

1           **Title: METABOLIC RESPONSES TO TEMPERATURE STRESS UNDER**  
2           **ELEVATED  $p\text{CO}_2$  IN THE SLIPPER LIMPET *CREPIDULA FORNICATA***

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27          **Short running head:** *C. fornicata* respiration under high  $p\text{CO}_2$

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## ABSTRACT

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In the current context of environmental change, ocean acidification is predicted to affect the cellular processes, physiology and behavior of all marine organisms, impacting survival, growth and reproduction. In relation to thermal tolerance limits, the effects of elevated  $p\text{CO}_2$  could be expected to be more pronounced at the upper limits of the thermal tolerance window. Our study focused on *Crepidula fornicata*, an invasive gastropod which colonized shallow waters around European coasts during the 20<sup>th</sup> century. We investigated the effects of 10 weeks' exposure to current (380  $\mu\text{atm}$ ) and elevated (550, 750, 1000  $\mu\text{atm}$ )  $p\text{CO}_2$  on this engineer species using an acute temperature increase ( $1^\circ\text{C } 12\text{h}^{-1}$ ) as the test. Respiration rates were measured on both males (small individuals) and females (large individuals). Mortality increased suddenly from  $34^\circ\text{C}$ , particularly in females. Respiration rate in *C. fornicata* increased linearly with temperature between  $18^\circ\text{C}$  and  $34^\circ\text{C}$ , but no differences were detected between the different  $p\text{CO}_2$  conditions either in the regressions between respiration rate and temperature, or in  $Q_{10}$  values. In the same way, condition indices were similar in all the  $p\text{CO}_2$  treatments at the end of the experiment but decreased from the beginning of the experiment. This species was highly resistant to acute exposure to high temperature regardless of  $p\text{CO}_2$  levels, even though food was limited during the experiment. *C. fornicata* appears to have either developed resistance mechanisms or a strong phenotypic plasticity to deal with fluctuations of physico-chemical parameters in their habitat. This suggests that this invasive species may be more resistant to future environmental changes compared to its native competitors.

**Keywords:**  $\text{CO}_2$  stress, invasive species, ocean acidification,  $Q_{10}$ , respiration, temperate waters

## INTRODUCTION

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As part of global change, ocean acidification is caused by increasing anthropogenic CO<sub>2</sub> emissions which have increased since the beginning of the industrial revolution (Solomon *et al.*, 2007). Future *p*CO<sub>2</sub> increases are predicted to reduce the pH of surface waters by 0.3 - 0.4 units by the end of the century (Caldeira & Wickett, 2003). Such decreases will produce changes in carbon and carbonate seawater chemistry through decreased carbonate ion concentrations (CO<sub>3</sub><sup>2-</sup>) and a lower calcium carbonate saturation state ( $\Omega$ ). These changes are predicted to have major consequences for marine life (Fabry *et al.*, 2008; Kroeker *et al.*, 2013b) and, especially, could have broad impacts on physiological functions of heterotrophic marine organisms (Pörtner, 2008; Hofmann & Todgham, 2010).

The decrease in pH is likely to have a wide range of effects on marine invertebrates via shifts in acid-base homeostasis, changes in metabolism and energy balance (Pörtner *et al.*, 2005), leading to effects on somatic growth (Berge *et al.*, 2006; Thomsen & Melzner, 2010), respiration (Melatunan *et al.*, 2011; Schalkhauser *et al.*, 2013), excretion (Liu & He, 2012), calcification (Gazeau *et al.*, 2007; Wood *et al.*, 2008; Watson *et al.*, 2012) or feeding rates (Bamber, 1990; Navarro *et al.*, 2013). Many marine invertebrates exposed to elevated *p*CO<sub>2</sub> have exhibited metabolic depression (Willson & Burnett, 2000; Michaelidis *et al.*, 2005; Navarro *et al.*, 2013) as a decrease in respiration rate while others have remained unaffected (Gutowska *et al.*, 2008; Lannig *et al.*, 2010; Clark *et al.*, 2013) or even increased their metabolic rate (Wood *et al.*, 2008; Beniash *et al.*, 2010). These responses are highly species-specific and may vary with organism size (Beniash *et al.*, 2010). The resilience of the species studied, and the capacity to regulate metabolism under stressful conditions are also important (Pörtner, 2008). These physiological impacts are likely to have broad effects on the survival, growth and reproduction of marine species (Shirayama & Thornton, 2005; Byrne, 2011),

83 which would lead to changes in community structure from altered diversity and abundances  
84 (Hale *et al.*, 2011; Kroeker *et al.*, 2013a).

85         These physiological impacts are likely to be modulated by temperature because  
86 temperature is a primary driver of physiological function in ectotherms (Hofmann &  
87 Todgham, 2010). Increasing temperature affects the rate of all biochemical reactions, and  
88 hence cellular processes and physiological functions (Clarke, 1983; Pörtner, 2012), increasing  
89 metabolic costs within a limited thermal tolerance window (Peck *et al.*, 2002; Marshall *et al.*,  
90 2003). The interactive effects of increased temperature and elevated CO<sub>2</sub> concentrations are  
91 predicted to impair physiological processes (Clarke, 2003; Pörtner, 2008) by narrowing the  
92 thermal tolerance window of the organisms (Metzger *et al.*, 2007; Lannig *et al.*, 2010) and  
93 elevating vulnerability to extreme temperature (Schalkhausser *et al.*, 2013).

94         In a context of global change, non-indigenous species are expected to be favored in  
95 their introduced area (Dukes & Mooney, 1999; Occhipinti-Ambrogi, 2007) mainly because  
96 robustness to abiotic variation is often a trait that determines the success of invasive species  
97 (Hellmann *et al.*, 2008; Lenz *et al.*, 2011). Climatic changes in the physical environment will  
98 be likely to affect the distribution, spread, abundance, impacts and interactions of species,  
99 possibly to the advantage of introduced organisms (Occhipinti-Ambrogi, 2007). Thus our  
100 study focused on the response of an invasive Calyptraeidae gastropod living on western  
101 European coasts, but which originates from North East America. The slipper limpet,  
102 *Crepidula fornicata* (Linné 1758) was introduced in Europe at the end of the 19<sup>th</sup> century,  
103 mainly with oysters (*Crassostrea gigas*) which were imported for farming (Blanchard, 1995),  
104 and has subsequently colonized European coasts from southern Sweden to southern France  
105 (Blanchard, 1997). *C. fornicata* has significant impacts on biodiversity and ecosystem  
106 functioning where it has established (De Montaudouin *et al.*, 1999; Decottignies *et al.*, 2007;  
107 Martin *et al.*, 2007). It lives in shallow sites, especially in bays and estuaries where very high

108 densities of over one thousand individuals  $m^{-2}$  have been reported (Blanchard, 1995). *C.*  
109 *fornicata* is known to be strongly resistant to environmental variations, particularly  
110 temperature and salinity (Blanchard, 1995; Blanchard, 1997; Diederich & Pechenick, 2013).  
111 In light of the different ecological and physiological characteristics of *C. fornicata*, it is  
112 important to investigate the impact of future  $pCO_2$  levels, and determine its resistance  
113 capacities to high levels of stress to assess the likely future impact of this engineer species in  
114 the ecosystems to which it was introduced.

