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## Responses of early life stages of European abalone (*Haliotis tuberculata*) to ocean acidification after parental conditioning: Insights from a transgenerational experiment

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### Abstract :

CO<sub>2</sub> absorption is leading to ocean acidification (OA), which is a matter of major concern for marine calcifying species. This study investigated the effects of simulated OA on the reproduction of European abalone *Haliotis tuberculata* and the survival of its offspring. Four-year-old abalone were exposed during reproductive season to two relevant OA scenarios, ambient pH (8.0) and low pH (7.7). After five months of exposure, abalone were induced to spawn. The gametes, larvae and juveniles were then exposed for five months to the same pH conditions as their parents. Several biological parameters involved in adult reproduction as well as in larval, post-larval and juvenile fitness were measured. No effects on gametes, fertilisation or larval oxidative stress response were detected. However, developmental abnormalities and significant decreases in shell length and calcification were observed at veliger stages. The expression profile of a GABA A receptor-like gene appeared to be regulated by pH, depending on larval stage. Larval and post-larval survival was not affected by low pH. However, a lower survival and a reduction of growth were recorded in juveniles at pH 7.7. Our results confirm that OA negatively impacts larval and juvenile fitness and suggest the absence of carry-over effects on abalone offspring. This may compromise the survival of abalone populations in the near future.

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## Highlights

► Abalone has experienced severe population decline worldwide due to overfishing, disease and climate change. ► OA effects were evaluated on reproduction and early life stages of *H. tuberculata* through a transgenerational experiment. ► No carry-over effects were observed on abalone offspring following parental exposure to OA. ► Larval and juvenile fitness were affected by a pH decrease of 0.3 unit. ► Species dispersion and survival may be compromised in the near future, with potential negative consequences for European abalone populations.

**Keywords:** European abalone, *Haliotis tuberculata*, ocean acidification, carry-over effects, developmental abnormality, shell growth, calcification, gene expression

## 1 **1. Introduction**

2 The absorption of anthropogenic carbon dioxide emissions by seawater is responsible for a  
3 decrease in the pH of the global ocean, a process known as ocean acidification (Doney et al.,  
4 2009; Gattuso et al., 2015; IPCC, 2014). Current projections suggest that, by the year 2100,  
5 seawater pH will have decreased by 0.1 to 0.3 units, threatening marine calcifiers such as corals,  
6 molluscs and echinoderms (Hendricks et al., 2010; Hofmann et al., 2010; Kroeker et al., 2013;  
7 Widdicombe and Spicer, 2008; Wittmann and Pörtner, 2013). Molluscs are among the  
8 invertebrates considered the most vulnerable to OA, with pronounced sensitivity at larval and  
9 juvenile stages (Beniash et al., 2010; Gazeau et al., 2013; Melzner et al., 2009; Orr et al., 2005;  
10 Przeslawski et al., 2015; Ross et al., 2011). In marine shelled molluscs, OA has been shown to  
11 reduce larval survival, lengthen development time, alter morphology and/or impair shell  
12 formation and calcification (Byrne et al., 2011; Byrne and Fitzner, 2019; Duquette et al., 2017;  
13 Ellis et al., 2009; Fitzner et al., 2014; Gazeau et al., 2010; Kurihara 2008; Noisette et al., 2014).  
14 Since many mollusc species are sources of commercially important foods, the negative impacts  
15 of OA may also result in significant economic losses (Ekstrom et al., 2015; Gazeau et al., 2007).

16 Abalone are ecologically and economically important shelled gastropods, which are grazers  
17 in the marine ecosystem and a delicacy for human consumers (Cook, 2016; Huchette and Clavier  
18 2004). Many abalone species worldwide have experienced severe population decline due to  
19 overfishing, disease and environmental perturbations such as global warming (Cook, 2016;  
20 Nicolas et al., 2002; Travers et al., 2009). Understanding the effects of climate change on abalone  
21 physiology is an important issue for the management of abalone populations in natural and  
22 aquaculture environments (Aalto et al., 2020; Morash and Alter, 2015).

23 The European abalone *Haliotis tuberculata* (Linnaeus, 1758) is a commercially important  
24 species in Europe, for which rearing over the whole life cycle is controlled in aquaculture  
25 (Courtois de Viçose et al., 2007). The impacts of OA on several stages of *H. tuberculata* have  
26 been well examined by experimental studies (Auzoux-Bordenave et al., 2020; Avignon et al.,

27 2020; Wessel et al. 2018). As with most marine molluscs, abalone have a pelago-benthic life  
28 cycle with a larval planktonic stage followed by a critical metamorphosis into benthic juveniles,  
29 making them highly sensitive to environmental changes (Byrne et al., 2011). Several studies have  
30 also focused on early life stages of abalone, especially larvae, demonstrating adverse effects of  
31 elevated CO<sub>2</sub>, such as reduced survival, developmental delay, body and shell abnormalities and  
32 reduced mineralization (Byrne et al., 2011; Crim et al., 2011; Guo et al., 2015; Kimura et al.,  
33 2011; Onitsuka et al., 2018; Wessel et al., 2018; Zippay and Hofmann 2010). More recently,  
34 Kavousi et al. (2021) investigated the combined effects of OA and ocean warming on *H.*  
35 *tuberculata* larvae in a full factorial experiment. Despite no interaction between elevated  
36 temperature and low pH being observed, the results provided additional evidence for the  
37 sensitivity of abalone larvae to OA (Kavousi et al., 2021). Only one study has yet reared abalone  
38 embryos in acidified conditions from the fertilisation stage (Byrne et al., 2011); this demonstrated  
39 that resulting larvae were more severely affected by OA than those exposed at a later stage. In  
40 all the above-mentioned papers, experiments were carried out on a single life-history stage using  
41 short-term or acute exposure to pH decrease, that provided information on the vulnerabilities of  
42 embryos and larvae (Parker et al., 2015; Ross et al., 2016). More recently, a multigenerational  
43 experiment conducted on the red abalone *Haliotis rufescens* (Swezey et al., 2020) demonstrated  
44 that larval mortality under OA was strongly correlated with differences in lipid provisioning and  
45 metabolism. The differences were linked to population-specific variation in the maternal  
46 provisioning of lipids with a positive correlation between lipid concentrations and survival under  
47 lower pH. This relationship also persisted in experiments on second-generation animals (Swezey  
48 et al., 2020), confirming the importance of parental exposure when studying the effects of OA.

49 To better understand the effects of OA on abalone reproduction and the survival of its  
50 offspring, we carried out a 10-month transgenerational experiment exposing adult abalone to two  
51 climatically relevant OA scenarios. Four-year-old abalone *H. tuberculata* were exposed during  
52 reproductive season to ambient seawater pH (8.0) or to a lower pH value (7.7) corresponding to  
53 the decrease of -0.3 pH units expected for the end of the century (RCP 8.5 climate change

54 scenario, Gattuso *et al.*, 2015; IPCC, 2014). The offspring resulting from the reproduction of  
55 these adults were then kept in the same conditions and studied until the age of 5.5 months. Several  
56 biological parameters involved in adult reproduction (gamete evaluation and fertilisation rate) as  
57 well as larval, post-larval and juvenile fitness (survival, growth, calcification) were measured  
58 throughout the experimental period. To our knowledge, this is the first study that examines the  
59 acclimation potential of European abalone to simulated OA, considering carry-over effects being  
60 transmitted from adults to their offspring.

61

## 62 **2. Material and Methods**

### 63 **2.1. Abalone collection and conditioning**

64 Adult *H. tuberculata* ( $n = 260$ ,  $48.5 \pm 4.2$  mm shell length) were selected at random from an  
65 offshore sea-cage structure at the France Haliotis abalone farm ( $48^{\circ}36'50\text{N}$ ,  $4^{\circ}36'3\text{W}$ ;  
66 Plouguerneau, Brittany, France) in January 2017, as previously described in Avignon *et al.*  
67 (2020). These abalone were distributed without any selection among ten 45-L open-circuit aquaria  
68 ( $n = 26$  abalone per aquarium) supplied with a minimum of 15 L/h of 3  $\mu\text{m}$  filtered seawater at  
69 ambient temperature. They were conditioned for three weeks in the laboratory under ambient  
70  $\text{pCO}_2/\text{pH}$  conditions and fed *ad libitum* with the macroalgae *Palmaria palmata*.

71

### 72 **2.2. Experimental set-up**

73 The experimental system used to test the two pH treatments (ambient pH 8.0 and reduced pH  
74 7.7) on adult abalone was previously described in detail in Avignon *et al.* (2020). Briefly, each  
75 experimental aquarium ( $n = 10$ ) was supplied from a separate header tank where  $\text{pCO}_2$  was  
76 adjusted by bubbling  $\text{CO}_2$  (Air liquid, France) through electro-valves controlled by a pH-stat  
77 system (IKS Aquastar, Germany). Five replicate aquaria, were used per pH condition. The adult  
78 experiment was conducted for five months between January and June 2017, during reproductive  
79 conditioning.

80 The maturity of the broodstock was checked during routine hatchery operations before  
81 induction of spawning. After four months of exposure, 10 abalone per pH treatment were  
82 sacrificed for the assessment of gonad maturity (Avignon et al. 2020). Experimental animals  
83 were maintained for one additional month before spawning induction. At maturity (i.e. after five  
84 months of exposure to pH treatments), adult abalone were induced to spawn using a temperature  
85 shock of +4°C and UV-irradiated seawater. For ambient pH, 18 males and 19 females were used;  
86 for reduced pH, 18 males and 16 females were used (n = 3–4 abalone of each sex per aquarium).  
87 Abalone were placed individually in 1 L buckets with continuous water renewal at ambient pH  
88 and allowed a maximum of 5:30 h from the start of the spawning induction in which to spawn.  
89 During spawning induction, the pH was not adjusted,  
90 due to the small volume of the buckets and to minimise the stress for abalone during spawning.  
91 An experimenter continuously observed spawning behaviour to spot the onset of gamete  
92 emission. Once the spawning started, the water renewal was halted to avoid gamete loss. When  
93 they had finished spawning, the adults were removed from the buckets. The water containing the  
94 gametes was gently stirred to ensure homogeneous distribution through the water column. Two  
95 replicate samples of 0.4 ml were taken to estimate the number of gametes released by females  
96 and males and to determine spermatozoon morphology. Oocytes were fixed in 70% ethanol and  
97 spermatozoa in 2% glutaraldehyde. Furthermore, for analysis of spermatozoon movement, a  
98 sample of 0.5 ml of male gametes was taken, placed in a microcentrifuge tube and transferred to  
99 the Argenton laboratory within 45 min. Male gametes of abalone are known to be resistant to  
100 transport, even without oxygenation (Pereira et al., 2007).

