Short-term effects of CO_2-induced low pH exposure on target gene expression in *Platynereis dumerilii*

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Abstract

Objective: Increasing atmospheric CO_2 concentration are causing changes to the seawater carbonate chemistry, lowering the pH and we study potential impacts of these changes at the molecular level in a non-calcifying, marine polychaete species *Platynereis dumerilii*. Methods: we investigate the relative expression of *carbonic anhydrase* (*CA)*, *Na^+/H^+ exchangers* (*NHE)*, and *calmodulin* (*CaM)* genes from *P. dumerilii* under acidified seawater conditions (pH 7.8) induced by CO_2 using qPCR. Results: mRNA expression of *CA* in the CO_2-induced worms was significantly up-regulated at low pH conditions (pH 7.8, 1h), suggesting changes in acid-base balance. In contrast, the expression of *NHE* and *CaM* showed no significant change. In addition, we compare these results to a previous study using inorganic acid (HCl)-induced pH changes. Conclusions: results suggest that carbonate chemistry has an impact on gene expression that differs from pH-associated change. To our knowledge, this is the first study that compares low pH exposure experiments using HCl and CO_2 as the inducing agents.

Introduction

Atmospheric CO_2 concentrations are rising faster than ever recorded in the past 800 000 years [1], which may lead to a decrease in seawater pH. The ocean surface pH has dropped by 0.1 pH-units, equivalent to a 26% increase in H^+ ion concentration [2], leading to a current average pH of ocean surface water of around pH 8.1 [3]. Global ocean surface pH is predicted to decrease to ~7.8 by 2100 [2] representing unprecedented fast present-day changes. Increased adaptation pressure is consequently predicted to act on marine species [4], especially on physiological processes such as acid-base regulation, growth and reproduction [5-8].

To predict how ocean acidification (OA) will biologically impact organisms, understanding and characterising the underpinning molecular level changes of physiological processes is required. Recently, a number of transcriptomic and proteomic studies have been conducted using marine organisms such as sea urchins (*Strongylocentratus purpuratus*, *Lytechinus pictus* and *Paracentrotus lividus*) [9-13], corals (*Acropora millepora* and *Pocillopora damicornis*) [14,15], barnacle (*Balanus amphitrite*) [16], oysters (*Saccostrea glomerata, Crassostrea virginica*, *C. gigas* and *C. hongkongensis)* [17-20], fish (*Oryzias latipes*) [21] and the coccolithophore (*Emiliania huxleyi*) [22] all exposed to experimental OA conditions for various exposure durations and at different life stages. While the species used in these studies mainly represent calcifying organisms, and the main focus has been on biomineralisation processes [11,12,14,19], the effects on acid-base regulation [12,14], ion transport [11,12,15] and energy metabolism [11,12,15,16] have also been observed. For instance, the down-regulation of metabolic genes has been observed in sea urchin larvae, oyster larvae, primary coral polyps, adult corals and fish [9,12,14,19,21,23]. Yet other studies using sea urchin larvae (*S. purpuratus*) and mussels, *Mytilus edulis*, report an up-regulation of metabolic genes [10,24]. There are also calcifying species, such as the marine coccolithophore, *E. huxleyi*, that show no changes in key metabolic processes [22].

In addition to changes in metabolic processes, ion regulation and acid-base pathways have been reported as impacted. For instance, in sea urchin *L. pictus* larvae, both pathways were up-regulated [12], whereas the sea urchin *S. purpuratus* showed both up- regulation and down-regulation of ion regulation genes [10]. Taken together, these studies show that responses to low pH/ high pCO_2 are species specific and that many cellular processes, in addition to calcification, may be impacted by pH in marine organisms.

