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Title: Thermal tolerance and gene expression characterization in Manila clams (*Ruditapes philippinarum*) exposed to elevated carbon dioxide

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Abstract

Global climate change, including ocean acidification, resulting from anthropogenic carbon dioxide (CO_2) emissions, poses a risk to the ecological landscape of intertidal and shallow subtidal communities. Organisms that inhabit these waters will have to cope with changing environmental conditions, including increases in partial pressure CO_2 (pCO_2) and temperatures. The ability of a species to maintain homeostasis under changing environmental conditions will confer resilience and ecosystems could shift based on each species' ability to adapt. In this study, juvenile Manila clams were exposed to elevated pCO_2 conditions for 3 weeks and physiological impacts were assessed by characterizing gene expression levels and evaluating thermal tolerance. Elevated pCO_2 conditions did not significantly influence expression of candidate genes involved in ion metabolism, protein activity, and oxidative stress. Exposure to elevated pCO_2 did not significantly affect mortality of juvenile clams after an acute heat shock. These data suggest that Manila clams are capable of tolerating stressors associated with environmental change.

Introduction

Atmospheric carbon dioxide (CO_2) levels have increased from 280 parts per million (ppm) prior to the industrial revolution to present day levels of approximately 400 ppm, higher than they have been in the past 800,000 years (Lüthi et al. 2008). Atmospheric pCO_2 concentrations have fluctuated historically (Tyrrell 2008), but current atmospheric pCO_2 concentrations are increasing at an unprecedented rate of 0.5% per year (Caldeira and Wickett, 2003; Orr et al. 2005; Zeebe 2012). Increasing atmospheric levels of CO_2 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 CO_2 emissions (Sabine et al. 2004) so that the partial pressure of CO_2 (pCO_2) in the oceans is approximately equal correlated to that in the atmosphere. When CO_2 from the atmosphere equilibrates with surface water of the oceans it disassociates in water to form hydrogen and bicarbonate ions. This equilibrium reaction increases the oceanic concentration of bicarbonate and decreases the amount of carbonate available to calcifying organisms while also increasing the concentration of free hydrogen ions, causing the pH of water to decrease (Zeebe and Wolf-Gladrow, 2001). If the current rate of fossil fuel emissions continues, atmospheric levels of CO_2 will reach 750-1000 ppm 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).

Organisms that inhabit intertidal and shallow subtidal waters are thought to be at risk due to ocean acidification, particularly those dependent on carbonate-based structures for stability, defense, and survival (Fabry et al. 2008; Cooley and Doney, 2009). Temperature fluctuations associated with increased CO_2 emissions (the greenhouse effect) are also predicted to impact near shore communities as temperature changes more rapidly in these shallower waters (Levitus et al, 2000; Nixon et al. 2004). Elevated pCO2 could cause a shift in physiological thresholds as an organism is faced with another stressor, such as increased temperature. Changes in an organism’s ability to cope with environmental stress will in turn provide insight into potential changes to the ecological landscape of intertidal and subtidal communities.

The Manila clam, Ruditapes philippinarum is a calcifying marine mollusc that inhabits intertidal and subdital zones and will be subject to changing environmental conditions ~~as~~ associated with climate change. Manila clams are indigenous to the Philippines, South China and East China Seas, up to the Sea of Okhotsk and the southern Kuril Islands (Scarlato, 1981). Since being introduced to the west coast of the United States in the 1930s (Magoon and Vining, 1981), they have become an economically important aquaculture species (Dumbauld et al. 2009). Manila clams are resilient to a wide range of temperatures and salinities (Numaguchi 1998), however, little is known concerning their tolerance to ocean acidification.

The objective of this study is to examine the impact of elevated pCO_2 on juvenile Manila clam physiology. Expression level of candidate genes involved in ion metabolism, protein activity, and oxidative stress were measured during a three-week exposure to elevated pCO_2 conditions. In addition, the effect of elevated pCO_2 conditions on juvenile Manila clam thermal limits was assessed. Our hypothesis was that ocean acidification would negatively impact other physiological responses, so that exposure to a heat shock would result in higher mortality in clams exposed to elevated pCO_2 compared to ambient pCO_2 conditions.

