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## **Ocean acidification impacts growth and shell mineralization in juvenile abalone (*Haliotis tuberculata*)**

Stéphanie Auzoux-Bordenave, Nathalie Wessel, Aïcha Badou, Sophie Martin, Saloua M'Zoudi, Solène Avignon, Sabine Roussel, Sylvain Huchette, Philippe Dubois

### ► To cite this version:

Stéphanie Auzoux-Bordenave, Nathalie Wessel, Aïcha Badou, Sophie Martin, Saloua M'Zoudi, et al.. Ocean acidification impacts growth and shell mineralization in juvenile abalone (*Haliotis tuberculata*). *Marine Biology*, 2020, 167 (1), pp.11. 10.1007/s00227-019-3623-0 . hal-02558510

**HAL Id: hal-02558510**

**<https://hal.science/hal-02558510>**

Submitted on 5 May 2020

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# Marine Biology

## Ocean acidification impacts growth and shell mineralization in juvenile abalone (*Haliotis tuberculata*) --Manuscript Draft--

<b>Manuscript Number:</b>	MABI-D-19-00091R4	
<b>Full Title:</b>	Ocean acidification impacts growth and shell mineralization in juvenile abalone ( <i>Haliotis tuberculata</i> )	
<b>Article Type:</b>	Original paper	
<b>Keywords:</b>	ocean acidification; abalone; juvenile; growth; shell mineralization	
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<b>Abstract:</b>	<p>Ocean acidification is a major global driver that leads to substantial changes in seawater carbonate chemistry, with potentially serious consequences for calcifying organisms. Marine shelled molluscs are ecologically and economically important species, providing essential ecosystem services and food sources for other species. Due to their physiological characteristics and their use of calcium carbonate (CaCO<sub>3</sub>) to build their shells, molluscs are among the most vulnerable invertebrates with regard to ocean acidification, with early developmental stages being particularly sensitive to pH changes. This study investigated the effects of CO<sub>2</sub>-induced ocean acidification on juveniles of the European abalone <i>Haliotis tuberculata</i>, a commercially important gastropod species. Six-month-old juvenile abalones were cultured for 3 months at four pH levels (8.1, 7.8, 7.7, 7.6) representing current and predicted near-future conditions. Survival, growth, shell microstructure, thickness and strength were compared across the four pH treatments. After three months of exposure, significant reductions in</p>	

juvenile shell length, weight and strength were revealed in the pH 7.6 treatment. SEM observations also revealed modified texture and porosity of the shell mineral layers as well as alterations of the periostracum at pH 7.6 which was the only treatment with an aragonite saturation state below 1. It is concluded that low pH induces both general effects on growth mechanisms and corrosion of deposited shell in *H. tuberculata*. This will impact both the ecological role of this species and the costs of its aquaculture.

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## 1 **Ocean acidification impacts growth and shell mineralization in juvenile** 2 **abalone (*Haliotis tuberculata*)**

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19

20

### 21 **Abstract**

22 Ocean acidification is a major global driver that leads to substantial changes in seawater  
23 carbonate chemistry, with potentially serious consequences for calcifying organisms. Marine  
24 shelled molluscs are ecologically and economically important species, providing essential  
25 ecosystem services and food sources for other species. Due to their physiological  
26 characteristics and their use of calcium carbonate (CaCO<sub>3</sub>) to build their shells, molluscs are  
27 among the most vulnerable invertebrates with regard to ocean acidification, with early  
28 developmental stages being particularly sensitive to pH changes. This study investigated the  
29 effects of CO<sub>2</sub>-induced ocean acidification on juveniles of the European abalone *Haliotis*  
30 *tuberculata*, a commercially important gastropod species. Six-month-old juvenile abalones  
31 were cultured for 3 months at four pH levels (8.1, 7.8, 7.7, 7.6) representing current and  
32 predicted near-future conditions. Survival, growth, shell microstructure, thickness and  
33 strength were compared across the four pH treatments. After three months of exposure,  
34 significant reductions in juvenile shell length, weight and strength were revealed in the pH 7.6

35 treatment. SEM observations also revealed modified texture and porosity of the shell mineral  
36 layers as well as alterations of the periostracum at pH 7.6 which was the only treatment with  
37 an aragonite saturation state below 1. It is concluded that low pH induces both general effects  
38 on growth mechanisms and corrosion of deposited shell in *H. tuberculata*. This will impact  
39 both the ecological role of this species and the costs of its aquaculture.

40 **Keywords:** ocean acidification; abalone; juvenile; growth; shell mineralization

41

## 42 **Introduction**

43

44 Anthropogenic carbon dioxide emission and its subsequent absorption by the ocean is  
45 responsible for seawater pH decrease and reduced availability of carbonate ions, leading to a  
46 decrease of calcium carbonate saturation state, two processes known as ocean acidification  
47 (OA) (Doney et al. 2009; Gattuso et al. 2015; IPCC 2014). Future projections suggest there  
48 will be a pH reduction of 0.3 units by the year 2100, threatening marine organisms that  
49 produce calcium carbonate exoskeletons and shells, such as corals, molluscs and echinoderms  
50 (Kroeker et al. 2010; Hendricks et al. 2010; Hofmann et al. 2010; Widdicombe and Spicer  
51 2008; Wittmann and Pörtner 2013). Because they do not compensate for acid-base  
52 disturbances, molluscs are considered to be among the most vulnerable invertebrates with  
53 respect to OA, with a pronounced sensitivity in larval and juvenile stages (Beniash et al.  
54 2010; Gazeau et al. 2013; Melzner et al. 2009; Orr et al. 2005; Przeslawski et al. 2015, Ross  
55 et al. 2011). In marine shelled molluscs, OA has been shown to reduce larval survival,  
56 lengthen development time, alter morphology and/or impair shell formation and calcification  
57 (Byrne et al. 2011; Duquette et al. 2017; Ellis et al. 2009; Fitzner et al. 2014a; Gazeau et al.  
58 2010; Kurihara 2008; Noisette et al. 2014). Since many mollusc species are sources of

59 commercially important foods, negative impacts of OA may also result in significant  
60 economic loss (Ekstrom et al. 2015; Gazeau et al. 2007).

61 Abalone are ecologically and economically important shelled gastropods, which are  
62 grazers in the marine ecosystem and a food delicacy for humans (Cook 2014; Huchette and  
63 Clavier 2004). Many abalone species worldwide have experienced severe population decline  
64 due to both overfishing and environmental perturbations, such as global warming and disease  
65 (Nicolas et al. 2002; Travers et al. 2009). In the context of the growth of worldwide  
66 aquaculture (Cook 2014), understanding the effects of stress on abalone physiology would  
67 allow the farmers to adapt their practices to minimize stress, prevent mortalities and produce  
68 higher quality shellfish (Morash and Alter 2015).

69 The abalone *Haliotis tubercula* is a commercially important species, for which rearing over  
70 the whole life cycle is controlled in aquaculture (Courtois de Viçose et al. 2007). It displays a  
71 pelago-benthic life cycle with a larval planktonic stage followed by a critical metamorphosis  
72 into the benthic juvenile, making this species highly sensitive to environmental changes  
73 (Byrne et al. 2011). Several studies have focused on early life-history stages of abalone,  
74 especially on larvae, and have demonstrated adverse effects of elevated CO<sub>2</sub>, such as reduced  
75 survival, developmental delay, body and shell abnormalities and reduction in mineralization  
76 (Byrne et al. 2011; Crim et al. 2011; Guo et al. 2015; Kimura et al. 2011; Onitsuka et al.  
77 2018; Wessel et al. 2018; Zippay and Hofmann 2010). Shell formation has been extensively  
78 studied in *H. tuberculata*, revealing that aragonite is the main CaCO<sub>3</sub> polymorph (Auzoux-  
79 Bordenave et al. 2010, 2015). Since aragonite is a less stable polymorph than calcite and is  
80 likely to be more susceptible to dissolution (Morse et al. 2007), the abalone shell is expected  
81 to be more sensitive to OA (Gazeau et al. 2013). The juvenile stage is a relevant model to  
82 study the effects of OA since (i) it corresponds to the growing stage between larvae and adults  
83 and (ii) it is a critical period where abalone are submitted to strong predation (Shepherd  
84 1973). So far, only two studies investigated the responses of juvenile abalone to decreased

85 seawater pH. In juvenile *Haliotis iris* from New Zealand, significant reduction of shell  
86 growth was observed under lower pH (0.3 to 0.5 pH units less than the control), but no effect  
87 on respiration rate (Cunningham et al. 2016). More recently, the effects of low pH (- 0.3 to -  
88 0.5 units below control pH) on juvenile *H. discus hannai* resulted in eroded shell surfaces,  
89 reduced growth rates and altered biochemical composition and energy metabolism (Li et al.  
90 2018). The impact on shell microstructure or resistance to fracture was not investigated.