101 A full factorial fertilisation was carried out per aquarium. Gametes of each female were  
102 transferred to 5-L buckets (two to four buckets of gametes per female depending on the number  
103 of males from the same aquarium that spawned), which were topped up with seawater at pH 8.0  
104 or pH 7.7 once the adults stopped spawning. Spermatozoa of each male were added to the buckets  
105 containing the oocytes of the females from the same aquarium. To avoid spermatic competition,

106 this was done at an optimal ratio of approximately 100 000 spermatozoa / oocyte (Huchette et  
107 al., 2004). Because oocytes are fragile, a maximum of 30 min was allowed between spawning  
108 and fertilisation. Two minutes after fertilisation, three aliquots of 0.5 ml were taken per family  
109 after gentle homogenisation of the bucket and placed in 12-well plates to study the percentage of  
110 fertilisation per female. Ethanol (final concentration 70%) was added to the well after two hours  
111 to block egg development until later analysis. Two hours after fertilisation, the 5-L egg buckets  
112 of females from the same aquarium were pooled and placed in food-safe polystyrene boxes,  
113 containing seawater of appropriate pH, until the eggs reached the hatching stage at about 18 hours  
114 post fertilisation (hpf). To avoid flushing away any eggs, no water renewal was done during this  
115 period. The pH was registered in the hatching boxes for the two pH treatments (n= 5 per pH  
116 treatment). At 18 hpf, all swimming larvae were transferred through a pipe to 350-L larval tanks  
117 supplied with filtered seawater (n = 5 tanks per pH treatment). Samples of larvae were taken from  
118 the tanks at three key stages: (1) the trochophore stage (20 hpf), which is characterised by the  
119 formation of the larval shell, to evaluate the initial number of swimming larvae; (2) the veliger  
120 stage (48 hpf); and (3) the pre-metamorphic veliger stage (96 hpf), which is the last pelagic life  
121 stage before larval settlement (Auzoux-Bordenave et al., 2010; Jardillier et al., 2008). At each  
122 key stage, three 10-mL replicates were sampled per tank to measure the total number of  
123 swimming larvae in each. In addition, 10–15 L of seawater were collected from the tank water  
124 columns to study larval development and birefringence of larval shell. Collection of swimming  
125 larvae in the water column allowed us to sample only live larvae from the tank. The larval  
126 samples were then filtered through a 40- $\mu$ m sieve and aliquoted into 15-mL tubes. Larvae were  
127 concentrated at the bottom of each tube by adding a few drops of 70% ethanol. The samples were  
128 then fixed and stored in 70% ethanol until analysis.

129 Since abalone larvae are lecithotrophic, they were not fed during the experiment. At the end  
130 of the planktonic phase (96 hpf), when the veliger larvae reached the pre-metamorphic stage, all  
131 swimming larvae were collected from the 350 L tanks using a syphon and a 40- $\mu$ m sieve and



132 placed in buckets containing 5 L of seawater adjusted to the same pH as in their respective  
133 treatments. The larval density at 96 hpf was calculated from eight 1-mL replicate samples per  
134 bucket. After density evaluation, a total of 10 000 larvae was collected using a pipette and gently  
135 poured into ten 45-L aquaria. These larvae were allowed to settle on 10 plastic plates covered  
136 with the green alga *Ulvela lens*, which is a major inductive cue for the settlement of abalone  
137 larvae (Courtois de Viçose et al. 2012; Daume et al., 2004). Post-larvae were sampled at 5 and 9  
138 days after larval fixation on the plates and juveniles were collected at the mid-term (2.5 mo) and  
139 end of the experiment (5.5 mo). By using this overall procedure, the juveniles sampled in one  
140 aquarium corresponded to the offspring of the abalone of the same treatment conditioned in the  
141 same aquarium.

142

### 143 **2.3. pH and carbonate parameters**

144 The desired low pH value was obtained by adjusting the pH to be 0.3 units lower than the  
145 ambient pH value, which was characterised by natural pH fluctuations reported for coastal  
146 seawater of northern Brittany (pH<sub>T</sub> range of 7.9–8.2; Qui-Minet et al., 2018). The pH-stat system  
147 values were adjusted from daily electromotive force measurements in the header tanks using a  
148 pH meter (Metrohm 826 pH mobile, Metrohm AG, Herisau, Switzerland) with a glass electrode  
149 (Metrohm, Primatrode, Switzerland). The electromotive force values were converted to pH units  
150 on the total scale (pH<sub>T</sub>) after calibration with Tris-HCl and 2-aminopyridine-HCl (AMP) buffers  
151 (Dickson et al., 2007). Temperature and salinity were measured daily using a portable  
152 conductivity meter (ProfiLine Cond 3110, WTW, Oberbayern, Germany).

153 Total alkalinity (AT) was measured monthly on 50-mL samples taken from each experimental  
154 aquarium (n = 5 per pH treatment). Seawater samples were filtered through 0.7-µm Whatman  
155 GF/F membranes, immediately poisoned with mercury chloride, and stored in a dark place at  
156 room temperature for later analysis. Values of AT were then determined from approximately 50  
157 g of weighed samples using a potentiometric titration at 25°C with 0.1 M HCl and an automatic

158 titrator (Titrimo 847 plus, Metrohm). The balance point was determined by the Gran method  
159 (Gran, 1952), according to Haraldsson et al. (1997). The accuracy of this method was  $\pm 2$   
160  $\mu\text{mol}\cdot\text{kg}^{-1}$  and was verified using Certified Reference Material 182, provided by A. Dickson  
161 (Scripps Institute of Oceanography, University of South California, San Diego, United States).

162 The seawater carbonate chemistry analysis included dissolved carbonate ( $\text{CO}_3^{2-}$ ), bicarbonate  
163 ( $\text{HCO}_3^-$ ), dissolved inorganic carbon (DIC),  $\text{pCO}_2$ , aragonite saturation state ( $\Omega_{\text{aragonite}}$ ) and  
164 calcite saturation state ( $\Omega_{\text{calcite}}$ ). These values were determined by entering the values of  $\text{pH}_T$ , AT,  
165 temperature and salinity into  $\text{CO}_2\text{SYS}$  software (Pierrot et al., 2006) using constants from  
166 Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

167 The effects of reduced pH were examined at different sampling times corresponding to the  
168 different life stages of abalone: gametes, eggs, larvae, post-larvae and juveniles. Several  
169 biological parameters were measured at each life stage and compared between the pH treatments.

170

#### 171 ***2.4. Number of gametes released and spermatozoon shape***

172 Oocytes were counted under a photonic microscope (Zoom 2000, Leica, Germany). For the  
173 spermatozoon samples, SYBR Green fluorescent dye (4  $\mu\text{l}$ , Molecular Probes,  $10^{-3}$  dilution of  
174 the commercial stock solution) was added before flow cytometry (Guava EasyCyte Plus  
175 equipped with a 488-nm laser). Data were analysed using the Guava ExpressPro program. The  
176 total number of spermatozoa was estimated as was their volume (Forward Scatter, FSC) and  
177 complexity (Side Scatter, SSC). Duplicates were averaged per male ( $n = 12$  males for pH 7.7;  $n$   
178  $= 15$  males for pH 8.0) and per female ( $n = 14$  females for each pH level). Fecundity was  
179 calculated as the number of oocytes and spermatozoa released per gram of abalone (wet weight)  
180 that spawned (Bilbao et al., 2010).

181

182

### 183 **2.5. Spermatozoon motility**

184 Aliquots of 5  $\mu\text{L}$  of spermatozoon suspension were diluted in 500  $\mu\text{L}$  of activating solution  
185 (1- $\mu\text{m}$  fresh seawater, 0.3% pluronic acid, 15 mM Tris, pH 8.0., Boulais et al., 2018). Then, 12  
186  $\mu\text{L}$  of this diluted suspension were transferred to a Fast-Read 102 cell (Biosigma, Italy), and  
187 spermatozoon movement was triggered under a microscope (Olympus BX51, X200  
188 magnification) connected to a camera (Qicam Fsr 1394) as described in Boulais et al. (2015).  
189 Two aliquots per male were analysed, with an average of 200 spermatozoa tracked (minimum of  
190 56 spermatozoa observed per male).

191 The percentage of motile spermatozoa and their velocity (VAP: velocity of the average path)  
192 were quantified using a CASA plug-in developed for ImageJ software adapted to Pacific oyster  
193 spermatozoa (Boulais et al., 2015) and averaged per male. Calibration settings were defined as  
194 follows: minimum spermatozoon size (pixels) = 1; minimum track length (frames) = 15;  
195 maximum spermatozoon velocity between frames (pixels) = 8; minimum straight-line velocity  
196 for motile spermatozoa ( $\mu\text{m sec}^{-1}$ ) = 5; minimum VAP for motile spermatozoa ( $\mu\text{m sec}^{-1}$ ) = 10;  
197 minimum curvilinear velocity for motile spermatozoa ( $\mu\text{m sec}^{-1}$ ) = 13; low VAP speed ( $\mu\text{m sec}^{-1}$ )  
198 = 2; maximum percentage of path with zero VAP = 1; low VAP speed 2 ( $\mu\text{m sec}^{-1}$ ) = 12; low  
199 curvilinear velocity speed ( $\mu\text{m sec}^{-1}$ ) = 15; and frame rate (frames  $\text{sec}^{-1}$ ) = 25.

200

### 201 **2.6. Fertilisation percentage**

202 The number of eggs at 1-cell (non-fertilised eggs), 2-cell and 4-cell stages were counted with  
203 a photonic microscope (Zoom 2000, Leica, Germany). The percentage of fertilisation  
204 corresponds to the total number of cells at the 2- or 4-cell stages divided by the total number of  
205 eggs counted. The fertilisation percentage was calculated for each female by averaging the value  
206 obtained with the different males from the same aquarium ( $n = 12$  for pH 7.7;  $n = 15$  for pH 8.0).

207

208

## 209 **2.7. Larval survival**

210 The developmental timing was verified under a binocular microscope at 20, 48 and 96 hpf  
211 before sampling the larvae. At each sampling time, the total number of swimming larvae was  
212 estimated by counting the number of larvae under a binocular microscope. Larval survival at 48  
213 hpf and 96 hpf was calculated with the following formula:

$$214 \quad \text{Larval survival} = (\text{total number of swimming larvae at } t+1) / 100 \times (\text{total number of} \\ 215 \text{ swimming larvae at } t)$$

216

## 217 **2.8. Slide preparation for larval morphometry and birefringence**

218 Larval samples were ethanol-fixed and whole-mounted on microscope slides for morphometry  
219 and birefringence analysis. Microscope slides were prepared with ethanol-fixed larvae from the  
220 different pH conditions, following the method described in Wessel et al. (2018). Larvae were  
221 whole-mounted in about 500  $\mu\text{L}$  of glycerol, keeping the amount of ethanol transferred to a  
222 minimum. The slides were kept at room temperature for 5 to 10 min allowing the ethanol to  
223 evaporate and the larvae to settle. Six spots of vacuum gel were deposited at the corners and  
224 middle edges of a square coverslip to prevent the larvae from being crushed. After the coverslip  
225 was placed over the larval samples, the slides were gently sealed with clear nail varnish. Each  
226 slide contained approximately 100 larvae per treatment per larval stage.

227 Approximately 40 larvae per tank ( $n = 200$  larvae per pH condition), regardless of their  
228 orientation, shape and development were photographed with an Olympus binocular microscope  
229 (Olympus, Hamburg, Germany) under phase contrast and polarized light. The same microscope  
230 was equipped with polarizing filters for the birefringence analysis. Microscopic observations  
231 were fully blinded to prevent bias arising from any a priori knowledge of larval treatment. All  
232 images were taken with a digital camera (DS-R11, Nikon, Japan) at 20X magnification and 40-  
233 ms exposure. Images were acquired with NIS-element and analysed using ImageJ software (v  
234 1.52a).