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 on San Juan Island, WA. Clams were exposed to seawater equilibrated to ambient (400 µatm; pH 8.03) or elevated (1000 µatm; pH 7.67) pCO_2. Gas equilibration was achieved as previously described by Timmins-Schiffman et al., 2012. Briefly, seawater filtered to 0.2 µm and CO_2 was stripped using a membrane contactor under vacuum pressure. Pure CO_2 gas was then mixed with CO_2-free air using gas proportionators. The prepared gas mixtures were then equilibrated with seawater using solenoid valves attached to Venturi injectors. Treatments were monitored using a Honeywell controller connected to a Durafet pH probe adjusted to maintain the desired

pH. Durafet calibration was ensured daily using spectrophotometric pH. Total alkalinity (A_T) measurements were performed prior to the addition of animals to the system and once per week following the addition of animals. The water chemistry inside the animal chambers (A_T and spectrophotometric pH) was also monitored once a week. Carbonate chemistry measurements, including partial pressure CO_2 as well as aragonite and calcite saturation, were calculated from A_T, spectrophotometric pH, and salinity using the CO_2 calculator "CO_2Calc" and adjusting for ambient temperature of the experiment (13°C) (Robbins et al. 2010) with the following parameters: CO_2 constants: Lueker et al. 2000, $KHSO_4$: Dickson (1990), pH Scale: Total scale (mol/kgSW), Air-Sea Flux: Wanninkhof, 1992.

Each experimental treatment contained 8 replicate 3-L 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 in liquid nitrogen. A total of eight clams were sampled from each pCO_2 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 to 38 or 39°C. During thermal treatments, clams were completely submerged in their designated pCO_2 treatment seawater. Clams were then returned to 13°C at their respective pCO_2 treatment conditions and mortality was monitored for one week. A total of 14 clams (two replicate groups of seven animals) were used for each

pCO_2 and temperature combination. Mortality was assessed based on gaping behavior. Clams that failed to close their shells in response to mechanical stimulation were considered dead.

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 20 ul reaction volumes consisting of 1x Ssofast EvaGreen Supermix (Bio-Rad, Hercules, CA), 0.2 μM of each primer, and 2 ul 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: $1/(1+\text{Efficiency})^{Ct}$. 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 similar experiments (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-1α expression between ambient and elevated pCO_2 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 in the elevated pCO_2 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 α=0.05. All statistical analysis was conducted using SPSS statistical software (IBM, Somers, NY).

Results/Discussion

Elevated pCO_2 treatment

Partial pressure CO_2 conditions were maintained at two different levels for the duration of the experiment (Figure 1A). Conditions representing present day (ambient) pCO_2 concentrations were maintained at 424±45µatm (mean±SD) corresponding to a pH of 8.01±0.04. Elevated levels of pCO_2 were maintained at 1146±312µatm corresponding to a pH 7.63±0.10 (Figure 1), 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 pCO_2 was observed in the elevated treatment (Figure 1). No mortalities occurred as a result of the different CO_2 t~~reatment conditions~~. A summary of results from the carbonate chemistry sampling is provided in Table 3.

Gene expression analysis

One of the most commonly studied processes in organisms facing elevated pCO_2 conditions is calcification. Most reports to date have documented a negative effect of elevated pCO_2 conditions on calcifying organisms (Kroeker et al. 2010; Gazeau et al. 2007; Orr et al. 2005). Perlucin 6 is a C-type lectin (Mann et al, 2000) that has been shown to facilitate the formation calcium carbonate structures (Blank et al. 2003) and

may therefore be an important component of calcification in Manila clams. Perlucin 6 transcript levels were not significantly different in juvenile clams exposed to elevated pCO_2 (Figure 2A). We expected to observe differences in Perlucin expression. There are several explanations for the lack of influence including limited impacts of elevated pCO_2 a disparate function of Perlucin in clams, or expression could be affected in other tissues. However this result is consistent with observations in larval abalone in which changes in pCO_2 had no effect on the expression of genes involved in biomineralization (Zippay and Hofmann 2010).

Ion transport is necessary for maintaining ion homeostasis, particularly in gill tissue. Calmodulin is a Ca^{2+} dependent messenger protein that moderates the activity of enzymes involved in several vital cellular processes, including ATPase driven ion pumps (Klee 1980). Calmodulin transcripts in corals decreased under elevated pCO_2 conditions (Kaniewska et al. 2012). There was no effect of elevated pCO_2 on calmodulin transcript levels in juvenile Manila clams (Figure 2B), suggesting that ion homeostasis in the gills may not be impacted under the experimental conditions.

Increased expression of genes associated with protein translation and proteolysis can occur during periods of increased metabolic demand as an means to provide alternative energy sources through the break down of amino acids (Hawkins and Day 1996; Stumpp et al. 2011). Cathepsin L and elongation factor 2 (EF2) are involved in mediating protein synthesis and degradation. Elevated pCO_2 conditions did not influence cathepsin L or EF2 transcript levels in juvenile clam gill tissue (Figure 2C).