115         The present study was designed to investigate the metabolic responses of *C. fornicata*  
116 to high  $pCO_2$  conditions during temperature stress. Short-term experimental approaches using  
117 faster temperature elevations than natural changes provide valuable insight into physiological  
118 responses of marine invertebrates in term of their ability to resist high levels of stress or their  
119 lethal temperature (Sokolova & Pörtner, 2003; Peck *et al.*, 2004; Pörtner *et al.*, 2006; Richard  
120 *et al.*, 2012). Following the hypothesis that  $CO_2$  stress will increase sensitivity to temperature  
121 change, we evaluated changes in oxygen-consumption of *C. fornicata* individuals previously  
122 reared under elevated  $pCO_2$  for 10 weeks during a rapid temperature increase ( $1^\circ C\ 12h^{-1}$ ).  
123 Respiration rates were measured as a proxy for metabolism on males (small individuals) and  
124 females (large individuals), as in this species there is sexual dimorphism in size

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## MATERIAL & METHODS

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### *Biological material*

129         *Crepidula fornicata* stacks were collected by SCUBA divers on 4 February 2010, in  
130 Morlaix Bay (northwest Brittany, France), at the “Barre des Flots” site ( $3^\circ 53.015'W$ ;  
131  $48^\circ 40.015'N$ ) at a depth of 10 meters and at an *in situ* temperature of  $11.6^\circ C$  (SOMLIT:  
132 *Service d’Observation de la Mer et du Littoral* data). They were transferred directly to

133 aquaria at the Station Biologique de Roscoff where they were held in natural unfiltered  
134 seawater at a temperature around 10°C, until they were used in experiments starting on 10  
135 March 2010.

136 Males and females at the top and the bottom of stacks respectively, were selected,  
137 separated and individually labelled. Small males ( $23.31 \pm 0.16$  mm length), which were still  
138 slightly mobile, were placed individually on 3 cm Petri dishes one month before the beginning  
139 of the trials. Dead individual shells at the base of stacks were kept as the substratum under the  
140 largest living immobile females ( $47.53 \pm 0.25$  mm length). In *C. fornicata*, size cannot be  
141 discriminated from sex because this is a protandrous hermaphroditic organism, changing sex  
142 with age and size (Coe 1938). All individuals were gently brushed to remove epibionts and  
143 biofilm from their shells before proceeding to the metabolic measurements.

144 Condition indices (CI) were calculated on a pool of 20 specimens in March, before the  
145 beginning of the experiment, and on all remaining living and recently dead individuals (male  
146  $n = 74$ ; female  $n = 99$ ) at the end of the temperature increase on 29 May 2010. Shell dry  
147 weight ( $DW_{\text{Shell}}$ ), shell length and tissue dry weight ( $DW_{\text{Tissue}}$ ) were determined separately on  
148 each individual after drying at 60°C for 48h. Specimens were then ignited in a muffle furnace  
149 at 520°C for 6 h, with tissue ash-free dry weight ( $AFDW_{\text{Tissue}}$ ) being obtained by difference.  
150 CI were calculated as:

$$151 \quad CI = (AFDW_{\text{Tissue}} / DW_{\text{Shell}}) \times 100.$$

152 Mortality was checked daily during the experiment. Individuals with no reaction when  
153 the foot was stimulated were classed as dead and removed from the tanks.

154

#### 155 *Experimental conditions and set-up*

156 After distributing randomly in each of twelve 10-L aquarium tanks comprising the  
157 experimental flow-through system (as described in Noisette *et al.*, 2013), 120 males and 120

158 females (i.e. 10 individuals of each sex per aquarium) were held under different  $p\text{CO}_2$   
159 conditions between 13 March and 29 May 2010. At the beginning of the experiment, pH was  
160 gradually decreased (by bubbling  $\text{CO}_2$ ) over four days at 0.1 pH units  $\text{day}^{-1}$  from 8.1 until the  
161 required pH was reached. Specimens were subsequently held for ten weeks in four different  
162  $p\text{CO}_2$  conditions: a current  $p\text{CO}_2$  of 380  $\mu\text{atm}$  ( $\text{pH}_\text{T} = 8.07$ ), and three elevated  $p\text{CO}_2$  levels of  
163 550  $\mu\text{atm}$  ( $\text{pH}_\text{T} = 7.94$ ), 750  $\mu\text{atm}$  ( $\text{pH}_\text{T} = 7.82$ ) and 1000  $\mu\text{atm}$  ( $\text{pH}_\text{T} = 7.77$ ). The elevated  
164  $p\text{CO}_2$  values corresponded to different scenarios predicted by the Intergovernmental Panel on  
165 Climate Change (IPCC) for the end of the century (Solomon *et al.*, 2007) and were selected  
166 according to the recommendations of Barry *et al.*, (2010).  $p\text{CO}_2$  was adjusted by bubbling  
167  $\text{CO}_2$ -free air (current  $p\text{CO}_2$ ) or pure  $\text{CO}_2$  (elevated  $p\text{CO}_2$ ) in four 100 L header tanks (1 per  
168  $p\text{CO}_2$  condition) supplied with natural unfiltered seawater pumped from the sea, directly at the  
169 foot of the Station Biologique de Roscoff. Seawater was continually delivered by gravity from  
170 each header tank to three aquaria per  $p\text{CO}_2$  condition at a constant rate of 9  $\text{L h}^{-1}$  (renewal  
171 rate: 90% total aquarium volume  $\text{h}^{-1}$ ),  $p\text{CO}_2$  was monitored and controlled by a feedback  
172 system (IKS Aquastar, Karlsbad, Germany) that regulated the addition of gas in the header  
173 tanks. pH values of the pH-stat system were adjusted from daily measurements of pH on the  
174 total scale ( $\text{pH}_\text{T}$ ) in the aquaria using a pH meter (HQ40D, Hach Lange, Ltd portable LDO<sup>TM</sup>,  
175 Loveland, Colorado, USA) calibrated using Tris/HCl and 2-aminopyridine/HCl buffers  
176 (Dickson *et al.*, 2007). The twelve aquaria were placed in four thermostatic baths where  
177 temperature was controlled to  $\pm 0.2$  °C using 100 - 150 W submersible heaters.