91 The goal of the present study was to investigate the effects of CO<sub>2</sub>-induced ocean  
92 acidification on survival, growth and shell mineralization and mechanics of juveniles of the  
93 European abalone *H. tuberculata*. Six-month-old juvenile abalones were cultured for three  
94 months under current and near-future pH conditions (8.1, 7.8, 7.7 and 7.6). Biological  
95 responses such as survival rate, growth performance (shell length, weight and shell growth)  
96 and shell biomineralization were compared across the different pH treatments. SEM  
97 microscopy and fracture force analyses were performed to assess whether reduced pH has an  
98 influence on shell microstructure, thickness and resistance.

99

## 100 **Materials and methods**

101

### 102 **Abalone collection and rearing**

103

104 Farmed, 6-month-old juvenile abalones *H. tuberculata* (n = 540 in total, 6.0 ± 0.5 mm total  
105 shell length) were collected from nursery tanks at the France *Haliotis* abalone farm  
106 (48°36'46N, 4°33'30W; Plouguerneau, France) in January 2013 and transported to the  
107 laboratory at the marine station, Concarneau (MNHN, France). The abalone were randomly  
108 distributed into 12 experimental aquaria (12 L) supplied with filtered 3µm natural seawater  
109 renewed at a rate of 6 L.h<sup>-1</sup> and aerated with ambient air. Temperature was the local value in  
110 January, i.e., 9.3°C ± 0.5 °C. Animals were conditioned in the laboratory, at ambient pCO<sub>2</sub>, at

111 a density of 45 abalones per aquarium, for 3 weeks. Juvenile abalone shells usually have a  
112 green coloration, resulting from their grazing on *Ulvelia sp.* algae at the farm. At the start of  
113 the experiment, the abalone diet was changed from green algae *Ulvelia* to red *Palmaria*  
114 *palmata* which is a high quality alga giving the best growth performance for *H. tuberculata*  
115 (Mercer et al. 1993). This changeover is part of standard procedure in aquaculture when  
116 juveniles reach 6–10 mm and results in a change of shell colouration from greenish to red  
117 (Marchais et al. 2017). Shell marking by algal feeding can be used as a proxy to determine  
118 growth in long term stock enhancement program (Gallardo et al. 2003). This transition  
119 allowed us to mark the start (T0) of the experimental period for the evaluation of shell  
120 growth. The abalones were fed once a week *ad libitum* with the red macroalgae *Palmaria*  
121 *palmata*.

122

## 123 **Experimental design**

124

125 Experiments were carried out between March and June 2013 at the Concarneau marine station  
126 (France) according to an experimental design adapted from Martin et al. (2011). Juvenile  
127 abalones were exposed to four pH levels (Total scale) for 3 months. The treatments consisted  
128 of a control at present-day pH, pH<sub>T</sub> 8.1 (pCO<sub>2</sub> ≈ 400 μatm), and three levels of predicted pH<sub>T</sub>  
129 according to different climate change scenarios, as outlined in Riebesell et al. (2010): 7.8  
130 (pCO<sub>2</sub> ≈ 750 μatm), 7.7 (pCO<sub>2</sub> ≈ 1000 μatm) and 7.6 (pCO<sub>2</sub> ≈ 1400 μatm). Three replicate  
131 aquaria were set up per pH treatment.

132

133

134

135



## 136 **pH control and carbonate chemistry**

137

138 Low seawater  $\text{pH}_T$  levels were obtained by bubbling  $\text{CO}_2$  (Air Liquide, France) into three 100  
139 L header tanks supplied with filtered through-flowing seawater, continuously aerated with  
140 ambient air. Each header tank supplied three experimental 12 L aquaria at a rate of  $6 \text{ L}\cdot\text{h}^{-1}$ .  
141  $\text{pCO}_2$  in each tank was regulated through electro-valves controlled by a pH-stat system (IKS  
142 Aquastar, Germany). pH values of the IKS system were adjusted from daily measurements of  
143 the electromotive force (emf) in each aquarium using a pH meter (Metrohm 826 pH mobile,  
144 Metrohm, Switzerland) with a glass electrode (Metrohm electrode plus), converted to total  
145 scale pH units ( $\text{pH}_T$ ) using Tris/HCl and 2-aminopyridine/HCl buffers (Dickson 2010). At the  
146 start of the experiment, pH was gradually decreased over 10 days by  $0.05 \text{ pH unit}\cdot\text{day}^{-1}$  until  
147 the different target pH levels were reached.

148 Seawater parameters were monitored throughout the 3-month experiment. Temperature and  
149  $\text{pH}_T$  were recorded almost daily in each of the 12 experimental aquaria using a pH meter as  
150 described above. Salinity was measured twice a week using a conductivity meter (3110,  
151 WTW, Germany). Total seawater alkalinity ( $A_T$ ) was measured every two weeks on 100 mL  
152 samples taken from each experimental aquarium. Seawater samples were filtered through  $0.7$   
153  $\mu\text{m}$  Whatman GF/F membranes, immediately poisoned with mercury chloride and stored in a  
154 cool dark place pending analyses.  $A_T$  was determined potentiometrically using an automatic  
155 titrator (TitroLine alpha, Schott SI Analytics, Germany), calibrated with the National Bureau  
156 of Standards scale.  $A_T$  was calculated using a Gran function applied to pH values ranging  
157 from 3.5 to 3.0, as described by Dickson et al. (2007), and corrected by comparison with  
158 standard reference material provided by Andrew G. Dickson (CRM Batch 111). Seawater  
159 carbonate chemistry, i.e., dissolved inorganic carbon (DIC),  $\text{pCO}_2$  and aragonite saturation  
160 state ( $\Omega_{\text{aragonite}}$ ) were calculated from  $\text{pH}_T$ ,  $A_T$ , temperature and salinity with the  $\text{CO}_2\text{SYS}$

161 program (Pierrot et al. 2006) using constants from Mehrbach et al. (1973) refitted by Dickson  
162 and Millero (1987).

163

#### 164 **Juvenile survival and sampling**

165

166 Survival of the juvenile abalones was assessed almost daily during the 3-month experiment.

167 Any dead individuals were removed from the tanks immediately. Mortality was calculated as

168 the proportion of the number of dead abalones vs the total number of abalones per tank and

169 then averaged over the three replicates to calculate the mean survival for each pH treatment.

170 Juvenile individuals were sampled at the start of the experiment (T0) and after 3 months of

171 exposure (T3m). Soft tissues were dissected while shells were rinsed with distilled water,

172 dried and stored at room temperature until analysis.

173

#### 174 **Biometric measurements**

175

176 Shell length was measured with a Vernier calliper to the nearest 0.05 mm on 12 abalones

177 from each replicate (n = 36 per pH treatment). Shell dry weight was measured on an

178 analytical laboratory balance (Ohaus, Switzerland) to the nearest 0.001 mg (n = 21 per pH

179 treatment, except for pH 7.6, n = 13). For shell weight, the lower number of individuals in

180 pH<sub>T</sub> 7.6 is due to the high percentage of broken shells in this condition; indeed, only the

181 whole shells were used for shell weight determination and further SEM analysis on cross

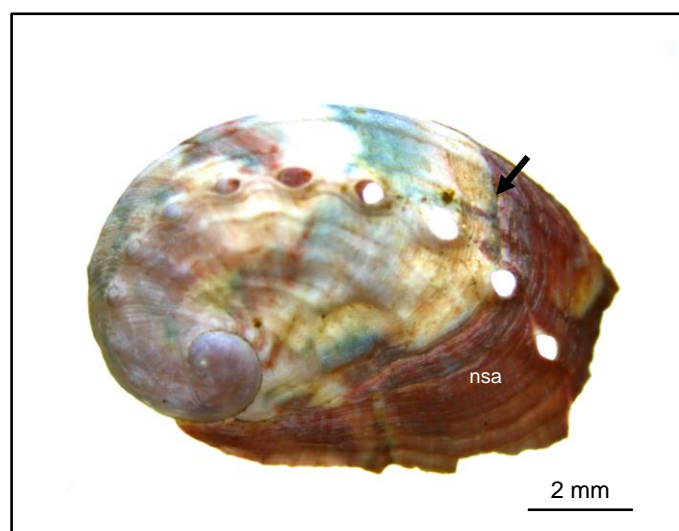
182 sections. The remaining shell samples were used for shell strength measurements. Shell index

183 was calculated as the ratio total shell weight/(shell length)<sup>3</sup> (n = 21 per pH treatment , except

184 for pH 7.6, n = 13).

