

Characterizing the effects of ocean acidification in larval and juvenile Manila clam,
Ruditapes philippinarum, using a transcriptomic approach

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Abstract

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Ocean acidification as a result of anthropogenic carbon dioxide (CO₂) emissions and global climate change poses a risk to the ecological landscape of intertidal and shallow subtidal communities. The organisms that inhabit these waters will have to cope with changing environmental conditions through the appropriate modulation of physiological processes. Calcifying organisms are particularly at risk, as increased atmospheric levels of CO₂ in the atmosphere increase the partial pressure of CO₂ (pCO₂) in the oceans. Increased pCO₂ reduces the saturation of carbonate minerals required to form calcified structures. Being able to cope with the increased energetic demand of maintaining these structures, in addition to other vital physiological processes, will be the key driver that determines which organisms will persist. Assessment of larval and juvenile Manila clam mortality and physiology in this study suggests that this species is capable of coping with elevated pCO₂ conditions. The use of high throughput sequencing and RNA sequence analysis in larval clams revealed several physiological processes that play important roles in the Manila clam's ability to tolerate elevated pCO₂ conditions during this life stage. Exposure of juvenile Manila clams, acclimated to elevated pCO₂ conditions, to a thermal stress revealed that this species might also be capable of coping with multiple stressors associated with global climate change. Manila clams could therefore represent a model for studying physiological mechanisms associated with successful acclimation of populations to ocean acidification.

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CHAPTER I

Introduction

The Manila clam (*Ruditapes philippinarum*) is an ecologically and commercially important bivalve mollusc found along the west coast of North America, Europe, and throughout Asia. Adult Manila clams are infaunal and produce planktonic larvae that remain in the water column for about two weeks. The larval period is considered a particularly vulnerable life stage given the small size, lack of a robust shell, and degree of morphological and physiological changes experienced during this period. Changes in environmental conditions that negatively impact larval survival could have ecosystem-wide consequences and disrupt aquaculture production. Studies on Manila clam larvae have documented sensitivity to several stressors including temperature, salinity, pathogens, and food availability (Numaguchi 1998; Paillard 2004; Inoue et al. 2007; Yan et al. 2009), however there are no studies investigating the impact of ocean acidification.

Ocean acidification refers to the reduced pH in oceans as a result of increased anthropogenic CO₂ emissions (Caldeira and Wickett 2003; Feely et al. 2004; Sabine et al. 2004). Increasing partial pressure of CO₂ (pCO₂) in seawater alters the carbonate chemistry, effectively reducing the saturation state of biologically important carbonate compounds such as aragonite and calcite (Feely et al. 2004). Coastal waters are of particular concern as other natural and anthropogenic processes can exacerbate these changes in water chemistry (Feely et al. 2008 and 2010). Environmental conditions associated with ocean acidification could be devastating to local organisms, particularly marine calcifiers that use carbonate minerals to form shells (Caldeira and Wickett 2003). Knowledge of the physiological mechanisms underlying species response to elevated pCO₂ conditions will provide a better understanding of the organismal and ecological impacts of ocean acidification (Pörtner 2008; Widdicombe and Spicer 2008).

The use of transcriptomic approaches can provide valuable information of the organismal response to changing environmental conditions. Previous studies have investigated the impact of ocean acidification on gene expression. Many of these studies have focused on sea urchins, which have significant genomic resources. One technique is to select a set of target genes and perform quantitative PCR analysis. This method has

been used in sea urchins to characterize a suite of genes involved in stress response, biomineralization and metabolism (O'Donnell et al. 2009; Stumpp et al. 2011, Martin et al. 2011). Todgham and Hofmann (2009) and O'Donnell et al. (2010) used a more global approach examining 1,057 sea urchin genes on a microarray platform providing insight into the transcriptomic response of critical processes including acid-base balance and biomineralization. Most recently, Moya et al (2012) utilized Illumina RNA sequencing and RNA-Seq (Wang et al. 2009) to characterize the transcriptome of the coral *Acropora millepora*. Consistent with previous microarray studies by Todgham and Hofmann (2009) and O'Donnell et al. (2010), the authors noted a majority of transcripts were decreased in corals exposed to elevated CO₂, however, the authors noted a more variable response of taxon-specific genes involved in biomineralization and skeletal organic matrix (Moya et al. 2012).

Advancements in high throughput sequencing and bioinformatic analysis have allowed for the transcriptomic characterization of organisms with limited genomic information. Examples include the characterization of differentially expressed genes in Pacific oyster populations (Gavery and Roberts 2011), transcripts involved in shell deposition in arctic clam (Clark et al. 2010), and biological processes associated with thermal stress and development in coral (Meyer et al. 2011). A value in this approach is that there are no *a priori* assumptions concerning the physiological response to a particular treatment or condition. This characteristic is particularly advantageous in characterizing the effects of ocean acidification as studies in other taxa have shown that there is often a reallocation of resources that might not be readily evident. For example, in brittle stars increased calcification in response to elevated pCO₂ resulted in a decrease in muscle mass (Wood et al. 2008), while in mussels, a decrease in calcification was associated with an increase in energy demand required for maintaining pH homeostasis (Thomsen and Melzner 2010).

In this study, growth, mortality, and the underlying transcriptomic response of larval Manila clams are characterized in elevated pCO₂ conditions. Characterization of the underlying processes affected by elevated pCO₂ will provide valuable information towards elucidating the physiological response of Manila clam larvae to ocean

acidification, and insight for future research investigating exposure of bivalves to elevated pCO₂ conditions.

Materials and Methods

Recirculating seawater system

This experiment was conducted at the NOAA Northwest Fisheries Science Center in Seattle, Washington in a laboratory designed to study the impacts of ocean acidification on marine resources (McElhany et al. in prep). Seawater for the experiment was collected from Elliott Bay near Seattle and filtered to 2 μm, degassed using Liqui-Cel membrane contactors (Membrana, Weppertal, Germany) under partial vacuum, and added to individual recirculating seawater systems. Each system contained a gas exchange reservoir (492L) in which ambient or elevated pCO₂ conditions were created by bubbling CO₂, air, or CO₂-free air in seawater, with gas additions controlled by a program built in LabView software (National Instruments, Austin, TX). This program adjusted gas additions to maintain a treatment pH that equates to our target pCO₂, given seawater temperature (18°C), salinity (29.4psu), and alkalinity (2069μmol/kg). Water chemistry in each system was continuously monitored using a Durafet pH electrode, dissolved oxygen transmitter, and conductivity and temperature probes (Honeywell Process Solutions Bracknell, Berkshire, UK) in a reservoir separate from the gas exchange reservoir. Gas-equilibrated seawater flowed into six replicate, 4.5L, CO₂-impermeable larval chambers per treatment with an average flow rate of 3L/hour. Water was delivered through the base of each chamber and exited through a port in the lid that was covered by a 50μm bag filter to retain larvae. Effluent from the chambers and water bath was pumped through a 2μm filter, UV treatment and heat pump before returning to the gas exchange reservoir. Water from each treatment system was continuously exchanged with the laboratory's main reservoir (103% of each treatment exchanged/day), so that no treatments were isolated.

Experimental Design

One system maintained near present day (ambient) oceanic sea surface levels of pCO₂ (355±16.85μatm pCO₂; pH 8.07±0.02) and the second system maintained an

elevated pCO₂ concentration (897.74±47.61 μatm pCO₂; pH 7.71±0.02) resulting in a difference of 0.36 pH units, a change that is expected to occur by the year 2100 (Caldiera and Wickett, 2005). Spectrophotometric pH was measured on each sampling day in larval chambers within each system. Spectrophotometric pH was also used to confirm Durafet pH probe readings. 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). All samples were analyzed according to the Department of Energy guidelines (DOE 1994). A summary of the carbonate chemistry parameters monitored during this experiment is provided in Table 1.

Manila clam larvae (5 days old) were wrapped in moist cheesecloth and shipped overnight from Taylor Shellfish in Kona, Hawaii. Larvae were distributed in twelve 4.5L chambers containing ambient pCO₂ seawater to a density of approximately 11 larvae/mL (48,600 larvae/chamber). Larval chambers were placed in the appropriate recirculating seawater treatment system (6 chambers/treatment) where seawater containing either 400 μatm (ambient) or 900 μatm (elevated) pCO₂ was distributed to each chamber. Larval clams were fed a mixture of algae (*Nannochloris sp.*, *Chaetoceros muelleri*, *Isochrysis 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.

