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 (CO2) 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, particularly increases in partial pressure CO2 (pCO2) and in temperatures, through the appropriate modulation of physiological processes. The ability of a species to maintain homeostasis under changing environmental conditions will confer tolerance to organisms when faced with environmental change. In this study, juvenile Manila clams were exposed to elevated pCO2 conditions for 3 weeks and physiological impacts were assed by characterizing gene expression levels and evaluating thermal tolerance. Elevated pCO2 conditions did not significantly influence expression of candidate genes involved in thermal stress, protein translation, or oxidative stress. Exposure to elevated pCO2 did not significantly affect mortality of juvenile clams after an acute heat shock. These data suggest that Manila clams are capable of tolerating some environmental stressors associated with global climate change.

Introduction

Atmospheric carbon dioxide (CO2) levels have increased from 280 parts per million (ppm) prior to the industrial revolution to present day levels of 400 ppm, higher than they have been in the past 800,000 years (Lüthi et al. 2008). Atmospheric CO2 concentrations have fluctuated historically (Tyrrell, 2008), but current atmospheric CO2 concentrations are increasing at an unprecedented rate of 0.5% per year (Caldeira and Wickett, 2003; 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) so that the partial pressure of CO2 (pCO2) in the oceans is correlated to that in the atmosphere. When carbon dioxide from the atmosphere equilibrates with surface water of the oceans it reacts with water to form hydrogen and bicarbonate ions (Feely et al. 2004; Orr et al. 2005). 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 water to become more acidic (Zeebe and Wolf-Gladrow, 2001). If the current rate of fossil fuel emissions continues, atmospheric levels of CO2 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 from 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 CO2 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 limitations 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 coastal communities.

One species that is susceptible to changes in environmental conditions is the Manila clam, Ruditapes philippinarum. The Manila clam is 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 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.

The objective of this study is to examine the impact of elevated pCO2 on juvenile Manila clam physiology. Expression level of candidate genes involved in calcification, translation, stress response, and oxidative stress were measured during a three-week exposure to elevated pCO2 conditions. In addition, the minimum lethal temperature (MLT) for juvenile Manila clams was determined and the effect of elevated pCO2 conditions on juvenile Manila clam thermal limits assessed. Our hypothesis was that ocean acidification would negatively impact other stress-related physiological processes, so that exposure to a heat shock would result in higher mortality in clams exposed to elevated pCO2 compared to ambient pCO2 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) pCO2. Gas equilibration was achieved by stripping seawater filtered to 0.2 µm 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. Treatments were monitored by measuring pH 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 (AT) 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 (AT and spectrophotometric pH) were also monitored once a week. Carbonate chemistry measurements, including partial pressure CO2 as well as aragonite and calcite saturation, were calculated from AT, spectrophotometric pH, and salinity using the CO2 calculator “CO2Calc” and adjusting for ambient temperature of the experiment (13°C)(Robbins et al. 2010) with the following parameters: 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 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 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. You need to say something here about finding the lethal heat treatment.

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 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 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+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 pCO2 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 pCO2 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 pCO2 treatment

Partial pressure CO2 conditions were maintained at two different levels for the duration of the experiment (Figure 1A). Conditions representing present day (ambient) pCO2 concentrations were maintained at 424±45μatm (mean±SD) corresponding to a pH of 8.01±0.04. Elevated levels of pCO2 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 pCO2 was observed in the elevated treatment (Figure 1). 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.

Gene expression analysis

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 elevated pCO2 conditions on calcifying organisms (Kroeker et al. 2010; Gazeau et al. 2007; Orr et al. 2005). The gene encoding perlucin 6, a calcium carbonate nucleation factor (Weiss et al. 2000; Blank et al. 2003), was recently identified in larval Manila clams (Metzger et al. in review). 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.

In order to maintain normal growth, or minimally prevent reduced shell mass or reduced shell integrity under corrosive high pCO2 conditions, altered expression of genes associated with calcification and shell growth would be expected. Perlucin 6 transcript levels were not significantly different in gill tissue of juvenile clams exposed to elevated pCO2 (Figure 2A). This result suggests that the pCO2 conditions used in this study do not significantly affect the processes of shell deposition in juvenile Manila clams.

Juvenile Manila clams are sedentary infaunal organisms that lived buried in sediment where pCO2 is typically higher than that of the surrounding water. Therefore, Manila clams could be adapted to the elevated pCO2 conditions used in this experiment. Alternatively, it is possible that perculin activity is altered, but only in specific tissues. For instance, expression of genes involved in bio mineralization has been shown to vary between tissues in other calcifying organisms (Hüning et al. 2012).

