBase are annotated by Gene Ontology and protein (NCBI nr database) BLAST results. RNA-seq analysis was performed using the following parameters; unspecific match limit = 10, maximum number of mismatches = 2, minimum number of reads = 10. Expression values were measured in RPKM (reads per kilobase of exon model per million mapped reads; see Mortazavi et al., 2008). Differentially expressed genes were identified as having $\geq$ 1.5 fold difference between libraries and a p-value <0.10 (Baggerly et al., 2003). Hypergeometric tests on annotations were performed to identify enriched biological themes in the differentially expressed genes as compared to the whole transcriptome. This test procedure was performed using CLC Genomics Workbench V4.0 and is similar to the unconditional GOstats test of Falcon and Gentleman, 2007. Significantly enriched (p<0.10) GO terms and their associated p-values were uploaded to REViGO (Reduce + Visualize Gene Ontology http://revigo.irb.hr/) for visualization (Supek et al. 2011).

Treatment	pH	TA (µmol/kg)	DIC (µmol/kg)	ΩAr	ΩCa	CO3-- (µmol/kg-SW)	HCO3- (µmol/kg-SW)	pCO2 (µatm)
Ambient	8.07±.02	2068.09±21.43	1865.73±9.82	2.31±.12	3.63±.19	146.25±8.0	1706.98±4.05	355.04±16.85
Elevated	7.71±.02	2069.46±21.87	1996.43±12.85	1.12±.08	1.76±.12	71.09±5.1	1893.68±9.56	897.74±47.61

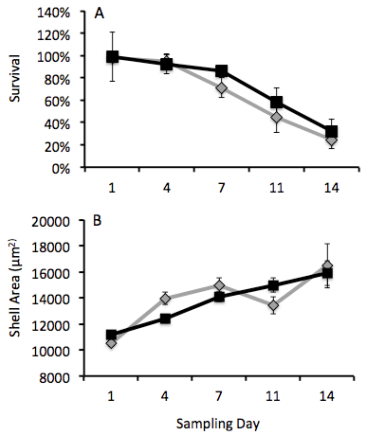
Water Chemistry

Ambient conditions were maintained at an average of 355.04 µatm with a pH of 8.07. The elevated treatment maintained at an average of 897 µatm with a pH of 7.71. Aragonite levels dropped from 3.63 in the ambient treatment to 1.12 in the elevated pCO_2 treatment,. Similarly, the concentration of calcite decreased from 3.63 in ambient to 1.76 in the elevated pCO_2 treatment. All water chemistry parameters for both the ambient and elevated treatment are available in **Table 1**.

Larval growth and survival

There was no detectable effect of elevated pCO_2 conditions on *R. philippinarum* growth and survival (**Figure 1**). Larval shell size increased steadily in both treatments over the course of the experiment.

Fig1



RNA-Seq analysis

After quality trimming, 240 million sequence reads (average length: 36 bp) remained from the combined transcriptome libraries. All data are available in the NCBI Short Read Archive database (Sample ID: SRS283130). RNA-Seq using Ruphibase as the scaffold identified 3954 differentially expressed contigs. Of those,162 contigs were expressed at a lower level in the library constructed form larvae exposed to high pCO2 conditions, and 3792 were expressed at an elevated level (**Supplemental Table 1**). A subset (781) of the differentially

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expressed contigs were annotated in Ruphibase. Hypergeometric tests on annotations revealed 55 biological processes to be significantly enriched in the differentially expressed gene set. The most significantly enriched processes were associated with translation followed by development and hydrogen peroxide catabolism (**Figure 2**), Other enriched processes of note include ATP synthesis coupled proton transport and respiratory electron transport chain (Figure 2). Contigs corresponding with enriched biological processes are denoted (*) in **Supplemental Table 1**.