Heat shock proteins (hsp) are molecular chaperones that bind and stabilize

proteins, aiding in protein synthesis or the repairing of damaged proteins. Analysis of hsp90 expression in gill tissue of juvenile clams exposed to elevated pCO_2 conditions was not significantly different from those compared to ambient pCO_2 conditions (Figure 2E). These data imply that there is limited negative impact on protein viability under these particular experimental conditions.

Antioxidant defense is a primary response of Eastern oysters exposed to elevated pCO_2 conditions as a result of increased metabolism (Tomanek et al. 2011). In this study, elevated pCO_2 conditions did not impact the expression of Glutathione peroxidase 3, an important antioxidant compound (Figure 2F). While limited in nature, given what is known about the physiological impact of ocean acidification, the gene expression data provided here is consistent with a specific response. In this case, juvenile Manila clams are not dramatically impacted at the transcriptome level.

Thermal tolerance

No differences in survival, onset of mortality (OM), or mean day of death (MDD) were observed at 39°C (the pre determined minimum lethal temperature) or 38°C heat shock ($p>0.05$) (Figure 3). The OM in animals heat shocked at 39°C occurred on day 3 in both the ambient and elevated pCO_2 treatment with a MDD for animals exposed to 39°C of 4.1 days for animals treated with ambient pCO_2 seawater while the elevated pCO_2 treatment was slightly lower at 3.8 days. The OM at 38°C occurred on day 4 in both the ambient and elevated pCO_2 treatments. The MDD for animals heat shocked at 38°C was 6.6 and 6.7 for ambient and elevated pCO_2 treated animals respectively with 64.3%

surviving until day 7 in the ambient pCO_2 group and 71.4% surviving in the elevated pCO_2 treatment.

Concurrent stressors associated with temperature and carbonate chemistry have been shown to vary across species. For example in larval red abalone, Haliotis rufenscens, the combination of elevated pCO_2 and thermal treatments increased mortality (Zippay and Hofman 2010). In the tropical sea urchin, Tripneustes gratilla, elevated pCO_2 conditions reduced calcification and nullified increased growth associated with warmer temperatures (Brennand et al. 2010). In this study, elevated pCO_2 had no effect on the mortality of juvenile Manila clams. These results are similar to what has been observed in juvenile M. mercenaria where tissue and shell growth were monitored for 45 days in clams exposed to elevated temperature and pCO_2 with no significant effect (Talmage and Gobler 2011).

Conclusions

Ocean acidification conditions did not alter the response of Manila clams to temperature stress. In addition, the expression of several genes associated with processes such as ion metabolism, protein activity, and oxidative stress were not different in juvenile clams exposed to elevated pCO_2. These data indicate juvenile clams could be resilient to elevated pCO_2 conditions expected to occur this century. This resiliency is consistent with their life history as infaunal adults, where pCO_2 is typically higher due to respiration and decomposition processes. Resiliency attributed to later developmental stages has been suggested in the M. mercenaria, where increased calcification rates

associated with later developmental stages can overcome the dissolution pressures of elevated pCO_2 conditions (Waldbusser et al. 2010). Further studies are needed to examine the physiological response of species across all life stages in order to gain a better understanding of how species will respond. For example in this study we only examine the response of juveniles to acute changes in conditions. There is the possibility that negative impacts might not be realized until later life stages (i.e. adult). Having a comprehensive understanding of how species will be respond will allow us to better predict impacts at the coastal ecosystem level.

