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1 **From reproductive behaviour to responses to predators: ocean acidification does not**  
2 **impact the behaviour of an herbivorous marine gastropod.**

3

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26

27 **ABSTRACT**

28 Ocean acidification (OA), which reduces ocean pH and leads to substantial changes in seawater  
29 carbonate chemistry, may strongly impact organisms, especially those with carbonate skeletons.  
30 In marine molluscs, while the physiological effects of OA are well known, with a reduction of  
31 growth and shell calcification, there are few studies on behavioural effects. A large marine  
32 gastropod, *Haliotis tuberculata*, was exposed to ambient (pH<sub>T</sub> 8.0) or low pH (pH<sub>T</sub> 7.7) during  
33 a 5-month experiment. Because animal fitness can be affected through various behavioural  
34 changes, a broad spectrum of behavioural parameters was investigated, including situations  
35 involving no stress, responses to predators, righting to evaluate indirectly the level of energy  
36 reserves, and finally, reproductive behaviour. In addition, we measured the expression profile  
37 of the GABA A-like and serotonin receptor genes, often described as central neuromodulators  
38 of sensory performance and behaviour and known to be affected by OA in molluscs. No  
39 significant effect of low pH as compared to ambient pH was observed on abalone behaviour for  
40 any of these behavioural traits or gene expressions after either one week or several months of  
41 exposure to OA. The significance tests were corroborated by estimating the size of pH effects.  
42 The behaviour of this mollusc appears not to be affected by pH decrease expected by the end  
43 of the century, suggesting some resilience of the species to OA at the adult stage. This is  
44 probably related to the ecological niche of this abalone, where important pH variations can be  
45 observed at tidal, diurnal or seasonal scales.

46

47 **KEYWORDS:** ocean acidification, behaviour, predator, diurnal rhythm, reproductive  
48 behaviour, abalone, *Haliotis tuberculata*

49

50

## 51 **1. Introduction**

52

53 Rising atmospheric carbon dioxide (CO<sub>2</sub>) reduces ocean pH and causes a shift in seawater  
54 carbonate chemistry (Orr et al., 2005; Doney et al., 2009). The average ocean surface water pH  
55 is expected to decrease at least by 0.3 pH unit at the end of the century if annual CO<sub>2</sub> emissions  
56 continue to increase and are not stabilised rapidly (scenarios with high and very high  
57 greenhouse gas concentrations, SSP3 -7.0 and SSP5 – 8.5) (IPCC, 2021). These changes will  
58 take place at an unprecedented rate for marine organisms (Gattuso et al., 2015) threatening  
59 calcifying species such as corals and molluscs (Hofmann et al., 2010; Kroeker et al., 2013).

60

61 One way animals may respond to a changing environment is through modifications of their  
62 behaviour (Nagelkerken et Munday, 2016). Ocean acidification (OA) affects a plethora of  
63 marine animal behavioural traits. A majority of the studies have been done on fish behaviour,  
64 focusing specifically on predator-prey interactions: larval, juveniles or adult fishes become less  
65 risk averse, and are even attracted to the odour of predators. In addition, a reduction of the  
66 ability to find settlement sites and to find food or prey was reported (reviews from Briffa et al.  
67 2012; Nagelkerken and Munday 2016; Ashur et al. 2017). Results are much less consistent in  
68 marine invertebrates, with contrasting results depending on taxon, stage, pCO<sub>2</sub> exposure and  
69 the behaviour studied (Clements et Comeau, 2019). Some experiments have reported negative  
70 effects on predator avoidance (Manriquez et al., 2014; Jellison et al., 2016; Manriquez et al.,  
71 2016), predation rate (Dodd et al., 2015), foraging performance (Leung et al., 2015; Horwitz et  
72 al., 2020; Park et al., 2020), shelter use (Park et al., 2020), swimming behaviour (Gravinese et  
73 al., 2019), response to flow (Cohen-Rengifo et al., 2019), righting time (Manriquez et al., 2013)  
74 or decision-making (de la Haye et al., 2011). However, others found no detectable effects on  
75 the ability to right (Schram et al., 2014; McCarthy et al., 2020), capacity to detect the position

76 of the prey/food (Manriquez et al., 2014), escape ability (Schram et al., 2014) or motility  
77 (Jellison et al., 2016). Some even observed increased avoidance behaviour to predators (Bibby  
78 et al., 2007), increased activity (Watson et al., 2017) or righting ability (Manriquez et al., 2016).