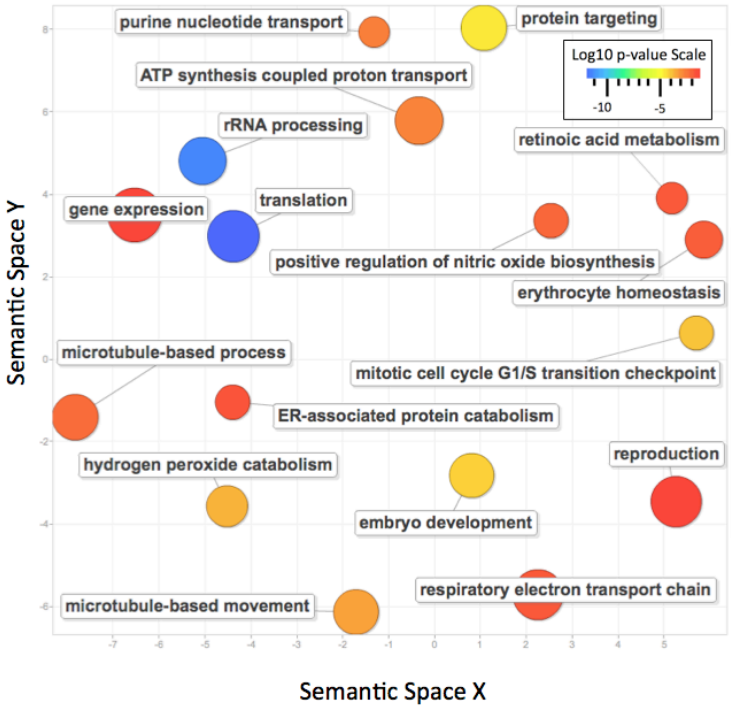

Figure 2.

Discussion

Increased dissolution of atmospheric carbon dioxide compounded by natural processes such as coastal upwelling and estuarine runoff are contributing to dramatic changes in estuarine water chemistry (Feely et al 2010). Resulting conditions could be potentially devastating to local organisms, particularly marine calcifiers (Caldiera and Wickett 2003). While there have been a number of studies on the effects of ocean acidification on shellfish, studies investigating the

influences of increased CO2 at the transcriptome level are limited. To our knowledge this is the first report of using high throughput sequencing technology to evaluate the physiological effects of elevated pCO_2 n a bivalve mollusc. While no impact on larval growth and mortality was observed in this study, a number of biological process were identified as influenced by elevated carbon dioxide. The combined results reported here provide important information on how shellfish respond to ocean acidification and demonstrate species specific responses to ocean acidification.

Examination of *R. philipinarum* larvae in this study revealed no effect of elevated pCO2 on growth and survival. These results are consistent with a recent study in the juvenile clam *Ruditapes decussatus* where no difference in growth was observed in reduced pH environments (Range et al., 2011). Likewise, no mortality in juvenile *R. decussatus* was reported (Range et al., 2011). These results are in contrasts to a majority of other studies in bivalves where it has been shown that ocean acidification negatively affects larval survival (Fabry et al., 2008; Raven et al., 2005; Talmage and Gobler 2009). Negative impacts reported for other species include reduced growth rate (Michaelidis et al, 2005) reduced calcification and shell formation (Orr et al, 2005; Gazeau et al, 2007; McDonald et al, 2009; Miller et al, 2009) and reduced respiration rates and metabolism (Michaelidis et al, 2005). Larval tolerance to increased CO_2 is most likely taxa and age dependent. It should be noted that this study was designed to assess the physiological response of manila clam larvae to an acute change in carbonate chemistry at a state in which they were fully calcified. Analysis of fertilization rates and the immediate survival after fertilization may not coincide with the results presented in this study. These data do suggest that the impact of transient exposure to acidified water by planktonic larvae would not significantly impact manila clam larvae survival.

While no impact on growth or survival was identified during this study, the altered environmental conditions associated with ocean acidification did significantly alter gene expression patterns as revealed by RNA-Seq analysis. Overall, a majority of the differentially expressed genes were upregulated in larvae exposed to elevated pCO_2 and the most significantly enriched biological processes were associated with translation and ribosomal activity. The physiological plasticity necessary for an organism to respond to environmental stress is largely dependent on the ability to effectively regulate expression of genes required to maintain homeostasis (Somero 2009; Gracey 2007). Increased expression of genes associated with translational activity in larvae exposed to elevated CO_2 could indicate an overall increase in