The majority of these OA studies alter the pH with CO_2 to mimic predicted environmental pH levels, relating the findings as the effects induced by acidification of the seawater [e.g. 20,25,26]. While CO_2 from the atmosphere does lead to a lower pH of the seawater it also causes several changes in carbonate chemistry, specifically an increase in HCO_3^- concentrations [27]. It is therefore difficult to assess whether any observed changes are causally linked to pH or carbonate level changes. Previously, we examined low seawater pH effects induced by inorganic acids (HCl) on specific genes: *CA*, *NHE*, and *CaM*, involved in biomineralisation, ion transport, acid-base regulation and energy metabolism on *P. dumerilii* [28]. *P. dumerilii* is a marine model, which is simple to establish in laboratory cultures and its genome is available (PLATYpopsys, transcriptomic and genomic data base: http://hydra.cos.uni-heidelberg.de/pps/styled-2/) [29]. It is primarily used for the study of molecular development, evolution, neurobiology, ecology and toxicology [30-32] and can be found in naturally occurring acidified habitats [33]. Herein we report the effects of short term low pH exposure (1 hour and 7 days) induced by CO_2 gas on the same transcripts, as previously studied with HCl, in the marine polychaete *P. dumerilii* [28], allowing a comparison of the two approaches.

Material and Methods

Animals and experimental exposure.

P. dumerilii (mean body mass ± s.e.m.: pH 8.2 worms 1 h: 24.22 ± 2.43 mg, n=10; pH 7.8 worms 1h 21.86 ± 3.19 mg, n=10; pH 8.2 worms 7 d: 31.34 ± 4.80 mg, n=10; pH 7.8 worms 7 d: 30.33 ± 3.34 mg, n=10) were used from a laboratory culture obtained from the EMBL Heidelberg (Germany). The worms were originally collected from shallow depth in the Mediterranean Sea (Ischia, Italy) and Atlantic Ocean (Roscoff, France). Worms were kept in filtered natural seawater (~pH 8.2) in culture tanks (2000cm^3; approximately 50 individuals per tank) at a light regime of 16 h light/ 8 h dark in a temperature controlled room at 18°C. Worms were fed twice a week with either organic spinach or a fish food-microalgae-mix (e.g. *Tetraselmis marinus* or *Isochrysis galbana*). A complete water change was conducted fortnightly or earlier if the water quality failed to meet standard.

To reduce effects induced by physiological changes during reproduction and metamorphosis, only adult and sexually immature worms were used in the experiment. For the exposure experiment, 20 worms were transferred into plastic containers (2000 cm^3) with 800 ml filtered natural seawater (salinity 33.23 ± 0.21 ppt; temperature 17.81 ± 0.13°C) of pH 8.2 (control), and another 20 worms were transferred into plastic containers (2000 cm^3) with approximately 800 ml filtered natural seawater (salinity 33.19 ± 0.22 ppt; temperature 17.81 ± 0.13°C) of pH 7.8 (treatment) kept at the same light regime. The containers were covered with a lid to prevent evaporation. The pH was adjusted by bubbling CO_2 gas into the water, and a complete water change was conducted every 24 h to ensure that the desired pH was maintained. At two time points (1 h and 7 days), 10 individuals from both the treatment and control containers were taken and placed in RNALater solution (Sigma-Aldrich Company Ltd., Gillingham, U.K.). The worms were stored at -80°C until further processing. The sampling time points were chosen to show an initial stress response (1 h) and an acclimation response (at 7 days).

Target gene isolation and characterisation.