79

80 Several factors may explain these contrasted effects. The difference in the reduction of pH  
81 might be a factor explaining the contrasted results. In some experiments, molluscs were exposed  
82 to a pH reduction similar (0.3-0.4 pH unit) to the one expected at the end of century in scenarios  
83 with intermediate or high greenhouse gas concentrations in subtidal environment (Manriquez  
84 et al., 2014; McCarthy et al., 2020; Park et al., 2020) while in other experiments, pH reduction  
85 was stronger with fluctuations up to pH observed in intertidal rock pools, estuaries or during  
86 upwelling processes (Dodd et al., 2015; Jellison et al., 2016; Manriquez et al., 2016). Some  
87 species may have been subjected to greater pH fluctuations in estuarine and tidepool  
88 environments than the overall level expected by the end of the century, and thus have evolved  
89 resilience. Another explanation is the difference between experiments of exposure time to low  
90 pH: some short-term experiments have exposed species to low pH for only a few hours or days  
91 (for a review, see Briffa et al. 2012, Clements and Comeau 2019), while other exposure times  
92 involved a few weeks (Bibby et al., 2007; Schram et al., 2014) or several months (Manriquez  
93 et al., 2013; Manriquez et al., 2014). In addition, most of these studies were based on only few  
94 behavioural variables, which probably are not representative of the complex behavioural  
95 responses of an animal facing environmental stress.

96

97 *Haliotis spp.* are calcifying gastropods in the order Vetigastropoda, and some are among the  
98 largest marine herbivores in the world. Most species live on rocky shores, in narrow crevices  
99 or under boulders, in low-tidal zone and shallow subtidal environments. On the Atlantic coast  
100 of France, *H. tuberculata* is naturally exposed to variable seawater pH for several hours during

101 emersion of spring tides in rock pools of the low tide zone. In rock pools of the mid-tidal zone,  
102  $pH_T$  can decrease down to 7.5 at night and increase up to 10 during day-light periods (Legrand  
103 et al., 2018). However, most of the individuals are found in subtidal zones in a much less  
104 variable pH environment. For instance, in the Bay of Brest, one of this species natural habitats,  
105  $pH_T$  ranges between 7.9 in autumn up to 8.2 during winter and spring periods (Qui-Minet et al.,  
106 2018).

107

108 With this study, we aimed to elucidate the effects of OA using a high greenhouse gas emission  
109 scenario (IPCC, 2021) on a wide range of key behavioural parameters that determine the  
110 persistence of this mollusc found worldwide in coastal area. An experimental approach with  
111 different time-scales and extensive behavioural measurements was undertaken. The tests were  
112 performed one week and up to five months after the start of OA exposure. In addition to stress  
113 responses under different pH conditions, unstressed abalone behaviour was studied in the  
114 experimental aquariums, in the absence of any handling stress. Because reproductive behaviour  
115 directly impacts the fitness of a species, spawning behaviour was also measured under ambient  
116 and decreased pH. Furthermore, because the effects of OA on behaviour might be a  
117 consequence of chemosensory impairment, altered central processing impairment, or both  
118 (Ashur et al., 2017), we measured the expression profile of two genes involved in neurosensory  
119 transmission (the GABA A-like receptor and the serotonin receptor). The consequences of OA  
120 on behaviour of marine species has recently been quite controversial (Clark et al., 2020). To  
121 take into the “decline effect” in OA impacts reported on fish behaviour (i.e. the decrease in  
122 significant negative effects found in recent studies compared to initial studies), the estimated  
123 size effect was calculated for each test performed in the present study (Clements et al., 2022)  
124 to highlight the magnitude of any difference, if any was present.