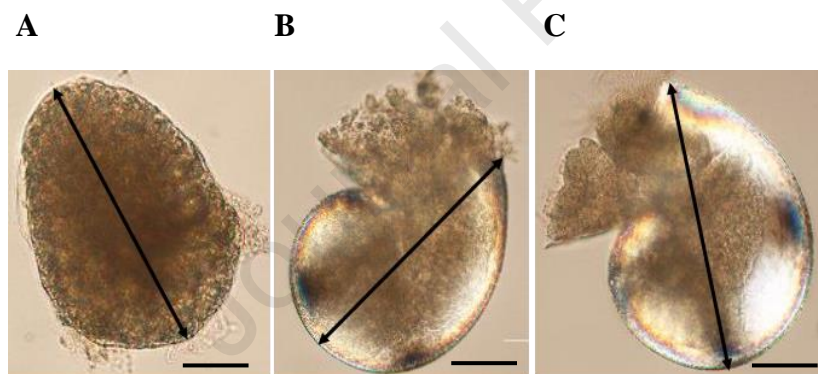
## 235 **2.9. Morphometric analysis**

236 Tissue morphogenesis, shell formation and shell size were analysed on larvae lying laterally  
 237 on their sides (n = 200 larvae per pH condition). A qualitative categorisation was used to assess  
 238 larval morphology and growth according to the method previously developed for *H. tuberculata*  
 239 larval assessment (Wessel et al., 2018). The phenotype of each larva was scored according to the  
 240 following degrees of soft tissue morphogenesis and shell formation (see **Fig. 2** for more details):

241 - *Normal tissue development/ normal shell = normal phenotype*

242 - *Abnormal or partially developed tissues/ partial or no shell = altered phenotype*

243 The maximum larval length at 20 hpf and total shell length of fully shelled larvae at 48 and  
 244 96 hpf were measured using ImageJ software and used as indicators of larval size, according to  
 245 the method described in Kavousi et al. (2021) (**Fig. 1**). The mean larval length was calculated  
 246 based on 40 larvae per tank (n = 200 per pH treatment).



**Fig. 1.** Measurements of larval length at 20 hpf (A), and shell length at 48 hpf (B) and 96 hpf (C). Scale bar: 50µm

## 247 **2.10. Birefringence analysis**

248 Birefringence intensity has been previously used as a proxy for the assessment of CaCO<sub>3</sub>  
 249 mineralisation within the larval abalone shell (Kavousi et al., 2021; Wessel et al., 2018).  
 250 Birefringence was measured on cross-polarized images taken of 48 and 96 hpf larval stages (n =  
 251 200 per pH treatment). The younger 20 hpf larvae were not considered because the shell at this  
 252 stage lacks sufficient crystallized CaCO<sub>3</sub> to calculate birefringence (Wessel et al., 2018). The  
 253 mean greyscale level (0–255 pixels) was determined for each area of the larval shell showing

254 birefringence (i.e. 2 to 3 areas per larval shell). The values recorded for each area were averaged  
255 into a global mean greyscale value, providing the birefringence intensity (in %) for each larval  
256 shell.

### 257 **2.11. Oxidative stress**

258 Since OA and warming can cause significant alterations in cellular antioxidant responses  
259 (Grilo et al., 2018; Zhang et al., 2021), three parameters related to oxidative stress, i.e. lipid  
260 peroxidation, superoxide dismutase (SOD) and catalase, were measured in abalone larvae.

261 About 4000 larvae per tank ( $n = 5$  tanks per treatment) were sampled at 96 hpf. The samples,  
262 composed of larvae and seawater (200–300  $\mu\text{L}$  per tube), were flash frozen in liquid nitrogen and  
263 then stored until analyses. These larval samples were then sonicated in two volumes of lysis  
264 buffer (50 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Igepal,  
265 1 tablet of complete EDTA-free protease inhibitor cocktail in 25 ml of buffer, phosphatase  
266 inhibitor cocktail III) and centrifugated (45 min, 10 000 rpm, 4°C). The same lysates were used  
267 for quantification of total proteins and parameters related to oxidative stress, i.e. lipid  
268 peroxidation, superoxide dismutase (SOD) and catalase.

269 All enzyme assays were performed in triplicate in 96-well microplates (Nunc™, Thermo  
270 Scientific) and read using a microplate reader (Bio-Tek® Synergy™ HT). Total proteins, MDA  
271 and SOD were quantified using Gen5 software to compare the results with the calibration curves  
272 provided with the respective assay kits. Total protein contents were quantified in each lysate (i.e.  
273 1/10 dilution) according to Lowry et al. (1951) using the DC protein assay (Bio-Rad, Hercules,  
274 CA, USA). Results are expressed in mg/mL.

275 Lipid peroxidation was approximated by MDA contents, quantified in the lysates (i.e. pure  
276 samples) using the *OxisResearch*™ commercial kit (BIOXYTECH® MDA-586, TebuBio, Le  
277 Perray-en-Yvelines, France) following the manufacturer's instructions. Results are expressed in  
278 nmol per mg protein. Superoxide dismutase contents were quantified in the lysates (i.e. 1/10  
279 dilution) using a CAS No. RN 905-89-1 commercial kit (S7446, Sigma Aldrich, Saint-Louis, MI,

280 USA) following the manufacturer's instructions. Results are expressed in units per mg protein  
281 (U/mg), where 1 U of SOD is the amount of enzyme necessary to inhibit the formation of  
282 xanthine/XO complex by 50%. Catalase activity was quantified in lysates at room temperature  
283 following the method of Curd et al. (2019). Briefly, 10  $\mu$ L of pure samples were added to 195  $\mu$ L  
284 of hydrogen peroxide solution (10 mM) to initiate the reaction. Absorbance was immediately  
285 recorded every 15 s for 4 min using a Synergy HT microplate reader (BioTek, Winooski VT,  
286 USA). CAT activity is expressed in mU/mg proteins, where 1 U is the amount of enzyme  
287 necessary to catalyse 1  $\mu$ mole of  $H_2O_2$  per minute (using  $\epsilon_{H_2O_2}$ , 39.4  $mM^{-1} cm^{-1}$ ).

288

### 289 **2.12. Gene expression analysis**

290 The expression profiles of selected genes were analysed in the abalone larvae at 48 and 96 hpf  
291 (pooled per treatment from 5 tanks and corresponding to about 50 000 larvae/sample). The genes  
292 were chosen with respect to their putative functions in shell biomineralization (lustrin A; carbonic  
293 anhydrase 1, CA1; carbonic anhydrase 2, CA2) and neurosensory transmission (GABA A  
294 receptor-like; serotonin receptor, 5HTR; **Table 1**). 18S-rRNA (18S) and elongation factor 1  
295 (EF1) were chosen as reference genes. Specific primers for each gene were designed as detailed  
296 in **Table 1**. To search for potential candidate hits for the GABA A receptor in *Haliotis*  
297 *tuberculata*, tblastx (Basic Local Alignment Search Tool, NCBI) research was carried out using  
298 the GABA A receptor sequence of *Haliotis asinina* (EF222254.1) as a query sequence against  
299 the unfiltered transcriptome of *H. tuberculata* published by Harney et al. (2016). Then, blastx  
300 (Basic Local Alignment Search Tool, NCBI) research against the non-redundant protein  
301 sequences database was performed with all 38 candidate sequences previously obtained. The  
302 sequence TR57267\_c2\_g1 (GenBank accession number GEAU01240864.1) was chosen as the  
303 GABA A receptor-like sequence of *H. tuberculata* based on sequence similarities to the GABA  
304 A receptor sequence of *Aplysia californica* (XP005111291.2). The *H. tuberculata* serotonin  
305 receptor sequence (GEAU01264085.1) was identified by using serotonin receptor sequences  
306 available in *Haliotis* against the unfiltered transcriptome of *H. tuberculata* (Harney et al., 2016).



307 The PCR products obtained with the primers targeting the serotonin receptor and the GABA  
308 A receptor-like were sequenced to verify potential errors in the predicted sequences and primers.  
309 The same primers were subsequently used in RT-qPCR to target the specific genes of the *H.*  
310 *tuberculata* serotonin receptor and GABA A receptor-like sequence. Total RNA was extracted  
311 from pools of larvae using Extract-all reagent (Eurobio, Courtaboeuf, Essonne, France) followed  
312 by chloroform phase separation and isopropanol precipitation, including a step of DNase  
313 treatment (RTS DNase Kit, MoBio). The concentration and quality of RNA were evaluated using  
314 an ND-1000 NanoDrop® spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA) and  
315 an Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA). cDNA  
316 synthesis was performed by reverse transcription (RT) using an iScript™ cDNA Synthesis kit  
317 (Bio-Rad Laboratories Inc., Hercules, CA, USA) strictly following recommendations from the  
318 supplier. Negative RT controls were performed for all samples (RT reaction without retro-  
319 transcriptase enzyme). Transcript expression was quantified using the CFX96 Touch Real-Time  
320 PCR Detection system (Bio-Rad Laboratories Inc.) according to the protocol previously  
321 described by Mazurais et al. (2020). The relative quantity of messenger was normalized with the  
322  $\Delta$ Ct method using the same CFX Manager software (Bio-Rad Laboratories Inc.).

323

### 324 ***2.13. Evaluation of post-larvae and juveniles***

325 Survival at settlement was evaluated at 9 days and 2 months after settlement. Biometric  
326 measurements of juvenile abalone were performed at 2.5 and 5.5 mo of exposure to the different  
327 pH conditions. At 2.5 months, 2-3 juveniles were sampled per aquarium. To avoid experimenter  
328 bias during collection, a first individual was randomly observed on the plate. The next closest  
329 one was then sampled, and images were taken with a digital camera (DS-Ri1, Nikon) and size  
330 was analysed using ImageJ software (1.52a) with 0.01 mm precision. At the end of the  
331 experiment (i.e. at 5.5 months of age), all the surviving abalone were photographed and sized  
332 using digital imaging. The shells were weighed to the nearest 0.01 mg using an analytical balance.



**Table 1.** Specific primers used for gene expression analysis of *Haliotis tuberculata* larvae: GenBank accession number, primer sequences and references.