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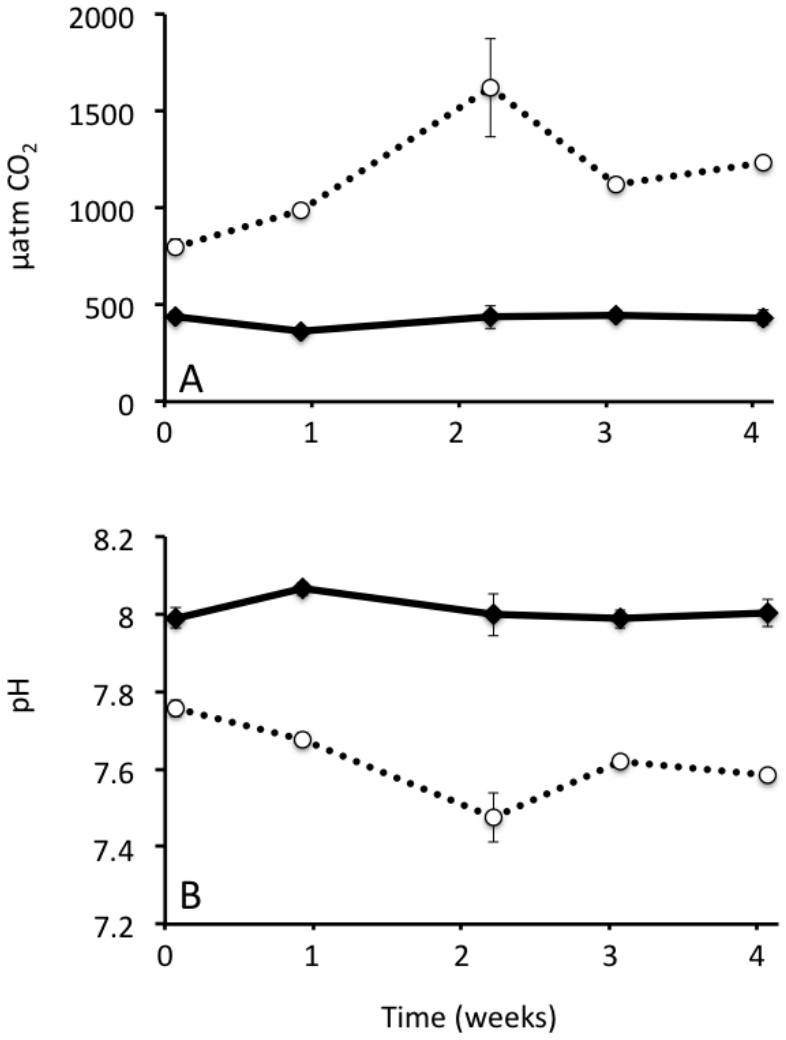

Figure 1. Summary of pH and concentration of dissolved CO_2 (µatm) of the ambient (solid line) and elevated CO_2 (dashed line) treated seawater. Concentrations of dissolved CO_2 were calculated using total alkalinity, salinity, and pH measurements. Final pH measurements were adjusted to correspond to the 13°C treatment water. The plotted data are means ± standard deviation. Give overall mean and SD for each treatment.