185

186 For the estimation of relative shell growth, the external mark on the shell induced by the  
187 change in algal diet (Fig. 1) was used as a proxy to estimate the shell area newly formed  
188 during the 3-month experiment. Shell surfaces were examined under a binocular microscope  
189 (Leica, Germany) and imaged with NIS elements software (Nikon, Japan). For the  
190 determination of relative shell growth, the newly formed area (red coloured) was measured by  
191 tracing its outline using Image J software. The ratio of red shell area/total shell area was  
192 calculated for each abalone shell and used as a proxy to determine relative shell growth (n =  
193 36 per pH treatment). All measurements were made by a single researcher, with the origin of  
194 each shell anonymised to eliminate subjective bias.



203 **Fig. 1** Shell of a 9-month-old abalone showing the transition from greenish to red colouration  
204 induced by the change of algal diet (black arrow); the transition mark was used as a proxy to  
205 estimate the shell area newly (nsa) formed during the 3-month experiment.

### 207 Scanning electron microscopy (SEM)

209 Randomly chosen abalone shells from control pH and pH 7.6 (n = 5 per pH treatment) were  
210 analysed by SEM. Outer and inner shell surfaces were investigated on whole, gold-coated

211 (JEOL JFC 1200 fine coater) samples. Shell thickness and microstructure were examined on  
212 shell cross-sections cut using a razor blade along the longitudinal growth axis of the shell.  
213 Samples were gold-coated (JEOL JFC 1200 fine coater) and observed at 5–15 kV with either  
214 a JEOL JSM-840A or a Sigma 300 FE-SEM scanning electron microscope (SEM, Plateau  
215 Technique de Microscopie Electronique, MNHN, Paris and Concarneau, France). Shell  
216 thickness (total, outer spherulitic and nacre layer thickness) measurements were made on  
217 SEM images of the cross sections using Image J software from 6 to 9 transects per shell  
218 section (Fig. S1, electronic supplementary material).

219

## 220 **Biomechanical tests**

221

222 Shell strength (resistance) of abalone shells was measured individually (n = 10 shells/pH  
223 treatment) using a simple compression method. The shells were placed on a metal block with  
224 the opening downwards (i.e., in what would be their natural position on a rocky substrate) and  
225 the mechanical test was carried out using a second metal block fixed on the load frame of the  
226 force stand (Instron 5543), which was then lowered onto the shell at a speed of 0.3 mm.min<sup>-1</sup>  
227 (simple compression test) until fracture. Displacement and force were recorded continuously  
228 at a frequency of 10 Hz using a 100N force cell (Instron 2530-100N). The failure force was  
229 recorded for each specimen. A representative curve of compressive force vs displacement is  
230 presented in supplementary Fig. S2. A stress-strain relationship was not established because  
231 of the absence of symmetry in the abalone shell and the difficulty to define the surface on  
232 which the force is applied.

233

234

## 235 **Statistical analysis**

236

237 All statistical analyses were performed with Rstudio software (R Core Team, 2015).  
238 Differences in juvenile survival, shell length and weight, relative growth and shell index  
239 across pH treatments were assessed using generalized linear model (GLM) ANOVAs after  
240 testing the normality of data, normality of residuals and homogeneity of variances (pH: fixed  
241 factor, tank: random factor nested into pH). In the few cases where data distribution deviated  
242 from normality and/or variances were not homogeneous, data were Log-transformed to ensure  
243 compliance with ANOVA assumptions. If the homogeneity of variance was not verified, a  
244 Welch test was performed, as recommended by Day and Quinn (1989). Post-hoc HSD Tukey  
245 tests, using the appropriate mean square error, were used to test the differences between the  
246 group means. To detect any significant differences in shell thickness, unpaired Student's t-  
247 tests were performed. To assess the effect of decreased pH on shell strength, regression  
248 analyses were performed to look at the relationships between fracture force and shell weight.  
249 ANCOVA model was used to compare linear regression models and evaluate the effect of pH,  
250 using weight as a covariate. All data are presented as means  $\pm$  SD, except where otherwise  
251 stated. Differences were considered significant at  $P < 0.05$ .

252

## 253 **Results**

254

### 255 **Seawater parameters**

256

257 Mean values of seawater carbonate chemistry parameters for the four pH treatments are given  
258 in Table 1. Seawater temperature followed the natural variations found in the bay of  
259 Concarneau, ranging from  $9.3 \pm 0.5^{\circ}\text{C}$  at the start of experiment (early March) to  $16.1 \pm$

260 0.5°C at the end (mid-June), with the mean ranging from  $12.1 \pm 1.8^\circ\text{C}$  to  $12.4 \pm 1.8^\circ\text{C}$   
261 according to treatment. The  $\text{pH}_T$  of the experimental aquaria was maintained close to the  
262 nominal values throughout the experiment, at  $\text{pH}_T = 8.10$  ( $\text{pCO}_2 = 347 \pm 5 \mu\text{atm}$ ),  $\text{pH}_T = 7.81$   
263 ( $\text{pCO}_2 = 746 \pm 10 \mu\text{atm}$ ),  $\text{pH}_T = 7.73$  ( $\text{pCO}_2 = 934 \pm 13 \mu\text{atm}$ ) and  $\text{pH}_T = 7.65$  ( $\text{pCO}_2 = 1157$   
264  $\pm 35 \mu\text{atm}$ ). Mean total alkalinity ( $A_T$ ) measured in the experimental aquaria ranged from  
265  $2305 \pm 28 \mu\text{Eq.kg}^{-1}$  to  $2312 \pm 30 \mu\text{Eq.kg}^{-1}$  ( $n = 8$  per pH treatment) over the course of the  
266 experiment and presented only slight differences between treatments. Salinity was  $35.2 \pm 1.7$   
267 in all experimental aquaria.  $\Omega_{\text{calcite}}$  was always greater than 1 in all four pH treatments, while  
268  $\Omega_{\text{aragonite}}$  only reached values below 1 in the lowest  $\text{pH}_T$  treatment (7.6).

269

## 270 **Survival**

271 The mortality of juvenile abalones was very low, with a survival percentage ranging between  
272  $90.9 \pm 10.3\%$  and  $96 \pm 3.3\%$  at the end of the experiment. There were no significant  
273 differences in survival among the four pH treatments (ANOVA,  $F(3,8) = 0.367$ ,  $P = 0.78$ ).

274

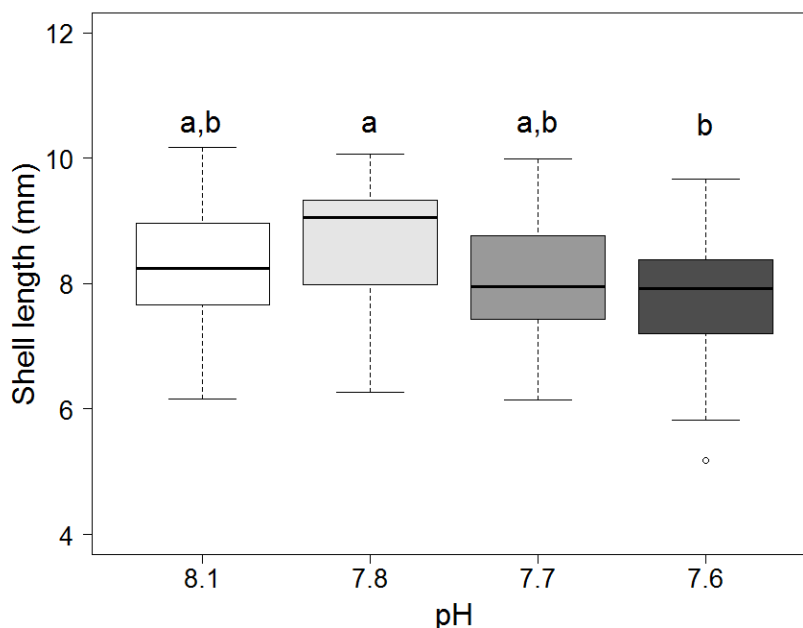
## 275 **Shell growth**

276

277 Abalones grew in all treatments, from  $6.0 \pm 0.5$  mm at the start of the experiment to  $8.3 \pm 1.0$   
278 mm ( $\text{pH}_T$  8.1),  $8.7 \pm 1.0$  mm ( $\text{pH}_T$  7.8),  $8.1 \pm 1.0$  mm ( $\text{pH}_T$  7.7) and  $7.8 \pm 1.1$  mm ( $\text{pH}_T$  7.6)  
279 after 3 months. Total shell length after 3 months differed significantly according to pH  
280 treatment (ANOVA,  $F(3,8) = 4.78$ ,  $P = 0.034$ , Fig. 2): juveniles exposed to  $\text{pH}_T$  7.6 were  
281 significantly smaller than those exposed to  $\text{pH}_T$  7.8 (Post-hoc Tukey,  $t_8 = -3.656$ ,  $P = 0.027$ ,  
282 Table 2). No significant differences were observed between juveniles exposed to  $\text{pH}_T$  7.7, 7.8  
283 and the control  $\text{pH}_T$  (8.1).