Gene	Accession number	Sequence 5'-3'	References
<i>Lustrin A</i>	HM852427.2	F-ATCTGTCCGGCAGTTCCTAC R-CTGGGGCACTGTAAGTTGGT	Gaume <i>et al.</i> (2014)
<i>Carbonic anhydrase 1</i>	HQ845770.1	F-ATGGCAGCTGATAAAGCAAC R-AGGGAAATGAGTGTGCATGT	Avignon <i>et al.</i> (2020)
<i>Carbonic anhydrase 2</i>	HQ845771.1	F-CGCCGACTTTATCTGAGAGC R-GTCTCCCACGAAGTGGTTGT	Le Roy <i>et al.</i> (2012)
<i>18S</i>	AF120511.1	F-GGTTCCAGGGGAAGTATGGT R-AGGTGAGTTTTCCCGTGTTG	Gaume <i>et al.</i> (2014)
<i>EF1</i>	FN566842.1	F-ATTGGCCACGTAGATTCTGG R-GCTCAGCCTTCAGTTTGTCC	Gaume <i>et al.</i> (2014)
<i>GABA A receptor-like sequence</i>	GEAU01240864.1	F-GCACACCGAGTGTGAGAAGA R-CAACACTGAATGTGGCGAAC	Designed for this study
<i>Serotonin receptor</i>	GEAU01264085.1	F-GCTACCACGACGACCATATC R- CCTTTCACGCTTTAGTTCCA	Designed for this study

333

334 **2.14. Statistical analysis**

335 All statistical analyses were performed with R software (R Core Team, 2015). Differences in  
336 fertilisation rate, larval survival, total length, shell birefringence, and juvenile length, width and  
337 weight were tested using the linear mixed model (LMM) with the lmerTest package (Kuznetsova  
338 *et al.*, 2017) based on the method described by Winter (2013). This model used pH as a fixed  
339 factor and aquarium as a random factor nested within pH. The normality of residuals and  
340 homogeneity of variances were verified using Shapiro–Wilk tests and Levene tests based on  
341 medians, respectively. When assumptions of homogeneity of variances and normal distribution  
342 of residuals were not confirmed, the data were log or inverse transformed before analysis. If these  
343 assumptions were not validated, generalized linear mixed models were applied using the GLMM  
344 package with gamma inverse family for continuous data (Knudson *et al.*, 2021) with Wald Chi-  
345 squared tests. If the GLMM model failed to converge, a non-parametric Welch test was

346 performed using the mean value per aquarium. To compare MDA, enzyme activities, gene  
347 expression and juvenile survival between the two pH treatments, Student t-tests were used. A  
348 Wilcoxon test was applied to 9-day post-larvae survival because non-normality of data was  
349 observed. The morphological parameters of the larvae were evaluated using a Chi-squared ( $\chi^2$ )  
350 homogeneity test. Differences were considered significant at  $p < 0.05$ . Data are presented as least  
351 square means of squares  $\pm$  standard error of means unless otherwise indicated.

### 352 **3. Results**

#### 353 **3.1. Seawater parameters**

354 Mean seawater carbonate chemistry parameters for the different periods (i.e. the reproductive  
355 period and the larval and juvenile period) of abalone exposure to the pH treatments are given in  
356 **Table 2.**

357 During the reproductive conditioning (January to June 2017), seawater temperature followed  
358 natural variations and ranged from  $12.6^\circ\text{C} \pm 0.7^\circ\text{C}$  at the start of the experimental period to  
359  $19.2^\circ\text{C} \pm 0.2^\circ\text{C}$  at the end. Salinity was  $34.6 \pm 0.6$  in all experimental aquaria and remained stable  
360 over the experiment. Total alkalinity (AT) measured in the experimental tanks was  $2355 \pm 9 \mu\text{Eq.}$   
361  $\text{kg}^{-1}$  and remained stable throughout the experiment and among all aquaria. The seawater  $\text{pH}_\text{T}$   
362 was maintained close to the nominal value throughout the experiment, with  $8.01 \pm 0.05$  ( $\text{pCO}_2$   
363  $439 \pm 57 \mu\text{atm}$ ) in the ambient pH aquaria and  $7.71 \pm 0.06$  ( $\text{pCO}_2$   $951 \pm 138 \mu\text{atm}$ ) in the low pH  
364 aquaria. In pH 8.0 and pH 7.7 conditions,  $\Omega_{\text{aragonite}}$  was  $2.30 \pm 0.31$  and  $1.25 \pm 0.19$  and  $\Omega_{\text{calcite}}$   
365 was  $3.59 \pm 0.46$  and  $1.95 \pm 0.28$ , respectively (**Table 2**).

366 During the larval and juvenile period (July to December 2017), the seawater temperature  
367 ranged from  $19.2^\circ\text{C} \pm 0.2^\circ\text{C}$  at the start to  $10.5^\circ\text{C} \pm 0.05^\circ\text{C}$  at the end of the experiment. Salinity  
368 over the larval/juvenile period was  $35.2 \pm 0.2$  in all experimental aquaria and remained stable  
369 throughout the experiment. Total alkalinity (AT) was  $2323 \pm 19 \mu\text{Eq. kg}^{-1}$  and remained stable  
370 throughout the experiment and among all aquaria. The mean  $\text{pH}_\text{T}$  was  $8.10 \pm 0.06$  ( $\text{pCO}_2$ :  $351 \pm$

371 54  $\mu\text{atm}$ ) in ambient pH aquaria and  $7.71 \pm 0.03$  ( $\text{pCO}_2$ :  $951 \pm 138 \mu\text{atm}$ ) in low pH aquaria. In  
 372 pH 8.0 and pH 7.7,  $\Omega_{\text{aragonite}}$  was  $2.78 \pm 0.41$  and  $1.26 \pm 0.13$  and  $\Omega_{\text{calcite}}$  was  $4.32 \pm 0.63$  and  $1.97$   
 373  $\pm 0.19$  conditions, respectively (**Table 2**).

**Table 2.** Seawater  $\text{pH}_T$  temperature and parameters of the carbonate system in each pH treatment (i.e., pH 8.0. and pH 7.7,  $n = 25$  per pH treatment). Seawater pH on the total scale ( $\text{pH}_T$ ), temperature, salinity and total alkalinity (AT) were used to calculate  $\text{CO}_2$  partial pressure ( $\text{pCO}_2$ ;  $\mu\text{atm}$ ), dissolved inorganic carbon (DIC;  $\mu\text{mol}/\text{kg}$ ),  $\text{HCO}_3^-$  ( $\mu\text{mol}/\text{kg}$ ),  $\text{CO}_3^{2-}$  ( $\mu\text{mol}/\text{kg}$ ), aragonite saturation state ( $\Omega_{\text{aragonite}}$ ) and calcite saturation state ( $\Omega_{\text{calcite}}$ ) by using the  $\text{CO}_2\text{SYS}$  program. Values are means  $\pm$  s.d.

Experimental period	Nominal pH	$\text{pH}_T$	Temperature ( $^{\circ}\text{C}$ )	AT ( $\mu\text{mol}\cdot\text{kg}^{-1}$ )	$\text{pCO}_2$ ( $\mu\text{atm}$ )	DIC ( $\mu\text{mol}\cdot\text{kg}^{-1}$ )	$\text{HCO}_3^-$ ( $\mu\text{mol}\cdot\text{kg}^{-1}$ )	$\text{CO}_3^{2-}$ ( $\mu\text{mol}\cdot\text{kg}^{-1}$ )	$\Omega_{\text{aragonite}}$	$\Omega_{\text{calcite}}$
Reproductive period <sup>1</sup> (5 months)	8.0	$8.01 \pm 0.05$	$14.4 \pm 1.4$	$2355 \pm 9$	$439 \pm 57$	$2150 \pm 28$	$1984 \pm 48$	$151 \pm 20$	$2.30 \pm 0.31$	$3.59 \pm 0.46$
	7.7	$7.71 \pm 0.06$	$14.4 \pm 1.5$	$2355 \pm 9$	$951 \pm 138$	$2277 \pm 25$	$2154 \pm 30$	$82 \pm 12$	$1.25 \pm 0.19$	$1.95 \pm 0.28$
Larval and juvenile period <sup>2</sup> (5.5 months)	8.0	$8.10 \pm 0.06$	$15.4 \pm 2.2$	$2323 \pm 19$	$351 \pm 54$	$2069 \pm 41$	$1875 \pm 64$	$181 \pm 26$	$2.78 \pm 0.41$	$4.32 \pm 0.63$
	7.7	$7.71 \pm 0.03$	$15.3 \pm 2.1$	$2323 \pm 19$	$966 \pm 69$	$2237 \pm 15$	$2119 \pm 20$	$83 \pm 8$	$1.26 \pm 0.13$	$1.97 \pm 0.19$

<sup>1</sup>Data from Avignon *et al.* 2020; <sup>2</sup> 5 time points per month ( $n = 25$  per treatment)

### 374 3.2. Gametes and fertilisation

375 The number of gametes released (oocytes and spermatozoa) per gram of abalone was not  
 376 different between individuals exposed to pH 8.0 and pH 7.7 (**Table 3**). No significant differences  
 377 in spermatozoon characteristics (i.e. SSC, FSC, percentage of motile spermatozoa and velocity)  
 378 were observed between abalone exposed to pH 8.0 and pH 7.7 ( $p > 0.05$ , **Table 3**). In addition,  
 379 fertilisation success was not significantly different between low and ambient pH ( $p > 0.05$ , **Table**  
 380 **3**).

381

### 382 3.3. Larval survival

383 Survival did not differ between larvae exposed to pH 8.0 and 7.7 at 48 hpf or 96 hpf (survival  
 384 at 48 h for pH 8.0 vs pH 7.7:  $9.3 \pm 1.69\%$  vs  $12.7 \pm 2.32\%$ ,  $t = 1.165$ ,  $df = 8$ ,  $p = 0.2778$ ; survival

385 at 96 h for pH 8.0 vs pH 7.7:  $78.8 \pm 17.83\%$  vs  $56.2 \pm 11.74\%$ ,  $t = -1.057$ ,  $df = 8$ ,  $p = 0.321$ ,  
 386 Student t-test, mean  $\pm$  s.e.).

387

**Table 3.** Number of gametes released, spermatozoon characteristics and percentage of fertilisation of abalone *H. tuberculata* after 5 months of low (7.7) or ambient (8.0) pH exposure. <sup>α</sup> Linear mixed model analysis with the Satterthwaite method otherwise stated. <sup>β</sup>GLMM analysis fit by maximum likelihood (Laplace approximation) with gamma inverse family and Wald Chi-squared test with no over-dispersion. Least square mean  $\pm$  s.e.m. presented.

	pH 8.0	pH 7.7	F	p-value
<b>Number of gametes released</b>				
Number of oocytes released/g <sup>α</sup>	4187 $\pm$ 623	3708 $\pm$ 625	F <sub>1,10.7</sub> = 0.295	0.598
Millions of spermatozoa released/g <sup>α</sup>	885 $\pm$ 164.7	857 $\pm$ 163.4	F <sub>1,9.0</sub> = 0.015	0.906
<b>Spermatozoa shape and motility</b>				
SSC (complexity) <sup>α</sup>	326 $\pm$ 10.3	315 $\pm$ 10.0	F <sub>1,9.0</sub> = 0.628	0.449
FSC (volume) <sup>α</sup>	180 $\pm$ 2.9	179 $\pm$ 2.9	F <sub>1,8.9</sub> = 0.045	0.836
Percentage of motile spermatozoa (%) <sup>α</sup>	64.0 $\pm$ 0.03	56.9 $\pm$ 0.03	F <sub>1,26</sub> = 2.241	0.146
Velocity ( $\mu$ m/s) <sup>α</sup>	75.6 $\pm$ 1.44	74.0 $\pm$ 1.44	F <sub>1,26</sub> = 0.626	0.436
<b>Gamete fertilisation success</b>				
Fertilisation percentage (%) <sup>β</sup>	53.1 $\pm$ 5.55	62.9 $\pm$ 6.26	$\chi^2 = 0.814$ , $df = 1$	0.361

### 388 3.4. Larval morphology

389 At 20 hpf, larvae exposed to pH 7.7 showed less alteration of tissue organogenesis than those  
 390 exposed to pH 8.0 (25% for pH 7.7 vs 43% for pH 8.0,  $\chi^2 = 15.2$ ,  $p < 0.001$ , Pearson Chi-squared  
 391 test). On the contrary, 20 hpf-larvae showed a significant increase in shell abnormalities at pH  
 392 7.7 compared with larvae reared in ambient conditions (69% for pH 7.7 vs 59% for pH 8.0,  $\chi^2 =$   
 393 4.31,  $p = 0.038$ , Pearson Chi-squared test, **Fig. 2A**).