Ion transport is necessary for maintaining ion homeostasis, particularly in gill tissue. 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). Calmodulin transcripts in corals decreased under elevated pCO2 conditions (Kaniewska et al. 2012). There was no effect of elevated pCO2 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). Previous studies have identified changes in protein concentration of larval barnacles (Wong et al 2011) exposed to elevated pCO2. Cathepsin and elongation factor (EF) proteins are involved in mediating protein synthesis and degradation. Elevated pCO2 conditions did not influence cathepsin L or EF2 transcript levels in juvenile clam gill tissue (Figure 2C). These results could indicate that juvenile Manila clams possess adequate energetic resources via routine metabolic activity to compensate for the additional stress of an elevated pCO2 environment.

Heat shock proteins (hsp) are involved in several physiological processes including protein metabolism, response to thermal stress, immune response, and apoptosis (e.g. Feder and Hofmann, 1999; Roberts et al. 2010). HSPs are molecular chaperones that bind and stabilize proteins, aiding in protein synthesis or the repairing of damaged proteins from processes such as oxidative stressed. 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). 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 regulated during early larval periods while juvenile and adults do not actively express hsp90 to the same degree.

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 2E). 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.

Elevated pCO2 conditions can also invoke an oxidative stress response (Tomanek et al. 2011). Oxidative stress is caused by the production of reactive oxygen species (ROS). Antioxidant defense is a primary response of Eastern oysters exposed to elevated pCO2 conditions (Tomanek et al. 2011). Glutatione peroxidase 3 (GPx3) catabolizes ROS to more benign compounds. In this study, elevated pCO2 conditions did not impact the expression of GPx3 transcripts (Figure 2F) suggesting that juvenile Manila clams are not experiencing increased levels of oxidative stress as a result of elevated pCO2 conditions. Variability between species’ oxidative stress response may be an important component regarding their tolerance to elevated pCO2 conditions, however a side-by-side comparison of species is needed before conclusions regarding species specific oxidative stress response regulation can be determined.  
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Thermal tolerance

Juvenile Manila clams held in ambient or elevated pCO2 seawater for three weeks were heat shocked to assess the influence of elevated pCO2 conditions on thermal tolerance (Figure 3).

No differences in survival, OM, or MDD were observed at 39°C (the pre-determined minimum lethal temperature) 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.

Global climate change’s associated effects, such as ocean acidification and increasing sea surface temperatures, will occur in concert with other naturally occurring environmental stressors. Some of these naturally occurring stressors may be benign under normal environmental conditions, but could become stressful or lethal when an organism is faced with a novel, sustained stressor. 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). 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 highly dynamic ecosystems may be more adapted to harsh conditions. Depending on the species and developmental period, the effects 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 rufenscens, 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). Similar to the results presented for juvenile Manila clams, mortality of juvenile *M. mercenaria* was unaffected by elevated temperatures suggesting a potential resistance of infaunal species to increased temperature and pCO2 conditions.

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 calcium ion binding, metabolism, translation, or stress response, were not different in juvenile clams exposed to elevated pCO2. While there is a need for additional studies that examine different life stages, these data indicate juvenile clams could be relatively more resilient to elevated pCO2 conditions expected to occur this century when compared with other aquatic invertebrates of the same life stage (xxxxxxxx). This resiliency is consistent with their life history as adults are infaunal and live buried in sediment where pCO2 is typically higher due to respiration and decomposition. Adaptation has been observed in other sub-benthic bivalves including the hard clam, Mercinaria mercenaria, where increased rates of calcification were attributed to high pCO2 conditions experience in sediment (Waldbusser et al. 2010). Further analysis of the mechanisms evoked by these organisms during metamorphosis into adults may provide insight into successful adaptation strategies to cope with the environmental conditions predicted to occur as a result of anthropogenic CO2 emissions and climate change.

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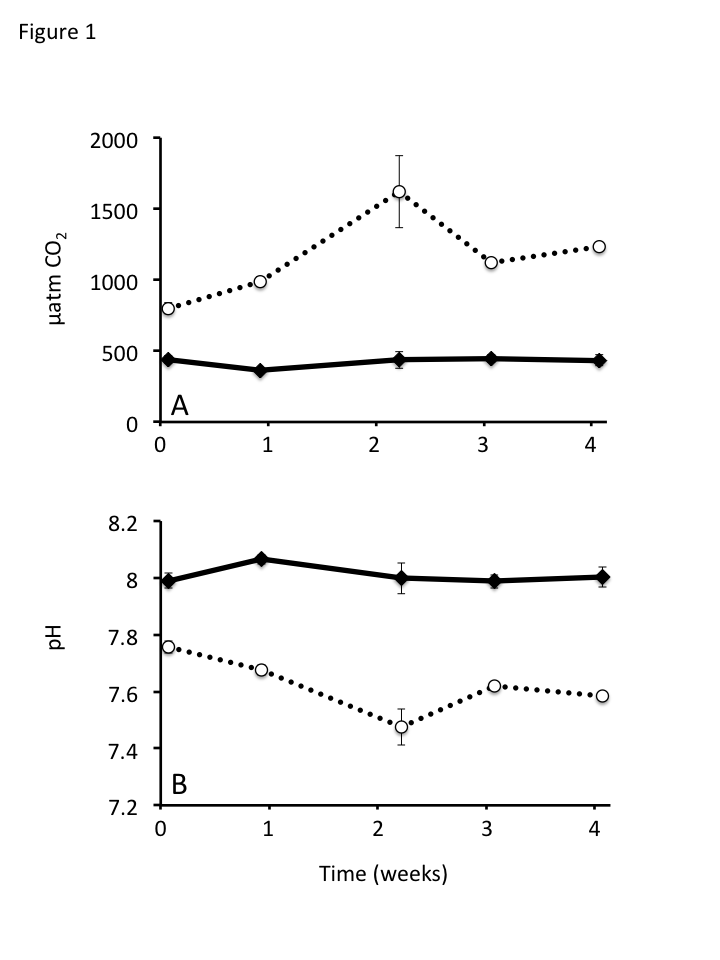