Larval mortality and size analysis

Three larval chambers from each treatment were sampled for mortality and size analysis on days 1, 4, 7, 11, and 14 of the study. To minimize potential impacts of reduced larval densities, consecutive sampling of chambers between sampling days was avoided. Sampling consisted of concentrating all larvae from a chamber on a 50 μm screen and adding 50 mL of the appropriate seawater. Two replicate samples of ~50 larvae each were then transferred into 12 well plates for subsequent mortality counts and size analysis. Larval mortality was determined by counting the number of dead larvae using an inverted compound microscope at 20x magnification (Nikon). Ethanol (75%) was then added to immobilize live larvae in order to count the 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. T-tests were conducted to compare mean larval sizes between treatments on each sampling day. Statistical significance was determined based on Bonferroni corrected alpha values (≤ 0.01). Differences in survival were assessed by generating the slope of the regressions based on larval abundance in each individual larval chamber. A generalized linear model was then applied to calculated slope values on each sampling day. All statistical analyses were conducted using SPSS statistical software (IBM, Somers, NY)

Larval RNA Isolation

Samples for RNA isolation were taken on day 7 by straining larval chambers onto a 50 μ m screen to remove seawater. Samples were snap frozen in liquid nitrogen and stored at -80°C. Two chambers from each treatment were harvested in this manner for a total of four samples consisting of ~30,000 larvae each. RNA was isolated using Tri-reagent (Molecular Research Center, Inc) following manufacturer protocols. Equal quantities of total RNA (20 μ g) from the replicate samples for each treatment were pooled and used for the 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 HiSeq platform (Illumina Inc., San Diego, CA) using standard protocols as outlined by the TruSeqTM RNA Sample Preparation Guide (part# 15001836 Rev A) and the HiSeq 2000 User Guide (part# 15011190 Rev. K). CLC Genomics Workbench version 4.0 (CLC bio) was used for all sequence analysis. Initially, sequences were trimmed based on a quality score of 0.05 (Phred; Ewing and Green, 1998; Ewing et al. 1998) and the number of ambiguous nucleotides (>2 on ends). Sequences smaller than 25bp were also removed.

Reference assembly

For the reference assembly, RuphiBase, a transcriptome database for *R. philippinarum* (<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 Expressed Sequence Tags (ESTs), and 51 publicly available mRNA sequences. Contigs in RuphiBase are annotated by Gene Ontology (GO) and protein (NCBI nr database) BLAST results. Sequences from the ambient and elevated pCO₂ library treatments were combined and mapped to the Ruphibase transcriptome database using the following parameters: ungapped alignment, mismatch cost = 2, limit = 8.

RNA-Seq analysis

RNA-Seq analysis was carried out to determine differential gene expression patterns between the two libraries. RNA-Seq 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 ≥ 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 processes. This test procedure was performed using CLC Genomics Workbench v4.0 and is similar to the unconditional GStats test of Falcon and Gentleman (2007). Significantly enriched (p < 0.10) GO terms and associated p-values were visualized using REVIGO (Reduce + Visualize Gene Ontology) (Supek et al. 2011).

Results

Larval growth and survival

Survival and growth of *R. philippinarum* were similar at both pCO₂ treatments (p >0.05) (Figure 1). Larval shell size increased at similar rates in both treatments over the course of the experiment. No difference was detected in larval size between pCO₂ treatments. Larval survival decreased at similar rates between pCO₂ treatments and no difference in survival was detected.

Characterization of short read sequences

A total of 244,082,559 reads were generated with an average length of 36 bases. After quality trimming, 99.7% of the reads were retained (Table 1). All data are available in the NCBI Short Read Archive database (Sample ID: SRS283130). Reference assembly of reads from the Illumina HiSeq libraries were mapped to 84% of the contigs in Ruphibase. Average coverage for the assembly was 17x with an average number of reads of 133 (Figure 2).

RNA-Seq analysis

RNA-Seq using Ruphibase as the scaffold identified 3954 differentially expressed contigs. Of those, 162 contigs were expressed at a lower level in larvae exposed to elevated pCO₂ conditions, and 3792 were expressed at an elevated level (Figure 3). Among differentially expressed contigs, 204 were expressed over 10-fold higher in elevated pCO₂ conditions including the calcification gene *perlucin 6*, which was expressed 133 fold higher under elevated pCO₂ conditions. Only 8 contigs were expressed 10-fold lower under elevated pCO₂ conditions. A complete list of all differentially expressed genes is provided in Supplemental Table 1.

Gene enrichment analysis

Differentially expressed contigs that were annotated in Ruphibase (n=781) were subjected to enrichment analysis to identify enriched biological processes. Hypergeometric tests revealed 55 biological processes significantly enriched in the differentially expressed gene set. The most enriched processes were associated with translation followed by development, hydrogen peroxide catabolism, ATP synthesis-coupled proton transport, and respiratory electron transport chain (Figure 4). Contigs corresponding with enriched biological processes are denoted (*) in Supplemental Table 1.

Discussion

Growth and survival of *R. philippinarum* larvae were similar between the pCO₂ treatments. These results are consistent with a recent study in juvenile *Ruditapes decussatus* for which no difference in growth or mortality was observed in elevated pCO₂

environments (Range et al. 2011). In contrast, a majority of studies in larval bivalves have demonstrated that ocean acidification negatively affects survival, growth, and physiology (Michaelidis et al. 2005; Orr et al. 2005; Gazeau et al. 2007; Talmage and Gobler 2009; McDonald et al. 2009; Miller et al. 2009). Larval tolerance to increased $p\text{CO}_2$ is likely taxa and age dependent. This study was designed to assess the physiological response of Manila clam larvae with fully calcified shells to an acute change in carbonate chemistry. Analysis of parameters such as fertilization rates, or the response of trochophore and early veliger stage clam larvae could reveal an impact of altered $p\text{CO}_2$ conditions. Similar growth patterns under the two $p\text{CO}_2$ conditions in the current study suggests that the impact of transient exposure of mid-stage veliger larvae to acidified water would not significantly impact Manila clam larval survival.

Elevated $p\text{CO}_2$ significantly altered gene expression patterns in Manila clam larvae, as revealed by RNA-Seq analysis. Increased global gene expression (Figure 3) as well as the enrichment of genes associated with translational activity (Figure 4; Supplemental Table 1), are consistent with an increase in the protein synthesis necessary to maintain homeostasis in an elevated $p\text{CO}_2$ environment. Increased expression could also be a response to repairing or replenishing damaged protein products. Regardless of the primary reason for the upregulation of this suite of genes, protein synthesis is an energetically demanding process that could impair other critical physiological processes by limiting available energy resources. It is possible that the effects of the physiological stress induced by elevated $p\text{CO}_2$ might become apparent only in a later stage of development. Thus any species fitness predictions based solely on the absence of an impact on larval growth and survival should be regarded with caution.

Another group of differentially expressed genes identified are associated with ATP-coupled proton transport (Figure 4). ATP-coupled proton transport is an integral part of the electron transport chain and the generation of ATP (Senior et al. 2002). These genes are also involved in several other vital biological processes including the maintenance of hemolymph pH (Byrne and Dietz 1997), and regulation of ion concentrations involved in calcification (Liang et al. 2007; McConnaughey and Gillikin 2008). Maintenance of hemolymph pH would be required under decreased seawater pH conditions as bivalves possess an open circulatory system. In fact it has been suggested

that the ability to control extracellular acid-base balance can determine species tolerance to elevated pCO₂ levels (Widdicombe and Spicer 2008). ATP-coupled proton transport genes are also involved in regulating ion transport, including Ca²⁺. Increased expression of genes associated with ATP-coupled proton transport could be indicative of an organism increasing scavenging efforts as a result of reduced calcium carbonate ions. It is possible that by increasing scavenging efforts, Manila clams could increase their tolerance for lower concentrations of calcium carbonate, as they would be more adept at obtaining these molecules at lower concentrations.

