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1 **Physiological responses to ocean acidification and warming synergistically**
2 **reduce condition of the common cockle *Cerastoderma edule***

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12
13
14 **Abstract**

15 The combined effect of ocean acidification and warming on the common cockle *Cerastoderma*
16 *edule* was investigated in a fully crossed laboratory experiment. Survival of the examined adult
17 organisms remained high and was not affected by elevated temperature (+3°C) or lowered pH
18 (-0.3 units). However, the morphometric condition index of the cockles incubated under high
19 pCO₂ conditions (i.e. combined warming and acidification) was significantly reduced after six
20 weeks of incubation. Respiration rates increased significantly under low pH, with highest rates
21 measured under combined warm and low pH conditions. Calcification decreased significantly
22 under low pH while clearance rates increased significantly under warm conditions and were
23 generally lower in low pH treatments. The observed physiological responses suggest that the
24 reduced food intake under hypercapnia is insufficient to support the higher energy requirements
25 to compensate for the higher costs for basal maintenance and growth in future high pCO₂ waters.

26 **Key words:** Future ocean, ocean acidification, ocean warming, *Cerastoderma edule*,
27 ecophysiology

28
29 **1. Introduction**

30 Estuaries are among the most productive marine ecosystems, supporting a high abundance and
31 diversity of organisms (Beck et al., 2001). Other ecosystem services provided by these systems

32 include disturbance regulation (e.g. flood control, storm protection), nutrient cycling, biological
33 control, habitat creation and others (e.g. Barbier et al., 2011; Meire et al., 2005), contributing
34 to an estimated total annual monetary value of 4.1 trillion US\$ (Costanza et al., 1997). However,
35 these habitats are gradually degraded by increasing human activities compromising their
36 function as feeding, nursery and breeding habitats (Seitz et al., 2014). In addition, coastal
37 habitats like estuaries are in the frontline of current environmental change, including climate
38 change (Scavia et al., 2002). The excess of CO₂ emissions produced by the burning of fossil
39 fuels, cement production, and deforestation (Sabine et al., 2004) are interacting with the global
40 climate and the ocean, causing warming and changes in ocean carbonate chemistry (Doney et
41 al., 2009). To date, approximately 30% of the anthropogenic CO₂ in the atmosphere is being
42 absorbed by oceans and is altering their chemistry, a process referred to as ocean acidification
43 (OA) (Caldeira and Wickett, 2003; Sabine et al., 2004), with an estimated reduction in pH of
44 0.3 - 0.4 units by the end of the 21st century for the open ocean (Caldeira and Wickett, 2003;
45 Feely et al., 2004; Orr et al., 2005). High temporal fluctuations in pH (Wootton et al., 2008)
46 may mask the effect of rising pCO₂ in coastal habitats in the short-term; yet recent analyses
47 show that rates of acidification are an order of magnitude higher in coastal habitats as compared
48 to the open ocean (Provoost et al., 2010; Wootton et al., 2008), suggesting that these shallow
49 water marine habitats might be particularly vulnerable to rising pCO₂ concentrations.

50 Over the last two decades, the consequences of changes in ocean carbonate chemistry on
51 various life stages of calcifying marine organisms have been intensively investigated, including
52 studies of OA effects on calcification, survival, acid-base regulation, metabolism, reproduction
53 and immunity (reviewed in Gazeau et al., 2013; Kroeker et al., 2010; Parker et al., 2013).
54 Calcifying organisms like corals, shellfish and crustaceans have been shown to be particularly
55 vulnerable to OA (Kroeker et al., 2010). For example, the reduction in the success of
56 fertilization and embryogenesis, and in the number, growth and survival of hatchlings in the
57 Baltic tellin *Macoma balthica* at pH 7.5 (Van Colen et al., 2012) illustrates the adverse impacts
58 OA can have on early life history processes. Another example is an 87-day study by Range et
59 al. (2014) demonstrating that juvenile clam *Ruditapes decussatus* decreased their rates of
60 respiration, clearance and ingestion, whereas excretion rates were increased under mimicked
61 acidic conditions (e.g. 7.8 and 7.5). Furthermore, OA was shown to disrupt behavioural
62 processes (e.g. finding shelter, ability to detect food, prey and predators), for example in adult
63 hermit crabs (*Pagurus bernhardus*), that might potentially affect the population fitness and their

64 effects on ecosystem functioning (Briffa et al., 2012). In short, OA does impact marine
65 calcifying organisms in term of morphology, physiology and behaviour.

66 Ocean acidification does not occur in isolation (Byrne and Przeslawski, 2013). The
67 Intergovernmental Panel on Climate Change (IPCC) has predicted an increase in global mean
68 temperature ranging between 1.0 - 4.1°C based on four CO₂ Representative Concentration
69 Pathway (RCP) scenarios (Collins et al., 2013). While the separate effects of changes in
70 seawater temperature and pH are well documented, comparatively few studies have hitherto
71 focused on the combined effects of seawater temperature rise and acidification. Both stressors
72 may act synergistically, antagonistically or additively on the condition and physiology of
73 marine animals (Darling and Cote, 2008), and their combined effects can reduce the functional
74 performance of species (e.g. foraging, growth, reproduction, competitiveness and behaviours)
75 at ecosystem levels through narrowing of species' thermal windows (Pörtner, 2008; Pörtner and
76 Farrell, 2008). Ocean acidification coupled with warming increased the energy metabolism of
77 the adult Pacific oyster *Crassostrea gigas* (Lannig et al., 2010). Furthermore, Talmage and
78 Gobler (2011) showed additive negative effects of both stressors on the mortality of bay
79 scallops *Argopecten irradians* and hard clam *Mercenaria mercenaria* larvae, and larvae reared
80 under high temperature and low pH conditions accumulated less lipids, were smaller and had
81 an extended time to metamorphosis. Some studies on bivalves found that elevated temperatures
82 had more pronounced impacts than lowered seawater pH on survival, immune response, growth
83 and development (e.g. *Mytilus galloprovincialis* and *Mytilus edulis* (Gazeau et al., 2014;
84 Mackenzie et al., 2014; Vihtakari et al., 2013)). On the other hand, Duarte et al. (2014)
85 concluded that calcification rates and total weight of adult Chilean mussels (*Mytilus chilensis*)
86 decreased more in response to lowered pH than to elevated temperature. In short, the effects of
87 OA and warming are thus highly species-specific and can depend on the life stage,
88 biogeography and environmental context.