Figure 1. Summary of pH and concentration of dissolved CO2 (μatm) of the ambient (solid line) and elevated CO2 (dashed line) treated seawater. Concentrations of dissolved CO2 were calculated using total alkalinity, salinity, and pH measurements. Final pH measurements were adjusted to correspond to the 13°C treatment water. The plotted data are means ± standard deviation. Give overall mean and SD for each treatment.

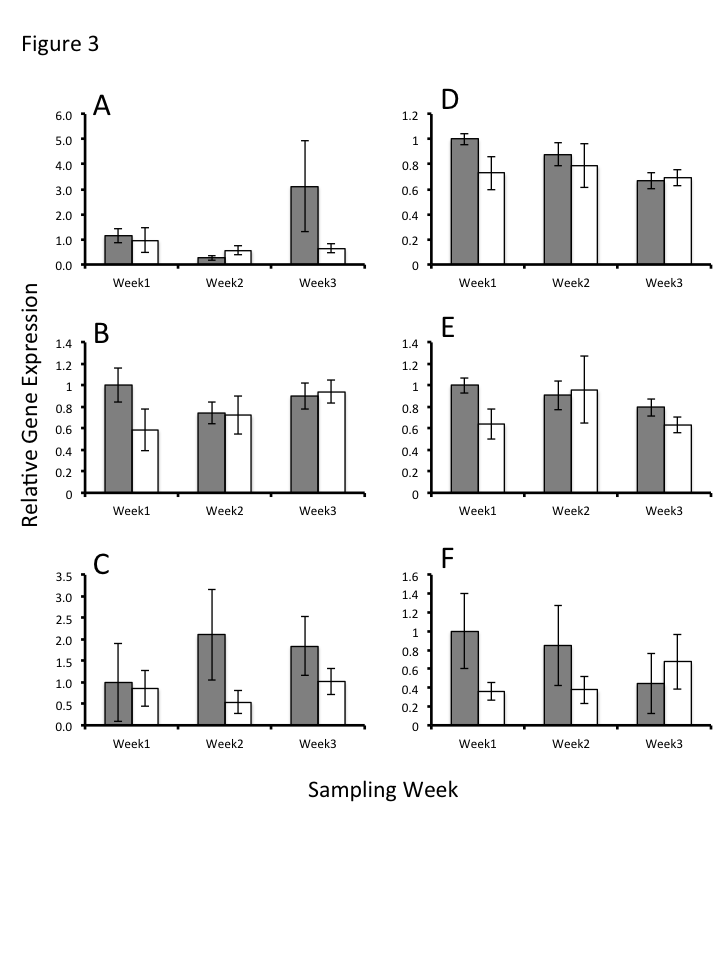


Figure 2. Quantification of transcript abundance in gill tissue of juvenile Manila clams exposed to ambient CO2 (grey bars) and elevated CO2 (white bars) (n= x for each treatment). Gill tissue samples were taken after 1, 2, and 3 weeks of exposure to CO2 treated water and analyzed for expression of perlucin (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.



Figure 2. 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 pCO2 (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 pCO2 conditioned animals to a 40°C thermal stress.

|  |  |  |  |
| --- | --- | --- | --- |
| Gene Target | Oligo | Ruphibase ID | Primer sequence |
| Ef-1α | 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  AGTACAGGCACTGGATGGTGCGTA |
| GPX3 | Fwd  Rev | ruditapes2\_c3709 | ATTCTCGAGCGCTGGGGTAAAAGTG  TAGTTGTCGGCCGGCTCTTGCATT |
| Perlucin | Fwd  Rev | ruditapes\_lrc29501 | GCAGACGTCGACAGGATGTCCAAT  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 |  |  |  |  |  |
| TA | 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 |  |  |  |  |  |
| TA | 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 CO2 treatments.(mean±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 inputs..