284

285  
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292



293 **Fig. 2** Shell length of juvenile abalones *H. tuberculata* exposed to decreased pH<sub>T</sub> values.  
294 Centre lines of box plots show the medians; box limits indicate the first and third quartiles,  
295 respectively, with whiskers encompassing data within 1.5 times the spread from the median (n  
296 = 36 per pH treatment). Different letters indicate significant differences between pH  
297 treatments ( $P < 0.05$ ).

298

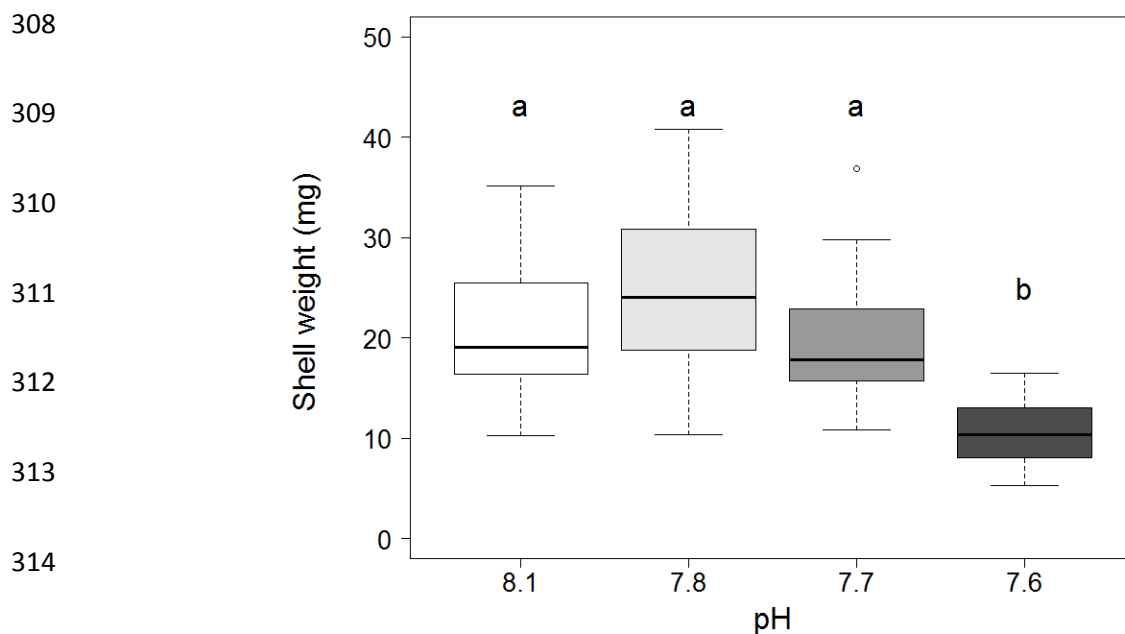
299 Shell weight was significantly affected by pH (ANOVA,  $F(3,8) = 24.43$ ,  $P < 0.001$ , Fig. 3),  
300 being lower for juveniles exposed to pH<sub>T</sub> 7.6 compared with those grown in other pH  
301 treatments (Post-hoc Tukey,  $P < 0.005$ , Table 2). Relative shell growth, determined as the  
302 ratio of red shell area/total shell area, did not differ significantly between pH treatments  
303 (ANOVA,  $F(3,8) = 0.626$ ,  $P = 0.618$ , Table 2).

304

305

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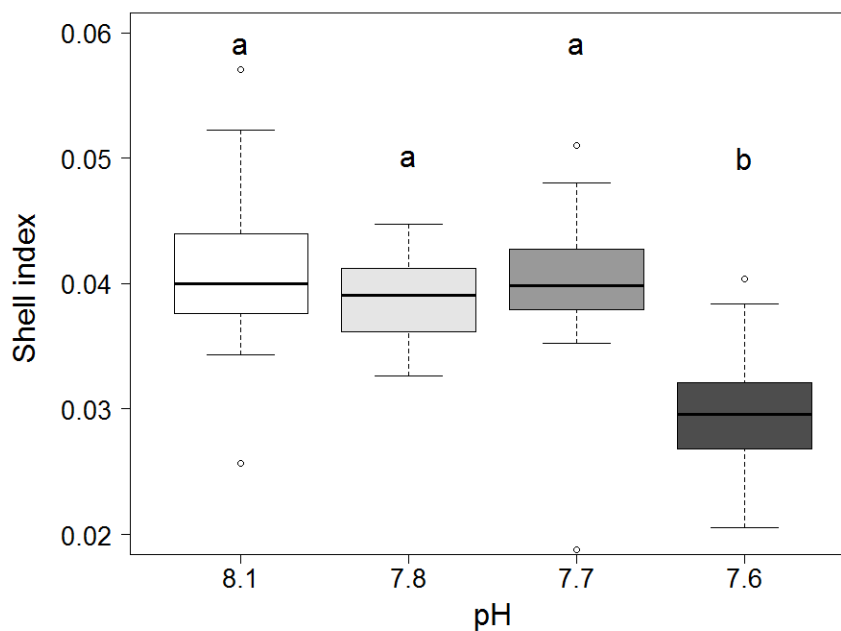
**Fig. 3** Shell weight of juvenile abalones *H. tuberculata* exposed to decreased pH<sub>T</sub> values. Centre lines of box plots show the medians; box limits indicate the first and third quartiles respectively, with whiskers encompassing data within 1.5 times the spread from the median (n = 22 per pH treatment, except for pH 7.6 n = 13). Different letters indicate significant differences between pH treatments ( $P < 0.05$ ).

### Shell calcification

Shell index was significantly affected by pH (Welch's F-test,  $F(3, 3.982) = 14.48$ ,  $P = 0.013$ ; Fig. 4). Abalones in the pH<sub>T</sub> 7.6 treatment had a reduced shell index compared to those grown in the other pH treatments ( $P < 0.005$ , Table 2).



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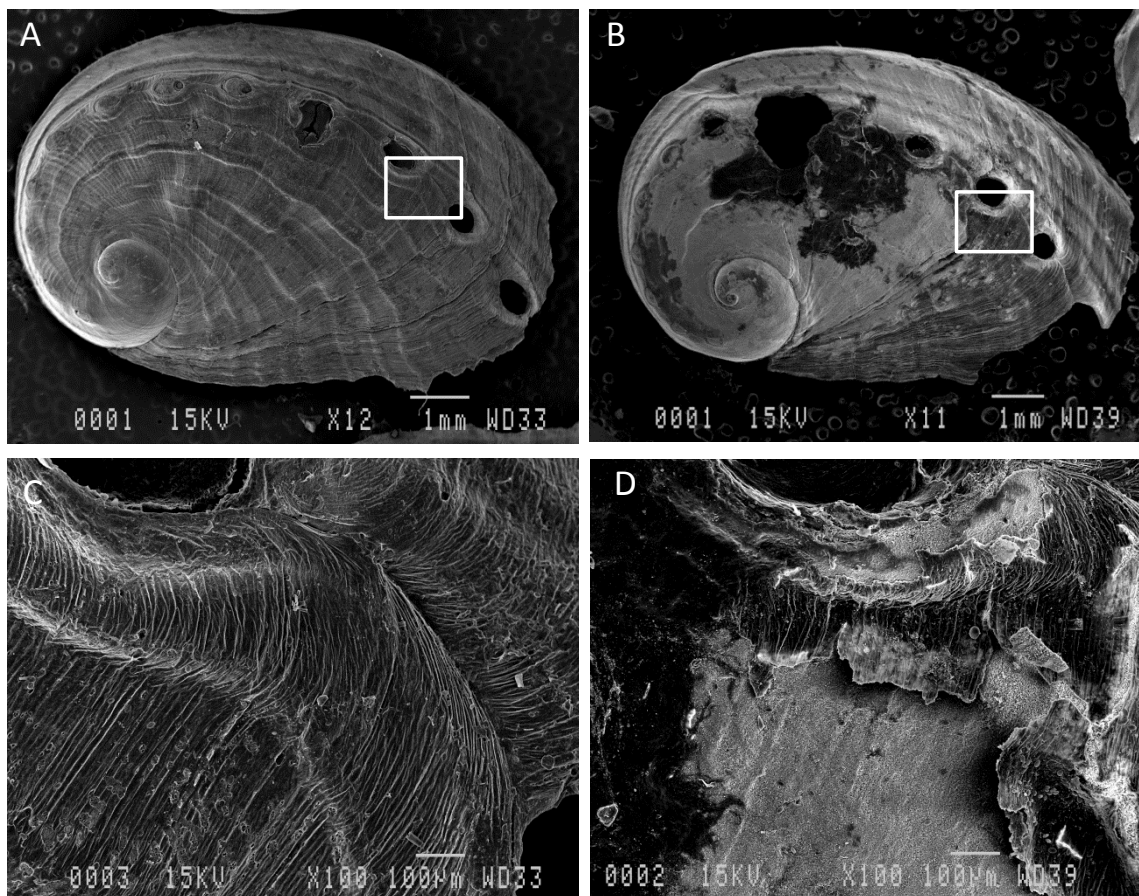
339 **Fig. 4** Shell index (determined as the ratio shell weight/shell length<sup>3</sup>) of juvenile abalones  
340 under different pH<sub>T</sub> conditions. Centre lines of box plots show the medians; box limits  
341 indicate the first and third quartiles, respectively, with whiskers encompassing data within 1.5  
342 times the spread from the median (n = 22 per pH treatment, except for pH<sub>T</sub> 7.6 n = 13).  
343 Different letters indicate significant differences between pH treatments ( $P < 0.05$ ).