89 To properly understand the impacts of future ocean scenario's on coastal ecosystems, it is
90 crucial to assess the combined effects of temperature change and OA on species that play a key
91 role in the ecosystem. The common cockle *Cerastoderma edule* is a sediment-dwelling filter-
92 feeding bivalve that represents an important dietary component for shorebirds (e.g. the
93 oystercatcher *Haematopus ostralegus* and the common eider *Somateria mollissima*) and
94 crustaceans (e.g. the brown shrimp *Crangon crangon* and the shore crab *Carcinus maenas*
95 (Beukema and Dekker, 2005; Sanchez-Salazar et al., 1987; Whitton et al., 2012)). Furthermore,
96 the species' subsurface crawling and shaking behaviour causes disturbance of the upper

97 sediment layer, which can induce erosion of the sediment bed (Ciutat et al., 2006) and
98 alterations in the benthic community (Flach, 1996; Van Colen et al., 2013). Common cockles
99 occur along the east Atlantic coast from Morocco to Norway, and along the Black,
100 Mediterranean and Baltic Seas (Malham et al., 2012), where they are extensively harvested by
101 fishermen. According to the Food and Agriculture Organization of the United Nations (FAO)
102 (<http://www.fao.org/fishery/statistics/global-aquaculture-production/en>), the average annual
103 harvest of common edible cockles was 17,073 tonnes (from 2008 till 2014). The shells of
104 cockles are solely composed of aragonite, which is a more soluble calcium carbonate
105 polymorph than calcite (Cubillas et al., 2005). Hence, cockle shell formation is expected to be
106 particularly vulnerable to OA and low pH conditions could therefore also reduce the species'
107 physiological tolerances to other environmental alterations such as ocean warming (Pörtner and
108 Farrell, 2008; Widdicombe and Spicer, 2008). Here we experimentally investigated the
109 physiology and condition of *C. edule* under current and future pCO₂ scenario's, i.e. warming
110 and acidification under both single and combined stressor conditions. By exposing cockles to
111 changes in pH and temperature we expected to find an enhanced energy demand related to the
112 support cost of tissue protection systems; for example, damage repair mechanisms, the
113 regulation of acid-base balance and ion transport (Sokolova et al., 2011). These compensatory
114 metabolic strategies can facilitate organisms to cope with stress at the short term but may disrupt
115 the energy budget balance at the longer term, ultimately affecting fitness of the population
116 (Sokolova et al., 2011).

117 **2. Material and methods**

118 *2.1 Collection and incubation of cockles*

119 In June 2015, adult cockles were collected during low tide in the lower intertidal zone at the
120 "Slikken van Viane", Oosterschelde estuary, The Netherlands (51° 37' N, 4° 2' E) and were
121 transported within two hours to the laboratory. Forty-four randomly chosen cockles (average
122 shell length of 27.7 ± 3.2mm (SD)) were cleaned and added to each of 12 aquaria (41cm x 31cm
123 x 40cm) that had been filled with sediment (median grain size 224 ± 1.7µm) to a height of 15cm
124 and allowed to acclimatize to preset laboratory conditions (15°C, salinity of 33 and pH of 8.1)
125 for 3 days, reflecting average seawater surface temperature and pH in June at the sampling
126 location (retrieved at location Zijpe (pH) and Bruinisse (temperature) from
127 <http://live.waterbase.nl> and [4](http://www.seatemperature.org/europe/netherlands/bruinisse-</p></div><div data-bbox=)

128 june.htm). All cockles burrowed in the upper first centimetres of the sediment within one hour
129 with their siphons flush or slightly extending from the sediment-water interface.

130 There were two factors in our fully orthogonal experiment; temperature (ambient or elevated)
131 and pH (current or lowered). Seawater was stored in four barrels (250L) and pumped from each
132 barrel to the three aquaria and circulated back to the barrel continuously. The seawater in the
133 aquaria and barrels was aerated and the seawater pH was manipulated in the barrels. A stirrer
134 was installed into each barrel in order to homogenise seawater. To maintain salinity a quarter
135 of the seawater was renewed per week which took less than 20 minutes for the whole setup (i.e.
136 4 treatments, 12 aquaria). All aquaria were subjected to a 12:12h light:dark regime. Cockles
137 were fed twice a week with 1ml of commercial Shellfish Diet 1800 (Reed Mariculture Inc.,
138 composed of 40% *Isochrysis*, 15% *Pavlova*, 25% *Tetraselmis* and 20% *Thalassiosira*
139 *weissflogii*) diluted with 3 liter of seawater and distributed equally in the barrels. We
140 acknowledge that the food quality and quantity used might not completely resemble to those
141 present in the field. However, we deliberately chose to use a mixture of dead microalgae to
142 avoid bias in our experiment related to temperature and pH effects on food quantity and quality
143 (Hinga, 2002; Thomas et al., 2012) that we were unable to account for.

144 After three days of acclimatization under ambient conditions, the pH of the seawater was
145 decreased by 0.1 pH unit and the temperature was increased by 1°C per day over 3 days, until a
146 pH value of -0.3 units and a temperature of +3°C was achieved (see below). These conditions
147 were maintained for 6 weeks (see Table 1 and Fig. A1). At the site of cockle collection field
148 pH data for mid-June and July ranged between 8.0 and 8.2 (data retrieved at location Zijpe from
149 2010 to 2015 from <http://live.waterbase.nl>) and the daily average seawater temperature ranged
150 from 15 to 18°C ([https://weatherspark.com/y/51298/Average-Weather-in-Bruinisse-](https://weatherspark.com/y/51298/Average-Weather-in-Bruinisse-Netherlands)
151 [Netherlands](https://weatherspark.com/y/51298/Average-Weather-in-Bruinisse-Netherlands)). The manipulated combined pH and temperature conditions thus enable us to
152 study realistic OA effects at two ambient temperatures, i.e. the average daily temperature and
153 the highest temperature during the period of the experiment.

154 ProMinent Dulcometers coupled with Hamilton glass pH electrodes were used to control the
155 seawater pH through the controlled bubbling of CO₂ in the lowered pH treatments. The pH
156 values were logged every 10 minutes using pH electrodes and a Consort data logger (Model:
157 C3040). All pH electrodes, including Hamilton (S/N: 16458 and 16451) and Consort electrodes
158 (SP10B-50), were calibrated weekly using Hanna instruments NBS buffer solutions (pH 4.01,
159 7.01 and 9.18). All pH measurements were reported according the NBS scale. Meanwhile,

160 seawater temperature was regulated by temperature heater/chiller controllers (Teco
161 Refrigeration technologies; Model: TK200H) and HOBO Pendant data loggers (Model: UA-
162 002-08) were used to record seawater temperature every 10 minutes. Vertical profiles of
163 sediment porewater pH (for a description on the profiling set up, see Braeckman et al., 2014)
164 obtained during pilot tests with the used sediment demonstrated a strong gradient in pH with a
165 steep and gradual decline from the sediment-water interface until 0.5 cm depth where the pH
166 stabilises. Importantly, figures A2a and b indicate that the applied pH manipulation created
167 similar differences (~0.3 pH units) between current and lowered pH treatments in both the water
168 column and sediment.

169 30-ml seawater samples were collected from each tank on a weekly basis and filtered through
170 GF/C filters for subsequent quantification of total alkalinity (TA). These samples were
171 temporally stored at 4°C prior to subsequent Gran titration using a Mettler Toledo G20 compact
172 titrator and a glass electrode calibrated using Hanna instruments buffer solutions (pH 4.01 and
173 7.01) and a TRIS buffer solution (pH 8.1 (MERCK Production Chemicals)). The obtained pH,
174 TA, temperature and salinity values were used to determine parameters of the carbonate
175 chemistry (e.g. partial pressure of carbon dioxide ($p\text{CO}_2$), saturation state of the seawater with
176 respect to aragonite (Ω_a), total inorganic carbon concentration (C_T) etc.) through CO₂SYS
177 software (Pierrot et al., 2006) using the thermodynamic constants of Mehrbach (1973).