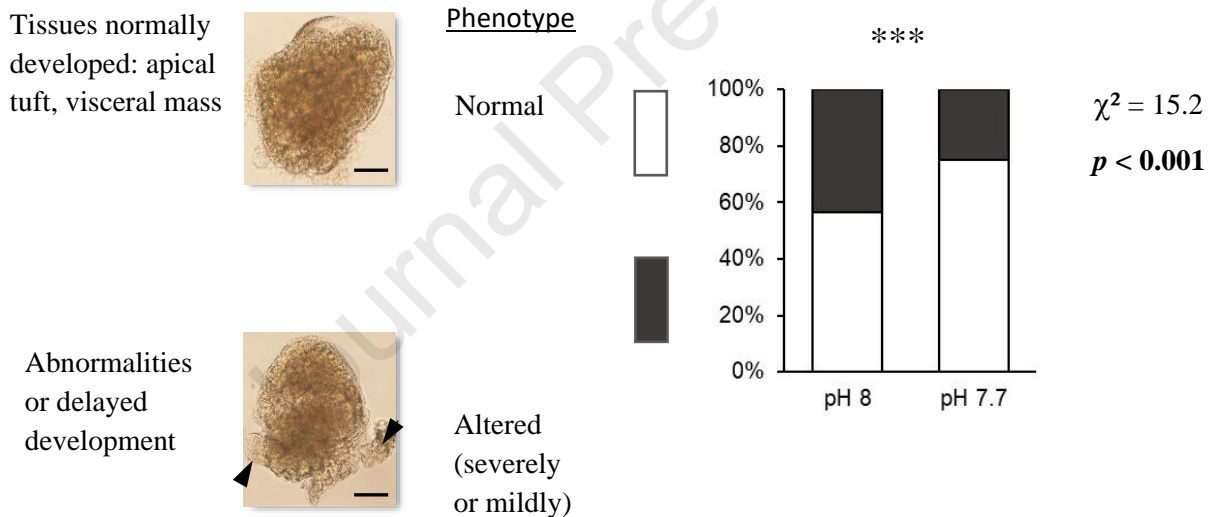
394 At 48 hpf, larvae exposed to pH 7.7 showed more tissue abnormalities than larvae exposed to  
 395 pH 8.0 (75% for pH 7.7 vs 64% for pH 8.0,  $\chi^2 = 6.27$ ,  $p = 0.012$ , Pearson Chi-squared test). In  
 396 addition, larvae exposed to pH 7.7 showed a significant increase in shell abnormalities compared

397 with those reared in ambient pH conditions (97% for pH 7.7 vs 83% for pH 8.0,  $\chi^2 = 20.7$ ,  $p <$   
 398 0.001, Pearson's Chi-squared test, **Fig. 2B**).

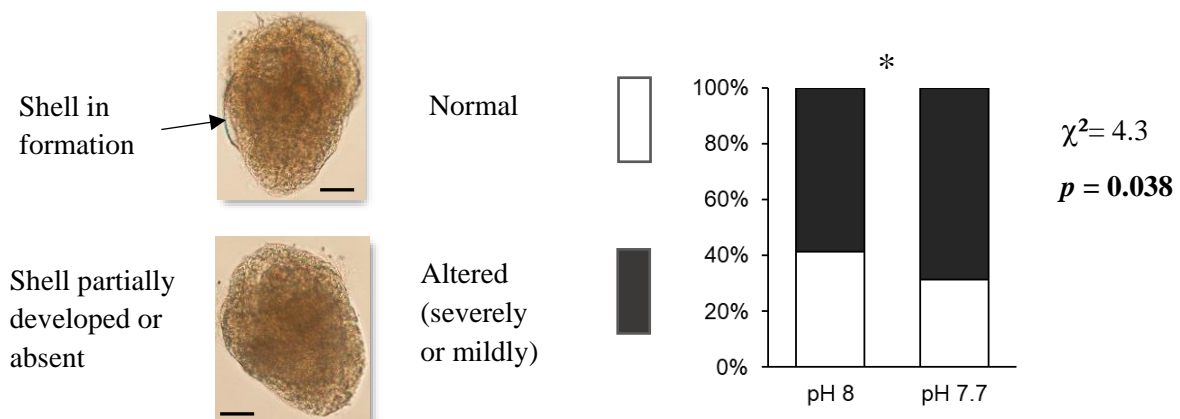
399 At 96 hpf, larvae exposed to pH 7.7 had more tissue abnormalities than those exposed to pH  
 400 8.0 (8.5% for pH 7.7 vs 3% for pH 8.0,  $\chi^2 = 5.58$ ,  $p = 0.018$ , Pearson Chi-squared test). Shell  
 401 formation was also affected by lower pH, with a significant increase in shell alterations at pH 7.7  
 402 compared with larvae reared in ambient pH conditions (97% for pH 7.7 vs 85% for pH 8.0,  $\chi^2 =$   
 403 16.56,  $p < 0.001$ , Pearson Chi-squared test, **Fig. 2C**).

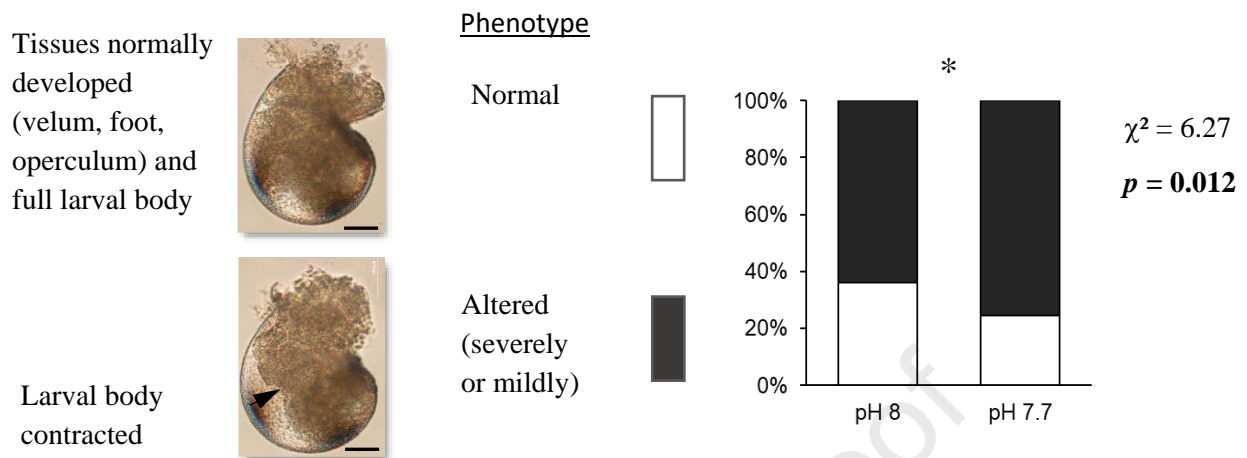
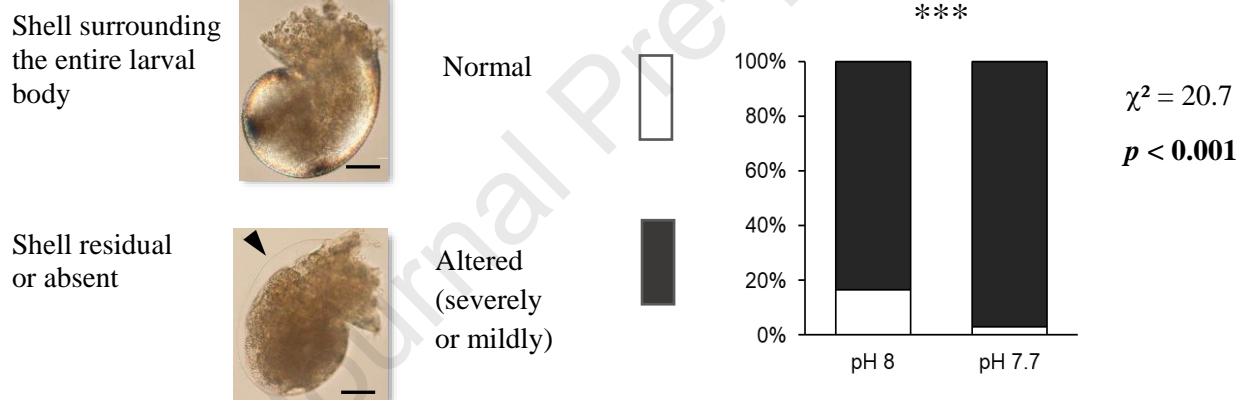
### A. 20 hpf larvae

#### SOFT TISSUE MORPHOGENESIS



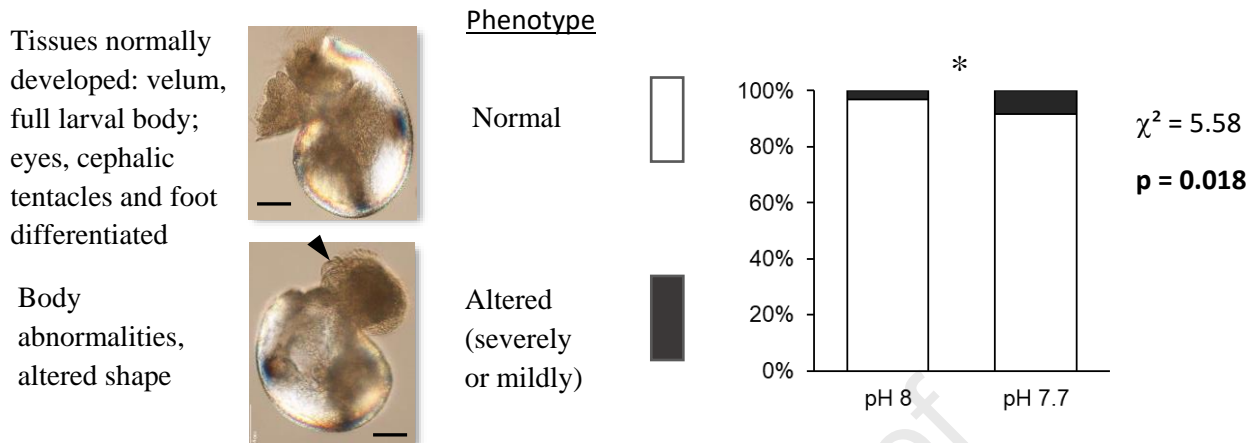
#### SHELL FORMATION



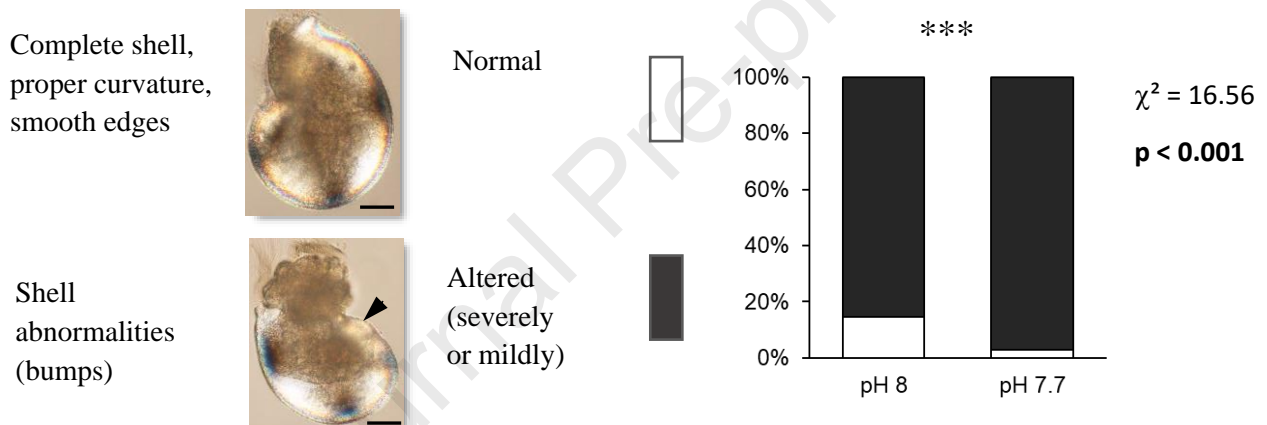
**B. 48 hpf larvae****SOFT TISSUES****SHELL**