Similarly, other proteins such as calmodulin are involved in scavenging and detecting Ca²⁺ ions. Calmodulin is known to be involved in Ca²⁺ metabolism and calcification in bivalves (Li et al. 2005). Calmodulin, and a regulator of calmodulin, G protein beta-subunit, were expressed at a higher level in larvae exposed to elevated pCO₂ concentrations (Supplemental Table 1). In addition, expression of *perlucin 6*, a gene involved in nucleation of calcium carbonate ions during shell formation (Blank et al. 2003; Hofmann et al. 2008) was 133-fold higher in larvae exposed to elevated pCO₂. Coordinated expression of these calcium-associated genes may be essential for larval tolerance of elevated pCO₂ conditions

Regulation of protein synthesis occurs on multiple levels including gene expression, translation and post-translational modifications, and it should be noted that changes in transcription do not necessarily correlate to changes in the corresponding protein concentration or activity (Feder and Walser 2005; Tomanek 2011). In fact, it is likely that responses to OA are regulated at multiple levels. For instance, a study in larval barnacles found that elevated pCO₂ induced changes in protein concentrations and posttranslational modifications (Wong et al. 2011). Thus, an integrated approach to evaluate physiological responses at both the transcriptional and translational level will improve our understanding of how species will respond to environmental perturbation.

In summary, this study illustrates that transcriptomic characterization provides important insight into organismal processes affected by elevated pCO₂ conditions that are not necessarily apparent with standard morphometric and survival analysis. Furthermore, these data indicate that increased translational activity and specific process associated with ion transport could contribute to short-term resilience in clams. Further research is

needed to determine how these transcriptomic changes and elevated pCO₂ will impact Manila clams at different developmental stages and if acute exposures to elevated pCO₂ has a long-term influence on survival

CHAPTER II

Introduction

Atmospheric carbon dioxide (CO₂) levels have increased from 280 parts per million (ppm) prior to the industrial revolution to present day levels of 400ppm, higher than they have been in the past 800,000 years (Lüthi et al. 2008). While historic levels of atmospheric CO₂ concentrations are known to fluctuate (Tyrrell, 2008), the current rate at which CO₂ levels are increasing is unprecedented (Caldeira and Wickett, 2003) at 0.5% per year (Orr et al. 2005). Increasing atmospheric levels of CO₂ are expected to increase global temperatures by 2 to 5°C (Houghton et al. 2001) and impact the carbonate chemistry of seawater (Feely et al. 2004, 2008).

Oceans have absorbed roughly one third of the anthropogenic CO₂ emissions (Sabine et al. 2004). When carbon dioxide from the atmosphere mixes in the oceans, it reacts with carbonate ions and water to form hydrogen and bicarbonate ions (Feely et al. 2004; Orr et al. 2005). By converting carbonate to bicarbonate, CO₂ dissolution decreases the saturation state of calcium carbonate minerals such as aragonite and calcite, and increases the concentration of free hydrogen ions causing the water to become more acidic (Zeebe and Wolf-Gladrow, 2001). By decreasing the saturation state of calcium carbonate, fewer calcium carbonate molecules are available for calcifying organisms to form shells. If the current rate of fossil fuel emissions persists, atmospheric levels of CO₂ will reach 750-1000ppm by 2100, corresponding to a pH decrease of 0.3-0.5 units in the oceans, a process known as “ocean acidification (Intergovernmental Panel on Climate Change 2007; Zeebe and Wolf-Gladrow 2001, Caldeira and Wickett 2005).

Elevated pCO₂ conditions can be found along coastal waters due to processes such as upwelling. Upwelling causes a shoaling of CO₂-enriched and carbonate deficient waters closer to coastal surface waters. While the source of CO₂ in these corrosive waters is largely due to natural processes such as respiration and decomposition of organic matter, surface waters enriched with anthropogenic CO₂ from the atmosphere exacerbate

shoaling of carbonate deficient waters. Consequently, recent observations in upwelling zones, such as those along the California coast, have recorded pCO₂ levels in surface waters exceeding levels not predicted to occur until 2050 (Feely et al. 2008). It takes approximately 50 years for water to cycle from the surface through deeper ocean currents and back to the surface again, suggesting that future upwelling events will contain higher levels of anthropogenically enriched CO₂ (Feely et al. 2008). Upwelling events are caused by strong northerly winds along eastern Pacific and Atlantic oceans that facilitate Ekman transport of surface waters away from the coasts allowing carbonate deficient ocean waters to rise to the surface. These winds are predicted to increase as a result of global warming, further increasing the intensity of upwelling events (Bakun 1990; Lachkar and Gruber 2012). Populations that inhabit these coastal waters are thought to be at risk from ocean acidification, particularly those dependent on calcium carbonate structures for stability, defense, and survival (Fabry et al. 2008; Cooley and Doney, 2009). For example, recent studies at shellfish hatcheries have correlated upwelling with increased larval oyster mortalities (Elston et al. 2008; Barton et al. 2012).

Temperature fluctuations associated with increased CO₂ emissions are also predicted to have a greater impact on coastal communities as these shallower waters equilibrate more readily to changes in atmospheric temperature (Levitus et al, 2000; Nixon et al. 2004). Concurrent changes in temperature and carbonate chemistry can have additive, synergistic, or antagonistic effects on physiological processes (Folt et al. 1999; Darling and Cote 2008; Hofmann and Todgham 2010). Depending on the species and developmental period, the mechanism of combined stressors can vary (Pörtner 2008). For example, antagonistic effects of combined thermal and elevated pCO₂ conditions were observed in the tropical sea urchin, *Tripneustes gratilla*, where elevated pCO₂ conditions reduced calcification and nullified the positive growth impact of warmer temperatures (Brennand et al. 2010). Combined high pCO₂ and thermal treatments increased mortality of larval red abalone, *Haliotis rufescens*, compared to either treatment alone (Zippay and Hofman 2010). A multispecies study among bivalves showed a variable effect of elevated pCO₂ and warmer temperatures on growth and survival of larval and juvenile eastern oysters, *Crassostrea virginica*, hard clams *Mercenaria mercenaria*, and bay scallops, *Argopecten irradians* (Talmage and Gobler 2011). Variability among species

may be due, in part, to differences in life history among species (Talmage and Gobler 2011). Intertidal and shallow subtidal species may already be functioning close to their physiological limits and thus may be more susceptible to changing environmental conditions as a result of climate change (Tomanek 2008; Somero 2010; Peck et al. 2009, 2010; Christensen et al. 2011). Alternatively, organisms inhabiting these ecosystems may be more adapted to harsh conditions. Detecting a shift in the physiological limitations, particularly in thermal tolerance, induced by elevated pCO₂ levels, will provide insight into an organism's ability to cope with changing conditions and potential changes to the ecological landscape of coastal communities.

The Manila clam, *Ruditapes philippinarum*, is an economically and ecologically important species (Dumbauld et al. 2009). Manila clams are indigenous to the Philippines, South China and East China Seas, and up to the Sea of Okhotsk and the southern Kuril Islands (Scarlato, 1981). Since being accidentally introduced to the west coast of the United States in the 1930's (Magoon and Vining, 1981), they have become an economically important aquaculture species (Dumbauld et al. 2009). Manila clams are tolerant of a wide range of temperatures and salinities (Numaguchi 1998), however, little is known concerning their tolerance to ocean acidification (López et al. 2010; Metzger et al. in review).

The objective of this study was to examine the impact of elevated pCO₂ on juvenile Manila clam physiology. Transcription of candidate genes involved in calcification, translation, stress response, and oxidative stress are measured during the acclimation period of juvenile clams to elevated pCO₂ conditions. In addition, the lethal temperature (LT) for juvenile Manila clams was determined and the effect of elevated pCO₂ conditions on juvenile Manila clam thermal limits was assessed. If ocean acidification alters physiological processes necessary for Manila clams to cope with thermal fluctuations, an increase in mortalities would be expected at lower temperatures compared to ambient pCO₂ conditions. However, if clams were able to cope with ocean acidification, utilizing mechanisms not associated with thermal stress response, increased susceptibility to lower temperatures would not be observed.