344

345 SEM examination of abalone shells grown at pH<sub>T</sub> 8.1 and 7.6 revealed differences in the  
346 texture and porosity of both outer and inner surface layers. Under control conditions, almost  
347 intact periostracum were observed with typical ornamentations (ridge and groove pattern) and  
348 regular organic sheets (Fig. 5a, c). At lower pH, the periostracum revealed bleached areas and  
349 a corroded surface with numerous small holes (Fig. 5b, d). In some individuals, large holes  
350 were observed between the apertures as the result of shell corrosion (Fig. 5b). The  
351 delamination of surface organic sheets revealed biominerals of the underlying spherulitic  
352 layer (Fig. 5d). In control shells, the inner nacreous layer had a homogeneous surface, with a  
353 gradual maturation of aragonite platelets (Fig. 6a, c, e). By contrast, juvenile shells from the  
354 low pH treatment (pH 7.6) were characterized by a partial degradation of the inner nacreous

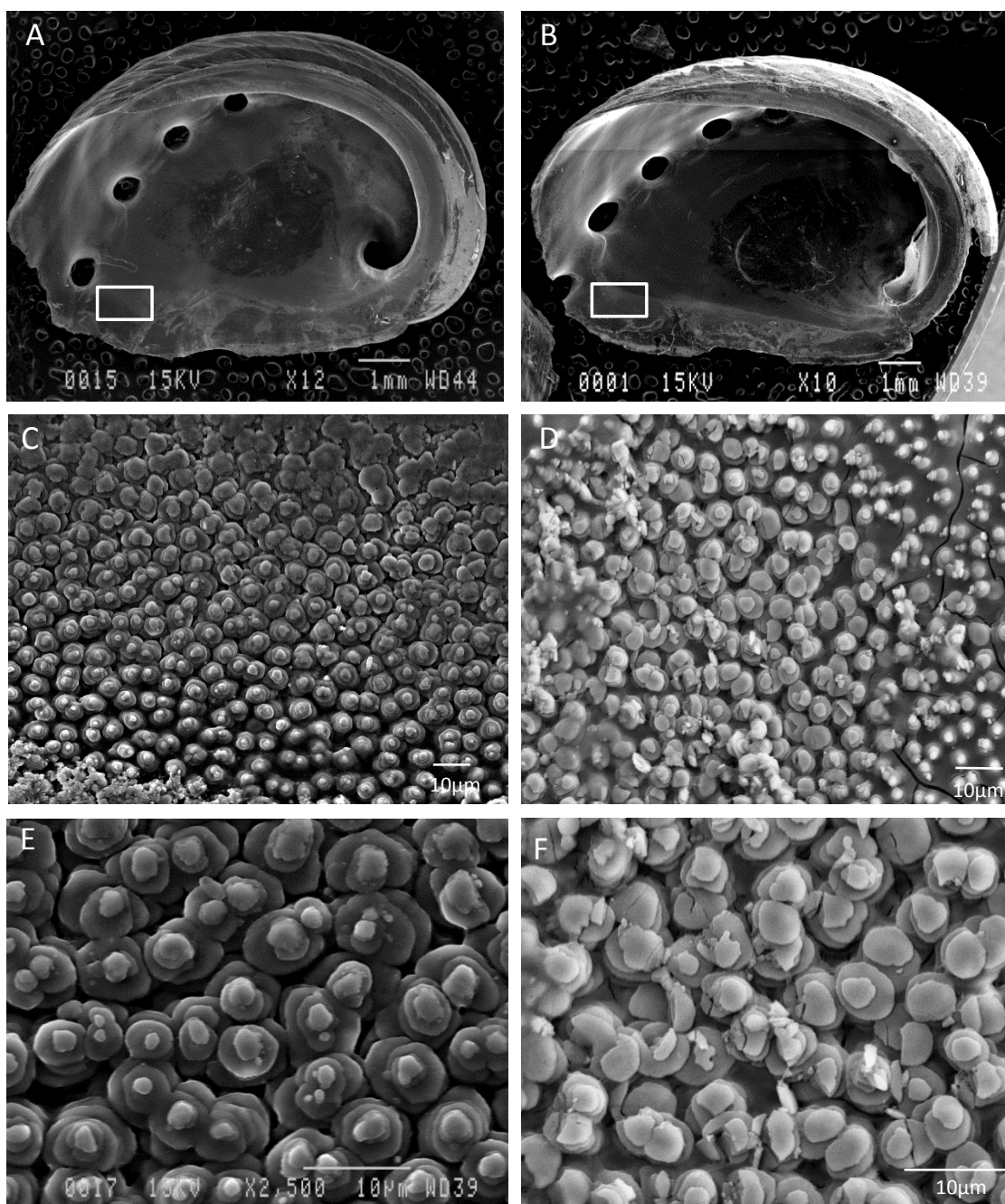
355 layer, resulting in decreased size of aragonite platelets and irregularities of their edges (Fig.  
 356 6b, d, f).

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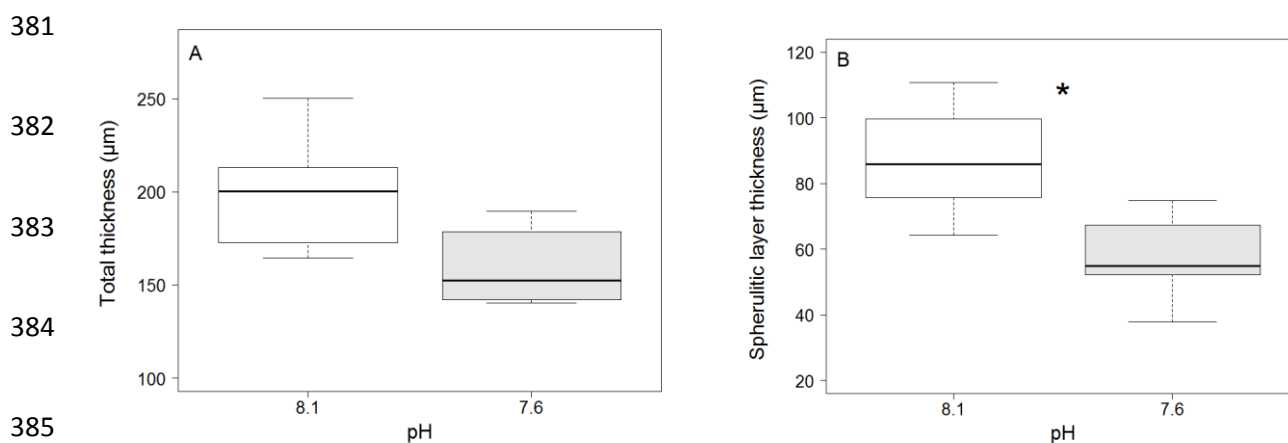
359 **Fig. 5** SEM images of abalone outer shell surface in control conditions ( $\text{pH}_T$  8.1: A, C) and  
 360 under lower pH ( $\text{pH}_T$  7.6: B, D). **A.** Periostracum in the control, showing a homogenous  
 361 surface with the typical ridge and groove patterns; **B.** Periostracum in the  $\text{pH}_T$  7.6 treatment,  
 362 showing bleached areas and corroded surface; **C.** Detail of the shell area boxed in A, showing  
 363 regular organic sheets; **D.** Detail of the shell area boxed in B, showing the delamination of  
 364 organic sheets and revealing biominerals of the underlying spherulitic layer.