125

126 **2. Materials and methods**

127

128 *2.1. Abalone collection and experimental set-up*

129

130 In January 2017, 3.5-year-old *H. tuberculata* were collected from an offshore sea-cage structure  
131 at the France Haliotis abalone farm (48°36'50N, 4°36'3W; Plouguerneau, Brittany, France)  
132 containing 600 individuals ( $48.5 \pm 4.2$  mm shell length,  $16.2 \pm 4.4$  g shell weight). During the  
133 sea-rearing procedure, fresh algae, mainly composed of a mixture of *Palmaria palmata*,  
134 *Laminaria digitata* and *Saccharina latissima*, were collected on the shore and provided *ad*  
135 *libitum* to each sea-cage once a month. Abalone were brought to the France Haliotis land-based  
136 facilities in the sea-cage in less than one hour, ensuring minimum stress during transport and  
137 minimum handling. After gently detaching the abalone from their support with a spatula, each  
138 abalone was measured and weighed. Because abalone are mostly nocturnal foragers (Roussel  
139 et al., 2020), a phosphorescent tag was glued to the shell with cyanoacrylate gel to record their  
140 night-time behaviour. In addition, numbered Hallprint® tags were glued to the shell to identify  
141 the abalone individually in tanks.

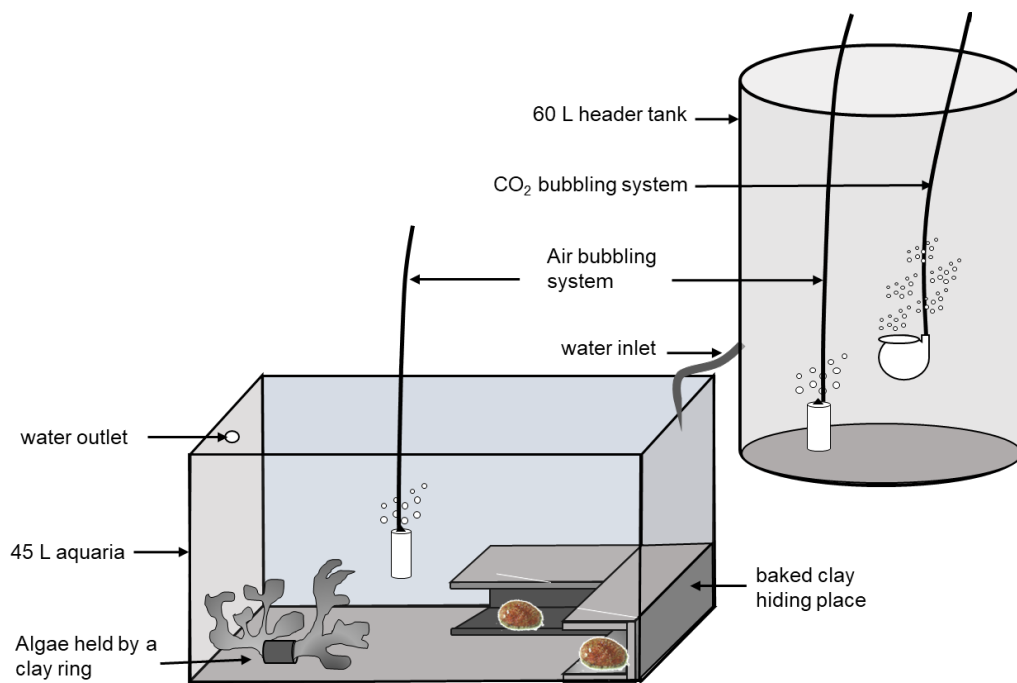
142

143 The individuals were randomly distributed into ten 45 L experimental rearing tanks (1 x w x h,  
144 50 x 30 x 35 cm) equipped with baked clay hiding places ( $n = 26$  abalone per tank) and the  
145 abalone were fed twice every week with macroalgae. A baked clay ring weight was used to  
146 hold the algae on the bottom of the tank, opposite the hiding place (Figure 1). The daily light  
147 versus dark regime was adjusted following the seasonal cycle (9 : 15 hr in February, 10 : 14 in  
148 March, 11 : 13 in April, 12 : 12 in May, and 13 : 11 in June). Temperatures were adjusted  
149 monthly from 12.5 C in January to 18.5 C in June to follow the natural seasonal change. To  
150 avoid stressful conditions during light/dark changes, a transition of light level was programmed

151 over 30 min during dawn and dusk using a dimmer (Gold Star, Besser Elektronik, Italy).  
152 Abalone were conditioned in the laboratory for three weeks before the start of the experiment  
153 under ambient pH/ $p\text{CO}_2$  conditions.