178 Before the rapid temperature increase experiment, *C. fornicata* individuals were  
179 maintained in the different  $p\text{CO}_2$  treatments for 10 weeks while temperature was raised  
180 successively to mimic the natural rate of temperature change from winter to summer.  
181 Temperature was maintained at 10°C from the beginning of the trial to 29 March. It was  
182 raised to 13°C from 5 to 19 April and to 16°C from 26 April to 18 May 2010. To reach these

183 set levels the temperature was increased by  $0.5^{\circ}\text{C day}^{-1}$  until the new set temperature was  
184 achieved. During the experiment, animals were naturally fed by the phytoplankton provided  
185 by unfiltered seawater.

186 The rapid temperature increase experiment was conducted between the 18 and 29 May  
187 2010. In all four  $p\text{CO}_2$  treatments, temperature was increased from 16 to  $36^{\circ}\text{C}$  at  $1^{\circ}\text{C } 12\text{h}^{-1}$ . *C.*  
188 *fornicata* oxygen consumption was measured (see below) both in small and large individuals  
189 in the different  $p\text{CO}_2$  treatments during this rapid temperature increase.

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#### 191 *Seawater parameters*

192 Seawater parameters were monitored throughout the experiment.  $\text{pH}_T$  and temperature  
193 were recorded daily in each of the 12 aquaria using a pH meter (HQ40D, Hach Lange, Ltd  
194 portable LDO<sup>TM</sup>, Loveland, Colorado, USA). Total alkalinity was determined every 3 weeks  
195 by 0.01N HCl potentiometric titration on an automatic titrator (Titroline alpha, Schott SI  
196 Analytics, Mainz, Germany). Seawater carbonate chemistry, *i.e.* exact  $\text{CO}_2$  partial pressure  
197 ( $p\text{CO}_2$ ) and saturation state of aragonite were calculated in each  $p\text{CO}_2$  condition using  
198  $\text{CO}_2\text{SYS}$  software (Lewis & Wallace, 1998) using constants from Mehrbach *et al.*, (1973)  
199 refitted by Dickson & Millero, (1987). Mean values ( $\pm$  standard error, SE) of the parameters  
200 in each  $p\text{CO}_2$  treatment are presented in Table 1.

201

#### 202 *Oxygen consumption measurements*

203 During the rapid temperature increase trial (18 - 29 May 2010), oxygen consumption  
204 of 6 randomly selected labeled individuals of each sex (2 per aquaria) was measured in each  
205 of the  $p\text{CO}_2$  treatments every two days, at 18, 22, 26, 30 and  $34^{\circ}\text{C}$ . Respiration rates were  
206 determined using closed incubations in 75 mL (males) or 180 mL (females) acrylic chambers  
207 (Engineering & Design Plastics Ltd, Cambridge, UK) filled with water from the same



208 aquarium (see methods in Morley *et al.*, 2007). Chambers were placed in their respective  
209 aquaria during incubations to keep the temperature constant. Incubations varied between 1 h  
210 and 3 h depending on temperature and were halted before oxygen saturation fell below 80%  
211 saturation. Control incubations without animals (n = 1 control incubation / aquarium /  
212 measurement) were carried out to allow correction for microbial activity in seawater.

213         Respiration rates were calculated from the differences in measurements of oxygen  
214 concentration during trials and controls using a non-invasive fiber-optical system (FIBOX 3,  
215 PreSens, Regensburg, Germany) made up of an optical fibre and reactive oxygen spots  
216 attached to the inner wall of the chambers. These spots were calibrated with 0% and 100%  
217 oxygen buffers made from the manufacturer instructions. 0% O<sub>2</sub> buffer was prepared by  
218 dissolving 10 g of Na<sub>2</sub>SO<sub>3</sub> in 1 L of seawater and 100% O<sub>2</sub> buffer was prepared by bubbling  
219 air in 1L of seawater for 20 min to achieve oxygen saturation. Previous experiments had  
220 demonstrated that oxygen consumption remained linear during all the incubation periods.  
221 Chamber contents were mixed gently by inverting chambers several times before each oxygen  
222 measurement. Respiration (R) rates (in μmol O<sub>2</sub> g<sup>-1</sup> AFDW h<sup>-1</sup>) were corrected for oxygen  
223 consumption in controls and calculated as:

$$224 \quad R = - (\Delta O_2 \times V) / (\Delta t \times AFDW_{\text{Tissue}})$$

225 where ΔO<sub>2</sub> (μmol O<sub>2</sub> L<sup>-1</sup>) is the difference between initial and final O<sub>2</sub> concentrations during  
226 the incubation, V (L) is the chamber volume minus the individual *C. fornicata* volume, Δt (h)  
227 is the incubation time and AFDW<sub>Tissue</sub> (g) is the tissue ash free dry weight of the slipper  
228 limpet incubated.

229         Q<sub>10</sub> coefficients were calculated by using the standard equation:

$$230 \quad Q_{10} = (R_H / R_L)^{10 / (T_H - T_L)}$$

231         where T<sub>L</sub> and T<sub>H</sub> were the lowest and highest temperature reached and R<sub>L</sub> and R<sub>H</sub> the  
232 respiration rates in these temperature respectively.

233

234 *Statistical analyses*

235 All statistical analyses were performed using R version 2.15.0 (R Core Team 2013)  
236 and STATISTICA software. A logistic regression (general linear model, GLM) was applied  
237 to test the differences in mortalities between the different  $p\text{CO}_2$  treatments and between sex  
238 with temperature as the linear variable. The effects of  $p\text{CO}_2$ , sex and the interaction of these  
239 two factors on condition index (CI) at the end of the experiment and on  $Q_{10}$  values were  
240 investigated by 2-way analysis of variance (ANOVA). Linear regressions between respiration  
241 rates and increasing temperatures were fitted in the four different  $p\text{CO}_2$  treatments for males  
242 and females separately. Differences between  $p\text{CO}_2$  treatments were explored using an  
243 ANCOVA with  $p\text{CO}_2$  and sex as fixed factors and temperature as co-variable.. Normality was  
244 assessed using the Kolmogorov-Smirnov test and Levene's test was used to ensure that  
245 variances were homogenous. All the results are presented as mean  $\pm$  standard error (SE).