365

366 **Fig. 6** SEM images of abalone inner nacreous surface in control conditions ( $\text{pH}_T$  8.1: A, C, E)  
 367 and under low  $\text{pH}_T$  7.6 (B, D, F). **A.** Inner nacreous layer of control shell; **B.** Inner nacreous  
 368 layer of shell exposed to  $\text{pH}_T$  7.6; **C.** Detail of the nacre growth region boxed in A showing  
 369 gradual maturation of aragonite platelets; **D.** Detail of the nacre surface boxed in B showing  
 370 disruption of the aragonite platelets; **E.** Magnification of the nacre surface showing  
 371 confluence of regular aragonite platelets; **F.** Magnification of the nacre platelets showing  
 372 breaks within the platelets and irregularities of their edges.

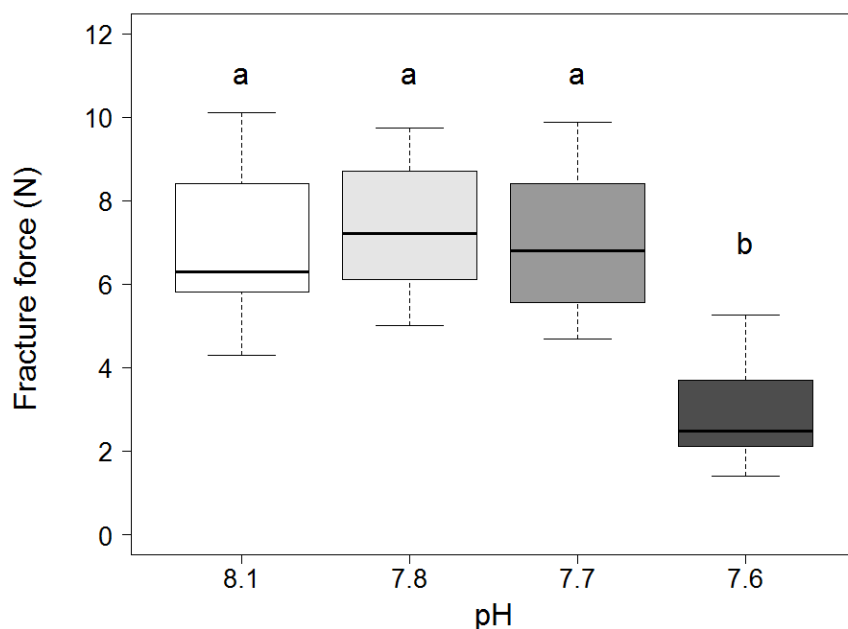
373 Measurements along the shell section showed a progressive decrease of total shell thickness  
 374 from older to newly formed shell regions (results not shown), which is in accordance with the  
 375 shell growth process. Total shell thickness tended to be smaller in juveniles exposed to pH 7.6  
 376 compared with those reared under control pH<sub>T</sub> (unpaired t test,  $t_8 = -2.1644$ ,  $P = 0.062$ , Fig.  
 377 7a). However, a significant reduction of the spherulitic layer thickness was observed at pH<sub>T</sub>  
 378 7.6 compared with the control group (unpaired t test,  $t_8 = -2.8522$ ,  $P = 0.021$ , Fig. 7b). There  
 379 was no significant differences in nacre shell thickness between abalones grown in these two  
 380 pH treatments (unpaired t test,  $t_8 = -0.4381$ ,  $P = 0.67$ ).



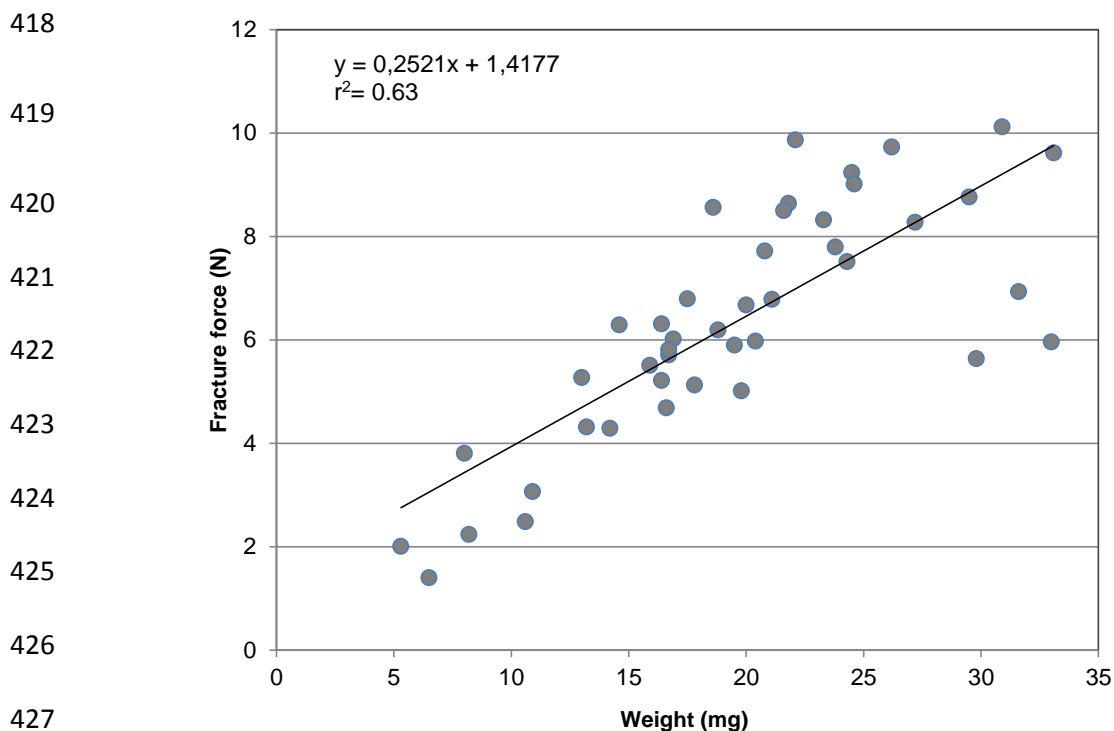
386 **Fig. 7** Total thickness (A) and spherulitic layer thickness (B) of juvenile abalone shells grown  
 387 under control pH<sub>T</sub> (8.1) and low pH<sub>T</sub> (7.6) treatment. Centre lines of box plots show the  
 388 medians; box limits indicate the first and third quartiles, respectively, with whiskers  
 389 encompassing data within 1.5 times the spread from the median (n = 5 per pH condition).  
 390 Asterisk indicates a significant difference between pH treatments (unpaired Student's t-test,  $P$   
 391  $< 0.05$ ).

392 The shell fracture force was significantly lowered at pH<sub>T</sub> 7.6 compared with shells that had  
 393 grown in the three other pH treatments (ANOVA,  $F(3,7) = 11.15$ ,  $P = 0.0047$ , Fig. 8, Table  
 394 3). However, because shell weight (and thickness) was reduced at low pH, the fracture force  
 395 was analysed according to weight. The regression of shell fracture force on abalone shell

396 weight showed a linear relationship (Linear regression,  $r^2 = 0.63$ ,  $F(1,41) = 68.42$ ,  $P = 0.031$ ,  
 397 Fig. 9). A test of homogeneity of slopes of the regression lines of shell fracture force vs  
 398 weight showed that these were not significantly different between pH treatments (interaction  
 399 pH \* weight,  $F(3,34) = 0.486$ ,  $P = 0.69$ , Table 4). Testing on the intercept with shell weight as  
 400 a co-variable, showed that the effect of pH on shell fracture force was not significant  
 401 (ANCOVA,  $F(3,7) = 1.021$ ,  $P = 0.44$ ). This indicates that the decreased fracture force at pH<sub>T</sub>  
 402 7.6 could be explained by the reduced amount of shell material only.



413 **Fig. 8** Fracture force of the shells of abalones reared under different pH conditions. Centre  
 414 lines of box plots show the medians; box limits indicate the first and third quartiles,  
 415 respectively, with whiskers encompassing data within 1.5 times the spread from the median (n  
 416 = 12 for pH<sub>T</sub> 8.1 and 7.8; n = 11 for pH<sub>T</sub> 7.7; n = 7 for pH<sub>T</sub> 7.6). Different letters indicate  
 417 significant differences between pH treatments ( $P < 0.05$ ).



428 **Fig. 9** Linear regression of shell fracture force against abalone shell weight (n = 43)

429 ( $r^2 = 0.63$ ,  $F(1,41) = 68.42$ ,  $P = 0.031$ ).