Nucleotide sequences for the reference and target cDNAs, *18S ribosomal RNA* (*18S*), *CA*, *NHE*, and *CaM* (previously described in [28]) were used. The reference gene *α-TUB* was re-optimised for use due to low efficiency under the new experimental conditions and the primer pair is shown in Table 1. Total RNA was extracted from each whole worm using High Pure RNA Tissue reagents and protocol (Roche Diagnostics Ltd., Burgess Hill, U.K.). An aliquot of each total RNA sample was run on a denaturing TAE agarose gel stained with ethidium bromide to test the integrity (Life Technologies, Paisley, U.K.). cDNA was synthesised from the total RNA using the SuperScript VILO cDNA Synthesis Kit reagents and protocol (Life Technologies, Paisley, U.K.). 4 µl 5× VILO™ Reaction Mix, 2 µl 10× SuperScript® Enzyme Mix were mixed with approximately 300 ng of RNA and incubated at 25°C for 10 min, followed by 60 min at 42°C and 5 min at 85°C. To degrade remaining RNA template RNase H (5 U/µl) (Thermo Scientific, Loughborough, U.K.) was used following the manufacturer's protocol. To generate the *α-TUB* PCR product, 1 µl of cDNA was combined with 0.5 µl of 10 pmol/ µl forward and reverse primer (Table 1), 0.25 µl of Herculase cDNA polymerase (Agilent Technologies, Wokingham, U.K.), 5 µl of 5× PCR buffer (Agilent Technologies, U.K.), 0.5 µL 40 mM dNTP mix (Thermo Fisher Scientific, U.K.), 0.5 µL DMSO (Agilent Technologies, U.K.), 0.5 µL 25 mM $MgCl_2$ (Thermo Fisher Scientific) and 16.25 µL sterile nuclease-free water (Fisher Scientific, U.K.) to prepare a total reaction volume of 25 µL. The PCR conditions for *18S rRNA*, *CA*, *NHE*, and *CaM* have been described previously in [28].

qPCR analysis of gene expression.

For each qPCR reaction, a CFX96 Real Time PCR Detection System (Bio-Rad, Hemel Hempstead, U.K.) was used to detect amplification. qPCR assays were optimised using the internationally implemented MIQE guidelines [34]. Several primer concentrations for each gene were tested to determine the optimal primer concentration. The primer pair concentration with the lowest Cq value

and a melt curve showing only a single distinct product was utilised. To amplify the cDNA, 10 µl of qPCR Fast Start SYBR Green Master Rox (Roche, Burgess Hill, U.K.), 1 µl of cDNA and 2 µl of primers (*18S* 50 nM; *α-TUB*$_2$ 700 nM; *CA*, *NHE*, *CaM* 100 nM) (Table 1) and 7 µl molecular grade water (Thermo Fisher Scientific, Loughborough, U.K.) were used to make up a final reaction volume of 20 µl. For each gene, a control reaction lacking template was included to determine the target cDNA amplification specificity. After an initial denaturation at 95°C for 2 min all reactions were carried out in the following 45 cycles: Denaturation at 95°C for 10 s, annealing at 60°C for 1 min and an extension step at 72°C for 1 min. In order to generate the melt curve a temperature gradient was created from 60°C to 95°C. To check the amplification specificity of the new *α-TUB* primer pair, the melting curve and gel picture was analysed. The amplification efficiency, based on a serial dilution of cDNA, showed 102.8% for *α-TUB*. The reference mRNAs *18S* and *α-TUB* were then used for the normalisation in pH 7.8 and pH 8.2 tissue of *P. dumerilii*. Both *18S* and *α-TUB* have previously been established as *reference* genes for *Nereis* species [28,35,36]. For the calculation of the relative expression levels of the target gene the $2^{-\Delta\Delta Ct}$ method was applied [37].

Statistical analyses

Statistical analysis was conducted using IBM® SPSS® Statistics 22.0 (Armonk, NY: IBM Corp., U.S.A.). All data were tested for normality and homogeneity of variances using the Kolmorgorov-Smirnov test. Significance for relative gene expression was tested using a two-way ANOVA. In cases of significance an independent t-test was used. Outliers were identified, and removed, if they differed by more than twice the standard deviation of the mean. For *NHE* one value of each treatment was identified as an outlier. For *CA* one value for pH 8.2, 1 h; two values of pH 7.8, 1 h and two values for pH 7.8, 7 d were identified as outliers and excluded from the statistical analysis. For all analyses, statistical significance was accepted at $p<0.05$. Values are presented as means ± S.E.M. All graphs were designed with XACT 8.03 (SciLab, Germany).