246

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## RESULTS

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249 Mortality occurred between 34 and 36°C for females and 22 and 36°C for males  
250 (Figure 1). There were no significant differences in mortality between the different  $p\text{CO}_2$   
251 treatments (GLM,  $df = 3$ ,  $F = 0.680$ ,  $p = 0.565$ ) or between sex (GLM,  $df = 1$ ,  $F = 0.580$ ,  $p =$   
252  $0.449$ ). Moreover, the interaction between factors  $p\text{CO}_2$  and sex of the individuals was not  
253 significant (GLM,  $df = 3$ ;  $F = 0.21$ ;  $p = 0.888$ ). At  $p\text{CO}_2$  levels of 380, 550, 750 and 1000  
254  $\mu\text{atm}$ , the total mortality at the end of the temperature increase was 29, 19, 19, and 24 for  
255 females and 28, 6, 8, and 6 for males . At the end of the acute temperature increase, nearly  
256 twice the number of females had died (91) compared with the males (48) ( $\chi^2$  test,  $p < 0.05$ ), all  
257  $p\text{CO}_2$  levels included.

258 . The mean condition indices before the start and at the end of the experiment are  
259 presented on Figure 2. There were no effects of  $p\text{CO}_2$  , sex or the interaction of these two  
260 factors on the condition index at the end of the trial (Table 2). However, the condition index  
261 from the beginning of the experiment ( $3.00 \pm 0.27$ ) was different from the mean condition  
262 index including all  $p\text{CO}_2$  conditions ( $2.11 \pm 0.07$ ) at the end of the trial (t-test,  $df = 181$ ,  $t =$   
263  $3.159$ ,  $p = 0.002$ ), which means that CI in both males and females decreased significantly  
264 from the start to the end of the experiment (Figure 2).

265 Female respiration rates varied between  $0.51 \mu\text{mol O}_2 \text{ g}^{-1} \text{ AFDW h}^{-1}$  at  $18^\circ\text{C}$  and  
266  $p\text{CO}_2$  of  $750 \mu\text{atm}$  and  $91.62 \mu\text{mol O}_2 \text{ g}^{-1} \text{ AFDW h}^{-1}$  at  $32^\circ\text{C}$  and  $p\text{CO}_2$  of  $380 \mu\text{atm}$ . Males  
267 had higher rates, which ranged between  $5.13 \mu\text{mol O}_2 \text{ g}^{-1} \text{ AFDW h}^{-1}$  at  $18^\circ\text{C}$  and  $p\text{CO}_2$  of  $380$   
268  $\mu\text{atm}$  and  $175.51 \mu\text{mol O}_2 \text{ g}^{-1} \text{ AFDW h}^{-1}$  at  $32^\circ\text{C}$  and  $p\text{CO}_2$  of  $380$  (Figure 3).

269 Relationships between respiration rate and temperature were linear at each  $p\text{CO}_2$  level  
270 (Figure 3). Respiration rose significantly with increasing temperature in all  $p\text{CO}_2$  treatments,  
271 for both males and females (Table 3, all p-values  $< 0.02$ ). There were no significant  
272 differences between the slopes of the different regressions among the  $p\text{CO}_2$  treatments or  
273 between sexes (analysis of slopes,  $df = 3$ ,  $F = 1.1$ ,  $p = 0.346$ ). The intercepts of the different  
274 regressions also did not significantly vary among  $p\text{CO}_2$  (ANCOVA,  $df = 3$ ,  $F = 0.350$ ,  $p =$   
275  $0.789$ ), but there were difference between males and females (ANCOVA,  $df = 1$ ,  $F = 62.63$ ,  $p$   
276  $< 0.001$ ).

277  $Q_{10}$  values ranged from 1.24 to 2.40 for females and from 1.36 to 2.77 for males  
278 among the different  $p\text{CO}_2$  treatments (Figure 4). There was no significant  $p\text{CO}_2$  effect on  $Q_{10}$   
279 values for either males or females (Table 2). Across all  $p\text{CO}_2$  treatments, females had  
280 significantly lower  $Q_{10}$  values than males with means of  $1.61 \pm 0.11$  and  $2.00 \pm 0.12$  for  
281 females and males, respectively (Table 2). The interaction between  $p\text{CO}_2$  and sex, however,  
282 was not significant (Table 2).

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284

## DISCUSSION

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286         Independently of the impact of  $p\text{CO}_2$  we planned to test, one of the major issues of this  
287 study was food limitation which was unintentionally imposed on the *C. fornicata* individuals  
288 in the experiments. This food limitation was detected because of the decrease in condition  
289 indices (CI) of both males and females from the beginning to the end of the experiment. Such  
290 decreases in CI are usually related to food quantity or quality supplied to organisms (Norkko  
291 & Thrush, 2006). Animals were maintained in unfiltered seawater which carried natural  
292 phytoplankton at a concentration between 0.2 and 1  $\mu\text{g Chl a L}^{-1}$  (*SOMLIT* data). The water  
293 renewal in the aquarium was maintained constant at a rate of 0.9 L h<sup>-1</sup> (i.e. 90% of the total  
294 volume of each aquarium changed per hour). Water supply in our experimental system was  
295 likely too low to provide sufficient food for the experimental animals, which thus relied on  
296 internal energy reserves and so decreased their CI. A similar outcome was reported for  
297 mussels by Mackenzie *et al.* (2014).

298         The use of stored reserves was similar in the different  $p\text{CO}_2$  conditions as CI at the end  
299 of the experiment did not differ between the different  $p\text{CO}_2$  treatments, and this was the case  
300 for both sexes. Previous studies have shown interspecific variability in the responses of  
301 condition indices under high  $p\text{CO}_2$  levels, ranging from a lack of effect (Cummings *et al.*,  
302 2011; Clark *et al.*, 2013; Sanders *et al.*, 2013) to large changes in condition under high  $p\text{CO}_2$   
303 levels (Hiebenthal *et al.*, 2013; Range *et al.*, 2014). Energy availability is a major component  
304 in mitigating the effects of ocean acidification (Pansch *et al.*, 2014). Studies have shown that  
305 an abundant food supply might counteract or even overcome the negative effects of high  
306  $p\text{CO}_2$  on adult and juvenile bivalves (Melzner *et al.*, 2011; Thomsen *et al.*, 2013). Thus, it is  
307 important to consider that in this study *C. fornicata* were under limited food conditions when

308 interpreting their metabolic responses to elevated  $p\text{CO}_2$  conditions during the temperature  
309 rise. The data here are representative of conditions where there is temperature stress and food  
310 supplies are limited, conditions that can occur in the field.