430

## 431 Discussion

432

433 The present study demonstrated that decreased pH negatively impacts the growth and shell  
 434 mineralization of juvenile European abalone *Haliotis tuberculata*. Almost all the tested  
 435 variables were significantly reduced at pH<sub>T</sub> 7.6 (0.5 units below control pH) while pH<sub>T</sub> 7.7  
 436 and 7.8 did not affect juvenile growth. These results are rather similar to those obtained in two  
 437 other species of farmed abalone. In juvenile *H. iris*, Cunningham et al. (2016) reported  
 438 significant effects on shell length and wet weight at pH<sub>NBS</sub> 7.6 in winter (corresponding to  
 439 pH<sub>T</sub> 7.5) and at pH<sub>NBS</sub> 7.8 (pH<sub>T</sub> 7.7; 0.3 units below control pH) and 7.6 (pH<sub>T</sub> 7.5; 0.5 units  
 440 below control pH) in summer. Similarly, shell growth and shell weight were significantly  
 441 lower in juvenile *H. discus hannai* after 3 months of exposure to pH<sub>NBS</sub> 7.7 and 7.9  
 442 (corresponding respectively to pH<sub>T</sub> 7.8 and 7.6, Li et al. 2018). These results are also in

443 accordance with previous studies on other marine shelled mollusc taxa, showing significant  
444 reductions in shell growth following medium to long term exposure to near-future pH  
445 (reviewed in Gazeau et al. 2013). In a two-month experiment, Thomsen and Melzner (2010)  
446 observed significant decreases in both shell mass and shell length in blue mussels exposed to  
447 lowered pH (- 0.3 to - 0.9 units). Similarly, the mussel *Mytilus galloprovincialis* exhibited a  
448 slow-down of shell growth when pH was reduced by 0.75 units, possibly caused by  
449 dissolution (Michaelidis et al. 2005). As suggested in previous studies, the effects of OA on  
450 shell growth and calcification would result either from a direct effect on CaCO<sub>3</sub> dissolution or  
451 from indirect metabolic effects, such as physiological and molecular processes regulating  
452 shell biomineralization (Beniash et al. 2010; Hüning et al. 2012; Klok et al. 2014). The  
453 increasing cost of maintaining acid-base balance combined with an impaired ability to calcify  
454 under OA may explain the decrease in the scope for growth among early life stages of  
455 molluscs experiencing acidified conditions (Gazeau et al. 2013; Parker et al. 2013; Thomsen  
456 et al. 2015). However, Cunningham et al. (2016) found no changes in respiration rate of *H.*  
457 *iris* juveniles subjected to reduced pH (- 0.3 to - 0.5 units), indicating that abalone do not up-  
458 regulate their metabolism. Nothing is known on the acid-base balance regulation abilities of  
459 abalones but molluscs (apart from cephalopods) are usually considered to have a poor ability  
460 to compensate for decreasing haemolymph pH in situations of seawater acidification (Melzner  
461 et al. 2009). According to Cyronak et al. (2016), elevated H<sup>+</sup> concentration and subsequent  
462 problems of homeostasis rather than carbonate ions concentration, would be more likely  
463 responsible for the reduction of calcification in marine organisms facing OA.

464 Our results revealed significant changes in shell microstructure of juvenile abalones grown  
465 at pH<sub>T</sub> 7.6, including corrosion of the outer periostracum and degradation of the inner  
466 nacreous aragonite layer. In juvenile *H. discus hannai*, corroded periostracum was found in  
467 individuals grown at pH<sub>NBS</sub> 7.7 (Li et al. 2018), but the effect of decreased pH on shell  
468 microstructure was not investigated. Shell surface corrosion and changes in biomineral

469 microstructure under low pH have been previously reported in juvenile bivalves and related to  
470 a direct effect on shell dissolution (Fitzer et al. 2014b; Hiebenthal et al. 2013; McClintock et  
471 al. 2009; Melzner et al. 2011; Waldbusser et al. 2011). It is noteworthy that we only recorded  
472 significant effects at  $\text{pH}_T$  7.6, the only treatment at which  $\Omega_{\text{aragonite}}$  was lower than one. The  
473 corrosion of the periostracum and degradation of the nacreous layer were observed both in the  
474 old part and in the newly formed shell under the low pH, indicating that direct dissolution is  
475 involved rather than indirect metabolic process affecting the synthesis of the new shell.  
476 However, the energetic cost for proton elimination during  $\text{CaCO}_3$  precipitation is increased  
477 under elevated  $\text{pCO}_2$ , suggesting a metabolic effect.

478 This finding also supports the hypothesis that the effects on shell length and weight may be  
479 mainly due to the shell dissolution. Indeed, the juvenile and adult shells of *H. tuberculata*  
480 consist of two biomineralized layers, the outer spherulitic and the inner nacreous layers, both  
481 composed of the aragonite  $\text{CaCO}_3$  polymorph (Auzoux-Bordenave et al. 2010, 2015; Jardillier  
482 et al. 2008). The predominance of aragonite in the *H. tuberculata* shell makes the species  
483 more susceptible to dissolution than other mollusc species with calcitic shells or a  
484 calcite/aragonite mixture (Gazeau et al. 2013; Parker et al. 2013). In the course of a trans-  
485 generational experiment on juvenile *M. edulis* exposed to elevated  $\text{pCO}_2$ , Fitzer et al. (2014a)  
486 revealed that mussels at high  $\text{pCO}_2$  (1000 $\mu\text{atm}$ ) did not produce aragonite, which is more  
487 vulnerable to carbonate under-saturation than calcite. Under lower  $\text{pCO}_2$  (550, 750  $\mu\text{atm}$ ), the  
488 nacreous tablets formed the usual ‘brick wall’ structure, although the nacre crystals appeared  
489 corroded and were not so closely stacked together as under normal conditions (Fitzer et al.  
490 2014a). This could indicate reduced biological control over biomineralization of aragonite by  
491 the organism and potentially raises questions about the integrity of mollusc shells composed  
492 only of aragonite under OA.

493 In addition to changes in shell microstructure, we found a significant reduction in the  
494 spherulitic shell thickness and, to a lesser extent, in the total thickness of abalone shells grown



495 at  $\text{pH}_T$  7.6. The reduction of shell thickness is likely due to  $\text{CaCO}_3$  dissolution of the outer  
496 aragonite layer, which would be more sensitive to OA after periostracum corrosion. In  
497 juvenile shells of *M. edulis*, a reduction in shell thickness was reported in the aragonite layer,  
498 but not in the calcite layer, after 6 months of exposure to elevated  $\text{pCO}_2$  (Fitzer et al. 2014b).  
499 Other juvenile bivalves (*Argopecten irradians* and *Mercenaria mercenaria*) grown under  
500 increased  $\text{CO}_2$  concentration (corresponding to a reduction of 0.4 to 0.6 pH units), also  
501 exhibited malformed and corroded shells with reduced thickness (Talmage and Gobler 2010).  
502 Welladsen et al. (2010) also reported a significant decrease in shell strength and nacre  
503 malformation in the adult pearl oyster *Pinctada fucata* exposed to acidified seawater ( $\text{pH}_{\text{NBS}}$   
504 7.6), suggesting shell dissolution.

505 The integrity of the periostracum (the organic coating of the mollusc shell) is very  
506 important for protecting the shell from corrosion induced by low pH (Thomsen et al. 2010). In  
507 juvenile abalone, we found that the periostracum was damaged under low pH, resulting in a  
508 higher vulnerability of the underlying mineralized layers. Indeed, a subsequent reduction in  
509 thickness of the spherulitic layer as well as changes in the nacreous layer microstructure were  
510 observed. These observations are consistent with those reported for other shellfish reared  
511 under lowered pH (Talmage and Gobler 2010). All of this suggests that processes leading to  
512 the synthesis of the periostracum are affected by low pH, implying that the kinetics of  
513 aragonite precipitation (evidenced by the reduced growth rate) and the thermodynamics of its  
514 dissolution (due to under saturated sea water in the  $\text{pH}_T$  7.6 treatment) are not the only aspects  
515 of the shell being affected. Potential effects of decreased pH on shell organic components  
516 (chitin and matrix proteins) may explain the corrosion of the periostracum and some of the  
517 changes observed in the nacre microstructure, but the underlying mechanisms are far from  
518 being understood (Fitzer et al. 2014b; Hüning et al. 2012). A number of indirect biological  
519 processes involved in shell calcification, such as matrix protein production, chitin synthesis  
520 and enzymatic control are influenced by changes in seawater  $\text{pCO}_2$  (Weiss et al. 2013). In the

521 mussel *M. edulis*, six months of incubation at 750  $\mu\text{atm}$   $\text{pCO}_2$  ( $\text{pH}_T$  7.5) significantly reduced  
522 carbonic anhydrase activity within the mantle tissue, explaining shell growth reduction (Fitzer  
523 et al. 2014b). In another study of *M. edulis*, a strong depression in the expression of mRNA  
524 for a chitinase, an enzyme involved in the calcification process, was correlated with a linear  
525 decrease in shell growth (Hüning et al. 2012). Interestingly, this study also found that  
526 expression of several genes, including some genes involved in shell protection and/or  
527 periostracum formation (tyrosinase) increased in response to elevated  $\text{pCO}_2$  (Hüning et al.  
528 2012). By contrast, despite evidence of shell dissolution, no difference was found in the  
529 organic content or periostracum integrity of adult *P. fucata* shells exposed to  $\text{pH}_{\text{NBS}}$  7.6  
530 (Welladsen et al. 2010). However, the authors noted that because the study was conducted  
531 over only a 28-day period, it did not allow acclimation of the oysters to the acidified  
532 conditions.