154

155 A detailed description of the experimental set-up can be found in Avignon et al. (2020). Briefly,  
156 five tanks were randomly assigned to each of two pH treatments: a control condition  
157 corresponding to the local seawater pH ( $\text{pH}_T$  of 8.0, corresponding to a  $p\text{CO}_2$  of  $\sim 460 \mu\text{atm}$ )  
158 and a lower pH value ( $\text{pH}_T$  of 7.7,  $p\text{CO}_2$  of  $\sim 1000 \mu\text{atm}$ ) corresponding to the projected decrease  
159 of -0.3 pH units under climate change scenario SSP3-7.0 (IPCC, 2021). Experimental design  
160 recommendations in OA research (Cornwall et Hurd, 2016) were followed by using header  
161 tanks (Figure 1) with independent  $\text{CO}_2$  bubbling and pH control as well as conversion to pH  
162 units on the total scale ( $\text{pH}_T$ ) after calibration with Tris/HCl and 2-aminopyridine/HCl buffers  
163 (Dickson, 2010).



164

165

166 **Fig. 1:** Experimental apparatus showing the experimental rearing tank and header tank where  
167  $p\text{CO}_2$  was adjusted to the target pH.



168

169

## 170 *2.2. Control and monitoring of pH and carbonate parameters*

171

172 In February 2017, pH values in the header tanks for the low pH treatments were gradually  
173 decreased over 6 days by 0.05 pH units / day until  $pH_T$  7.7 was reached. Thereafter, the pH  
174 treatments were maintained for 5 months between February and the end of June 2017. In the  
175 five  $CO_2$ -enriched header tanks,  $pCO_2$  was adjusted by bubbling  $CO_2$  (Air Liquide, France)  
176 through electro-valves controlled by a pH-stat system (IKS Aquastar, Germany). Experimental  
177 tanks were continuously supplied with seawater from the header tanks with a minimum of 15  
178  $l \cdot h^{-1}$ . Total alkalinity ( $A_T$ ) of seawater was measured monthly on 50-mL samples taken from  
179 each experimental tank according to the method described in Avignon et al. (2020).  
180 Calculations of  $pCO_2$ , dissolved inorganic carbon (DIC),  $HCO_3^-$ ,  $CO_3^{2-}$  concentrations, and  
181 saturation state of aragonite ( $\Omega_{ar}$ ) and calcite ( $\Omega_{ca}$ ) are detailed in Avignon et al. (2020).

182

## 183 *2.3. Behavioural test procedures*

184 A trained observer carried out behavioural analyses for the diurnal, hiding, righting, predator  
185 and foot contact tests described below, using the Observer program (Observer©XT, Noldus).

186 It was not possible to do blinded analyses as the abalone were identified with individual tags.

187 The diurnal rhythm, feed intake and foot contact tests, involving no or mild stress, were done  
188 in the experimental rearing tanks using all the abalone in the tank. Tests were performed at the  
189 same pH/ $pCO_2$  as those of the treatment just after reaching the target pH to measure acute stress  
190 responses to pH decrease (week 1, W1), and several months after exposure started to measure  
191 chronic stress effects (three, four or five months, respectively - M3, M4, M5) (see below for  
192 details).

193 Responses to acute stress situations such as the righting, hiding and predator tests were done in  
194 an aquarium or a raceway in the laboratory next to the experimental facility. Aquarium or  
195 raceway was cleaned 3 times and renewed with clean water before testing each individual  
196 abalone. It was filled with seawater taken from the abalone buffer tank from the same treatment.  
197 Four abalone from each experimental tank were tested one week after reaching the target pH  
198 (W1). Different abalone were tested for each behavioural test to avoid handling bias. The same  
199 individuals were tested again after 3 months of pH exposure (M3). A 3-month period is  
200 sufficiently long to allow a recovery from the experimental procedure for abalone. The four  
201 abalone from the same tank were tested consecutively and returned to their tank after all were  
202 tested to avoid confronting other abalone with stress cues. Treatments were tested alternatively  
203 but in a random sequence of the five replicate tanks.