311         The limitation of food supply was not markedly more important in any of our reduced  
312 pH conditions as there were no differences in mortality rates between the different  $p\text{CO}_2$   
313 treatments in *C. fornicata* males and females., This is a different outcome to that reported for  
314 some other mollusk species held in elevated  $p\text{CO}_2$  levels (Shirayama & Thornton, 2005;  
315 Beniash *et al.*, 2010). However, similarly to our study, Pansch *et al.*, (2014) showed that food  
316 availability had no impact on mortalities of the barnacle *Amphibalanus improvises* held in  
317 different  $p\text{CO}_2$  conditions. In the present study, important mortalities started to occur from  
318 32°C and they became larger at and above 34°C for both males and females. These values are  
319 consistent with the upper lethal temperature recorded for *C. fornicata* by Diederich &  
320 Pechenick, (2013) in a laboratory study investigating a population from Rhode Island, USA,  
321 in which only 40% of the adults survived after a 3 h exposure to 34°C, and all died after a 3 h  
322 exposure to 36°C. Mortality was higher in females (larger individuals) than in males (small  
323 individuals) even if, males started to die at lower temperatures than females. Similarly, Peck  
324 *et al.*, (2009) demonstrated for 14 species that smaller species survived to higher temperatures  
325 than large ones when temperature was raised at 1°C day<sup>-1</sup>, and Peck *et al.* (2013) showed that  
326 juveniles had higher upper temperature limits than adults in 4 species of marine invertebrates  
327 at warming rates of 1°C day<sup>-1</sup> and 1°C 3days<sup>-1</sup>. The mechanisms setting temperature limits at  
328 acute rates of warming may not be energy availability (Peck *et al.*, 2014) and females, which  
329 had more energetic reserves than males, may thus have not had an advantage.

330         Despite the decreases in CI, mean respiration rates of *C. fornicata* at 18°C and  $p\text{CO}_2$   
331 of 380  $\mu\text{atm}$  were 31 and 26  $\mu\text{mol O}_2 \text{ g}^{-1} \text{ AFDW h}^{-1}$  for males and females, respectively,  
332 which are close to the middle of the range of *in situ* values reported for wild individuals from

333 the Bay of Brest (Brittany, France) (6 to 63  $\mu\text{mol O}_2 \text{g}^{-1} \text{AFDW h}^{-1}$ ; Martin *et al.*, 2006). This  
334 indicates that animals in the experiments here had similar oxygen consumption to wild  
335 specimens and were not metabolically depressed under insufficient food supply. In both *C.*  
336 *fornicata* males and females, respiration rates increased with temperature, as previously  
337 demonstrated for this species by Newell & Kofoed, (1977) and most ectotherm metabolic  
338 rates are correlated positively with temperature (Cossins & Bowler, 1987). Respiration rates  
339 were higher in *C. fornicata* males than in females regardless of the temperature. Generally,  
340 mass-specific respiration rates of small individuals are higher than those of larger ones  
341 because metabolic rate (normalized to the biomass) decreases with increasing organisms size  
342 (von Bertalanffy, 1951; Parsons *et al.*, 1984).

343         The relationship between oxygen consumption and temperature here for *C. fornicata*  
344 was similar in all the different  $p\text{CO}_2$  treatments. The slopes and intercepts of the regressions  
345 were not significantly different across the four  $p\text{CO}_2$  conditions which means temperature  
346 effect on respiration rate was not affected by the different  $p\text{CO}_2$  levels in males or females. In  
347 contrast to our results, Lannig *et al.*, (2010) found that an acute temperature rise  
348 (1.25°C/12h) caused a more rapid increase in metabolic rate in *Crassostrea gigas* under  
349 elevated  $p\text{CO}_2$  conditions, and there was a synergistic effect of temperature and  $p\text{CO}_2$ . The  
350 lack of difference in respiration between animals held in different  $p\text{CO}_2$  conditions may be  
351 related to a stronger ability to up-regulate their metabolism under a temperature stress  
352 irrespective of  $p\text{CO}_2$ . Thus, under warming conditions, *C. fornicata* can generate sufficient  
353 energy to cope with any effects of decreased pH (Wood *et al.*, 2010).  $Q_{10}$  values were also  
354 similar across  $p\text{CO}_2$  treatments in both males and females and they were within the expected  
355 range of values recorded for marine invertebrates (Branch *et al.*, 1988; Marshall *et al.*, 2003).  
356 Even if *C. fornicata* individuals were food limited, their oxygen consumption remained  
357 unaffected by elevated  $p\text{CO}_2$ . A similar lack of  $p\text{CO}_2$  effect was reported for growth and shell

358 strength of the barnacle *A. improvisus* (Pansch *et al.*, 2014). In our study, the low food supply  
359 did not appear to affect the resistance or resilience of *C. fornicata* to CO<sub>2</sub> stress.

360 Several studies investigating the response of mollusk respiration to elevated *p*CO<sub>2</sub>  
361 have demonstrated metabolic depression under high *p*CO<sub>2</sub> in both bivalves and gastropods  
362 (Michaelidis *et al.*, 2005; Bibby *et al.*, 2007; Fernandez-Reiriz *et al.*, 2011; Melatunan *et al.*,  
363 2011; Liu & He, 2012; Navarro *et al.*, 2013). Conversely, others observed no *p*CO<sub>2</sub> effect on  
364 mollusk respiration and general metabolism (Gazeau *et al.*, 2007; Marchant *et al.*, 2010;  
365 Fernandez-Reiriz *et al.*, 2012; Clark *et al.*, 2013) as reported in our study. In some rare cases,  
366 O<sub>2</sub> consumption was reported to increase under high *p*CO<sub>2</sub> conditions (Wood *et al.*, 2010;  
367 Cummings *et al.*, 2011). The effects of high CO<sub>2</sub> concentrations on metabolism appear  
368 species-specific and depend on resistance capacities of the organisms (Melzner *et al.*, 2009).  
369 It has been widely reported that exposure to environmental high *p*CO<sub>2</sub> levels leads to changes  
370 in homeostasis and extracellular acid-base balance counterbalanced by metabolic depression  
371 in many cases (Pörtner *et al.*, 2005; Pörtner, 2008), although it should be noted, as above, that  
372 metabolic depression is often not seen in high *p*CO<sub>2</sub> conditions. Differences in acid-base  
373 regulatory capacities by increasing HCO<sub>3</sub><sup>-</sup> internal concentrations (Michaelidis *et al.*, 2005;  
374 Gutowska *et al.*, 2010) or H<sup>+</sup> excretion (Pörtner *et al.*, 2005) are taxon specific and are more  
375 or less effective in mitigating the effects of hypercapnia. It has also been suggested that  
376 organisms could maintain low metabolic rates without controlling internal pH by not using  
377 pH-sensitive oxygen-binding pigments (Thomsen *et al.*, 2010; Hiebenthal *et al.*, 2013). Such  
378 mechanisms may be crucial factors in explaining the observed variation in sensitivities and  
379 resistances of marine invertebrates to elevated *p*CO<sub>2</sub> conditions (Gutowska *et al.*, 2010).