Materials and Methods

Experimental design

Juvenile Manila clams (mean±SD; length =13.9mm±2.1; width=18.3mm±2.7; wet weight = 1.45g±0.6) were obtained from the Taylor Shellfish hatchery in Quilcene, WA and transported to the ocean acidification facility at the University of Washington Friday Harbor Laboratories. Clams were exposed to seawater equilibrated to ambient (400µatm; pH 8.03) or elevated (1000µatm; pH 7.67) pCO₂ levels. Gas equilibration was achieved by stripping seawater of CO₂ using a membrane contactor under vacuum pressure. Pure CO₂ gas was then mixed with CO₂-free air using gas proportionators. The prepared gas mixtures were then equilibrated with seawater using solenoid valves attached to Venturi injectors. Dissolved CO₂ was monitored by measuring pH using a Honeywell controller connected to a Durafet pH probe adjusted to maintain the desired pH. Total alkalinity measurements were performed prior to the addition of animals to the system and once per week following the addition of animals. Additionally, spectrophotometric pH samples were taken daily. Carbonate chemistry measurements, including partial pressure CO₂ as well as aragonite and calcite saturation, were calculated from alkalinity, spectrophotometric pH, and salinity using the CO₂ calculator “CO₂Calc” (Robbins et al. 2010) with the following parameters: physical data parameters °C=25, adjusted conditions °C= 13, CO₂ constants: Lueker et al. 2000, KHSO₄: Dickson (1990b), pH Scale: Total scale (mol/kgSW), Air-Sea Flux: Wanninkhof, 1992.

Each experimental treatment contained 8 replicate 3L chambers maintained at a constant temperature of 13°C and a flow rate of 1.9L/hr. Each chamber contained 10 clams for a total starting number of 80 juvenile clams for each treatment. At the end of each week, one clam from each chamber was sacrificed and gill tissue dissected and flash frozen. A total of eight clams were sampled from each pCO₂ treatment each week leaving a total of 56 clams at the end of the three-week sampling period. After the three weeks the remaining clams were exposed to a temperature stress.

Clams were transferred to a temperature-equilibrated treatment water bath and exposed for one hour at one of the following temperatures: 38, 39, or 40°C. During thermal treatments, clams were completely submerged in their designated pCO₂ treatment seawater. Clams were then returned to 13°C at their respective pCO₂ treatment conditions

and mortality was monitored for one week. A total of 14 clams (two replicate groups of seven animals) were used for each pCO₂ and temperature combination. Mortality was assessed based on gaping behavior. Clams that failed to close their shells in response to mechanical stimulation were considered moribund.

RNA extraction and cDNA synthesis

RNA was extracted from gill tissue using TriReagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's recommended protocol. Total RNA was DNase treated (DNA Free kit; Ambion, Austin, TX, USA) following the manufacturer's rigorous protocol to remove potential DNA carryover from RNA extractions. Purified RNA was quantified using a Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies Inc., Rockland, DE). Reverse transcription reactions were conducted using M-MLV reverse transcriptase (Promega, Madison, Wisconsin) and 0.5ug of total RNA to generate complementary DNA (cDNA).

Quantitative PCR analysis

Primers for quantitative PCR (qPCR) analysis were generated using Primer3 software (Rozen and Skaletsky, 2000) from sequences provided in the Manila clam transcriptome database (RuphiBase, <http://compgen.bio.unipd.it/ruphibase>). Primer sequences are provided in Table 2. All primers were ordered from IDT (Coralville, IA, USA).

Quantitative PCR reactions were carried out in 20ul reaction volumes consisting of 1x Ssofast EvaGreen Supermix (Bio-Rad, Hercules, CA), 0.2 μM of each primer, and 2ul of diluted (1:5) cDNA. Amplification reactions were carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with the following cycling parameters: 98°C 2min, followed by 40 cycles of 98°C for 2sec, 60°C for 5sec. Melt curve analysis was performed after cycle 40 by increasing the annealing temperature from 65°C to 95°C in 0.2°C increments and measuring fluorescence at each increment. All samples were run in duplicate. Efficiencies of qPCR reactions were calculated using PCR miner software (Zhao and Fernald, 2005). Expression values were calculated using the following equation: $1/(1+\text{Efficiency})^{\text{Ct}}$. Calculated expression values were then normalized to elongation factor-1 alpha (ef-1α). Ef-1α is a commonly used normalizing gene and has

previously been used as a reference gene in CO₂ manipulation (O'Donnell et al. 2009). The stability of ef-1 α was confirmed for this by a two-way ANOVA analysis, which showed no significant difference in ef-1 α between ambient and elevated CO₂ treatments.

Statistical Analysis

Prior to statistical analysis, normalized expression values (NEV) were transformed by taking the natural log of one plus the normalized expression value [ln(NEV+1)]. Two outliers were identified in the expression data from week 1 and week 2 for in the elevated CO₂ treatment for all qPCR assays and were omitted from further analysis resulting in an n=7 for the indicated sampling groups. A two-way ANOVA was conducted on the transformed expression data to test for significant effects of treatment and time. A Kaplan-Meier survivorship analysis was applied to survival data from the thermal stress trial and significance was determined using a log-rank test. Significance was determined based on p<0.05. All statistical analysis was conducted using SPSS statistical software (IBM, Somers, NY).

Results/Discussion

Elevated pCO₂ treatment

Partial pressure CO₂ conditions were maintained at two different levels for the duration of the experiment (Figure 5A). Conditions representing present day (ambient) pCO₂ concentrations were maintained at 424±45 μ atm corresponding to a pH of 8.01±0.04. Elevated levels of pCO₂ were maintained at 1146±312 μ atm corresponding to a pH 7.63±0.10 (Figure 5), which are within the projected changes expected to occur by 2100 (Caldeira and Wickett 2003). The greatest amount of variability was observed in samples taken during week 2 in which a spike in pCO₂ was observed in the elevated treatment (Figure 5). No mortalities occurred as a result of the different CO₂ treatment conditions. A summary of results from the carbonate chemistry sampling is provided in Table 3.

Thermal tolerance

Juvenile Manila clams held in ambient or elevated pCO₂ seawater for three weeks were heat shocked at the pre-determined lethal temperature (40°C) and at one and two degrees lower than the lethal temperature (39°C and 38°C) to assess any influence of elevated pCO₂ conditions on thermal tolerance (Figure 6). A greater number of more clams survived the 40°C heat shock from the ambient pCO₂ relative to the elevated pCO₂ treatment ($p < 0.05$). The onset of mortality (OM) occurred on day 2 in both the ambient and elevated CO₂ treatments. The mean day of death (MDD) for animals heat shocked at 40°C shifted from 3.4 days at ambient pCO₂ to 2.8 days post treatment (DPT) at elevated pCO₂ conditions.

No differences in survival, OM, or MDD were observed at 39°C or 38°C heat shock ($p > 0.05$). The OM in animals heat shocked at 39°C occurred on day 3 in both the ambient and elevated pCO₂ treatment with a mean day of death for animals exposed to 39°C of 4.1 DPT for animals treated with ambient pCO₂ seawater while the elevated pCO₂ treatment was slightly lower at 3.8 DPT. The OM at 38°C occurred on day 4 in both the ambient and elevated pCO₂ treatments. The MDD for animals heat shocked at 38°C was 6.6 and 6.7 for ambient and elevated pCO₂ treated animals respectively with 64.3% surviving until day 7 in the ambient pCO₂ group and 71.4% surviving in the elevated pCO₂ treatment.

An organism's survival and fitness capacity depends on their physiological plasticity to cope with changing environments (Hofmann and Todgham 2010). Plasticity is associated with the allocation of energetic resources to physiological processes required for maintaining vital cellular functions (Findlay et al. 2009). Understanding how a particular stressor impacts this energetic balance provides valuable insight into the potential mechanisms of coping with changing environmental conditions. Events associated with global climate change, such as ocean acidification and increasing sea surface temperatures, will occur in concert with other naturally occurring environmental stressors. The objective of the thermal stress study was to determine whether elevated pCO₂ has an effect on juvenile Manila clam thermal tolerance. Similar studies in other organisms have observed synergistic effects on stress response mechanisms (Liu et al. 2012; Zippay and Hoffman, 2010; Chapman et al. 2011; Metzger et al. 2007), and in

some, increased mortality (Zippay and Hofmann 2010). Elevated pCO₂ alone did not increase mortality of juvenile Manila clams compared to ambient pCO₂ conditions, however, the combination of elevated pCO₂ and thermal stress resulted in a shift in the juvenile Manila clam thermal tolerance window as seen in the 40°C trial. The effect of elevated pCO₂ was not severe enough to shift LT a degree below 40°C. This suggests that the thermal tolerance of juvenile Manila clams is not significantly affected by near future levels of atmospheric CO₂. However, increased sensitivity to a 40°C thermal suggests that the physiological mechanism necessary to cope with thermal stress are impacted by elevated pCO₂ conditions.