178 2.2 *Cockle condition and physiology*

179 Mortality of cockles was checked on a daily basis and dead cockles were removed. Cockle
180 physiology was measured as respiration rates, clearance rates and calcification rates after 3 and
181 6 weeks of incubation. To this purpose, 6 - 10 cockles corresponding to a total wet weight of
182 61 -70g were removed from the sediment of each of the three aquaria per treatment, and placed
183 inside their respective incubation chamber ($\emptyset = 19\text{cm}$, height = 30cm, volume = 8.2liter
184 (Braeckman et al., 2014)) along with the seawater from their respective barrels. Subsequently,
185 respiration, clearance and calcification rates were determined according to the methodologies
186 described below. Temperature was maintained throughout all incubations by working in a
187 climate room set at the desired temperature, i.e. 14.9 ± 0.5 and $17.9 \pm 0.5^\circ\text{C}$. After the
188 measurements, the soft tissues of individuals were separated from their shell. Individual shells
189 and soft tissues were allowed to dry in an oven at 60°C for 2 days and weighed. The *condition*
190 *indices* (CI) of cockles were determined using equation (1) from Lucas and Beninger (1985).

191
$$CI(\%) = \frac{\text{dry tissue weight}}{\text{dry shell weight}} \times 100 \quad (1)$$

192 *Respiration rates* of batches of 6-10 cockles with a total wet weight of 61-70g were measured
193 using Pyroscience sensor technology. Cockles were not fed 7 days prior to respiration
194 measurements, however, they were not starved as cockles were observed feeding before
195 measurements took place. Sensor spots were glued on the inner wall of incubation chambers;
196 when the sensor spots are excited by red light, they show an oxygen-dependent infrared
197 emission. Lens Spot Adapters were placed on the outside of incubation chambers facing the
198 sensor spot and connected with spot fibers to a FireSting O₂ logger, which allowed continuous
199 display and recording of the O₂ concentration (μmol l⁻¹) of the seawater. Seawater oxygen
200 concentrations were logged continuously for 2 hours and 40 minutes and respiration rates were
201 calculated as in equation (2)

202
$$R = \frac{V(R_0 - R_1)}{g(t_1 - t_0)} \quad (2)$$

203 where t₀ and t₁ represent initial and final time (h) of measurement, respectively; R₀ and R₁
204 represent the oxygen concentrations (μmol l⁻¹) at time t₀ and t₁; g is the dry flesh weight (g) of
205 cockles and V is the seawater volume (l) of the incubation chamber after correction for the
206 biovolume of the cockles. An additional incubation chamber without cockles was used as a
207 blank to correct for bacterial respiration.

208 Following the respiration measurements, oxygenation of the seawater in each incubation
209 chamber was restored and 1 ml of Shellfish Diet 1800 was injected into each incubation
210 chamber. After an initial mixing period of 30 minutes, seawater samples were collected to
211 quantify the algal cell density over time. Samples were collected by removing 10 ml of seawater
212 from the incubation chamber using a syringe, while at the same time injecting the same volume
213 of seawater using a second syringe in order to keep the seawater volume in the chamber constant.
214 Samples were collected at t₀ (30 minutes after the injection of food into seawater in order to
215 homogenise the mixture before samples were taken to avoid bias) and at t₁ (after 150 minutes
216 of incubation) from each chamber.

217 In order to calculate the volume of seawater cleared by cockles, algal cell concentrations were
218 quantified using a Coulter Multisizer equipped with a 100-μm aperture tube. Hence, *clearance*
219 *rates* were calculated from the exponential decline in the cell concentration in the incubation
220 chamber following equation (3), modified from Widdows and Navarro (2007),

221
$$CR(L\ h^{-1}\ dry\ weight^{-1}) = \frac{V*(\log_e C_1 - \log_e C_2)}{t*g} \quad (3)$$

222 where V represents the volume of water in the incubation chamber, t is the time interval in
 223 hours, C₁ and C₂ represent the shellfish diet cell concentration at the start and end of the
 224 measurement, and g represents the dry flesh weight (g) of cockles present in an incubation
 225 chamber.

226 *Calcification rates* were estimated using the alkalinity anomaly-method according to the
 227 modification proposed by Gazeau et al. (2015) to correct for other processes (e.g. mineralisation
 228 and assimilation) that can affect TA independent from calcification. This method has been
 229 broadly used for short-term incubation as it is non-destructive and based on parameters that are
 230 easily collectable and accurately quantifiable. Two samples of 25ml seawater were collected at
 231 the beginning and end of a 2-hour incubation period. These seawater samples were analysed to
 232 determine the total alkalinity (TA), concentrations of nutrients (NH₄⁺, NO₃⁻+NO₂⁻ and PO₄³⁻)
 233 and *calcification rates* were calculated as shown in equation (4) modified from Gazeau et al.
 234 (2015).

235
$$G*TA = \frac{\Delta NH_4^+ - \Delta TA - \Delta NO_x - \Delta PO_4^{3-}}{2*g*(t_1 - t_0)} \quad (4)$$

236 where t₁ and t₀ represent time (in hours) at the end and at the beginning of incubation,
 237 respectively; ΔNH₄⁺, ΔTA, ΔNO_x and ΔPO₄³⁻ (in μmol g⁻¹DW h⁻¹) are the differences in
 238 concentrations of ammonium, total alkalinity, nitrate plus nitrite and phosphate between t₀ and
 239 t₁, and g is the dry flesh weight (in g) of the cockles present in the incubation chambers.

240 2.3 Statistical analysis

241 A 2 x 2 contingency analysis was used to examine the difference in survival proportion between
 242 pH and temperature levels at the end of the experiment (i.e. after 6 weeks of incubation). In
 243 order to test the effects of temperature (ambient, elevated), pH (current, lowered) and time
 244 (three weeks, six weeks) on the conditional and physiological response variables (condition
 245 index, respiration, clearance and calcification rate), we used a series of linear mixed effects
 246 models with appropriate error structures for each response variable. Since cockles were taken
 247 on two occasions (after weeks three and six) from each aquarium, we accounted for the non-
 248 independence of these data by allowing random intercepts for aquaria identity. Prior to analysis
 249 data were Log₁₀ transformed to improve normality in case of non-normal data, and the model
 250 assumptions were checked using Q-Q plots. Data were modelled using a Gaussian error

251 distribution. For Gaussian models we calculated the F -statistic and associated P -values for each
 252 effect, using the Kenward-Roger method to estimate degrees of freedom. For non-Gaussian
 253 models we used log likelihood ratio tests. Analyses were conducted using the R software
 254 version 3.3.1 (Team, 2013) with packages lme4 (Bates et al., 2015), lmerTest and pbkrTest
 255 (Kuznetsova et al., 2015).

256 3. Results

257 Seawater carbonate chemistry, temperature, pH and salinity for each treatment throughout the
 258 experiment are shown in Table 1 and Figure A1. Temperature in the lowered pH-elevated
 259 temperature treatment and in the elevated temperature treatment were maintained at 2.57 ± 0.13
 260 (SD) °C and 2.74 ± 0.15 °C, respectively, above the control treatment (15.4 ± 0.68 °C). pH
 261 treatments were kept constant throughout the experiment with average values of 8.10 ± 0.01
 262 (SD) and 8.20 ± 0.01 for the control and elevated temperature treatment, respectively. Seawater
 263 pH was reduced by 0.37 ± 0.03 and 0.30 ± 0.02 pH units, respectively, in the lowered pH and
 264 lowered pH-elevated temperature treatments in comparison with the control (pH 8.11 ± 0.06).
 265 Throughout the experiment, total alkalinity (3568 ± 131 $\mu\text{mol kg}^{-1}$) and salinity (34.2 ± 1.0) did
 266 not differ between treatments (Table 1). Aragonite concentration remained favorable for
 267 calcification in all treatments, with average aragonite saturation states of 6.0, 3.7, 6.2, and 3.6
 268 in control, lowered pH, elevated temperature and lowered pH-elevated temperature treatments,
 269 respectively.