## C. 96 hpf larvae

## SOFT TISSUES



## SHELL

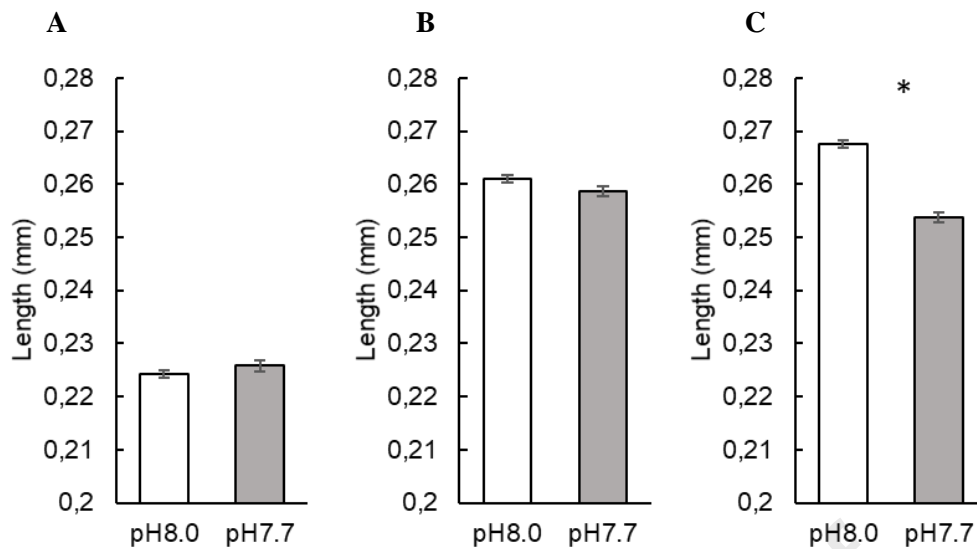


**Fig. 2. Percentage of larvae (%) presenting either normal (white squares) or altered phenotypes (black squares) based on the degree of soft tissue morphogenesis and shell formation after exposure to two pH conditions (8.0 and 7.7). (A) 20 hpf, (B) 48 hpf and (C) 96 hpf (n = 200 larvae per pH treatment, Chi-squared test). Arrowheads in the pictures indicate body or shell abnormalities. Scale bar: 50  $\mu$ m.**

404 **3.5. Larval length**

405 No differences were observed in larval length at 20 hpf or shell length at 48 hpf between larvae  
 406 exposed to pH 8.0 and pH 7.7 (20 hpf:  $\chi^2 = 0.197$ , df = 1,  $p = 0.657$ ; 48 hpf:  $\chi^2 = 0.250$ , df = 1,  
 407  $p = 0.617$ , GLMM analysis with gamma inverse family). However, larvae exposed to pH 7.7 had  
 408 a smaller shell length at 96 hpf than larvae exposed to pH 8.0 ( $\chi^2 = 10.13$ , df = 1,  $p = 0.001$ ,  
 409 GLMM analysis with gamma inverse family) (**Fig. 3**).

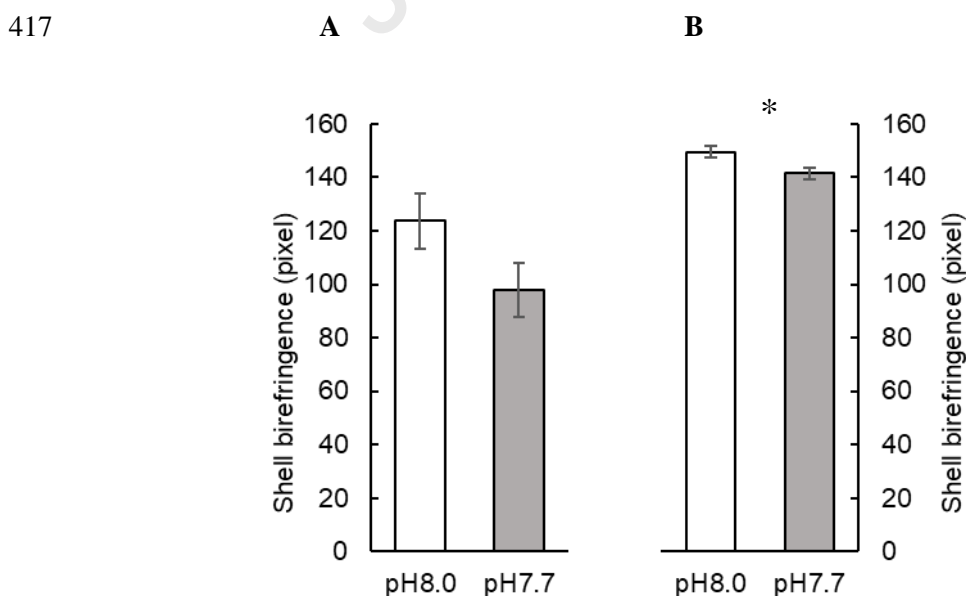
410



**Fig. 3.** Length of *H. tuberculata* larvae reared under ambient (pH 8.0) and low pH (7.7) at (A) 20 hpf, (B) 48 hpf and (C) 96 hpf (n = 200 larvae per pH treatment, n = 5 tanks per treatment). Larvae were exposed to the same pH as their parents. Ls mean  $\pm$  s.e.m. \*  $p < 0.05$  (GLMM analysis with gamma inverse family).

### 411 3.6. Birefringence analysis

412 The mean birefringence (corresponding to shell calcification level) of the larval shell at 48 h  
 413 did not differ significantly between individuals from the two pH treatments ( $F_{1,10} = 3.28$ ,  $p =$   
 414 0.099, LMM analysis, **Fig. 4A**). However, shell birefringence at 96 hpf was significantly lower  
 415 in the pH 7.7 treatment than in control larvae in ambient conditions at pH 8.0 ( $F_{1,10} = 7.46$ ,  $p =$   
 416 0.021, LMM analysis, **Fig. 4B**).



**Fig. 4.** Mean shell birefringence (number of greyscale pixels) of *H. tuberculata* larvae reared under ambient (pH 8.0) and low pH (7.7) at (A) 48 hpf and (B) 96 hpf. Larvae were exposed to the same pH as their parents. Ls mean  $\pm$  s.e.m. \*  $p < 0.05$  (LMM analysis).



### 418 **3.7. Oxidative stress**

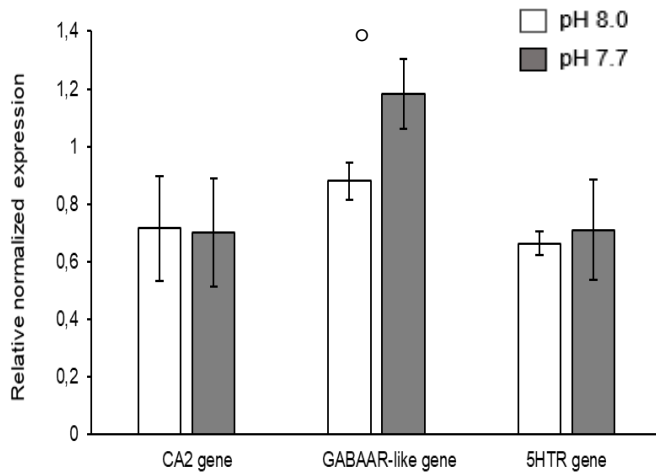
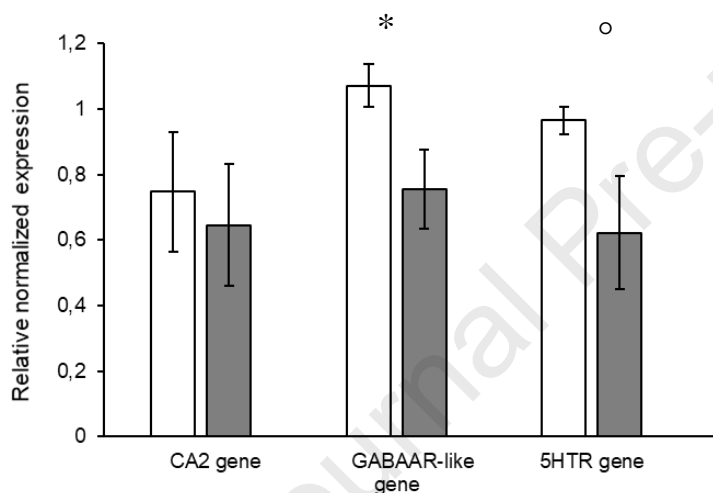
419 No difference in catalase activity was observed between 96hpf-larvae exposed to pH 8.0 and  
420 pH 7.7 ( $2.25 \pm 0.428$  vs  $2.09 \pm 0.408$  mU/mg prot, respectively,  $t = -2.26$ ,  $df = 8$ ,  $p = 0.800$ ,  
421 Student t-test, mean  $\pm$  s.e.), MDA ( $1.14 \pm 0.181$  vs  $1.28 \pm 0.152$  nmol/mg prot, respectively,  $t =$   
422  $-0.549$ ,  $df = 8$ ,  $p = 0.598$ , Student's t-test, mean  $\pm$  s.e.) or SOD activity ( $5.78 \pm 1.370$  vs  $6.48 \pm$   
423  $0.139$  U/mg prot, respectively,  $t = 0.370$ ,  $df = 5$ ,  $p = 0.727$ , Student t-test, mean  $\pm$  s.e.). Due to  
424 the small number of larvae contained in the samples, SOD could not be analysed for two samples  
425 from pH 7.7 and one sample from pH 8.0.

426

### 427 **3.8. Gene expression**

428 At 48 hpf, larvae exposed to pH 7.7 tended to have higher GABAAR-like gene expression  
429 than larvae exposed to pH 8.0 ( $t = -2.29$ ,  $df = 8$ ,  $p = 0.051$ , Student t-test). No difference was  
430 observed for 5HTR or CA2 gene expression between larvae exposed to the two pH treatments ( $t$   
431  $= -0.209$ ,  $df = 8$ ,  $p = 0.840$  and  $t = -0.46111$ ,  $df = 5$ ,  $p = 0.6641$ , respectively, Student t-test; **Fig.**  
432 **5A**).

