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2 Short-term effects of CO_2-induced low pH exposure on target gene 3 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].

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

72 The majority of these OA studies alter the pH with CO_2 to mimic predicted environmental
73 pH levels, relating the findings as the effects induced by acidification of the seawater [e.g.
74 20,25,26]. While CO_2 from the atmosphere does lead to a lower pH of the seawater it also causes
75 several changes in carbonate chemistry, specifically an increase in HCO_3^- concentrations [27]. It is
76 therefore difficult to assess whether any observed changes are causally linked to pH or carbonate
77 level changes. Previously, we examined low seawater pH effects induced by inorganic acids (HCl)
78 on specific genes: *CA, NHE,* and *CaM,* involved in biomineralisation, ion transport, acid-base
79 regulation and energy metabolism on *P. dumerilii* [28]. *P. dumerilii* is a marine model, which is
80 simple to establish in laboratory cultures and its genome is available (PLATYpopsys,
81 transcriptomic and genomic data base: http://hydra.cos.uni-heidelberg.de/pps/styled-2/) [29]. It is
82 primarily used for the study of molecular development, evolution, neurobiology, ecology and
83 toxicology [30-32] and can be found in naturally occurring acidified habitats [33]. Herein we report
84 the effects of short term low pH exposure (1 hour and 7 days) induced by CO_2 gas on the same
85 transcripts, as previously studied with HCl, in the marine polychaete *P. dumerilii* [28], allowing a
86 comparison of the two approaches.

87

88 # Material and Methods

89 **Animals and experimental exposure.**

90 *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
91 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:
92 30.33 ± 3.34 mg, n=10) were used from a laboratory culture obtained from the EMBL Heidelberg
93 (Germany). The worms were originally collected from shallow depth in the Mediterranean Sea
94 (Ischia, Italy) and Atlantic Ocean (Roscoff, France). Worms were kept in filtered natural seawater
95 (~pH 8.2) in culture tanks (2000cm^3; approximately 50 individuals per tank) at a light regime of
96 16 h light/ 8 h dark in a temperature controlled room at 18°C. Worms were fed twice a week with
97 either organic spinach or a fish food-microalgae-mix (e.g. *Tetraselmis marinus* or
98 *Isochrysis galbana*). A complete water change was conducted fortnightly or earlier if the water
99 quality failed to meet standard.

100 To reduce effects induced by physiological changes during reproduction and
101 metamorphosis, only adult and sexually immature worms were used in the experiment. For the
102 exposure experiment, 20 worms were transferred into plastic containers (2000 cm^3) with 800 ml
103 filtered natural seawater (salinity 33.23 ± 0.21 ppt; temperature 17.81 ± 0.13°C) of pH 8.2 (control),
104 and another 20 worms were transferred into plastic containers (2000 cm^3) with approximately
105 800 ml filtered natural seawater (salinity 33.19 ± 0.22 ppt; temperature 17.81 ± 0.13°C) of pH 7.8
106 (treatment) kept at the same light regime. The containers were covered with a lid to prevent
107 evaporation. The pH was adjusted by bubbling CO_2 gas into the water, and a complete water change
108 was conducted every 24 h to ensure that the desired pH was maintained. At two time points (1 h and
109 7 days), 10 individuals from both the treatment and control containers were taken and placed in
110 RNALater solution (Sigma-Aldrich Company Ltd., Gillingham, U.K.). The worms were stored at -
111 80°C until further processing. The sampling time points were chosen to show an initial stress
112 response (1 h) and an acclimation response (at 7 days).

113

114 **Target gene isolation and characterisation.**

115 Nucleotide sequences for the reference and target cDNAs, *18S ribosomal RNA* (*18S*), *CA, NHE*, and
116 *CaM* (previously described in [28]) were used. The reference gene *α-TUB* was re-optimised for use
117 due to low efficiency under the new experimental conditions and the primer pair is shown in Table
118 1. Total RNA was extracted from each whole worm using High Pure RNA Tissue reagents and
119 protocol (Roche Diagnostics Ltd., Burgess Hill, U.K.). An aliquot of each total RNA sample was
120 run on a denaturing TAE agarose gel stained with ethidium bromide to test the integrity (Life
121 Technologies, Paisley, U.K.). cDNA was synthesised from the total RNA using the SuperScript
122 VILO cDNA Synthesis Kit reagents and protocol (Life Technologies, Paisley, U.K.). 4 µl
123 5× VILO™ Reaction Mix, 2 µl 10× SuperScript® Enzyme Mix were mixed with approximately
124 300 ng of RNA and incubated at 25°C for 10 min, followed by 60 min at 42°C and 5 min at 85°C.
125 To degrade remaining RNA template RNase H (5 U/µl) (Thermo Scientific, Loughborough, U.K.)
126 was used following the manufacturer's protocol. To generate the *α-TUB* PCR product, 1 µl of
127 cDNA was combined with 0.5 µl of 10 pmol/ µl forward and reverse primer (Table 1), 0.25 µl of
128 Herculase cDNA polymerase (Agilent Technologies, Wokingham, U.K.), 5 µl of 5× PCR buffer
129 (Agilent Technologies, U.K.), 0.5 µL 40 mM dNTP mix (Thermo Fisher Scientific, U.K.), 0.5 µL
130 DMSO (Agilent Technologies, U.K.), 0.5 µL 25 mM $MgCl_2$ (Thermo Fisher Scientific) and
131 16.25 µL sterile nuclease-free water (Fisher Scientific, U.K.) to prepare a total reaction volume of
132 25 µL. The PCR conditions for *18S rRNA*, *CA, NHE*, and *CaM* have been described previously in
133 [28].