protein translation and modification associated with multiple signaling pathways directly or indirectly responding to changing conditions. Alternatively, the increased expression of these genes could be in response to replenishing damaged protein products. Based on the fact that 96% of the genes differentially expressed across several biological processes were in fact upregulated this would suggest that increased expression of genes associated with protein translation (and gene expression) are evidence of the general physiological response of the larvae necessary to maintain homeostasis. Regardless of the primary reason for the up regulation of this suite of genes, protein expression is an energetically demanding process that could paradoxically impair other critical physiological processes. It is therefore plausible that physiological stress of elevated CO_2 could have effects that might not become apparent until a later stage of development. For instance if resources (ATP) are limited, larvae might not be able to mount an effective immune response if exposed to a pathogen. Interestingly, this significant upregulation of genes involved in translational activity is in contrast to previous reports in larval sea urchins in which an overall decrease was observed (O'Donnell et al 2008). This difference could be related to development stage or simply differences in taxonomic lineages.

Another biological process that was found to be enriched in the suite of differentially expressed genes was ATP coupled proton transport. Genes associated with ATP coupled proton transport are involved in several vital biological processes such as the generation of ATP (Senior et al., 2002), maintenance of hemolymph pH (Byrne and Dietz, 1997), and regulation of ion concentrations involved in calcification (McConnaughey and Gilikin, 2008; Liang et al., 2007). Synthesis of ATP is vital for proper function of essential physiological processes. Increased expression of H+ transport ATP synthase subunits could lead to an increase in ATP production and thus provide the energy needed to compensate for propensity of for shell dissolution under acidified conditions. Maintenance of hemolymph pH would also be required under decreased seawater pH conditions. In fact it has been suggested that Iregulation of extracellular acid-base balance can determine a species' tolerance to elevated CO2 (Widdicombe and Spicer, 2008). These genes are also involved in the regulating ion transport including calcium. Increased expression of genes in associated with ATP coupled proton transport could be indicative of the organism increasing Ca+ scavenging efforts as a result of decreases in calcite concentrations. Similarly, other proteins such as calmodulin are involved in scavenging and detecting Ca+ ions. Calmodulin is known to be involved in Ca+ metabolism and calicification in bivalves (Li et al., 2005). Calmodulin is regulated by G protein beta-subunit, both of which are expressed higher in larvae exposed to elevated CO_2. Further evidence to support

the theory that manila clam larvae are able to effectively compensate for the decreased availability of calcium carbonate is the 133-fold increase in Perlucin -6 in larvae exposed to elevated pCO_2. Perlucin-6, a gene involved in nucleation of calcium carbonate ions during shell formation (Blank et al., 2003; Hoffman et al., 2008). While more research is required to illustrate the concerted regulation of these genes, transcriptomic analysis provides a powerful approach to begin to tease apart the regulatory pathways that are responsible for an organisms ability to tolerate stressful conditions.

Currently there is little information regarding the transcriptome level processes altered by increased CO_2 conditions in larval shellfish. Studies to date have focused on candidate genes that are predicted to be important components of processes such as calcification and metabolism. As molecular pathways typically function in a network of connections, a transcriptome wide approach provides valuable insight into the underlying molecular processes responsible for observed phenotypic response. In addition to characterizing the transcriptomic response and the associated physiological process of *R. philippinarum* larvae to increased CO_2 conditions, this study also illustrates that while standard morphometric and survival analysis might indicate tolerance to environmental stresses, characterizing the underlying molecular response can reveal novel processes. For instance, while there was no detectable affect of increase CO_2 on larval development based on the methods in this study, analysis of differentially regulated genes illustrates and enrichment of genes involved in developmental processes. This suggests that while there may be no observable effect of increase CO_2 during these stages, prolonged effects may persist and future studies should focus on taking an integrative approach to assessing the biological impact of ocean acidification.

Acknowlegements

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Table1

Summary of water chemistry measurements for ambient and elevated pCO2 treatments. Values are expressed as means ± standard deviations.

Fig1

Percent larval survival at ambient (diamonds) and high (squares) pCO_2conditions on days 1, 4, 7, 11, and 14 +/- SE (n=3/day)

Fig2

Visualization of enriched GO processes identified from significantly regulated genes. Distance between processes represents relatedness while size of the circles is represents –log10 p-value of the GO term enrichment