204

### 205 *2.3.1. Diurnal rhythm*

206 Diurnal rhythm was studied the first 48h after reaching the target pH (W1, using 26 abalone per  
207 tank and n = 5 tanks per treatment) and after 3 months of exposure to low pH (M3, using a  
208 minimum of 16 abalone per tank and n = 5 tanks per treatment). Measures were done with no  
209 handling stress: all experimental aquariums were continuously videotaped with 3 digital  
210 cameras (TS-WD6001HPSC, Sygonix GmbH, Germany), linked to a 24h-recording device  
211 (TVVR 40021, Abus, Germany). Videos were recorded over 48-h for all the aquariums and  
212 analysed using scan sampling with the Observer program. Every 10 minutes, the number of  
213 abalones moving (i.e. the number that had changed position between two scan samplings), the  
214 number of abalones eating algae (less than 2 cm from the algae with small movements of the  
215 algae observed), and the number of abalones in the open zone (not under the baked clay hiding  
216 place or next to the algae) were recorded. From these data, the following variables were

217 calculated per aquarium: time spent moving ( $\text{min.abalone}^{-1}.\text{day}^{-1}$ ), time spent feeding  
218 ( $\text{min.abalone}^{-1}.\text{day}^{-1}$ ) and time spent in open zone ( $\text{min.abalone}^{-1}.\text{day}^{-1}$ ).

219

### 220 2.3.2. Feed intake

221 Feed intake was measured over the first 3 months, two or three times a week (28 measures per  
222 tank,  $n = 5$  tanks per treatment). At each food distribution, fronds of *Palmaria palmata* were  
223 delicately dried on absorbent paper, weighed and then placed in the clay ring opposite to the  
224 hiding place. *P. palmata* is the most appropriate alga for *H. tuberculata* growth (Roussel et al.,  
225 2019a). The remaining algae were removed, dried with the same method and weighed. Two  
226 additional samples of this alga were placed in two extra aquariums submitted to normal and low  
227 pH but without abalone. The quantity of wet algae (in mg) ingested per gram of wet abalone in  
228 each aquarium was calculated, taking into account the degradation of the algae under each  
229 treatment in the extra aquariums. Weights of abalone in each aquarium were estimated using  
230 initial weight before acclimation, W1 and M2 measurements (Avignon et al., 2020).

231

### 232 2.3.3. Foot contact test

233 This test was performed 4 days after reaching the target pH (W1,  $n = 105$  abalone per treatment)  
234 and after 3 months of exposure (M3,  $n =$  minimum 83 abalone per treatment). The test involved  
235 gently touching the abalone resting (relaxed foot with epipodium visible) with a finger on the  
236 outer mantle edge border, and running the finger clockwise around the abalone in 10 s while  
237 the abalone were in their tanks. The test stopped once the abalone returned to their initial resting  
238 position (or after 90 s if they did not return). The following variables were measured: number  
239 of abalones doing a swivelling movement after touching (swivelling movement of the abalone  
240 shell from one side to the other, often observed to break the predator grip), number of abalones  
241 changing position after touching (epipodium i.e. appendages along the foot with sensory organs

242 or / and foot contraction) and number of abalone changing of position and returning to their  
243 initial position before the end of the observation period.

244

#### 245 2.3.4. *Righting test*

246 A glass aquarium ( $w \times l \times h$ ,  $20 \times 35 \times 20$  cm) was used, filled with 5 l of seawater. Individual  
247 abalone were placed on their back in the centre of the aquarium. The time to right was measured  
248 from the time the abalone were placed in the aquarium on their shell until they had fully turned  
249 over. The number of attempts to turn over (defined as the number of times the abalone placed  
250 its foot on the bottom and contracted its muscle) was also recorded. If an abalone did not  
251 successfully turn over after a delay of 4 min, a time to right of 4 min was recorded for this  
252 abalone.

253

#### 254 2.3.5. *Hiding test*

255 A glass aquarium ( $w \times l \times h$ ,  $20 \times 35 \times 20$  cm), filled with 5 l of seawater, and with a halogen  
256 light above, was equipped with a baked clay shelter positioned at one end of the aquarium.  
257 Abalone were placed on their foot at the side of the test aquarium opposite the shelter. The time  
258 until the first movement and the time before complete hiding were measured, with a maximum  
259 time of 15 min if an abalone did not move into the shelter.