134

135 **qPCR analysis of gene expression.**

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

140 and a melt curve showing only a single distinct product was utilised. To amplify the cDNA, 10 µl of
141 qPCR Fast Start SYBR Green Master Rox (Roche, Burgess Hill, U.K.), 1 µl of cDNA and 2 µl of
142 primers (*18S* 50 nM; *α-TUB*$_2$ 700 nM; *CA*, *NHE*, *CaM* 100 nM) (Table 1) and 7 µl molecular grade
143 water (Thermo Fisher Scientific, Loughborough, U.K.) were used to make up a final reaction
144 volume of 20 µl. For each gene, a control reaction lacking template was included to determine the
145 target cDNA amplification specificity. After an initial denaturation at 95°C for 2 min all reactions
146 were carried out in the following 45 cycles: Denaturation at 95°C for 10 s, annealing at 60°C for
147 1 min and an extension step at 72°C for 1 min. In order to generate the melt curve a temperature
148 gradient was created from 60°C to 95°C. To check the amplification specificity of the new *α-TUB*
149 primer pair, the melting curve and gel picture was analysed. The amplification efficiency, based on
150 a serial dilution of cDNA, showed 102.8% for *α-TUB*. The reference mRNAs *18S* and *α-TUB* were
151 then used for the normalisation in pH 7.8 and pH 8.2 tissue of *P. dumerilii*. Both *18S* and *α-TUB*
152 have previously been established as *reference* genes for *Nereis* species [28,35,36]. For the
153 calculation of the relative expression levels of the target gene the $2^{-\Delta\Delta Ct}$ method was applied [37].

154

155 **Statistical analyses**

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

165

166 # Results

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

168 The expression level of each target mRNA was analysed in worms maintained in normal or low pH
169 conditions using qPCR (Fig. 1). *CA* mRNA expression showed a significant effect of pH [F(1,
170 31) = 9.045; p=0.005] but no significant effect of time [F(1, 31) = 0.00; p=1.000] (Fig. 1A). *CA* was
171 significantly up-regulated [t(15) = -2.659; p=0.018] in worms maintained at pH 7.8 (1h) in
172 comparison to pH 8.2 and showed an up-regulation trend [t(16) = -1.899; p=0.076] in worms
173 maintained at pH 7.8 (7d) (Fig. 1A). There was no significant interaction between time and pH [F(1,
174 31) = 0.019; p=0.892]. For *NHE* expression, both pH [F(1, 32) = 3.528; p=0.069] and time [F(1,
175 32) = 2.448; p=0.128] showed no significant change on the relative gene expression and there was
176 also no significant interaction (p=1.000), though pH showed a clear down-regulation trend after 7 d
177 (Fig. 1B). Similarly to *NHE*, there was no significant interaction between time and pH [F(1,
178 31) = 0.019; p=0.892] for *CaM* with no significant relative gene expression change (pH [F(1,
179 36) = 0.061; p=0.806] and time [F(1, 36) = 0.812; p=0.374]) (Fig. 1C).

180

181 # Discussion

182 This study reports the relative gene expression of *CA, NHE* and *CaM* in response to CO_2-induced
183 acidified seawater (pH 7.8) on *P. dumerilii*. The exposure regime reflected the pH values predicted
184 for the year 2100 (~pH 7.8) [2] at two times points of 1 hour and 7 days. The selected *target* genes,
185 are involved in passive intra- and extracellular fluids buffering, ion exchange and transport,
186 transport of CO_2 in blood, biomineralisation and metabolic processes [38] and had previously been
187 shown to be affected in worms exposed to inorganic acid-induced pH changes to varying degrees
188 [28].

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

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

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

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

218 The relative gene expression of *CaM* was not changed under low pH conditions in
219 *P. dumerilii*, in contrast to previous studies using a range of species. For example, studies using the
220 coral *A. millepora* and Pacific oyster *C. gigas* larvae both showed a down-regulation of CaM in
221 response to low pH [19,23]. The commercial oyster *C. hongkongensis* showed a significant down-
222 regulation at moderate low pH (7.9) and a small up-regulation at low pH (7.6) [20]. However,
223 *A. millepora*, *C. gigas* and *C. hongkongensis* represent calcifying species unlike the polychaete.
224 Calcification is considered to be one of the most vulnerable physiological processes towards OA
225 [40]. It is therefore possible that the CaM is more important for calcification / biomineralisation
226 processes than other physiological processes, as indicated by the present results. Another relevant
227 factor that needs to be considered is the life stage of the animals. Many of the studies available in
228 the literature solely focus on the early life history stages [12,16,41]. This study used adult
229 specimens and *P. dumerilii* larvae may respond differently to the same exposure regime. Therefore,
230 further studies are needed to make predictions for different life stages.