270 **Table 1 The seawater carbonate chemistry parameters in four treatments during the 6-week**
 271 **experiment: temperature (Temp, in °C), pH, salinity, total alkalinity (TA in $\mu\text{mol kg}^{-1}$), partial**
 272 **pressure of carbon dioxide ($p\text{CO}_2$, in μatm), total inorganic carbon concentration (C_T in $\mu\text{mol kg}^{-1}$),**
 273 **concentration of bicarbonate ion and carbonate ion (HCO_3^- and CO_3^{2-} in $\mu\text{mol kg}^{-1}$) and saturation**
 274 **state of the seawater with respect to aragonite (Ω_a). Values between brackets represent**
 275 **maximum and minimum values during the experiment.**

Treatment	Control	Lowered pH	Elevated temperature	Lowered pH-elevated temperature
Temperature	15.4 (17.3; 13.1)	15.3 (16.3; 15.6)	18.2 (19.2; 15.6)	18 (19.0; 15.5)
pH	8.11 (8.20; 8.00)	7.75 (7.98; 7.50)	8.2 (8.38; 8.08)	7.82 (8.06; 7.53)
Salinity	34.4 (33; 36)	33.8 (33; 35)	34.5 (33; 36)	34.2 (33; 37)
TA ($\mu\text{mol/kg}^{-1}$)	3568 (3372; 3667)	3563 (3415; 3766)	3546 (3471; 3696)	3600 (3525; 3768)

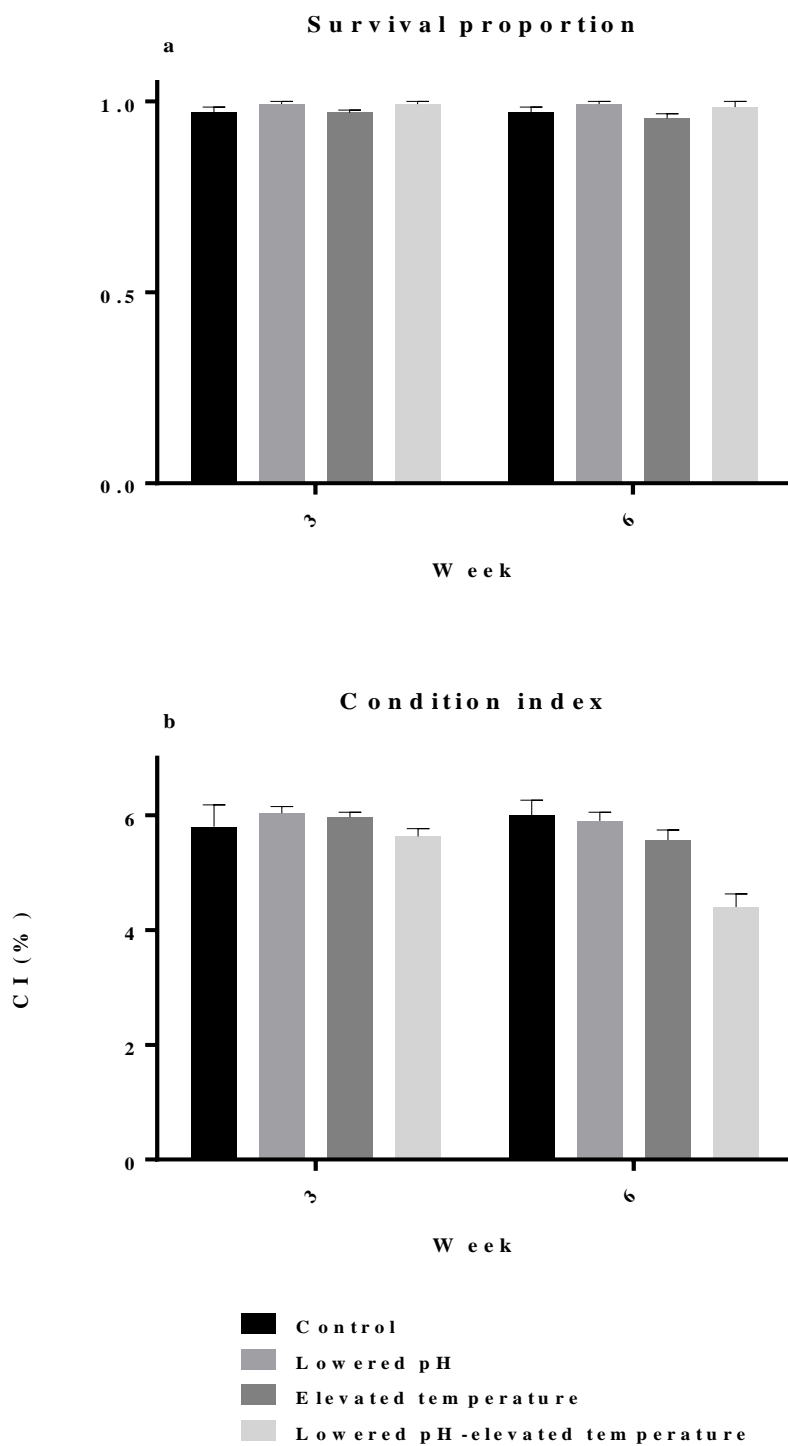
pCO₂ out (μatm)	325 (286; 367)	746 (288; 1065)	335 (280; 385)	870 (322; 1055)
C_T ($\mu\text{mol/kg}^{-1}$)	3069 (2917, 3507)	3283 (2965, 3557)	3039 (2924, 3142)	3332 (3211, 3406)
HCO₃⁻ ($\mu\text{mol/kg}^{-1}$)	2622 (2681; 2553)	3099 (3317; 2963)	2621 (2703; 2470)	3131 (3167; 3087)
CO₃²⁻ ($\mu\text{mol/kg}^{-1}$)	386	207	402	198
Ω_a	6 (5.4; 7.2)	3.7 (2.5; 6.1)	6.17 (5.6; 6.8)	3.6 (2.8; 6.6)

276

277 During the experiment, survival remained high (> 93%) and did not differ significantly among
278 treatments (Fig. 1a). Dead cockles laying on top of the sediment were removed immediately
279 during the daily observations. There was no difference in survival between current and lowered
280 pH treatments ($\chi^2_1 = 2.4$, $P = 0.12$) or between ambient and elevated temperature treatments (χ^2_1
281 = 2.4, $P = 0.12$) at the end of the 6-week incubation. There was no three-way interaction on
282 *condition index* (CI) of cockles between temperature, pH and time ($F_{1,8} = 0.69$, $P = 0.43$ (Fig
283 1b)). Similarly, there was no two-way interaction between pH and time ($F_{1,9} = 3.8$, $P = 0.082$).
284 However, there was a significant two-way interaction between temperature and pH ($F_{1,8} = 7.5$,
285 $P = 0.025$) indicating that reduced pH led to a decrease in CI in cockles held at 18°C but had
286 no effect on CI for cockles held at 15°C (Fig. A3a). Furthermore, there was a significant two-
287 way interaction between temperature and time ($F_{1,9} = 8.2$, $P = 0.020$), indicating that there was
288 no difference in CI between the two temperatures at week 3, but that by week 6 cockles held at
289 18°C had a lower CI than those held at 15°C (Fig. A3b). There were no main effects of time
290 ($F_{1,11} = 3.8$, $P = 0.077$) and pH ($F_{1,9} = 2.9$, $P = 0.12$), but there was a significant effect of
291 temperature ($F_{1,9} = 7.3$, $P = 0.025$).