380 It is important to note here that many of the studies to date on the effects of elevated  
381 *p*CO<sub>2</sub> on organisms are short-term and acute (e.g. Tomanek *et al.*, 2011), not reflecting the  
382 long-term trade off in energy balance and physiological changes associated with acclimation

383 of new environmental conditions (Clark *et al.*, 2013). For example, metabolic depression acts  
384 as a time-limited compensation strategy to survive unfavorable condition such as high CO<sub>2</sub>  
385 concentrations (Guppy & Withers, 1999; Willson & Burnett, 2000). Because *C. fornicata*  
386 were held for 10 weeks in the different pCO<sub>2</sub> treatments in this investigation, it is likely there  
387 was enough time for them to acclimate to the new pH, and no difference in oxygen  
388 consumption was detected between the different pCO<sub>2</sub> conditions. However, the energetic cost  
389 likely produced by the negative effects of elevated pCO<sub>2</sub> may either be relatively small , or  
390 difficult to maintain over longer time periods. This could be seen in impacts on other  
391 physiological processes than respiration (Catarino *et al.*, 2012). For example, Bibby *et al.*,  
392 (2008) demonstrated that exposure to hypercapnic conditions may compromise the ability to  
393 express an immune response in mussels. They showed that *Mytilus edulis* phagocytosis  
394 declined as function of decreased pH. In the same way, Matozzo *et al.*, (2012) showed that  
395 elevated pCO<sub>2</sub> and temperature may strongly affect haemocyte functionality in the bivalves  
396 *Chamelea gallina* and *Mytilus galloprovincialis*. Other cellular processes have also been  
397 shown to be negatively impacted by high CO<sub>2</sub> concentrations, including protein synthesis in  
398 the sipunculid *Sipunculus nudus* (Langenbuch *et al.*, 2006) or enzyme activities in *C. gallina*  
399 and *M. galloprovincialis* (Matozzo *et al.*, 2013). However, studies of the impact of reduced  
400 pH on immune systems have generally been of short duration and it would be interesting to  
401 investigate other physiological parameters than respiration (e.g. calcification, protein  
402 production, immunity regulation, fertility) in *C. fornicata* acclimated over several months in  
403 the different pCO<sub>2</sub> conditions predicted for the end of the century. As a coastal species  
404 adapted to relatively large fluctuations of abiotic parameters, *C. fornicata* in this study were  
405 strongly resistant to both elevated pCO<sub>2</sub> and increased temperature. Indeed, resistance to high  
406 pCO<sub>2</sub> levels can also come from pre-acclimation or pre-adaptation to fluctuations in the  
407 environment where species live (Burnett, 1997). Species living in environments with large



408 abiotic variation have a high phenotypic plasticity which can allow them to survive in  
409 stressful conditions (Hofmann & Todgham, 2010). Coastal organisms are more exposed to  
410 physico-chemical variations than their open-ocean counterparts that live in more stable  
411 thermal and pH environments (Berge *et al.*, 2006; Peck *et al.*, 2006). Species living in shallow  
412 waters tolerate not only seasonal and extreme temperature events but also periodic large  
413 fluctuations in seawater pH, driven by biological process that sequester and release large  
414 amounts of CO<sub>2</sub> (Beniash *et al.*, 2010). This exposure to a wide environmental variation has  
415 likely led to the evolution of resistance mechanisms to abiotic factors including variations in  
416 pCO<sub>2</sub> and/or pH (Lannig *et al.*, 2010).

417 *C. fornicata* is an invasive species which has successfully colonized European coastal  
418 shallow waters. This species is likely to have high phenotypic plasticity and resilience to  
419 physico-chemical variations that determined its success. Indeed, successful invasive species  
420 generally share characteristics that allow them to establish, colonize and expand their range.  
421 Among these characteristics, tolerance to environmental stress is one of the most common  
422 (Lenz *et al.*, 2011). In a global change context, the movement of physico-chemical conditions  
423 away from the optimum increases the energy required by marine species to fuel the extra  
424 processes entrained to resist the stresses involved and to maintain homeostasis. This may  
425 result in changes in overall physiological condition (Cummings *et al.*, 2011) that could impact  
426 ecological processes and community interactions. The high resilience to altered pCO<sub>2</sub>/low pH  
427 levels observed here for *C. fornicata* may confer a competitive advantage to this invasive  
428 species over taxonomically or functionally related species (Lenz *et al.*, 2011). For example,  
429 the performance of the scallop *Pecten maximus*, which is one of the *C. fornicata* competitors  
430 (Thouzeau *et al.*, 2000; Fresard & Boncoeur, 2006), has been shown to be negatively affected  
431 by high pCO<sub>2</sub> levels (Schalkhausser *et al.*, 2013). These different sensitivities to  
432 environmental factors will likely dictate “winners” and “losers” among marine species that

433 could lead to a restructuring of benthic communities. With other studies, our data suggest this  
434 restructuring could favor invasive species as evidence is building that shows they are more  
435 resistant to change than their native competitors (Dukes & Mooney, 1999; Occhipinti-  
436 Ambrogi, 2007).