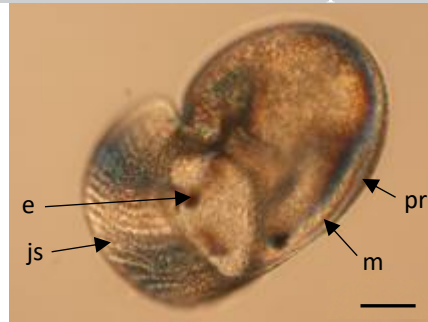
433 At 96 hpf, larvae exposed to pH 7.7 showed a downregulation of GABAAR-like gene expression  
434 ( $t = 2.43$ ,  $df = 7$ ,  $p = 0.046$ , Student t-test) and tended to have lower 5HTR gene expression ( $t =$   
435  $-2.16$ ,  $df = 7$ ,  $p = 0.068$ , Student t-test) compared with larvae exposed to pH 8.0. No significant  
436 difference was observed in CA2 gene expression levels ( $t = -0.38$ ,  $df = 7$ ,  $p = 0.712$ , Student t-  
437 test, **Fig. 5B**). The expression levels quantified for CA1 and lustrin A genes were below the  
438 detection limit for both larval stages.

**A****B**

**Fig. 5. Expression patterns of CA2, GABAAR-like and 5HTR genes of abalone larvae exposed to ambient (pH 8.0) and low pH (7.7). A: 48 hpf and B: 96 hpf. Larvae were exposed to the same pH as their parents. Mean  $\pm$  s.e. \*  $p < 0.05$ , °  $p < 0.10$  (Student t-test).**

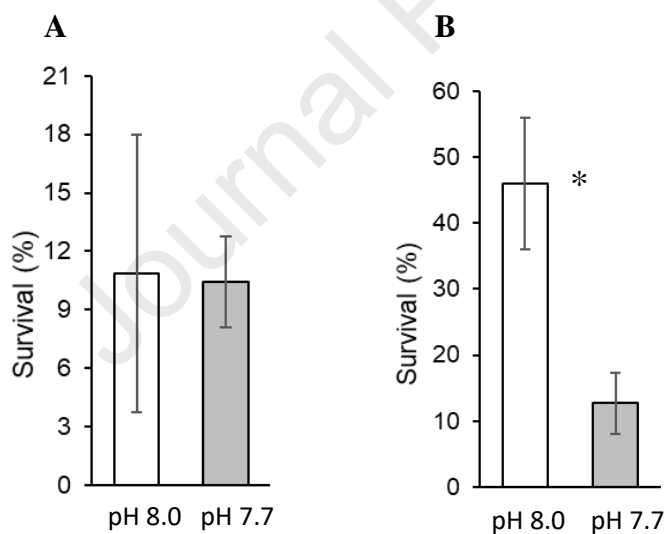
### 3.9. Post-larval and juvenile evaluation

439 The main characteristics of abalone post-larvae at 9 days after settlement are shown in **Fig. 6**.  
 440 After metamorphosis, the post-larval shell shows a well-defined transition between protoconch  
 441 (pr) and newly deposited sculptured, juvenile shell (js).



**Fig. 6. Microscopic image of post-larval abalone *H. tuberculata* 9 days after settlement.** The post-larval shell shows a well-defined transition between the protoconch (pr) and the newly deposited juvenile shell (js). Eyes (e) and mantle (m) are visible through the thin protoconch shell (pr). Scale-bar: 50  $\mu$ m.

442 No significant difference in abalone survival was observed between the two pH treatments 9  
 443 days after settlement ( $W = 17$ ,  $p = 0.42$ , Wilcoxon test, **Fig. 7A**). However, the survival of post-  
 444 larvae two months after settlement was significantly lower for individuals grown at pH 7.7  
 445 compared with those grown at pH 8.0. (13% and 46%, respectively,  $t = -3.02$ ,  $df = 8$ ,  $p = 0.017$ ,  
 446 Student t-test; **Fig. 7B**).



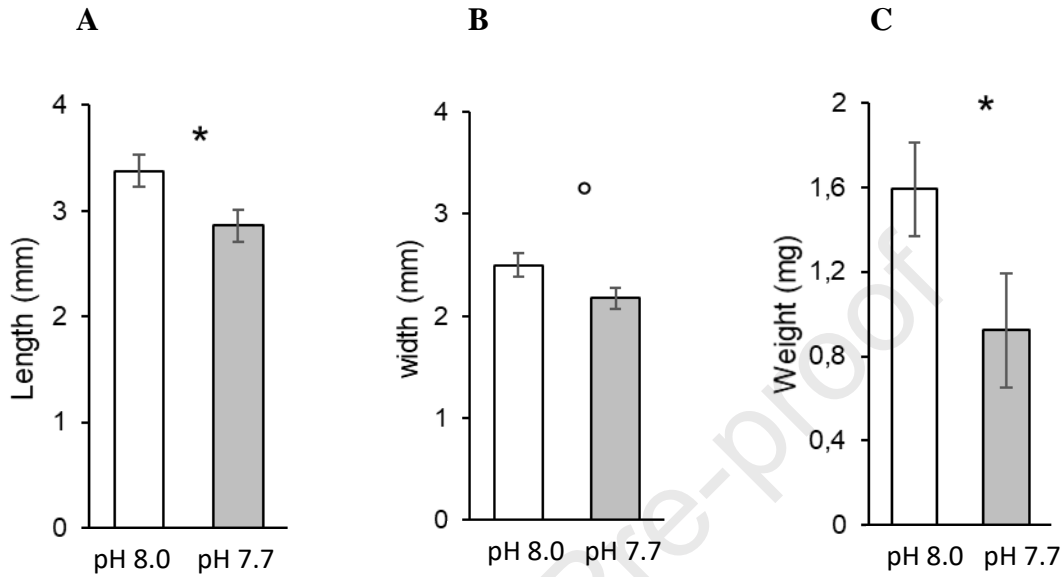
**Fig. 7. Survival of abalone post-larvae 9 days (A) and 2 months after settlement (B) in the two pH treatments (pH 8.0 and 7.7).** Post-larvae were exposed to the same pH as their parents. Mean  $\pm$  s.e. \*  $p < 0.05$  (Student t-test).

447 At 2.5 months, juveniles exposed to pH 7.7 had a lower length ( $F_{1, 9.9} = 5.89$ ,  $p = 0.036$ , LMM  
 448 analysis), tended to have a lower width ( $F_{1, 9.6} = 4.34$ ,  $p = 0.065$ , LMM analysis) and were lighter  
 449 in weight ( $F_{1, 5.2} = 6.94$ ,  $p = 0.045$ , log transformation, LMM analysis) compared with juveniles  
 450 kept at ambient pH (**Fig. 8 A–C**). At 5.5 months, shell length ( $F_{1, 21} = 16.56$ ,  $p < 0.001$ , LMM  
 451 analysis), width ( $F_{1, 20} = 19.78$ ,  $p < 0.001$ , LMM analysis) and weight ( $F_{1, 3.9} = 21.33$ ,  $p < 0.001$ ,

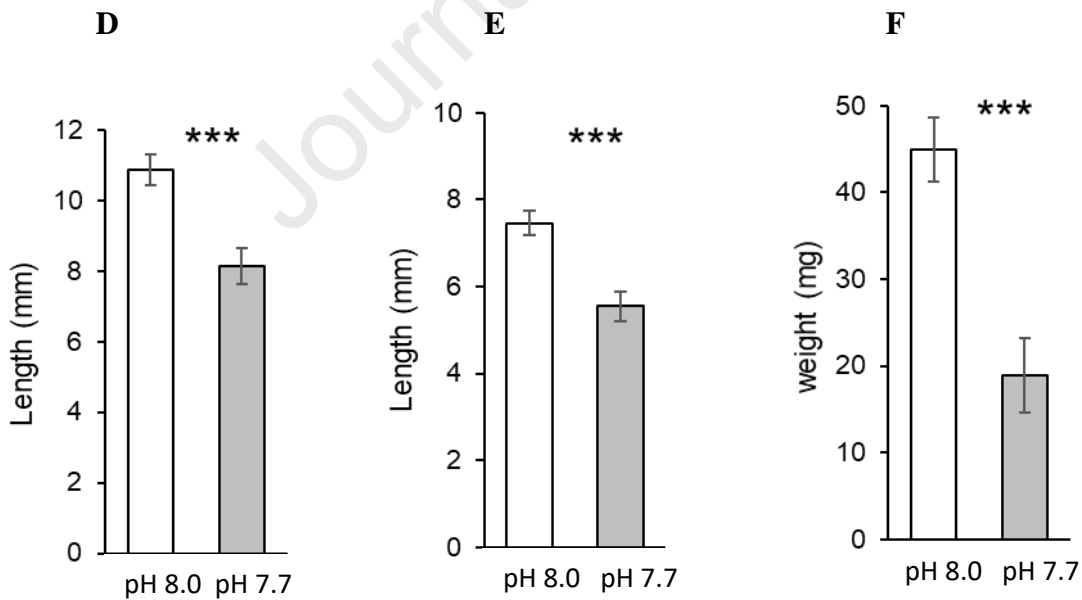
452 LMM analysis) were also significantly lower in juvenile abalones exposed to pH 7.7 than in those  
 453 exposed to ambient pH (Fig. 8 D–F).

454

### 2.5 months



### 5.5 months



**Fig. 8.** Shell length (A, D), width (B, E) and weight (C, F) of 2.5-month-old and 5.5-month-old abalones *H. tuberculata* in the two pH treatments (pH 8.0 and 7.7). Juveniles were exposed to the same pH as their parents. Ls mean  $\pm$  s.e.m. \*  $p < 0.05$ ; °  $p < 0.10$

#### 455 4. Discussion

456 This study investigated for the first time the effect of ocean acidification on the European  
457 abalone *Haliotis tuberculata* by following transgenerational exposure to two relevant OA  
458 scenarios (ambient pH and -0.3 pH unit decrease). Although the gametes and the fertilisation rate  
459 appeared unaffected by the decreased pH, several biological parameters involved in larval and  
460 juvenile fitness (survival at settlement, growth and calcification) were reduced at pH 7.7,  
461 suggesting a high sensitivity of abalone offspring exposed to the same pH conditions as their  
462 parents.

463 In adult *H. tuberculata*, no significant differences in gamete quantity or quality were found  
464 between the two pH treatments after five months of reproductive conditioning. In the same way,  
465 the fertilisation rate was not affected by this pH difference, suggesting that fertilisation success  
466 and resulting embryos are tolerant of OA. Such tolerance to pH stress in the early life stages has  
467 already been reported for many other species, including abalones (Byrne et al., 2010, 2011;  
468 Przeslawski et al., 2015). In early embryos of sea urchins, it has been suggested that the resilience  
469 to acidification stress might be due to protective proteins accumulated in the egg during  
470 embryonic development (Hamdoun and Epel, 2007). In *H. tuberculata*, the magnitude of OA (-  
471 0.3 pH units) is probably below the tolerance threshold of abalone embryo physiology.