Gene expression analysis

Transcriptomic processes can moderate an organism's physiological plasticity in response to changing environmental conditions (Hofmann and Todgham 2010). Previous studies that have examined molecular and cellular impacts of elevated pCO₂ have identified several vital physiological processes that are susceptible to changing pCO₂ conditions, including protein synthesis and metabolism, calcification, oxidative stress, and ion homeostasis (Metzger et al, in review; Moya et al, 2012; Todgham and Hofmann 2009; O'Donnell et al 2010). Candidate genes for the current study were selected from a previous study of Manila clam larvae in which RNAseq was employed to characterize the entire transcriptome of larvae exposed to elevated pCO₂ conditions (Metzger et al, in review).

One of the most commonly studied processes in organisms facing elevated pCO₂ conditions is calcification. Most reports to date have documented a negative effect of IPCC-projected CO₂ conditions on calcifying organisms (Kroeker et al. 2010; Gazeau et al. 2007; Orr et al. 2005). The gene encoding Perlucin 6 was recently identified in larval Manila clams (Metzger et al. in review). Perlucin is a C-type lectin (Mann et al, 2000) that has been shown to nucleate calcium carbonate ions (Weiss et al. 2000; Blank et al. 2003). *In vitro* studies have shown that Perlucin is incorporated into the calcium carbonate structure (Blank et al. 2003). Perlucin may therefore be an important component of calcification in Manila clams.

Perlucin-6 gene expression levels were not significantly different in juvenile clams exposed to elevated pCO₂ (Figure 7A). This result may not be surprising as mantle tissue is primarily responsible for laying down new shell. This study was designed to measure transcriptional changes in gill tissue as this represents the primary interface for gas and ion with the environment.

Calmodulin is a Ca²⁺ dependent messenger protein that moderates the activity of enzymes involved in several vital cellular processes including ATPase driven ion pumps (Klee 1980) necessary for maintaining ion homeostasis, particularly in gill tissue. Transcriptomic analysis in corals noted decreases in calmodulin transcripts in elevated CO₂ conditions (Kaniewska et al. 2012). We expected to see changes in calmodulin transcript levels if clams were regulating the activity of ATPase-driven ion channels and Ca²⁺ transport. However, an effect of elevated pCO₂ on calmodulin transcripts was not detected (Figure 7B).

Protein synthesis and degradation can be indicative of an organism's general physiological and metabolic status. The cathepsin family of proteins is involved in mediating protein synthesis and degradation. Increased activity of cathepsin L has been associated with proteolysis as an alternative source of energy during periods of increased metabolic demand (Hawkins and Day 1996). Elevated pCO₂ conditions did not influence cathepsin L transcription (Figure 7C) suggesting that juvenile manila clams are not experiencing metabolic stress under elevated pCO₂ conditions. In addition, there was no significant effect of elevated pCO₂ on elongation factor 2 (EF2) transcript levels between ambient and elevated pCO₂ conditions (Figure 7D). EF2 is involved in peptide elongation during protein synthesis. Previous studies have identified changes in protein concentration of larval barnacles (Wong et al 2011) and transcripts in larval Manila clams (Metzger et al, in review) suggesting that ef2 can play a role in moderating the physiological plasticity of organisms exposed to elevated pCO₂.

Also involved in protein metabolism, as well as thermal stress, immune response, and apoptosis (Feder and Hofmann, 1999; Roberts et al. 2010) are the heat shock protein (hsp) family. Named for their molecular mass (eg. hsp60, hsp70, hsp90) heat shock proteins are molecular chaperones that bind and stabilize proteins, aiding in protein synthesis or the repairing of damaged proteins from processes such as oxidative stressed.

Analysis of hsp90 expression in gill tissue of juvenile clams exposed to elevated pCO₂ conditions was not significantly different from those compared to ambient pCO₂ conditions (Figure 7E). It is possible that hsp90 is not required to cope with this particular stress response, or that regulation of hsp90 does not occur at the transcriptional level. Hsp90 is a ubiquitously expressed protein that undergoes an ATPase-dependent conformational change upon activation (Pearl and Prodromou 2006). Conditioning of the hsp stress response has been shown for several environmental conditions (Bierkens 2000), which may be the case here. Interestingly, expression of hsp90 has also been shown to decrease with age in the hard clam *Mercenaria mercenaria* (Farcy et al. 2007). Age dependent regulation of hsp90 might suggest that it is more highly expressed during early larval periods while juvenile and adults do not actively express hsp90 to the same degree.

Elevated pCO₂ conditions can also invoke an oxidative stress response possibly as the result of intracellular acidosis and the generation of reactive oxygen species (ROS) (Tomanek 2011). A study in the eastern oyster *Crassostrea virginica*, revealed antioxidant defense as a primary response of oysters exposed to elevated pCO₂ conditions (Tomanek 2011). Glutathione peroxidase 3 (GPx3) is selenium dependent and is highly expressed in gill tissue (Wong et al. 2011). Increased levels of GPx3 are typically associated with response to increases in ROS and oxidative stress (Wong et al. 2011). In this study, elevated pCO₂ conditions did not significantly affect the expression of GPx3 transcripts (Figure 7F), indicating that juvenile Manila clams may not be experiencing increased levels of oxidative stress as a result of elevated pCO₂ conditions. Glutathione peroxidases can also play a role in the immune response by protecting tissues from the ROS that are secreted as a defense mechanism. This experiment was not designed to assess the immunocompetency of juvenile clams under elevated pCO₂ conditions, however, these results suggest that the ability of juvenile Manila clams to cope with ROS secretion by their immune system may be unaffected by elevated pCO₂ conditions. Directed studies involving immune challenge with a pathogen under elevated pCO₂ are required to assess immunological effects of elevated pCO₂ before any conclusions can be made.

Conclusions

Preliminary results to assess the minimum lethal temperature for juvenile Manila clams determined that 40°C was the minimum lethal temperature that resulted in 100% mortality one week post-exposure (data not shown). Mortality of juvenile Manila clams exposed to 40°C was significantly affected by elevated pCO₂ conditions (Figure 6A). This increased sensitivity, however, did not translate to an increased sensitivity at lower temperatures (Figure 6B, C). While not significantly different, the OM and MDD of clams exposed to elevated pCO₂ was later than that of ambient clams (Figure 6). In addition, the expression of several genes associated with processes such as calcium ion binding, metabolism, translation, and stress response were not significantly different in juvenile clams exposed to elevated pCO₂. Together, these data suggest juvenile clams are capable of effectively coping with elevated pCO₂ conditions, possibly as a result of local adaptation to low-alkalinity coastal waters (Hofmann and Todgman 2010). Future experiments examining prolonged exposures of elevated pCO₂ on different populations of Manila clams are needed to determine whether projected environmental conditions as a result of climate change are within the physiological limits of the Manila clam. In addition, it would be of interest to examine the impact of larval exposure to elevated pCO₂ on physiological processes and gene expression in juvenile and adult animals.

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FIGURES

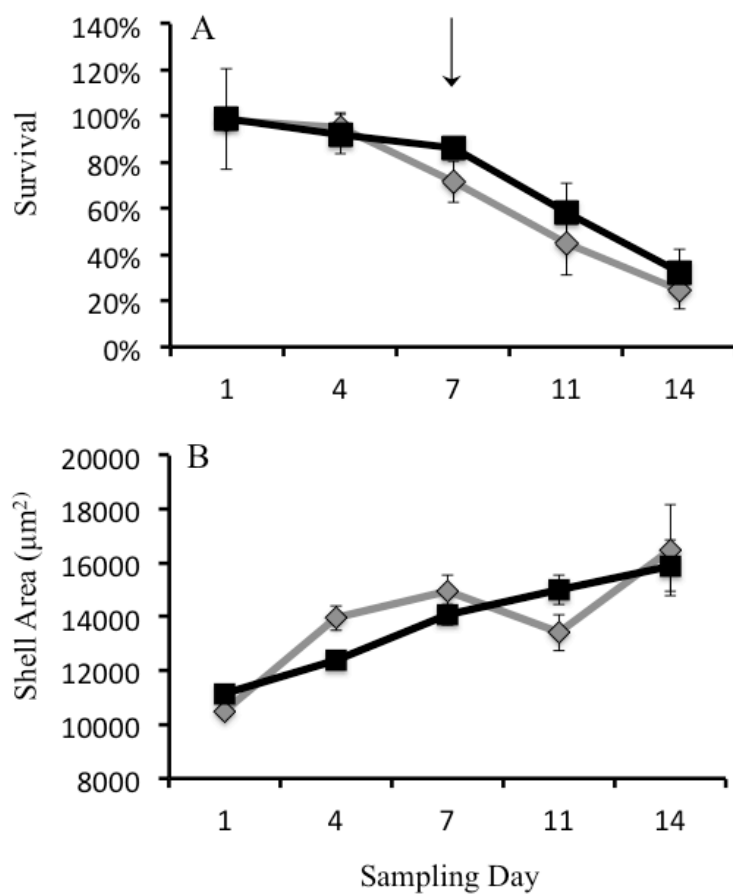


Figure 1. Average (\pm standard error) larval survival (A) and shell area (B) at ambient (diamonds) and elevated (squares) pCO₂ conditions (n=3). Arrows indicate sampling time point for RNA isolation and transcriptome library preparation.