Results

Quantitative real-time PCR analysis of *CA*, *NHE* and *CaM* mRNA expression.

The expression level of each target mRNA was analysed in worms maintained in normal or low pH conditions using qPCR (Fig. 1). *CA* mRNA expression showed a significant effect of pH [$F(1, 31) = 9.045$; $p=0.005$] but no significant effect of time [$F(1, 31) = 0.00$; $p=1.000$] (Fig. 1A). *CA* was significantly up-regulated [$t(15) = -2.659$; $p=0.018$] in worms maintained at pH 7.8 (1h) in comparison to pH 8.2 and showed an up-regulation trend [$t(16) = -1.899$; $p=0.076$] in worms maintained at pH 7.8 (7d) (Fig. 1A). There was no significant interaction between time and pH [$F(1, 31) = 0.019$; $p=0.892$]. For *NHE* expression, both pH [$F(1, 32) = 3.528$; $p=0.069$] and time [$F(1, 32) = 2.448$; $p=0.128$] showed no significant change on the relative gene expression and there was also no significant interaction ($p=1.000$), though pH showed a clear down-regulation trend after 7 d (Fig. 1B). Similarly to *NHE*, there was no significant interaction between time and pH [$F(1, 31) = 0.019$; $p=0.892$] for *CaM* with no significant relative gene expression change (pH [$F(1, 36) = 0.061$; $p=0.806$] and time [$F(1, 36) = 0.812$; $p=0.374$]) (Fig. 1C).

Discussion

This study reports the relative gene expression of *CA*, *NHE* and *CaM* in response to CO_2-induced acidified seawater (pH 7.8) on *P. dumerilii*. The exposure regime reflected the pH values predicted for the year 2100 (~pH 7.8) [2] at two times points of 1 hour and 7 days. The selected *target* genes, are involved in passive intra- and extracellular fluids buffering, ion exchange and transport, transport of CO_2 in blood, biomineralisation and metabolic processes [38] and had previously been shown to be affected in worms exposed to inorganic acid-induced pH changes to varying degrees [28].

Simulating the pH levels predicted for 2100, the mRNA expression of *CA* showed a significant up-regulation ($p=0.018$) under low pH conditions in the group exposed for 1 hour and an up-regulation trend ($p=0.076$) in the group exposed for 7 days in comparison to *P. dumerilii* kept at

control pH conditions (Fig. 1A). No effect of time ($p=1.000$) and no significant interaction ($p=0.892$) between time and pH was observed. The *NHE* expression showed no significant change after 1 hour or 7 days ($p=0.128$), yet a down-regulation trend ($p=0.069$) for the effect of pH was observed (Fig. 1B). *CaM* expression showed no significant changes at either time point relative to the control group (Fig. 1C).

In agreement with these results, *CA* expression in the coral species, *P. damicornis*, was found to be up-regulated under low pH (7.8 and 7.4) after an exposure time of 3 weeks [15]. In contrast, a study on the sea urchin larvae *S. purpuratus* kept at pH 7.7 for 2, 4 and 7 days reported no significant effect in related *CA*s [10]. Another study using sea urchin larvae *S. purpuratus* highlighted CAs as important for biomineralisation processes and reported *CA-7 like A* transcript levels as up-regulated and four other *CA* transcripts as down-regulated [9]. There are two further studies reporting *CA mRNA* or protein down-regulation: coral *A. millepora* after 3 days under elevated pCO_2 conditions [14], and mussel *M. edulis* after a six months exposure to high pCO_2 [39]. The different expressions of the *CA*s are likely caused by functional differences of the isoforms as well as different requirements for calcifying species compared to a non-calcifying species such as the polychaete *P. dumerilii*. The up-regulation in *P. dumerilii* under high pCO_2 concentrations could suggest an increased need of *CA* to maintain the acid-base balance in the blood and other tissues in helping to transport CO_2 out of the tissues.