533 In juvenile *H. tuberculata*, the significant reduction in shell fracture force at  $\text{pH}_T$  7.6 can be  
534 explained by the reduced shell weight. This probably results from both reduced growth and  
535 shell dissolution. A smaller (isometric) dome is submitted to a higher meridional compressive  
536 stress for the same external force applied and will therefore rupture at a lower applied force  
537 (Vogel 2003). Shell dissolution will induce an easier induction of cracks and cleavage, probably  
538 initiated by dissolution pits inducing stress concentration at their level (Mc Neill 1968). Both  
539 effects would increase the period during which abalone are exposed to strong predation pressure  
540 before they reach a refuge in size and shell strength. This would have an economic impact on  
541 abalone aquaculture as it would increase the time required to reach a marketable size and  
542 possibly enhance productivity losses through shell breakage during handling.

543 Marine molluscs exert a strong control on the calcification process, but their capacities to  
544 maintain optimal conditions at the site of calcification when facing OA stress remain largely  
545 unknown (Gazeau et al. 2013; Parker et al. 2013). Because shell calcification has a high  
546 energetic cost and is very sensitive to OA (especially in early stages), energy usually allocated

547 to growth and reproduction might be partially reallocated to calcification (Thomsen and  
548 Melzner 2010). The capacity of abalone to grow in the near future will depend on their  
549 potential to maintain their vital functions (reproduction, growth and biomineralization) under  
550 modified environmental conditions. Since responses to OA may also differ according to  
551 differences in physiology, habitat and behaviour of the species (Gazeau et al. 2013), the local  
552 seasonal variations in physico-chemical conditions should be considered in further studies on  
553 adaptive responses. The seawater  $\text{pH}_T$  along the Brittany coast in France naturally varies from  
554 7.9 to 8.2 (Qui-Minet et al. 2018), so that a future scenario testing a decrease of - 0.3 units  
555 from ambient  $\text{pH}_T$  is consistent with seasonal or diurnal variations presently experienced by  
556 abalone in the tidal zone. Indeed, seasonal variations in pH are close to 0.3 units in shallow  
557 subtidal environments (Qui-Minet et al. 2018), while diurnal variations of  $\text{pH}_T$  in the intertidal  
558 zone can be higher. For instance, pH can fall down to  $\text{pH}_T$  7.5 in rock pools along the Brittany  
559 coast during night-time emersion at low tide (Legrand et al. 2018). Thus, abalone from  
560 Brittany shoreline environments might already experience chronically pH similar to global  
561 average pH levels predicted for open ocean by 2100 (ie.  $\text{pH}_T$  7.7). Such fluctuations of pH in  
562 their coastal habitats might explain the potential resistance of abalone to  $\text{pH}_T > 7.6$ .

563 The results of our study indicate that a decrease of 0.5 pH units significantly reduces  
564 growth, shell calcification and shell strength of juvenile abalone *H. tuberculata*. In the natural  
565 environment, where juvenile stages are exposed to a strong predation pressure, abalone may  
566 be at greater risk under future pH conditions as their shells may not offer sufficient protection  
567 from predators and other external stressors. Understanding how different abalone life stages  
568 respond to OA will make it possible to identify bottlenecks for population persistence under  
569 near-future pH conditions. These findings also highlight the importance of monitoring  
570 seawater parameters in aquaculture systems, where effort can be directed to maintaining  
571 seawater pH at key periods of abalone culture to minimize stress and prevent production  
572 losses.

## 573 **Acknowledgements**

574

575 N. Wessel was supported by a postdoctoral fellowship from the MNHN (*Ministère de*  
576 *l'Enseignement Supérieur et de la Recherche*, Paris, France). This work was supported in part  
577 by the ATM program “Biom mineralization” of the MNHN funded by the *Ministère délégué à*  
578 *l'Enseignement Supérieur et à la Recherche* (Paris, France) and by the ICOBio project under  
579 the program “*Acidification des Océans*” funded by the Fondation pour la Recherche sur la  
580 Biodiversité (FRB) and the *Ministère de la Transition Ecologique et Solidaire* (MTES). We  
581 thank Dr. Chakib Djejat and Stéphane Formosa for their assistance in scanning electron  
582 microscopy (SEM, *Plateau technique de Microscopie Electronique*, MNHN, Paris and  
583 Concarneau, France). The Regional Council of Brittany, the General Council of Finistère, the  
584 urban community of *Concarneau Cornouaille Agglomération* and the European Regional  
585 Development Fund (ERDF) are acknowledged for the funding of the Sigma 300 FE-SEM of  
586 the Concarneau Marine Station. We thank Dr. Cedric Hubas for his valuable support for the  
587 statistical analyses and the Translation Bureau of the University of Western Brittany for  
588 improving the English of this manuscript. We also thank the 3 anonymous reviewers for their  
589 comments which have helped to improve this manuscript. Ph. Dubois is a Research Director  
590 of the National Fund for Scientific Research (Belgium).

591

## 592 **Compliance with ethical standards**

593 The authors declare that they have no conflicts of interest or competing financial interests.

594 The experiments complied with the current French laws. All applicable international, national,  
595 and institutional guidelines for the care and use of animals were followed.

596

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**Table 1** Parameters of seawater carbonate chemistry during the experiment (means  $\pm$  SD). pH on the total scale ( $\text{pH}_T$ ), temperature ( $^{\circ}\text{C}$ ), salinity and total alkalinity ( $\mu\text{Eq.kg}^{-1}$ ) were used to calculate  $\text{CO}_2$  partial pressure ( $\text{pCO}_2$ ;  $\mu\text{atm}$ ), Dissolved Inorganic Carbon (DIC;  $\mu\text{mol.kg}^{-1}$ ),  $\text{HCO}_3^-$ ,  $\text{CO}_3^{2-}$ , aragonite saturation state ( $\Omega_{\text{aragonite}}$ ) and calcite saturation state ( $\Omega_{\text{calcite}}$ ) by using the CO2SYS program.  $\text{pH}_T$  and temperature are the average values recorded almost daily throughout the experiment ( $n = 76$  per pH treatment). Salinity was measured twice a week (mean  $35.3 \pm 1.3$ ,  $n = 26$ ) and total alkalinity was measured every two weeks throughout the experiment ( $n = 8$  per pH treatment).

Nominal pH	$\text{pH}_T$	Temperature ( $^{\circ}\text{C}$ )	TA ( $\mu\text{Eq.kg}^{-1}$ )	$\text{pCO}_2$ ( $\mu\text{atm}$ )	DIC ( $\mu\text{mol.kg}^{-1}$ )	$\text{HCO}_3^-$ ( $\mu\text{mol.kg}^{-1}$ )	$\text{CO}_3^{2-}$ ( $\mu\text{mol.kg}^{-1}$ )	$\Omega_{\text{aragonite}}$	$\Omega_{\text{calcite}}$
8.1	$8.11 \pm 0.04$	$12.4 \pm 1.8$	$2305 \pm 28$	$335 \pm 33$	$2070 \pm 22$	$1890 \pm 35$	$167 \pm 14$	$2.53 \pm 0.22$	$3.96 \pm 0.34$
7.8	$7.81 \pm 0.05$	$12.1 \pm 1.7$	$2312 \pm 30$	$750 \pm 18$	$2209 \pm 18$	$2088 \pm 24$	$90 \pm 10$	$1.37 \pm 0.15$	$2.14 \pm 0.23$
7.7	$7.71 \pm 0.04$	$12.3 \pm 1.7$	$2308 \pm 37$	$958 \pm 108$	$2237 \pm 17$	$2124 \pm 20$	$74 \pm 8$	$1.12 \pm 0.13$	$1.75 \pm 0.2$
7.6	$7.60 \pm 0.05$	$12.1 \pm 1.8$	$2309 \pm 34$	$1259 \pm 140$	$2274 \pm 16$	$2164 \pm 17$	$58 \pm 7$	$0.88 \pm 0.1$	$1.38 \pm 0.16$



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