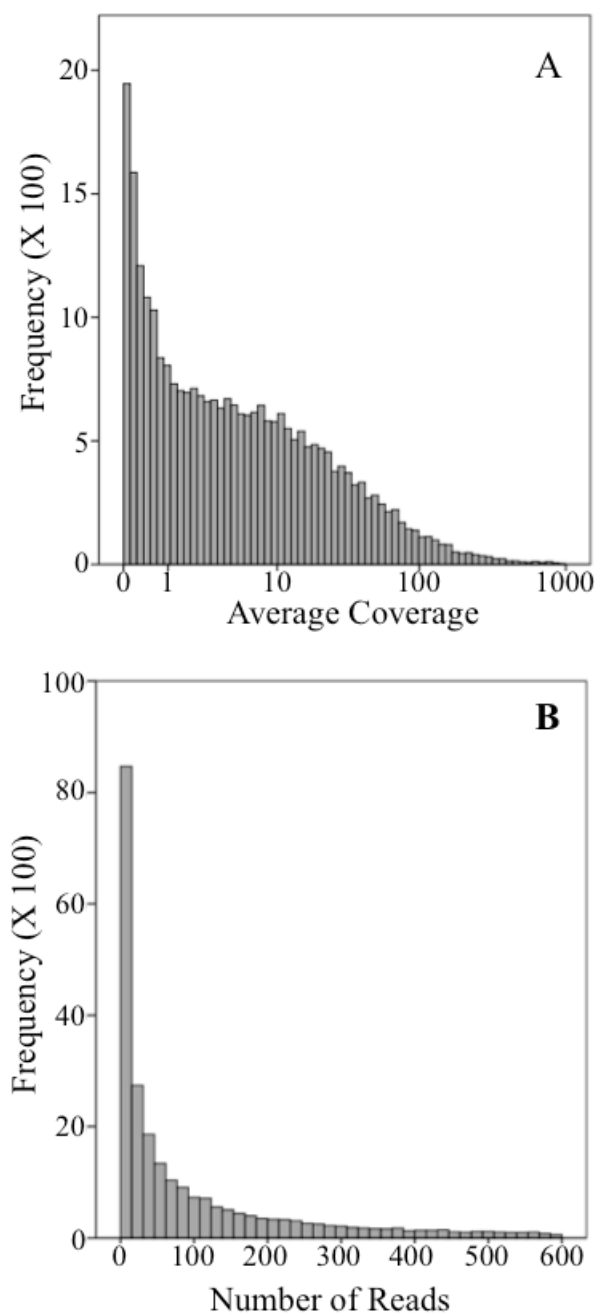


Figure 2. Histogram of average coverage (A) and number of reads (B) of the reference assembly of Manila clam larval transcriptome libraries to RUPhibase.

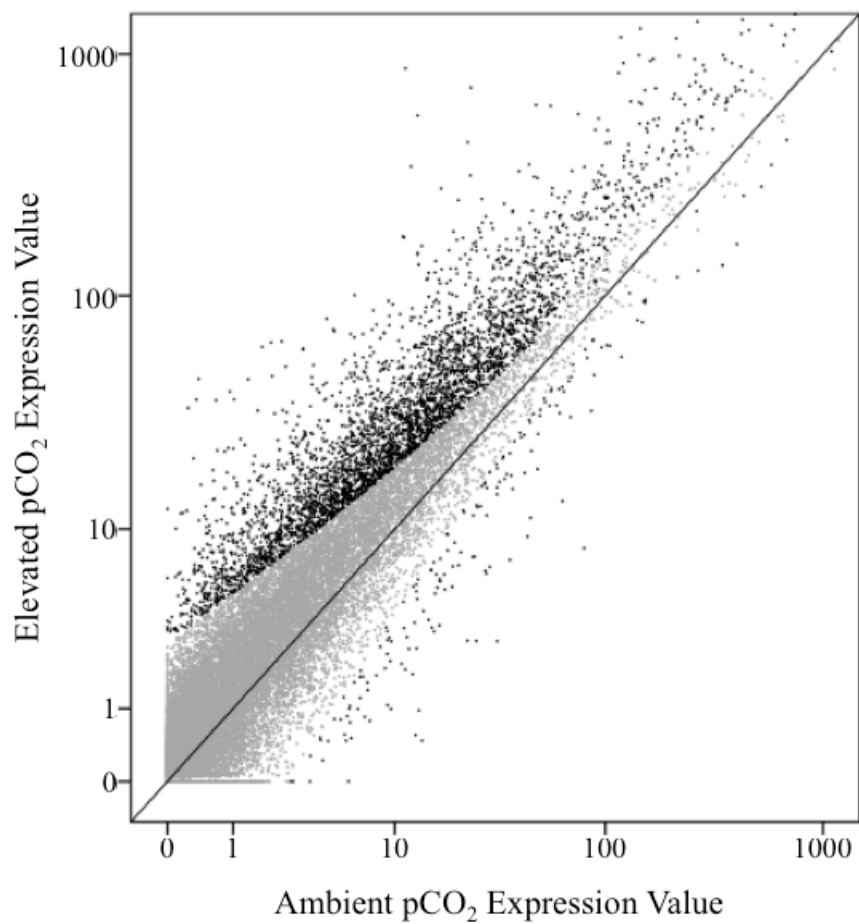


Figure 3. Contig expression values (RPKM) for ambient (x axis) and high (y axis) pCO₂ exposed Manila clam larvae libraries. Differentially expressed contigs ($p < 0.10$, fold ≥ 1.5) are shown in black.