Again, similarly to the results presented showing no changes in *NHE* mRNA expression levels, a study using the sea urchin *S. purpuratus* showed no significant change under acidified conditions (pH 7.88-7.96) within <72 hours [9]. In contrast, a different study on *S. purpuratus* larvae reported an up-regulation of *NHE3* after 2 days in low pH (7.7) conditions and a 45% down-regulation after 4 days [10]. The observed lack of significant changes in the *NHE* expression herein may suggest that the activity of existing protein levels were sufficient to regulate the pH under the exposure conditions, and that a costly increase in *NHE* was not required to maintain the acid-base balance.

The relative gene expression of *CaM* was not changed under low pH conditions in *P. dumerilii*, in contrast to previous studies using a range of species. For example, studies using the coral *A. millepora* and Pacific oyster *C. gigas* larvae both showed a down-regulation of CaM in response to low pH [19,23]. The commercial oyster *C. hongkongensis* showed a significant down-regulation at moderate low pH (7.9) and a small up-regulation at low pH (7.6) [20]. However, *A. millepora*, *C. gigas* and *C. hongkongensis* represent calcifying species unlike the polychaete. Calcification is considered to be one of the most vulnerable physiological processes towards OA [40]. It is therefore possible that the CaM is more important for calcification / biomineralisation processes than other physiological processes, as indicated by the present results. Another relevant factor that needs to be considered is the life stage of the animals. Many of the studies available in the literature solely focus on the early life history stages [12,16,41]. This study used adult specimens and *P. dumerilii* larvae may respond differently to the same exposure regime. Therefore, further studies are needed to make predictions for different life stages.

Previously we exposed *P. dumerilii* to the same pH levels for the same duration but using HCl to induce the pH change [28] finding different responses in the same gene expressions as follows. The relative gene expression of *CA* was significantly up-regulated under low pH conditions (1h) induced by CO_2, yet HCl-induced change resulted in a down-regulation trend of *CA* after 1 hour and no effect after 7 days [28]. To understand the difference in the gene expression response we must consider the impacts of the two methods of controlling pH. According to Le Châtelier's principle, any disturbance may have a cascading effect upon all components of the equilibrium whereby CO_2 dissolved in seawater changes the inorganic carbon and keeps the total alkalinity at a constant level, while mineral acid (HCl) changes the total alkalinity but not the concentration of inorganic carbon [42]. It is thus possible that total alkalinity and dissolved inorganic carbon levels influence the targeted gene expressions besides strict pH changes. A higher concentration of CO_2 in the seawater may have increased the quantity of *CA* mRNA to facilitate the catalytic reaction of the

interconversion of CO_2 and water to HCO_3^- and H^+, whereas this effect could not be observed when changing only the total alkalinity of the water.

Likewise, low pH induced by HCl showed a significant down-regulation of *NHE* in the worms exposed for 1 h and a significant up-regulation in the worms exposed to low pH for 7 d [28]. In contrast, CO_2 induced pH change resulted in a down-regulation trend only. In comparison to *CA* and *NHE*, *CaM* showed no change in the mRNA expression following both HCl and CO_2 induced pH level changes. Overall, the different gene expression results suggests that it is not necessarily the pH change that induces the expression changes observed in the animals kept at low pH in climate change simulation studies. Furthermore, the results indicate that non-calcifying species may also be affected by the seawater changes expected within this century and provide insight into the potential mechanisms of damage in the non-calcifying marine worm *P. dumerilii*.

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Competing interests

No competing interests declared.

Author contributions

JW carried out molecular work, statistical analysis and drafted the manuscript. SR conducted the exposure experiments. JDH participated in the design of the study. JMR participated in the design of the study and coordinated and contributed to the draft the manuscript.