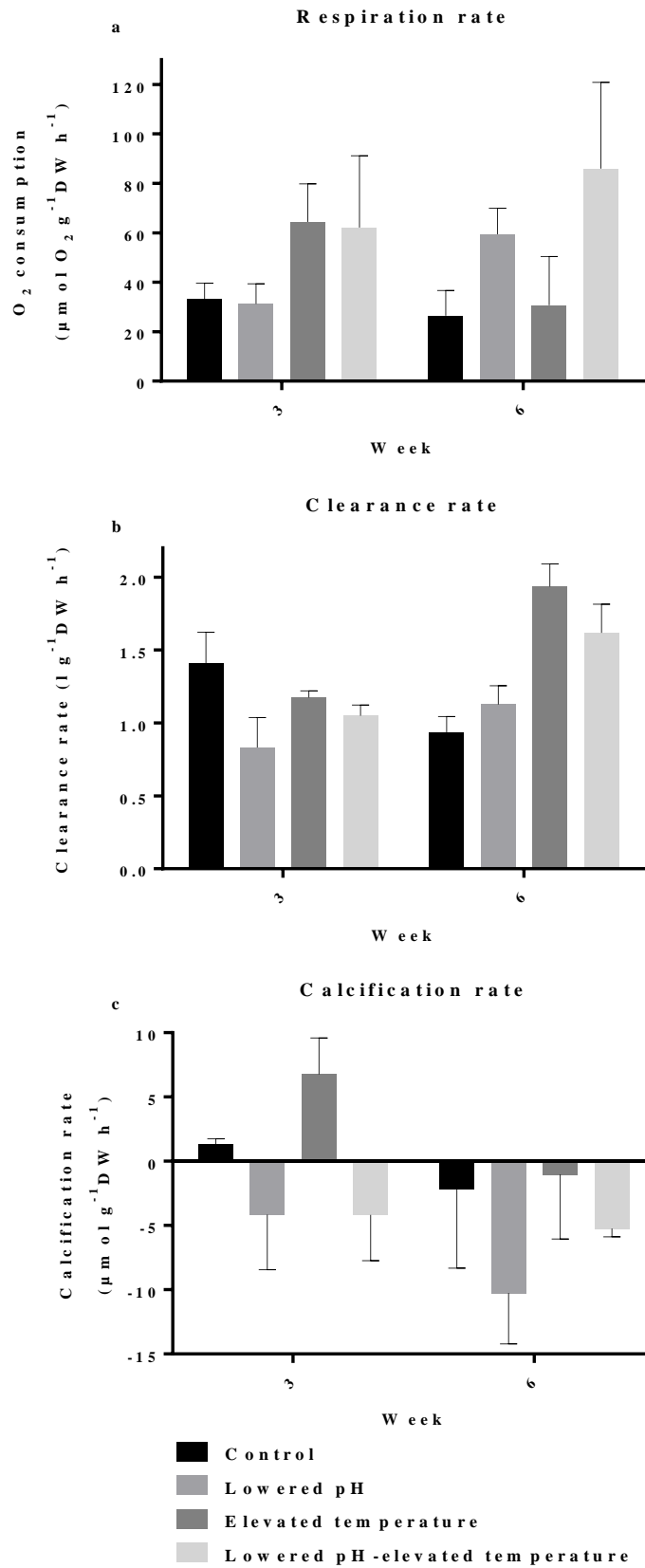
292 We observed no three-way interaction between temperature, pH and time on *respiration rates*
293 ($F_{1,8} = 0.096$, $P = 0.76$ (Fig. 2a)) and no two-way interactions between temperature and time
294 ($F_{1,9} = 0.96$, $P = 0.35$) or temperature and pH ($F_{1,8} = 0.020$, $P = 0.89$). However, a significant
295 interaction between pH and time ($F_{1,9} = 6.4$, $P = 0.033$) reflected that respiration rates decreased
296 between weeks three and six for the current pH treatment but increased between weeks three
297 and six for the lowered pH treatment (Fig. A4). There were no main effects of time ($F_{1,11} = 0.18$,
298 $P = 0.68$), pH ($F_{1,9} = 2.9$, $P = 0.12$) or temperature ($F_{1,9} = 1.4$, $P = 0.28$). In terms of *clearance*

299 *rates*, there was no three-way interaction ($F_{1,8} = 4.9, P = 0.057$) and no two-way interactions
300 between temperature and pH ($F_{1,8} = 0.061, P = 0.81$) or pH and time ($F_{1,9} = 3.1, P = 0.11$). There
301 was, however, a borderline non-significant trend for an interaction between temperature and
302 time ($F_{1,9} = 5.0, P = 0.053$), illustrating that there was no difference in clearance rate between
303 the two temperatures at week 3 but that by week 6 clearance rates were greater for cockles held
304 at 18°C compared to those held at 15°C (Fig. 2b). There were no main effects of time ($F_{1,11} =$
305 $3.9, P = 0.074$) and pH ($F_{1,9} = 2.2, P = 0.17$). Finally, a significant effect of temperature ($F_{1,9} =$
306 $6.8, P = 0.028$) indicated that clearance rates increased with elevated temperature treatment as
307 compared to the ambient temperature treatment at weeks three and six (Fig. A5). No three-way
308 interaction between temperature, pH and time ($F_{1,8} = 1.09, P = 0.33$), nor two-way interactions
309 between temperature and pH ($F_{1,8} = 0.016, P = 0.90$), temperature and time ($F_{1,9} = 0.0074, P =$
310 0.93) or pH and time ($F_{1,9} = 0.22, P = 0.65$) were found on *calcification rates* (Fig. 2c). There
311 was no main effect of temperature ($F_{1,9} = 0.97, P = 0.35$) on calcification rate but a significant
312 effect of pH ($F_{1,9} = 5.9, P = 0.038$) demonstrated a higher calcification rate under current pH as
313 compared to low pH conditions (Fig. A6a). Furthermore, there was a significant effect of time
314 ($F_{1,11} = 5.1, P = 0.045$) indicating a lower calcification rate at week 3 compared to week 6
315 (Figure. A6b).



316

317 **Fig. 1. Effects of temperature and pH on survival (1a) and condition index (1b) after 3 and 6**
 318 **weeks of incubation in the four treatments. Error bars represent standard errors (\pm SE).**



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Fig. 2. Physiological responses measurement of *C. edule* (respiration, clearance and calcification rates) at week 3 and week 6 incubated in four treatments. (Error bars represent standard errors (\pm SE), n = 3 replicates per treatment).

323 4. Discussion

324 According to Pörtner and Farrell (2008) and Pörtner (2012), OA may narrow the thermal
325 window of aquatic animals, probably through a reduction in functional capacity of tissue caused
326 by the accumulation of CO₂. Our study, which mimicked the conditions projected for a future
327 high pCO₂ ocean, i.e. warming and acidification, showed no effect of both applied stressors,
328 alone or in combination, on the survival of cockles. Schade et al. (2016) demonstrated equally
329 low mortality rates of the same species collected from the Baltic Sea in seawater with a pH of
330 7.4 to 7.8 (the latter being the ambient pH for the studied population). The lack of a mortality
331 effect under increased temperature and lowered pH might be due to temperatures used which
332 are well within the thermal window of the studied species (4 - 38°C (Compton et al., 2007;
333 Rygg, 1970)). Furthermore, the 6 week duration of our experiment might have been too short
334 to demonstrate mortality and could therefore rather represent a moderate level of environmental
335 stress (Sokolova et al., 2012).