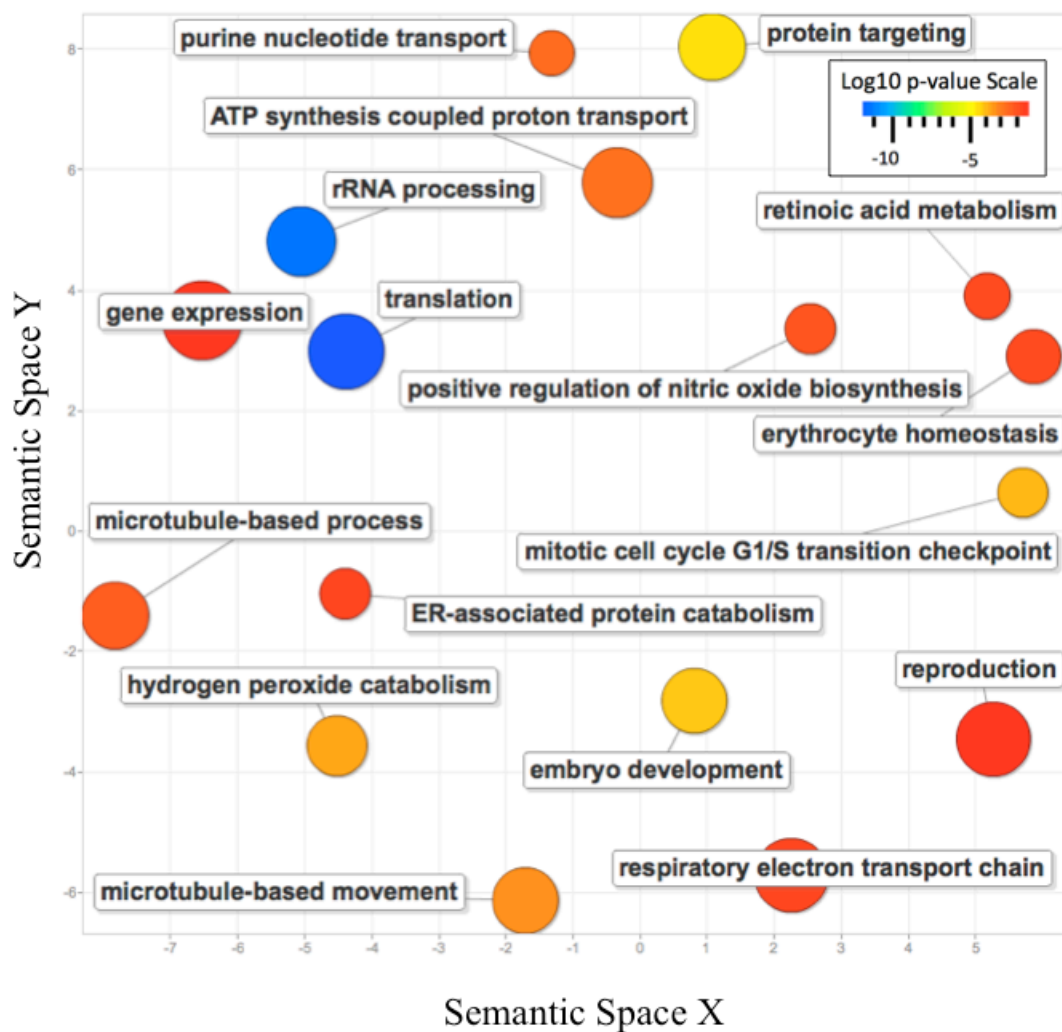


Figure 4. Gene ontology categories enriched in the differentially expressed gene set. Biological processes are plotted in semantic spaces where the proximity between circles represents relatedness of the processes. Color indicates degree of enrichment for each process presented as the p-value from the hyper geometric test. Spatial arrangement reflects a grouping of categories by semantic similarity.

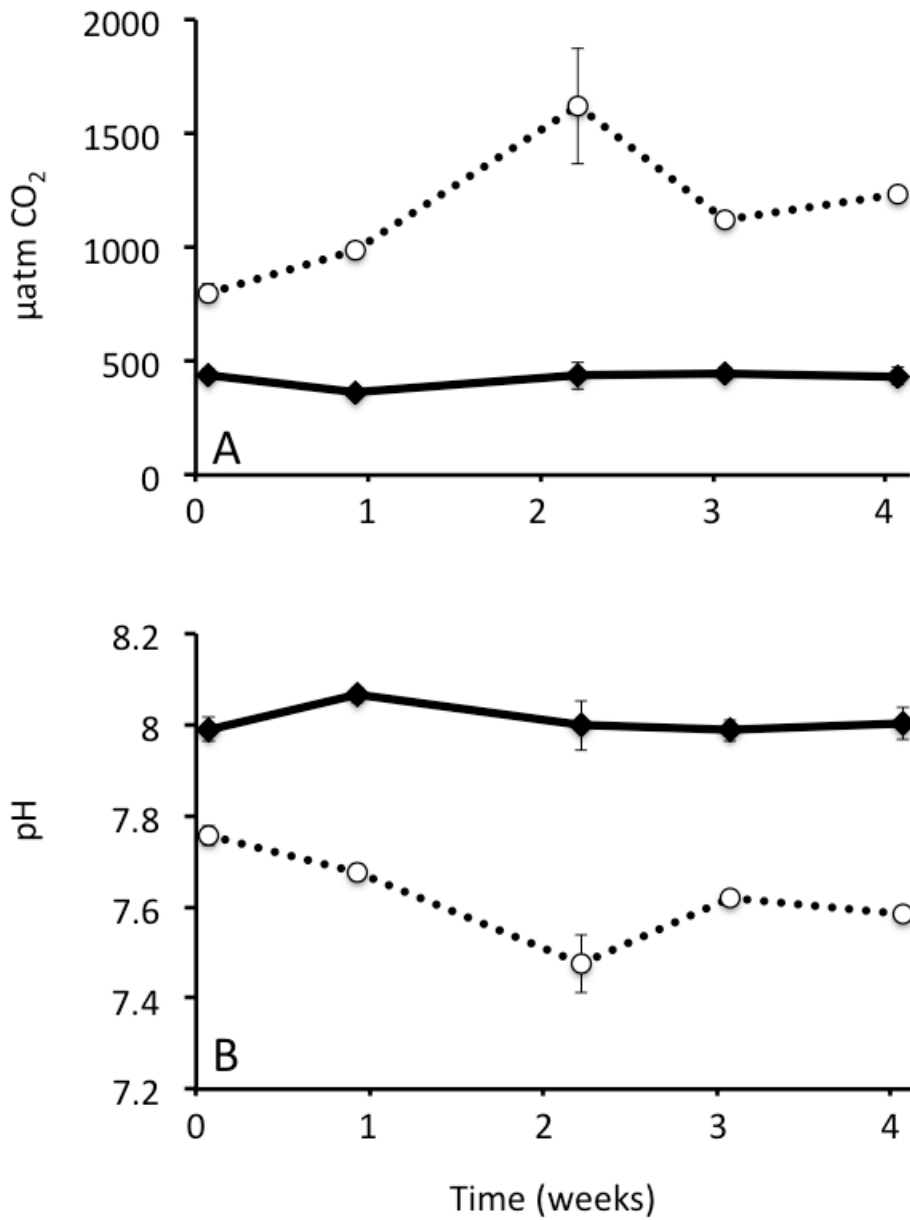


Figure 5. Summary of pH and concentration of dissolved CO₂ (µatm) of the ambient (solid line) and elevated CO₂ (dashed line) treated seawater. Concentrations of dissolved CO₂ were calculated using total alkalinity, salinity, and pH measurements. Final pH measurements were adjusted to correspond to the 13°C treatment water. Values represent means ± standard deviation.

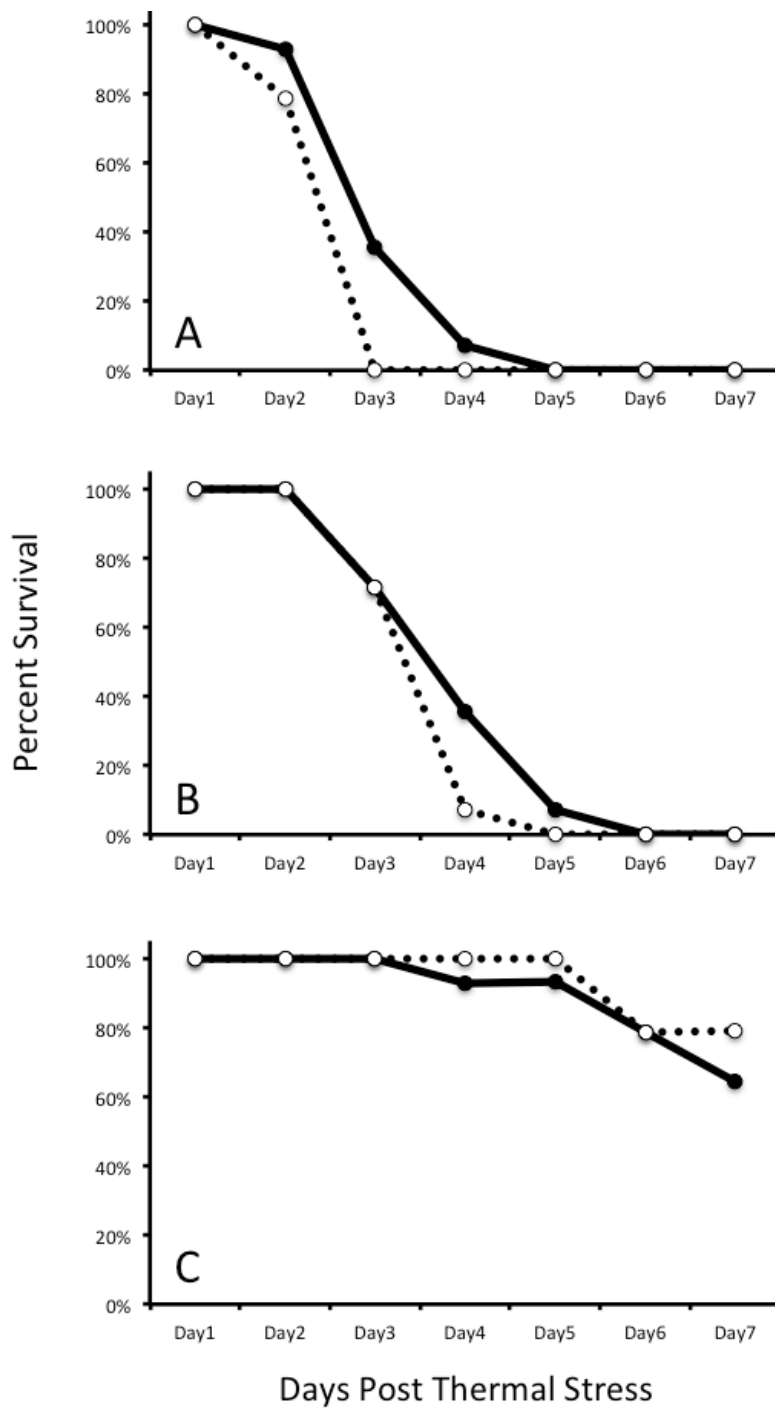


Figure 6. Percent survival of juvenile manila clams following a one hour exposure to a thermal stress at 40°C (A), 39°C (B), or 38°C (C). Clams were acclimated for 3 weeks at either ambient (solid line) or elevated CO₂ (dashed line) conditions prior to thermal stress. After thermal stress, animals were returned to the corresponding treatment water and mortality was monitored daily for one week. A significant effect on survival was detected in the elevated CO₂ conditioned animals to a 40°C thermal stress.