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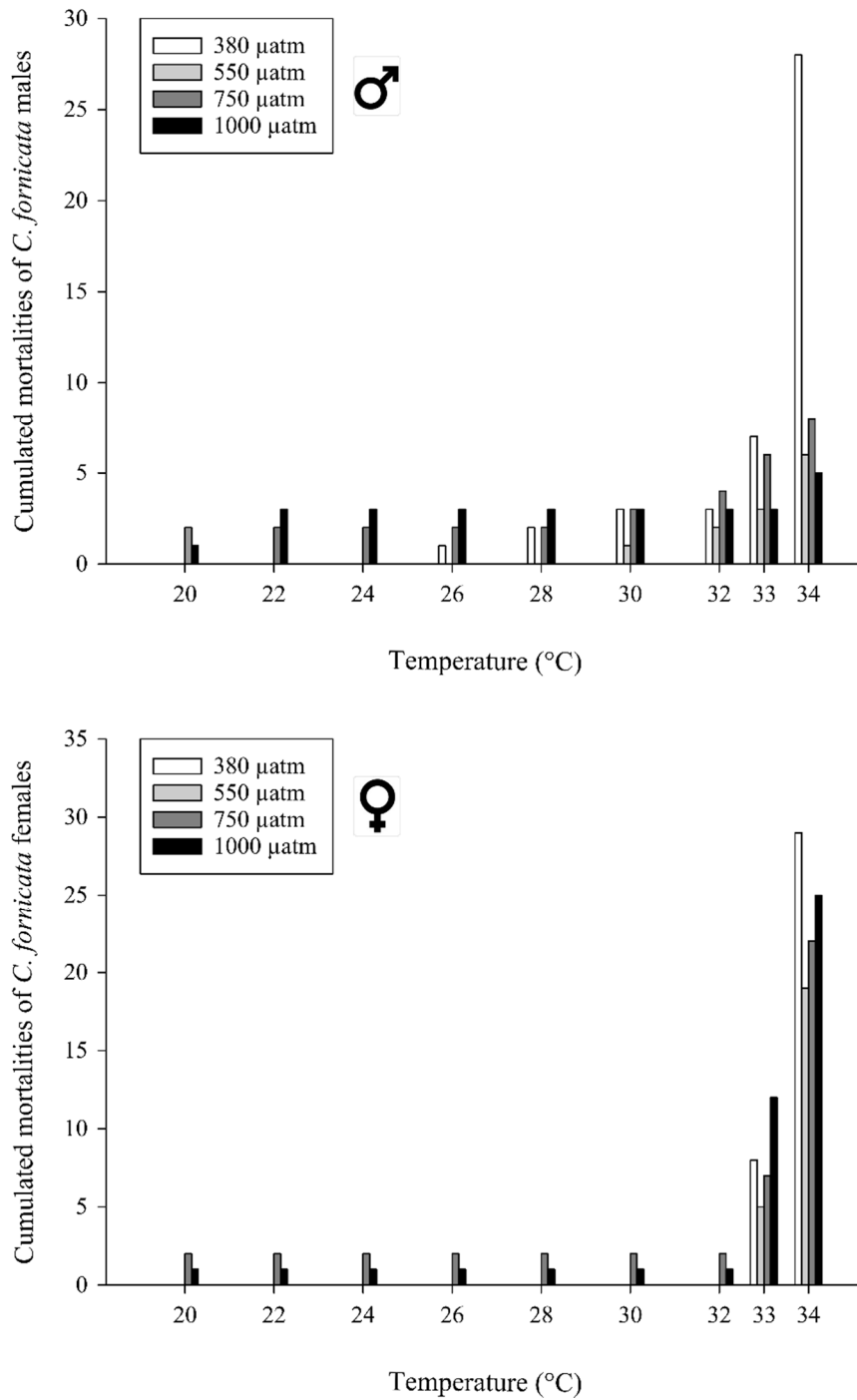
### FIGURES CAPTIONS

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763 **Figure1:** Cumulated mortalities of males (top) and females (bottom) of *Crepidula fornicata*

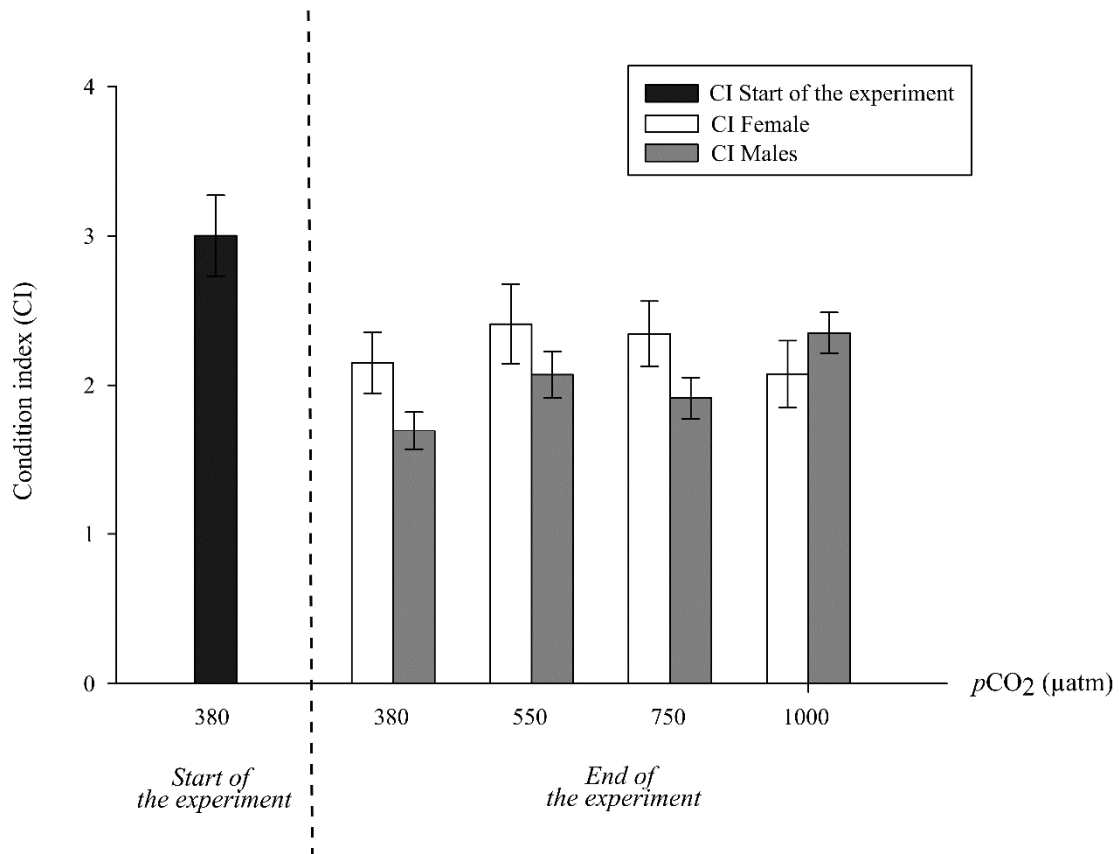
764 during temperature increase. Shading represents the different pCO<sub>2</sub> levels at which *C.*

765 *fornicata* individuals were held during the experiment.