472 Furthermore, our results reveal that the survival of abalone larvae was not affected by a 0.3  
473 pH unit decrease, as previously shown in 30 hpf *H. tuberculata* (Wessel et al., 2018). A recent  
474 multigenerational study on the red abalone *H. rufescens* demonstrated that larval mortality under  
475 OA was strongly correlated with differences in the maternal provisioning of lipids with a positive  
476 correlation between lipid concentration and survival under OA (Swezey et al., 2020). This  
477 relationship was maintained in experiments on second-generation animals. Our results agree with  
478 previous studies showing that early embryonic and larval stages are less vulnerable to pH stress  
479 than later stages (Przeslawski et al., 2015; Swezey et al., 2020). Indeed, later veliger stages (48  
480 and 96 hpf) of *H. tuberculata* exhibited a higher sensitivity to lower pH (7.7) with an increase of

481 developmental abnormalities, a decrease in length and reduced calcification, as previously shown  
482 in single-stage experiments on *H. tuberculata* larvae (Kavousi et al., 2021; Wessel et al., 2018).  
483 Such effects of OA have been already reported in marine mollusc larvae, as differences in  
484 sensitivity to pH stress according to stage of development (Gazeau et al., 2013; Przeslawski et  
485 al., 2015).

486 Our results did not, however, reveal any induction of oxidative stress response in 96 hpf  
487 veliger larvae exposed to the lowest pH, which overall showed no change in lipid peroxidation  
488 or similar antioxidant enzyme activities compared to control larvae. Yet, it has previously been  
489 demonstrated that ocean acidification and warming can cause significant alterations in cellular  
490 parameters related to antioxidant responses in marine gastropods (Grilo et al., 2018; Zhang et al.,  
491 2021). Here, the lack of antioxidant response highlights the ability of abalone larvae to counteract  
492 potential oxidative stress induced by OA, even though they showed developmental artefacts at  
493 96 hpf. Nevertheless, few studies have considered oxidative stress parameters as key factors in  
494 assessing the susceptibility of mollusc larvae to near-future OA. There are only, to our  
495 knowledge, two studies of this kind, which were done on clams (Gurr et al., 2021; Munari et al.,  
496 2016).

497 Although responses vary among marine organisms, the expression of genes involved in  
498 calcification, ion regulation, metabolism and behaviour have been shown to be influenced by  
499 decreased pH (Strader et al., 2020). To better understand the molecular processes involved in  
500 larval responses to OA, the present study investigated the expression profiles of genes involved  
501 in shell biomineralization (carbonic anhydrases CA1, CA2 and shell matrix lustrin A) on two  
502 larval stages (48 and 96 hpf). Only CA2 expression levels could be quantified in larval extracts  
503 and these did not show any regulation by pH treatment at the two larval stages studied. These  
504 results are consistent with those previously reported in adult *H. tuberculata*, where CA  
505 expression in the mantle did not vary across pH treatments (Avignon et al., 2020). In their study  
506 on larval red abalone *Haliotis rufescens*, Zippay and Hofmann (2010) also found that decreased

507 pH did not affect the expression pattern of two shell formation genes in any abalone larval stages.  
508 Although calcification gene expression showed no significant changes between pH treatments,  
509 larval shells showed significant alterations or a delay in development in individuals reared at low  
510 pH. These results support the hypothesis that the effects on shell formation may be mainly due  
511 to a direct effect of low pH on shell dissolution, as previously shown in juvenile and adult stages  
512 of *H. tuberculata* (Auzoux-Bordenave et al., 2020; Avignon et al., 2020).

513 As in vertebrates, GABA and serotonin signalling are major players in the nervous systems of  
514 gastropod molluscs, where they are involved in the control of motor activity and cognitive  
515 processes such as memory and learning (Aonuma et al. 2020; Miller, 2019;), but their specific  
516 role during larval development has not been clarified in abalone. GABA A receptors have often  
517 been proposed as responsible for the disruption of sensory transduction at the central level  
518 observed in fish exposed to OA (Nilsson et al., 2012). This hypothesis has only recently been  
519 extended to invertebrate species (Thomas et al., 2020). GABA is a ligand-gated ion channel  
520 permeable to chloride and bicarbonate ions and acts as a major inhibitory neurotransmitter in the  
521 central nervous system. In acidified seawater, because of the alteration of chloride and  
522 bicarbonate concentrations across the neuronal cells, GABA would act as stimulatory  
523 neurotransmitter (Nilsson et al., 2012). In the present study, the expression profile of two genes  
524 involved in neurotransmission and behaviour (GABA A receptor-like and serotonin receptors)  
525 were also analysed in abalone larvae exposed to OA conditions. Interestingly, the results showed  
526 that GABA A receptor-like was upregulated in 48 hpf larvae under lower pH but was  
527 downregulated in 96 hpf larvae in the same conditions. This could indicate that the genetic  
528 response of abalone to OA changes with ontogenetic stage. In several gastropod species, GABA  
529 signalling is involved in the induction of settlement and metamorphosis (Hatakeyama and Ito,  
530 2000; Hernádi, 1994; Jing et al., 2003; Lerusalimsky and Balaban, 2001). In our experimental  
531 conditions (temperature 15–16°C), the settlement of *H. tuberculata* larvae occurred around 96  
532 hpf, which is consistent with the opposite regulation shown for GABA A receptor-like at 48 and

533 96 hpf. Interestingly, the expression pattern of the serotonin receptor gene in abalone larvae  
534 broadly follows the one described for the GABA A receptor-like gene, at least at 96 hpf. This is  
535 in line with the observation that certain invertebrates possess a serotonin-gated chloride channel  
536 that may respond to an increase of pCO<sub>2</sub> in the same way as the GABA A receptor (Thomas et  
537 al., 2020).

538 Our results also showed that pH decrease did not have any impact on post-larval settlement 9  
539 days after fertilisation. These results are consistent with previous findings in *H. tuberculata*  
540 (Kavousi et al., 2021) and *H. kamtschatkana* (Crim et al., 2011), but contrast with two other  
541 studies, in the New Zealand abalone *H. iris* and the donkey's ear abalone, *H. asinina*, both of  
542 which showing a lower settlement rate of larval abalone under low pH conditions (Espinel-  
543 Velasco et al., 2021; Tahil and Dy, 2016). These contrasting effects among *Haliotis* species may  
544 be due either to differences in larval sensitivity or to other indirect effects of decreased pH on the  
545 settlement substrate, as previously shown in abalone larvae (O'Leary et al., 2017).

546 Juveniles maintained at pH 7.7 showed an increase of mortality at 2.5 months and significant  
547 reductions in total length, width and weight at 5.5 months. These results are consistent with those  
548 previously obtained on farmed abalone species including *H. tuberculata* (Auzoux-Bordenave et  
549 al., 2020; Cunningham et al., 2016; Li et al., 2018). In juvenile *H. iris*, Cunningham et al. (2016)  
550 reported significant effects on shell length and wet weight at lower pH<sub>T</sub> (0.3 to 0.5 units below  
551 ambient pH). Similarly, shell growth and shell weight were significantly decreased in juvenile  
552 *H. discus hannai* (Li et al., 2018) after 3 months of exposure to lower seawater pH (0.2 units  
553 below ambient pH). In six-month-old *H. tuberculata*, our previous studies revealed significant  
554 reductions in juvenile shell length, weight and strength in a pH 7.6 treatment (0.5 units below  
555 ambient pH) after three months of exposure (Auzoux-Bordenave et al., 2020). Furthermore, the  
556 decrease in shell strength and changes in texture and porosity of the biomineral layers suggested  
557 that low pH induced both general effects on growth mechanisms and corrosion of the carbonated  
558 shell in *H. tuberculata*.



559 Transgenerational experiments, in which parents are exposed to low pH conditions during  
560 reproductive conditioning, are currently performed to measure how this parental exposure may  
561 influence the responses of the offspring (Parker et al., 2015). For most of the species studied to  
562 date, especially bivalves, transgenerational exposure to OA resulted in positive carry-over effects  
563 being transmitted from adults to their offspring, influencing the resilience of molluscs to OA  
564 (Fitzer et al., 2014; Parker et al., 2015; Ross et al., 2016; Swezey et al., 2020). In the oyster *S.*  
565 *glomerata*, the exposure of adults to reduced pH (0.3 unit below ambient pH) during reproductive  
566 conditioning resulted in positive carry-over effects on larvae, including faster development and  
567 increased growth, compared with larvae spawned from adults reared at ambient pH (Parker et al.,  
568 2012). When the transgenerational exposure was repeated on the next generation (F2), an  
569 increase in the resilience of the larval and juvenile offspring was observed (Parker et al., 2015).  
570 These experiments demonstrate that marine molluscs may have the ability to acclimate or adapt  
571 to future acidification of seawater through transgenerational pathways.

572 In the present study, the adverse effects of seawater acidification on larval and juvenile F1  
573 abalone are in the range of those already observed in previous single-stage experiments (Auzoux-  
574 Bordenave et al., 2020; Kavousi et al., 2021; Wessel et al., 2018). The absence of significant  
575 carry-over effects on the offspring following parental exposure to OA suggests that larval  
576 dispersion of *H. tuberculata* in the natural environment may be at greater risk under pH stress,  
577 with potential negative consequences for abalone populations. However, considering the short  
578 duration of the abalone larval stage (5 days) and the huge morphological and physiological  
579 changes that occur concomitantly, it cannot be excluded that some transgenerational mechanisms  
580 may mitigate the adverse effects of OA on abalone larvae by acting on other biological variables  
581 or developmental processes that were not investigated in the present study.

582

583

584 In conclusion, this study demonstrated that OA may have detrimental consequences for  
585 abalone populations in natural and aquaculture environments. Because abalone is a slow-growing  
586 gastropod with a long transgenerational period, the possibility for this species to adapt to OA is  
587 probably lower than that of fast-growing molluscs with rapid life cycles. Consequently, wild  
588 abalone populations that are already weakened by anthropogenic pressure (e.g. pollution,  
589 overfishing and diseases), will probably be the most at risk over the next decades. In abalone  
590 aquaculture, various strategies could be developed to mitigate the effects of OA and help farmers  
591 to overcome the challenges posed by climate change. For example, the introduction of macroalgal  
592 culture into abalone farming (through IMTA system, Bolton et al., 2009) might be helpful to  
593 increase pCO<sub>2</sub> in seawater intake and control the pH in nursery tanks, enabling an upstream  
594 protection of the most vulnerable stages. In parallel, the identification of heritable traits involved  
595 in resilience to OA, and the subsequent selection of tolerant broodstock may be exploited in the  
596 future to ensure the sustainable development of abalone aquaculture.

597

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609

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613 Sabine Roussel, Sylvain Huchette: Investigation, Abalone breeding;

614 Stéphanie Auzoux-Bordenave, Apolline Ledoux, Sophie Martin, Aïcha Badou: Carbonate

615 chemistry analysis, Abalone sampling, Biological measurements;

616 Apolline Ledoux, Carole Di Poi : Oxidative stress measurements;

617 Carole Di Poi, Nelly Le Goïc, Marc Suquet : Gamete analysis;

618 Arianna Servili and Fanny Gaillard: mRNA extraction and gene expression

619 Stéphanie Auzoux-Bordenave, Sabine Roussel: Formal analysis, Visualization, Supervision,

620 Funding, Writing-Original Draft, Reviewing and Editing

621

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623

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Journal Pre-proof

- Abalone has experienced severe population decline worldwide due to overfishing, disease and climate change
- The effects of simulated OA were evaluated on reproduction and early life stages of European abalone (*Haliotis tuberculata*) through a transgenerational experiment.
- No carry-over effects were observed on abalone offspring following parental exposure to OA
- Larval and juvenile fitness were affected by low pH
- Species dispersion and survival may be compromised under future conditions, with potential negative consequences for European abalone populations.

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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