336 Bivalve condition indices represent a good reflection of the energy available for growth of
337 bivalves (Lucas and Beninger, 1985; Nilin et al., 2012). Consequently, such indices give a
338 realistic indication of bivalve fitness at longer time scales than can usually be studied under
339 laboratory conditions and are therefore used to assess the condition of bivalves under variable
340 environmental conditions (e.g. Dove and Sammut, 2007; Nilin et al., 2012; Norkko et al., 2005).
341 We found a temperature and time effect on the CI of cockles with lower CI at the high
342 temperature treatments after six weeks of incubation. The temperature effect corroborates
343 Hiebenthal et al. (2013) who reported that CI of *M. edulis* and *Arctica islandica* decreased with
344 increasing temperature. Furthermore, we also observed a synergistic effect of ocean warming
345 and acidification on cockle condition with a pronounced additive negative effect on CI related
346 to low pH in the combined treatment. This finding illustrates that physiological responses to
347 low pH (see below) aggravate the negative effect of ocean warming on cockle condition

348 This study shows that the respiration rates of cockles were significantly higher under acidified
349 conditions after 6 weeks of incubation, while such effect was absent after three weeks. We
350 hypothesise that different compensatory mechanisms for energy homeostasis under low pH can
351 explain this time-dependent effect. At the short term cockles may have compensated the
352 enhanced costs for basal maintenance through the allocation of energy from storage tissue (lipid
353 and glycogen), whereas at the longer term (e.g. after 6 weeks) when the storage material has
354 been depleted, increased respiration was required to maintain homeostasis (Sokolova et al.,

355 2012) and support the need for a higher expression in biomineralization-related enzymes and
356 acid-base regulation (Beniash et al., 2010). Similar upregulation of the aerobic respiration after
357 two months of incubation at a pH 7.70 was reported by Thomsen and Melzner (2010) for blue
358 mussels (*M. edulis*) and after an 11-weeks of incubation at pH 7.5 by Beniash et al., (2010) for
359 *C. virginica*. Furthermore, increased oxygen uptake with decreasing pH is found in other marine
360 taxa as well, e.g. ophiuroid brittlestar (*Amphiura filiformis*) and arctic pteropods (*Limacina*
361 *helicina* (Comeau et al., 2010; Wood et al., 2008)). Opposite effects have also been
362 demonstrated, e.g. for *M. chilensis* (Navarro et al., 2013) and juvenile and adult Mediterranean
363 mussels (*M. galloprovincialis*) (Michaelidis et al., 2005), but Gazeau et al. (2014) and
364 Fernández-Reiriz et al. (2012) found no effect of pH on respiration in the latter species.

365 Since no pseudofaeces production was observed during the measurements the rate of food
366 ingestion equals the rate of clearance multiplied by the cell concentration of the diet (Iglesias
367 et al., 1992; Lu and Blake, 1997). In this study, clearance rates were predominantly affected by
368 temperature with higher clearance rates in the elevated temperature treatments. Similarly,
369 Smaal et al. (1997) found a positive relation between cockle clearance rates and elevated
370 temperature in a field setting, which indicates that the cockles incubated under laboratory
371 conditions in this study reacted similarly to those from the field. Walne (1972) observed that
372 clearance rates of *Ostrea edulis* scaled down by 45%, and of *C. gigas* and *M. edulis* by 25%
373 when temperature was lowered from 20 to 10°C. In general, clearance rates in our study were
374 lower under low pH conditions but this effect was not statistically significant ($p = 0.17$).
375 However, both short and long-term observations on adult mussels (*M. chilensis*), juvenile clams
376 (*R. decussatus*), adult noble scallops (*Chlamys nobilis*) and adult green-lipped mussels (*Perna*
377 *viridis*) have demonstrated negative clearance rate responses to low pH (Fernández-Reiriz et
378 al., 2011; Liu and He, 2012; Navarro et al., 2013)). In general, marine bivalves thus seem to
379 reduce costs related to filtration in low-pH waters rather than to ingest more food to cope with
380 hypercapnia.

381 Shell dissolution rates of bivalves are often associated with the properties of a protective
382 external organic layer or periostracum that separates the shell from the ambient seawater (Ries
383 et al., 2009). As cockle has a thin periostracum of around 2µm (Hall-Spencer et al., 2008) and
384 its shell is solely composed of aragonite - the most soluble polymorph of carbonate (Cubillas et
385 al., 2005; Taylor, 1973), cockles may be particularly vulnerable to OA. We observed a
386 reduction in calcification rates at lowered pH despite the fact that the seawater remained
387 oversaturated with respect to aragonite ($\Omega_{\text{aragonite}} = 3.7$ and 3.6 in lowered pH and lowered pH-

388 elevated temperature treatments, respectively). Similarly, e.g. Beniash et al. (2010) and Ries et
389 al. (2009) found a reduction of calcification of juvenile and adult oysters (*C. virginica*) in low-
390 pH seawater that remained oversaturated with respect to calcite and aragonite. Clearly, the
391 reduction of calcification or dissolution of bivalves does not solely depend on the calcite and
392 aragonite saturation state. According to Cyronak et al. (2016), elevated proton concentrations
393 (H^+), rather than the concentration of carbonate ions (CO_3^{2-}), is likely to be responsible for the
394 reduction of calcification rates of marine calcifying organisms. Elevated $[H^+]$ in acidic seawater
395 alters the proton gradient between intracellular fluid and external seawater, thus, hampering
396 calcifying organisms from maintaining pH homeostasis (Cyronak et al., 2016). A recent study
397 on the blue mussel *M. edulis* indicated that seawater $[HCO_3^-]/[H^+]$ ratio is crucial in regulating
398 calcification rates of the mussel (Thomsen et al., 2015). Our result shows that calcification rates
399 were lower at week 3 (1st July 2015) than that of week 6 (28th July 2015 (Fig. A6b)). We
400 hypothesise that this reduction in calcification over the course of the experiment results from a
401 shift in energy allocation. Cockles start to spawn at the site of collection around July (Rueda et
402 al., 2005) and spawning cockles were observed in the last week of the experiment. It therefore
403 seems likely that cockles have allocated relatively more energy towards gonad production than
404 to calcification by the end of the 6-week incubation.

405 In summary, our results indicate synergistic effects of ocean warming and acidification on the
406 condition index of the common cockle *C. edule*. Since synergistic effects were not found for
407 the separate physiological responses addressed in this study, the interactive effect of OA and
408 warming on cockle condition must be explained by the cumulative impact of different responses.
409 We hypothesize in *C. edule* that the reduced food intake under low pH conditions is insufficient
410 to support the higher energy requirements in future high pCO_2 oceans to compensate for (1)
411 higher basal maintenance in warmer and more acidic waters, and (2) growth in low-pH waters.
412 Furthermore, cockles may allocate additional energy from energy storage pools to cover the
413 increasing maintenance demand (e.g. tissue repair and maintenance) but this mechanism will
414 leave less energy available for reproduction and growth, which will in turn likely have
415 repercussions on population of cockles and their roles in ecosystem functioning, e.g. their
416 influence on recruitment of other benthic species (Flach, 1996; Van Colen et al., 2013), the
417 mediation of benthic primary production (Swanberg, 1991), and as a food source for higher
418 trophic levels ((Beukema and Cadée, 1996)). In order to disentangle the different mechanisms
419 that will determine future population stability of bivalves, and of benthic invertebrates in

420 general, future research needs to address the different components that govern energy allocation
421 under multiple, combined stressor scenarios.