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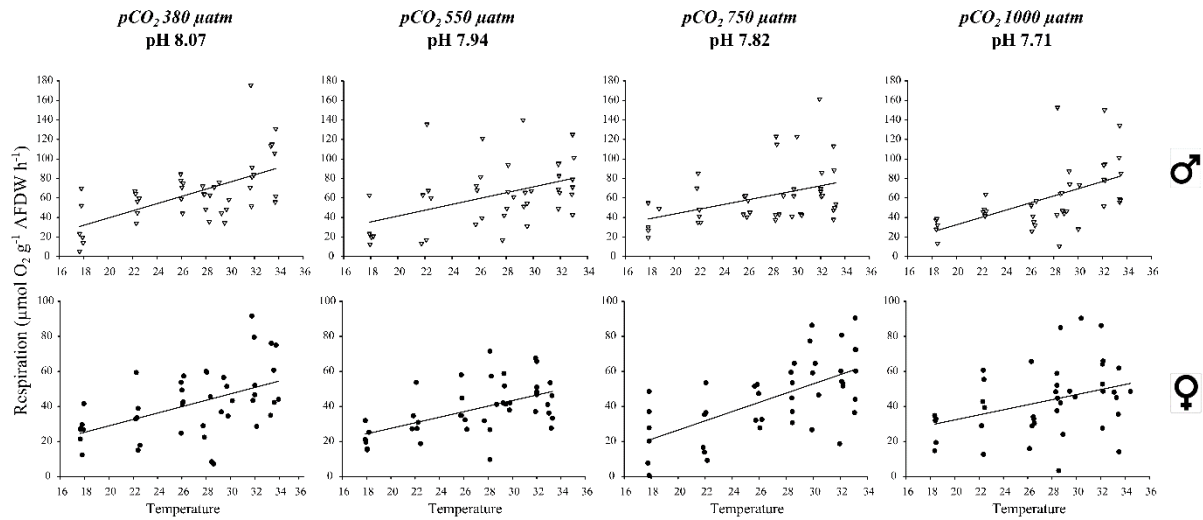
767 **Figure 2:** Mean (+SE) conditions indices at the beginning (black bar) and at the end of the  
768 experiment for *Crepidula fornicata* females (white bars) and males (grey bars) under the  
769 different pCO<sub>2</sub>. Sample sizes = 10–27.



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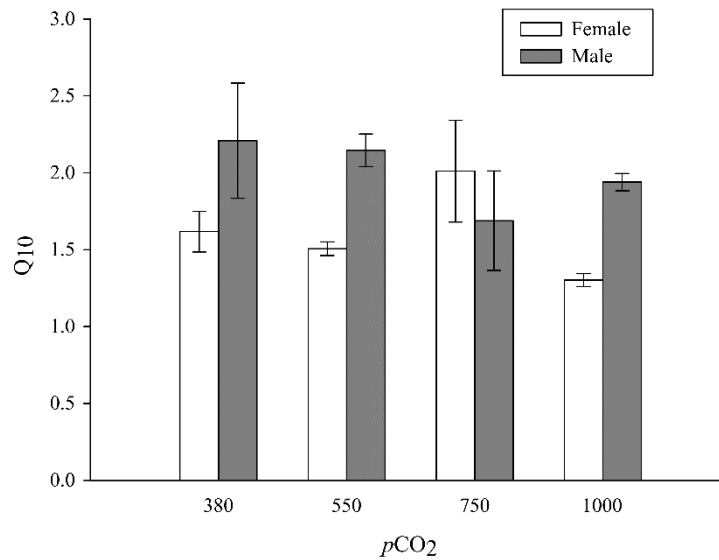


779 **Figure 3:** Respiration rates as a function of increasing temperature in each  $p\text{CO}_2$  treatment,  
 780 for *Crepidula fornicata* males (top, triangles) and females (bottom, circles). For statistical  
 781 analyses of regressions see Table 3.



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783 **Figure 4:** Mean ( $\pm$  SE)  $Q_{10}$  values for *C. fornicata* females (white bars) and males (grey bars)  
 784 in the different  $p\text{CO}_2$  treatments. N = 3



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## TABLES

789

790 **Table 1:** Mean ( $\pm$  standard error, SE) carbonate chemistry parameters for each  $p\text{CO}_2$   
 791 treatment. pH (on the total scale,  $\text{pH}_T$ ) was measured daily and total alkalinity ( $A_T$ ) was  
 792 measured every 3 weeks. Other parameters were calculated with CO2sys software.  $p\text{CO}_2$  :  
 793  $\text{CO}_2$  partial pressure;  $\Omega_{Ar}$  : saturation state of seawater with respect to aragonite.

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$p\text{CO}_2$ treatment	$\text{pH}_T$	$p\text{CO}_2$ ( $\mu\text{atm}$ )	$\Omega_{Ar}$	$A_T$ ( $\mu\text{Eq kg}^{-1}$ SW)
	n = 69	n = 69	n = 69	n = 76
380 $\mu\text{atm}$	$8.13 \pm 0.01$	$324 \pm 8$	$2.72 \pm 0.06$	$2333 \pm 1$
550 $\mu\text{atm}$	$7.89 \pm 0.01$	$619 \pm 16$	$1.69 \pm 0.04$	$2334 \pm 2$
750 $\mu\text{atm}$	$7.75 \pm 0.01$	$873 \pm 20$	$1.28 \pm 0.03$	$2335 \pm 2$
1000 $\mu\text{atm}$	$7.66 \pm 0.01$	$1138 \pm 65$	$1.05 \pm 0.02$	$2334 \pm 2$

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797 **Table 2:** Summary of two-way ANOVAs testing the effects of  $p\text{CO}_2$ , sex and their interaction  
 798 on the final condition indices (CI) and the  $Q_{10}$  values determined for *C. fornicata* males and  
 799 females under the different  $p\text{CO}_2$  conditions (380, 550, 750 and 1000  $\mu\text{atm}$ ). Bold numbers  
 800 indicate significant level greater than 95%.

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	df	CI		Q <sub>10</sub>	
		F-value	p-value	F-value	p-value
$p\text{CO}_2$	3	1.245	0.295	0.657	0.590
sex	1	2.472	0.118	6.124	<b>0.025</b>
$p\text{CO}_2$ x sex	3	1.371	0.254	2.293	0.117

802 **Table 3:** Relationships between *C. fornicata* male and female respiration rates and  
 803 temperature under each  $p\text{CO}_2$  treatment

	$p\text{CO}_2$	Regression equation	n	R	$R^2$	F	p
males	380	$y = 3.691 x - 34.455$	42	0.60	0.37	22.97	< 0.001
	550	$y = 2.993 x - 18.461$	42	0.46	0.21	10.56	0.002
	750	$y = 2.406 x - 4.543$	41	0.40	0.16	7.55	0.009
	1000	$y = 3.701 x - 41.556$	41	0.56	0.31	17.37	< 0.001
females	380	$y = 1.826 x - 7.635$	42	0.49	0.24	12.72	< 0.001
	550	$y = 1.585 x - 4.218$	42	0.55	0.30	16.89	< 0.001
	750	$y = 2.637 x - 26.240$	42	0.63	0.40	26.66	< 0.001
	1000	$y = 1.442 x + 3.435$	42	0.37	0.14	6.26	0.017

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