260

#### 261 2.3.6. *Starfish predator test*

262 Starfish *Marthasterias glacialis* (20 cm width) were collected from the offshore abalone sea  
263 cages of France Haliotis, placed in a 30 L aquarium and fed with dead, non-experimental  
264 abalone twice a week during the experimental test period. A raceway ( $l \times w \times h$ ,  $2.5 \times 0.4 \times$   
265  $0.15$  m) filled with 50 l of seawater was used, with a  $10 \times 10$  cm square grid printed on the  
266 bottom and on the side of the raceway. An abalone was placed in the centre of the raceway on

267 its foot. When the abalone had a semi-relaxed or relaxed foot attached to the bottom of the tank,  
268 the starfish was held in contact with the abalone foot for 10 s. While the starfish touched the  
269 abalone, any protective swivelling movement of the abalone shell was recorded as well as any  
270 mucus release, any turn-around behaviour and any movement directly away from the starfish.  
271 The number of abalone that performed these four escape behaviours was recorded. The time  
272 until the first movement was measured. In addition, the time spent moving, the number of  
273 squares crossed as well as the time to reach the edge of a circle of radius 20 cm away from the  
274 predator were recorded during the 5 min period of the test.

275

### 276 *2.3.7. Spawning behaviour*

277 The spawning behaviour was measured after 5 months of exposure (M5) in the spawning room  
278 of France Haliotis. A LED light covered with a red filter (Medium red, Rosco Supergel) allowed  
279 us to observe abalone behaviour while not disturbing them during the spawning process. On the  
280 day of spawning induction, a maximum of 4 female and 4 male abalone were selected per tank  
281 out of the 12 abalone available (i.e. abalone used for the righting, hiding and predator abalone  
282 tests). Sex-ratios were equalised as far as possible. In total, 34 abalone for  $\text{pH}_T$  7.7 and 37  
283 abalone for  $\text{pH}_T$  8.0 were studied. Abalone were placed individually in 5 L buckets with  
284 continuous water renewal. Spawning induction was performed by shining ultraviolet (UV) light  
285 and simultaneously heating the filtered seawater fed into the buckets from 17°C to 21°C over  
286 the course of 1h. Two buffer tanks were used connected to the 5 L buckets: one tank at  $\text{pH}_T$  8.0  
287 and one tank at  $\text{pH}_T$  7.7 to match the treatment applied to the abalone. The circulation of UV  
288 irradiated water was stopped as the abalone started to spawn. Abalone were allowed to spawn  
289 for 5.5 h from the start of the experiment. The time of active preparation movement (active  
290 movement in the bucket, crawling to the top of the bucket or a swivelling behaviour) as well as  
291 the duration of spawning (ejection of the gametes into the water column) and the number of

292 abalone spawning was recorded. Effect of pH on other reproduction parameters are presented  
293 in Avignon et al. (2020).

294

#### 295 2.4. Gene expression analysis

296 The expression profiles of selected genes were analysed in the head of one to two individual  
297 abalone per aquarium ( $n = 9$  for pH 8.0 and  $n = 10$  for pH 7.7) after four months (M4) of  
298 exposure to ambient and low pH. The GABA A-like receptor and the serotonin (5-HT) receptor  
299 genes were selected because of their putative functions in the neuroendocrine regulation of  
300 behaviour (notably locomotion, memory and learning) in gastropods. The methods of gene  
301 expression analysis are described in Avignon et al. (2020).