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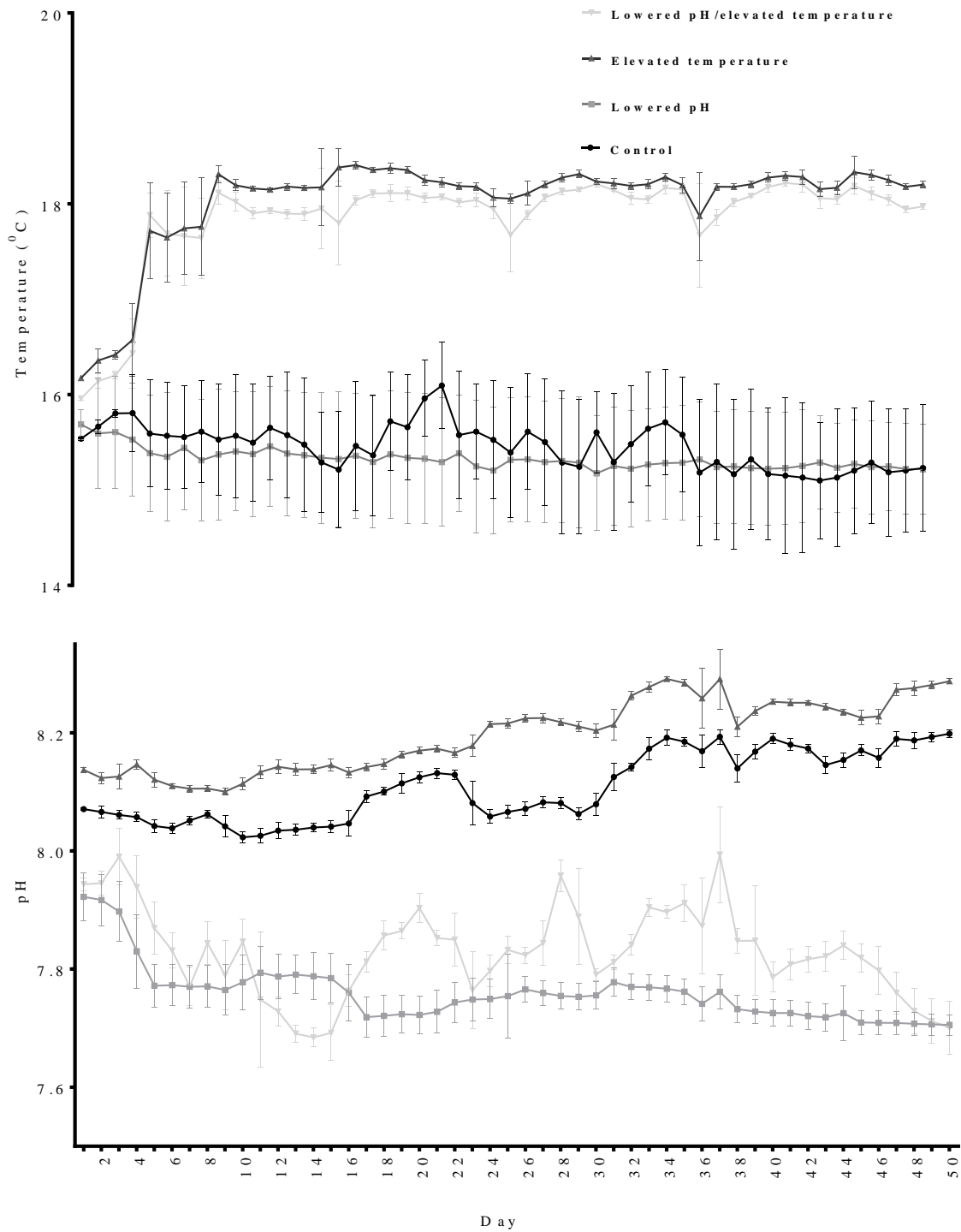
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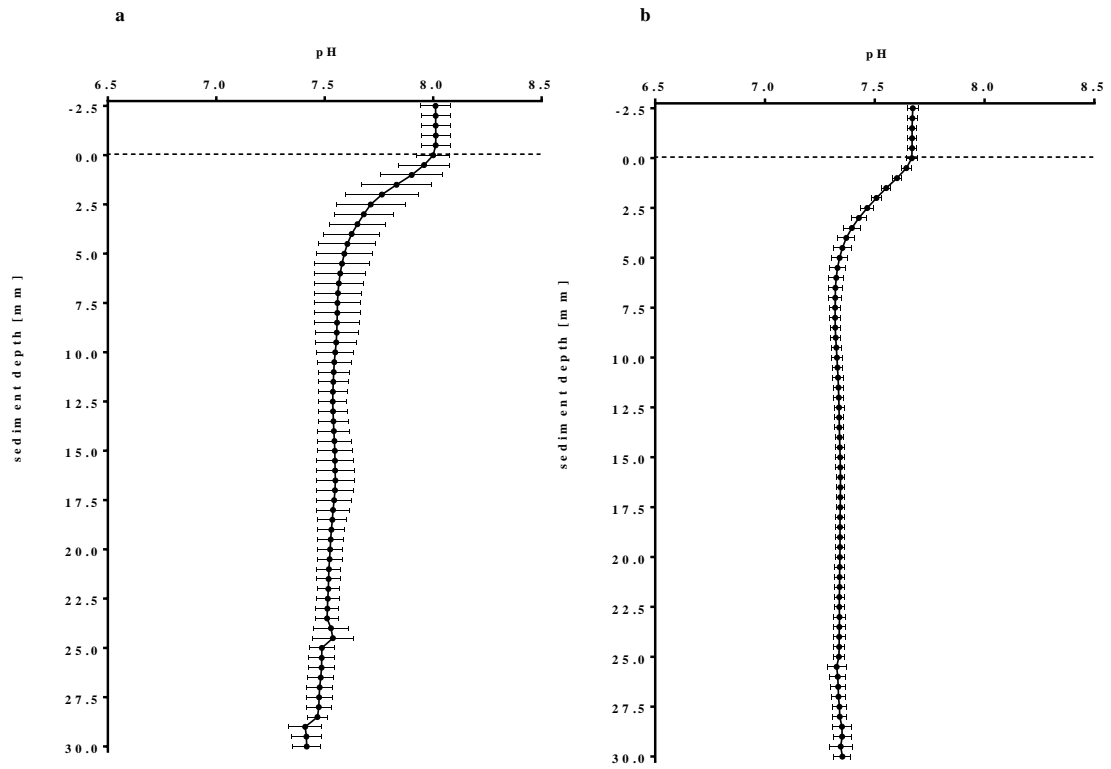
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686 **Appendices**