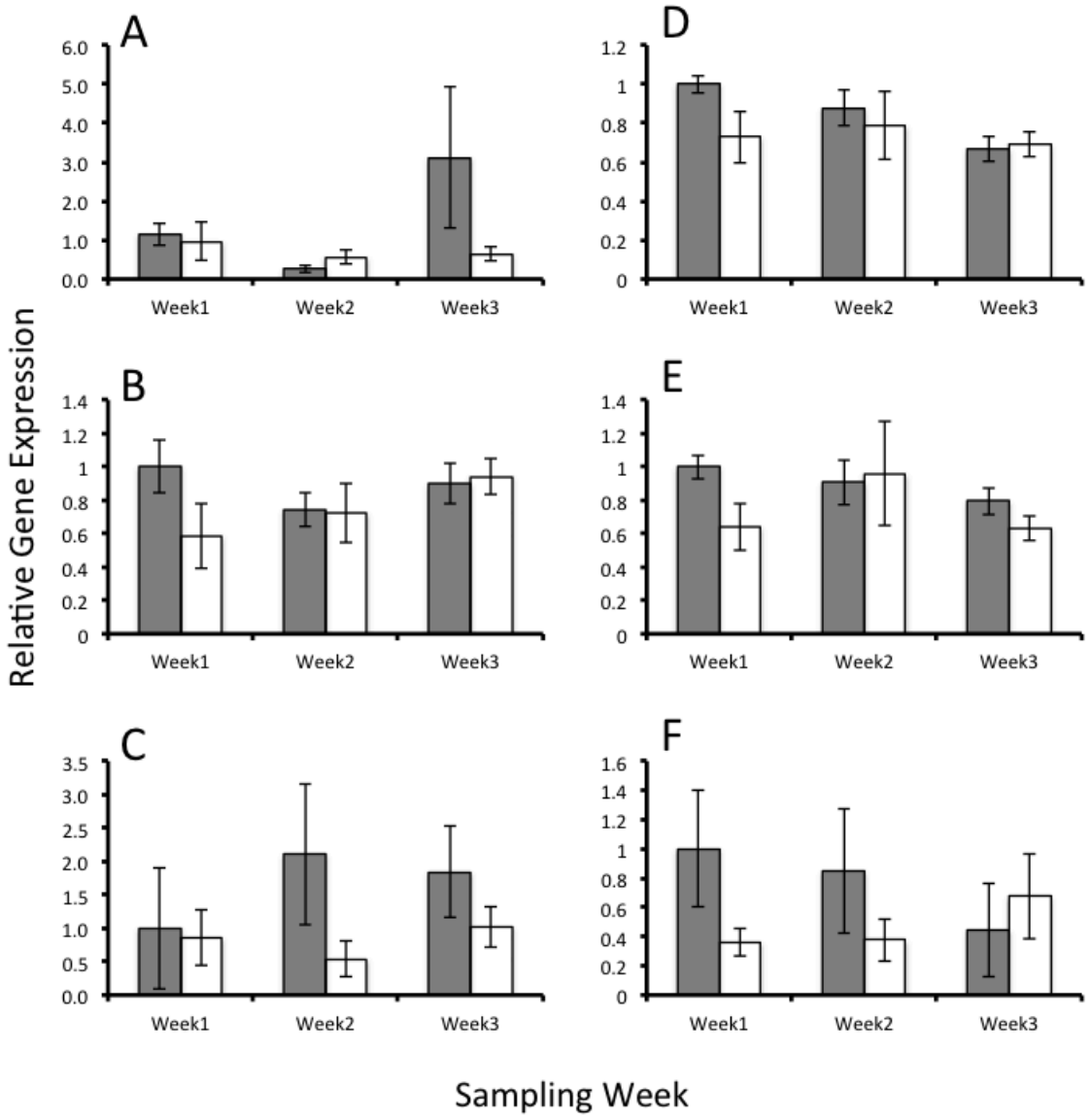

Figure 2. Quantification of transcript abundance in gill tissue of juvenile Manila clams exposed to ambient CO_2 (grey bars) and elevated CO_2 (white bars) (n= x for each treatment). Gill tissue samples were taken after 1, 2, and 3 weeks of exposure to CO_2 treated water and analyzed for expression of 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.

Figure 3. Percent survival of juvenile manila clams following a one hour exposure to a thermal stress at 39°C (A), or 38°C (B). Clams were acclimated for 3 weeks at either ambient (solid line) or elevated pCO_2 (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.

Gene Target	Oligo	Ruphibase ID	Primer sequence
Ef-1α	Fwd	ruditapes2_c4569	ACGCTCCACTTGGACGTTTTGCT
	Rev		TGTAGCCTTTTGGGCAGCTTTGGT
Hsp90	Fwd	ruditapes_c1528	TCTCCCTTGAAGAGCCAACAACCCA
	Rev		TCATCATCACCTTCCAATGGGGGCA
Cathepsin	Fwd	ruditapes_lrc32628	AGCCAAAGAACGGCCGATGTGA
	Rev		TCCTACTGTTGCTACAGCGGCTTG
Calmodulin	Fwd	ruditapes_c670	ACGACCAAGTGGACGAGATGTTGC
	Rev		AGTACAGGCACTGGATGGTGCGTA
GPX3	Fwd	ruditapes2_c3709	ATTCTCGAGCGCTGGGGTAAAAGTG
	Rev		TAGTTGTCGGCCGGCTCTTGCATT
Perlucin	Fwd	ruditapes_lrc29501	GCAGACGTCGACAGGATGTCCAAT

	Rev		ACAGTATGCCATAGCCTCCCACCA
EF2	Fwd Rev	ruditapes2_c46	GACAGTGTTGTTGCTGGCTTCCAGT TGTCCACCACCTCTGTGGATAGCA

Table 1. List of primers sequences developed from the designated contig sequence in Ruphibase. Primers were developed using Primer 3 software.

Ambient					
A_T	Salinity	pH	pCO2	Ω Ca	Ω Ar
2078.49	29.79	8.01	424.11	2.71	1.71
(±13.71)	(±0.25)	(±0.04)	(±44.90)	(±0.24)	(±0.16)
Elevated					
A_T	Salinity	pH	pCO2	Ω Ca	Ω Ar
2085.14	29.92	7.63	1146.11	1.24	0.78

(±13.37)	(±0.21)	(±0.10)	(±312.42)	(±0.26)	(±0.17)

Table 2. Summary of water chemistry measurements from ambient and elevated CO_2 treatments.(mean±SD) for the 28 days of the juvenile clam experiment. Total alkalinity (A_T), 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 inputs.