302 As a predicted sequence for the serotonin receptor of *Haliotis tuberculata* we used the sequence  
303 TR104080\_c0\_g1\_i2 from the transcriptome of this species (Harney et al., 2016) as  
304 homologous sequence of *Haliotis rubra* 5-HT receptor mRNA (GenBank accession number  
305 AY237917.1). We carried out a tblastx search of the *Haliotis asinina* GABA A receptor  
306 sequence GenBank (accession no. EF222254) against the unfiltered transcriptome of *Haliotis*  
307 *tuberculata*. This found 38 unigenes with hits, of which 13 had E-values  $< 1E-3$ . We carried  
308 out blastx against the non-redundant database of *Haliotis tuberculata* with all 38 candidate  
309 sequences. Based on sequence similarities to *Aplysia californica* GABA A receptor sequences  
310 (Moroz et al., 2006), we selected the predicted sequence TR57267\_c2\_g1\_i1 as the best *H.*  
311 *tuberculata* GABA A-like receptor candidate gene. The PCR products obtained with the  
312 primers shown in Table 1 were sequenced to verify potential errors in the predicted sequences  
313 and primers. Afterward, the same primers were used in RT-qPCR to target the specific genes  
314 of the *Haliotis tuberculata* serotonin receptor and GABA A-like receptor. 18S and EF1 were  
315 used as reference genes to normalise the values of expression levels.

316

317 **Table 1**

318 Specific primers used for gene expression analysis in *Haliotis tuberculata*: Genbank accession

319 number, primer sequences and reference.

Gene	Sequence 5'-3'	Reference
GABA A-like receptor	F- GCACACCGAGTGTGAGAAGA	(Auzoux-Bordenave et al., 2022)
	R- CAACACTGAATGTGGCGAAC	
Serotonin receptor	F- GCTACCACGACGACCATATC	(Auzoux-Bordenave et al., 2022)
	R- CCTTTCACGCTTTAGTTCCA	
18S	F- GGTTCAGGGGAAGTATGGT	Gaume <i>et al.</i> (2014)
	R- AGGTGAGTTTTCCCGTGTTG	
EF1	F- ATTGGCCACGTAGATTCTGG	Gaume <i>et al.</i> (2014)
	R- GCTCAGCCTTCAGTTTGTCC	

320

321

322 *2.5. Ethical notes*

323 All the abalone used for behavioural experiments were returned to the commercial sea-cages at  
324 the end of the experiment. At the end of the tests, starfish were released in the field where they  
325 were collected.

326

## 327 *2.6. Statistical analysis*

328 All statistical analyses were performed with R software (R Core Team, 2015). Where  
329 continuous data from individual abalone were recorded (Righting test, hiding test, starfish  
330 predator test, spawning behaviour, gene expression), differences between treatments were  
331 tested using linear mixed models with the lmerTest package (Kuznetsova et al., 2017) based on  
332 the methods described by Winter (2013). This model used the pH as a fixed factor and aquarium  
333 as a random factor. In addition, the abalone length was added as covariate for behavioural tests.  
334 For the diurnal rhythm, a mixed model was used with pH and days as a fixed factor, and the  
335 aquarium as a random factor. Statistical analysis was performed separately for the data obtained  
336 after reaching the target pH (W1) and after several months of low pH exposure (M3, M4 or M5  
337 tests). The denominator degrees of freedom and F statistic were computed using Satterthwaite's  
338 method. The normality of the residuals was verified with Shapiro's test and homogeneity of  
339 variance with Levene's test. When assumptions of homogeneity of variance and normal  
340 distribution of residuals were not confirmed, the data were log, inverse or square root  
341 transformed. If normality of residuals was not verified after data transformations, values from  
342 individual abalone were averaged by aquarium, and aquariums were used as the replicates (n =  
343 5 per treatment) in a Mann-Whitney U test (also called Wilcoxon rank sum test). If homogeneity  
344 of variance could not be verified after data transformations, a Welch's test was applied using  
345 averaged values per tank. Differences were considered significant at  $p < 0.05$ , and a trend at  $p$   
346  $< 0.10$ . Data are presented as means of treatments  $\pm$  standard error unless otherwise indicated.  
347 Where the data recorded were counts (i.e. foot contact test), contingency tables were used and



348 a Pearson's chi-square test was performed or a Fisher's exact test if there were less than 5  
349 expected counts per cell. No aquarium effect could be included when using contingency table  
350 analysis.

351 Effect size and effect size variances were estimated for the continuous and ratio-type  
352 behavioural variables of this experiment using the methodology of Clements et al. (2022) to  
353 evaluate how the difference between the two treatments was large. A logarithmic transformed  
354 response ratio was calculated using the formula:

$$355 \ln RR = \ln$$

356 where  $X_{pH7.7}$  and  $X_{pH8}$  are the average measured response in each pH treatment.