231 Previously we exposed *P. dumerilii* to the same pH levels for the same duration but using
232 HCl to induce the pH change [28] finding different responses in the same gene expressions as
233 follows. The relative gene expression of *CA* was significantly up-regulated under low pH conditions
234 (1h) induced by CO_2, yet HCl-induced change resulted in a down-regulation trend of *CA* after 1
235 hour and no effect after 7 days [28]. To understand the difference in the gene expression response
236 we must consider the impacts of the two methods of controlling pH. According to Le Châtelier's
237 principle, any disturbance may have a cascading effect upon all components of the equilibrium
238 whereby CO_2 dissolved in seawater changes the inorganic carbon and keeps the total alkalinity at a
239 constant level, while mineral acid (HCl) changes the total alkalinity but not the concentration of
240 inorganic carbon [42]. It is thus possible that total alkalinity and dissolved inorganic carbon levels
241 influence the targeted gene expressions besides strict pH changes. A higher concentration of CO_2 in
242 the seawater may have increased the quantity of *CA* mRNA to facilitate the catalytic reaction of the

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

245 Likewise, low pH induced by HCl showed a significant down-regulation of *NHE* in the
246 worms exposed for 1 h and a significant up-regulation in the worms exposed to low pH for 7 d [28].
247 In contrast, CO_2 induced pH change resulted in a down-regulation trend only. In comparison to *CA*
248 and *NHE*, *CaM* showed no change in the mRNA expression following both HCl and CO_2 induced
249 pH level changes. Overall, the different gene expression results suggests that it is not necessarily the
250 pH change that induces the expression changes observed in the animals kept at low pH in climate
251 change simulation studies. Furthermore, the results indicate that non-calcifying species may also be
252 affected by the seawater changes expected within this century and provide insight into the potential
253 mechanisms of damage in the non-calcifying marine worm *P. dumerilii*.
254

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258

259 Competing interests

260 No competing interests declared.
261

262 Author contributions

263 JW carried out molecular work, statistical analysis and drafted the manuscript. SR conducted the
264 exposure experiments. JDH participated in the design of the study. JMR participated in the design
265 of the study and coordinated and contributed to the draft the manuscript.
266

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383 # Figure and Table Legends

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

393

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

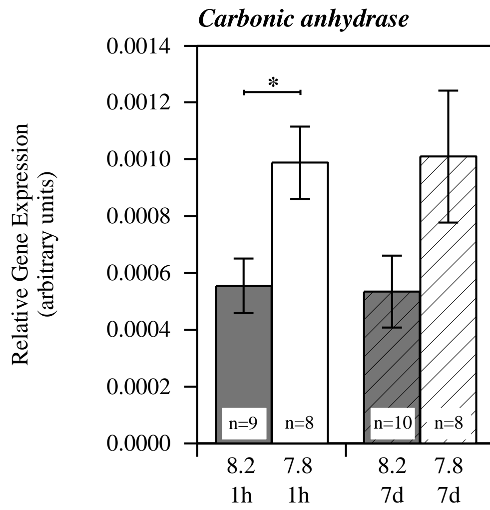
396

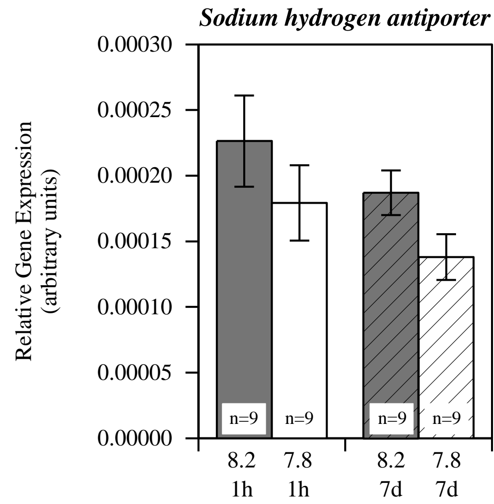
397

398 Figure 1.

399

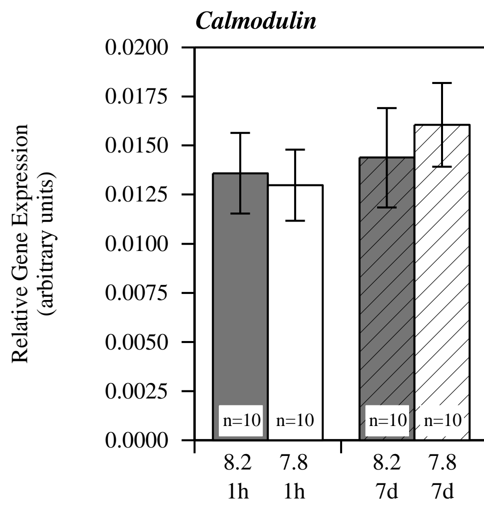
400 **A** *CA* **B** *NHE*





401

402 **C** *CaM*



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408 Table 1

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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