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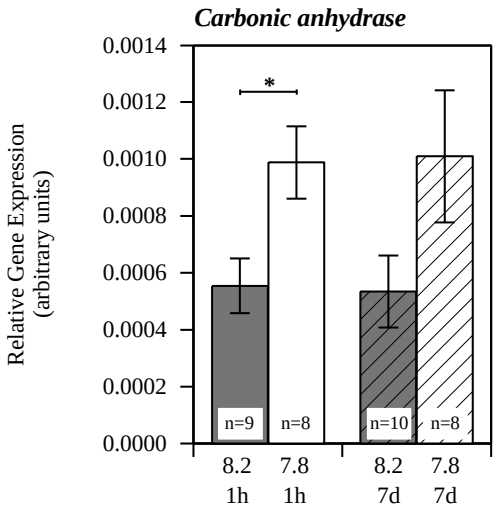
Figure and Table Legends

Figure 1. Normalised average relative mRNA transcription ± standard error of the mean in *P. dumerilii* for (A) *CA* (B) *NHE* and (C) *CaM* after 1 h and after 7 d in seawater with pH 8.2 and pH 7.8. For all groups (pH and time) 10 worms were exposed under laboratory conditions. The pH was adjusted by bubbling CO_2 gas into the water. All data were tested for normality and homogeneity of variances using the Kolmorgorov-Smirnov test. Statistical analysis was performed using a two-way ANOVA and, in the case of significance, an independent t-test was run. Outliers were identified, and removed, if they differed by more than twice the standard deviation of the mean. For all analyses, statistical significance was accepted at $*p<0.05$. Values are presented as means ± s.e.. All graphs were designed with XACT 8.03 (SciLab, Germany).

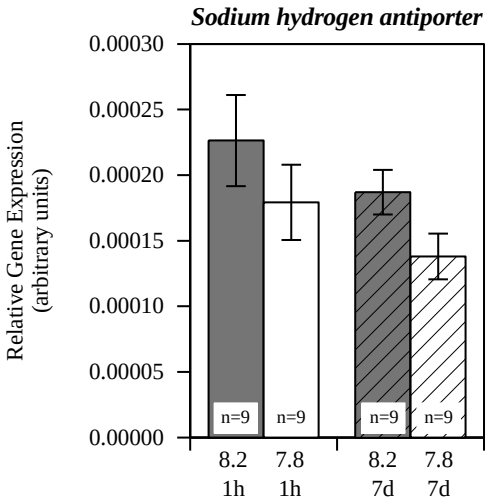
Table 1. Primer sequences of *target* genes for qPCR with their expected amplicon size (bp) and primer concentration (nM) used for the analysis.

Figure 1.

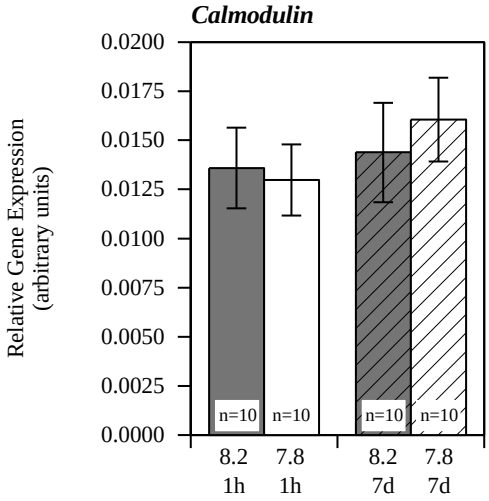
A *CA*



B *NHE*



C *CaM*



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Target gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Amplicon size (bp)**	**Primer concentration (nM)**
18S rRNA	GCGCATTTATCAGCACAAGA	CTTGGATGTGGTAGCCGTTT	239	50
α-TUB	TTGCTGTCTACCCAGCTCCT	AGATGGCCTCATTGTCAACC	123	700
CA	TAACCACCTCAACCGGAGAC	ATGGTGTGCTCTGAGCCTTT	118	100
NHE	CGCTCTGTTGCTGTCTTGAG	TGGCTACTAAGGCGAATGCT	130	100
CaM	AAGCTTTCCGAGTGTTCGAC	CCTCTTCGTCCGTCAATTTC	102	100

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