Mean temperature and pH of each treatment over 50-day incubation



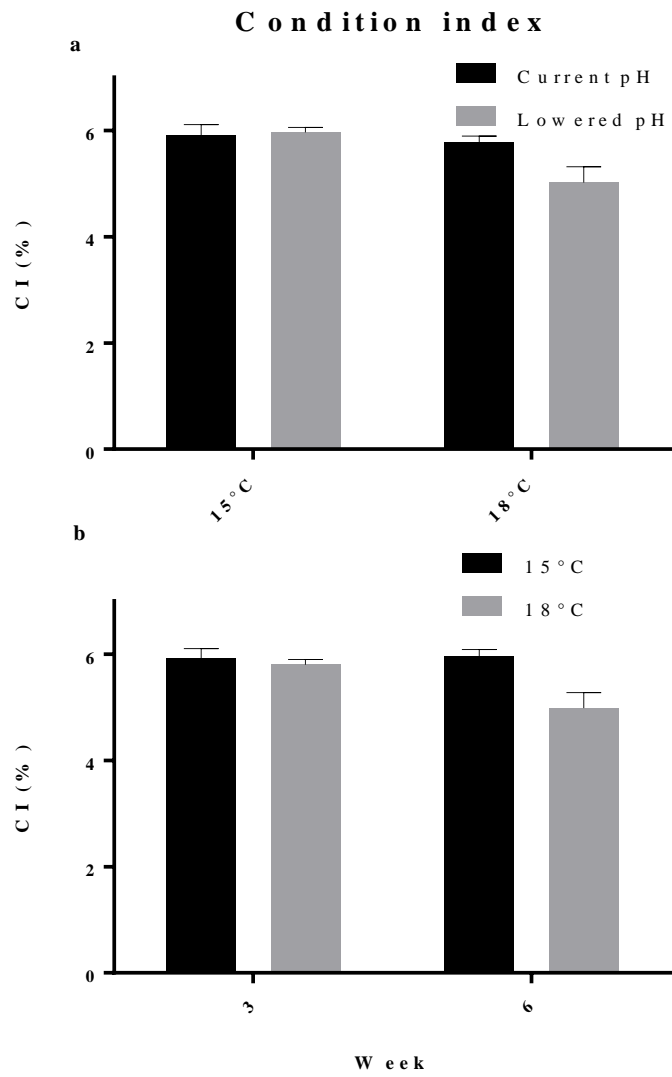
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 688 **Fig. A1. Variations of daily temperature and pH in four treatments for 50-day incubation.** The
 689 experiment started at 6th day of the incubation. Two conditional and physiological responses
 690 measurements were conducted at day-26 and day-47. Error bars represent standard deviation
 691 (\pm SD).



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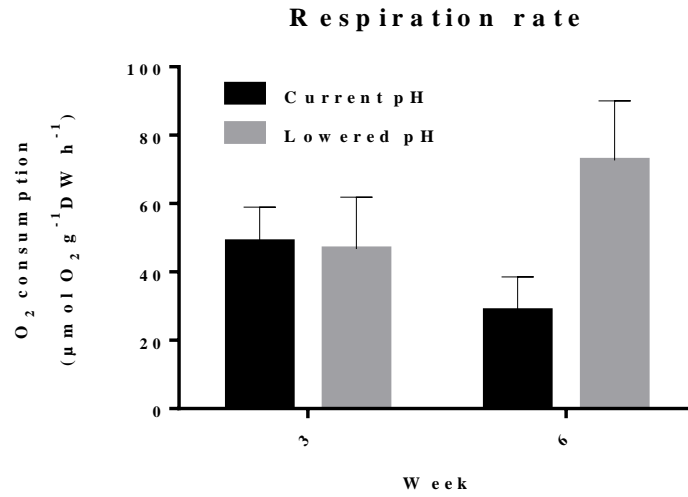
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Fig. A2. pH sediment profile of muddy sand sediment at current pH (a) and lowered pH (b).



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Fig. A3. Condition indices of cockles. a) The interaction effect between temperature and pH and b) the interaction effect between temperature and time. Error bars show standard errors.

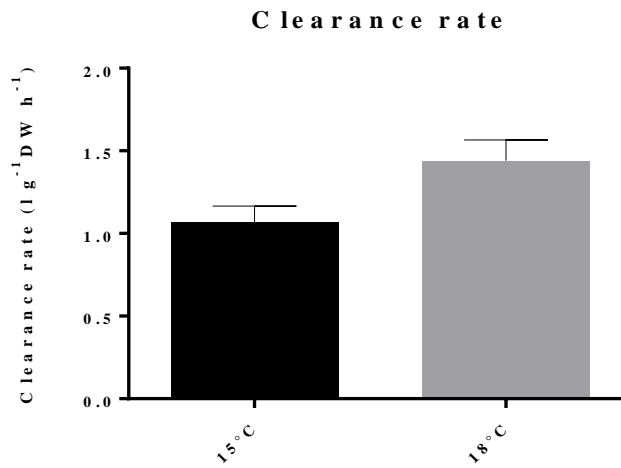


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Fig. A4. The interaction effect between time and pH. Analysis was conducted on Log_{10} transformed data but raw data are plotted here. Error bars show standard errors.



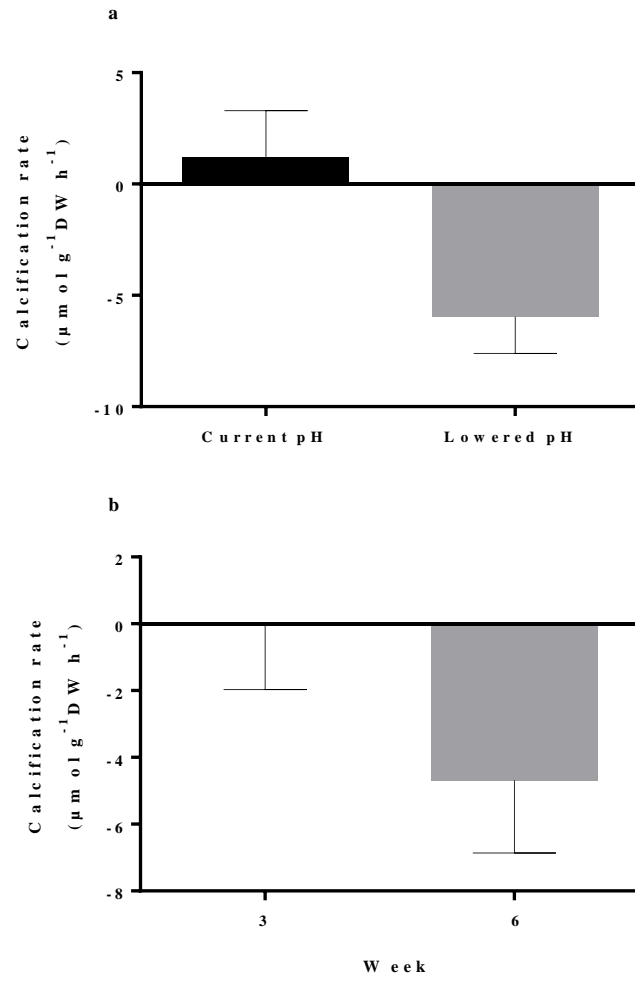
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Fig. A5. The main effect of temperature. Analysis was conducted on Log_{10} transformed data but raw data are plotted here. Error bars show standard errors.

Calcification rate



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Fig. A6. Calcification of cockles. a) The main effect between pH and b) the main effect of time. Error bars show standard errors.