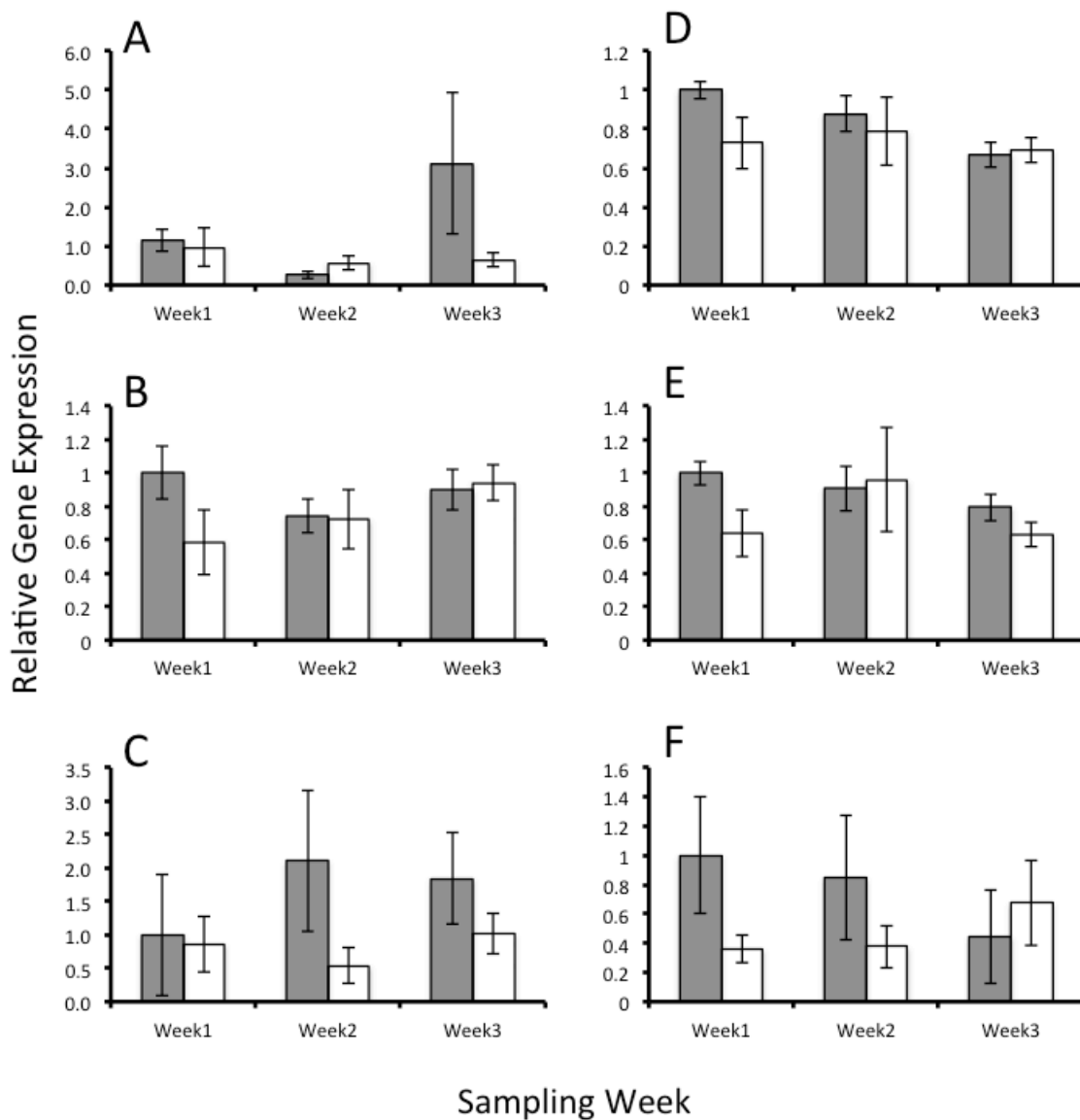


Figure 7. Quantification of transcript abundance in gill tissue of juvenile Manila clams exposed to ambient CO₂ (grey bars) and elevated CO₂ (white bars). Gill tissue samples were taken after 1, 2, and 3 weeks of exposure to CO₂ treated water and analyzed for expression of perlucin (A), calmodulin (B), cathepsin L (C), ef2 (D), hsp90 (E), and GPx3 (F). All values are normalized to Ef1- α and standardized to the average value of the corresponding ambient expression value from week one.

Table 1. Summary of water chemistry measurements for ambient and elevated pCO₂ treatments. Values are expressed as means ± standard deviations.

Treatment	pH	TA ($\mu\text{mol}/\text{kg}$)	DIC ($\mu\text{mol}/\text{kg}$)	Ω_{Ar}	Ω_{Ca}	CO ₃ ⁻⁻ ($\mu\text{mol}/\text{kg-SW}$)	HCO ₃ ⁻ ($\mu\text{mol}/\text{kg-SW}$)	pCO ₂ (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

Table 2. List of primers sequences developed from the designated contig sequence in Ruphibase. Primers were developed using Primer 3 software.

Gene Target	Oligo	Ruphibase ID	Primer DNA sequence
Ef-1 α	Fwd Rev	ruditapes2_c4569	ACGCTCCACTTGGACGTTTTGCT TGTAGCCTTTTGGGCAGCTTTGGT
Hsp90	Fwd Rev	ruditapes_c1528	TCTCCCTTGAAGAGCCAACAACCCA TCATCATCACCTTCCAATGGGGGCA
Cathepsin	Fwd REv	ruditapes_lrc32628	AGCCAAAGAACGGCCGATGTGA TCCTACTGTTGCTACAGCGGCTTG
Calmodulin	Fwd Rev	ruditapes_c670	ACGACCAAGTGGACGAGATGTTGC AGTACAGGCACTGGATGGTGC GTA
GPX3	Fwd Rev	ruditapes2_c3709	ATTCTCGAGCGCTGGGGTAAAAGTG TAGTTGTTCGGCCGGCTCTTGCATT
Perlucin	Fwd Rev	ruditapes_lrc29501	GCAGACGTCGACAGGATGTCCAAT ACAGTATGCCATAGCCTCCCACCA
EF2	Fwd Rev	ruditapes2_c46	GACAGTGTGTTGCTGGCTTCCAGT TGTCACCACCTCTGTGGATAGCA

Table 3. Summary of water chemistry measurements from ambient and elevated CO₂ treatments (\pm SD) for the 28 days of the juvenile clam experiment. Total alkalinity (TA), salinity, and pH were measured directly using techniques described in the methods. Other water chemistry parameters were calculated using CO2calc software with TA, salinity, and pH as the three required inputs. Values represent means \pm standard deviation.

Ambient					
TA	Salinity	pH	pCO ₂	Ω Ca	Ω Ar
2078.49 (\pm 13.71)	29.79 (\pm 0.25)	8.01 (\pm 0.04)	424.11 (\pm 44.90)	2.71 (\pm 0.24)	1.71 (\pm 0.16)
Elevated					
TA	Salinity	pH	pCO ₂	Ω Ca	Ω Ar
2085.14 (\pm 13.37)	29.92 (\pm 0.21)	7.63 (\pm 0.10)	1146.11 (\pm 312.42)	1.24 (\pm 0.26)	0.78 (\pm 0.17)