357

358 Effect size variance was calculated as

$$359 v = \frac{(S_{pH7.7})^2}{n_{pH7.7} X_{pH7.7}^2} + \frac{(S_{pH8})^2}{n_{pH8} X_{pH8}^2}$$

360

361 where S and n are the standard deviation and sample size for pH<sub>7.7</sub> and pH<sub>8</sub> treatment, and  $X_{pH7.7}$   
362 and  $X_{pH8}$  are the average measured response in each experimental and control treatment.

363 For continuous variables, behavioural data were averaged per tank to take into account potential  
364 tank effects (sample size, n = 5 per treatment). For binomial data (such as the number of abalone  
365 that changed their position), percentages were calculated per tank (n = 5 per treatment) and used  
366 to calculate the effect size variance and effect size.

367

### 368 **3. Results**

369

#### 370 *3.1. Behavioural tests*

371

##### 372 *Diurnal rhythm and feed intake*

373 No significant effect of decreased pH was observed at either W1 or M3 for the time spent  
 374 moving (W1:  $F_{1,8} = 0.259$ ,  $p = 0.624$ ; M3:  $F_{1,8} = 2.183$ ,  $p = 0.178$ , mixed model analysis), the  
 375 time spent feeding (W1:  $F_{1,8} = 3.416$ ,  $p = 0.102$ ; M3:  $F_{1,8} = 0.966$ ,  $p = 0.354$ , mixed model  
 376 analysis), and the time spent in the open area of the tank floor (W1:  $F_{1,8} = 0.564$ ,  $p = 0.474$ ; M3:  
 377  $F_{1,8} = 2.504$ ,  $p = 0.152$ , mixed model analysis) (Table 2). No significant effect of the covariate  
 378 abalone length was found. Similarly, the feed intake was not significantly affected by the pH  
 379 during the first 3-months ( $W_{1,8} = 0.083$ ,  $p = 0.780$ , Welch's test, Table 2).

380

381 **Table 2**

382 Behaviour responses of adult *Haliotis tuberculata* exposed to ambient pH ( $pH_T$  8.0) and low pH  
 383 ( $pH_T$  7.7). Diurnal behaviour was observed by scan sampling during a 48-h period after 2 days  
 384 (W1, 26 abalone per tank, 5 tanks per treatment) and after 3 months of exposure (M3,  $n = 17$ -  
 385 18 abalone per tank, 5 tanks per treatment). Feed intake was measured twice a week during the  
 386 first three months of exposure. Mixed model analysis and  $lsmeans \pm S.E.M$ , except otherwise  
 387 stated.

388

pH	pH 8.0	pH 7.7	$F_{1,8} / F_w$	P
<i>W1 diurnal behaviour (min. abalone<sup>-1</sup>.day<sup>-1</sup>)</i>				
Time spent moving	60.0 ± 6.09	55.7 ± 6.09	0.259	0.624
Time spent feeding	19.6 ± 2.22	13.8 ± 2.22	3.416	0.102
Time spent in open zone	56.1 ± 9.49	46.0 ± 9.49	0.564	0.474
<i>M3 diurnal behaviour (min.abalone<sup>-1</sup>.day<sup>-1</sup>)</i>				
Time spent moving	49.3 ± 5.44	37.9 ± 5.44	2.183	0.178
Time spent feeding	37.0 ± 6.71	27.6 ± 6.71	0.966	0.354
Time spent in open zone	54.1 ± 6.28	40.1 ± 6.28	2.504	0.152

---

*Feed intake during a 3-month period*

---

Quantity of algae ingested per gram of	31.0 ± 0.70	30.7 ± 0.70	0.083	0.780
abalone (mg algae. g <sup>-1</sup> abalone. day <sup>-1</sup> ) <sup>δ</sup>				

---

389 <sup>δ</sup> Welch's test, mean ± s.e.

390

391

392 **Table 3**

393 Behavioural responses of abalone *Haliotis tuberculata* exposed for 1 week (W1), three months (M3) or five months (M5) to ambient pH (pH<sub>T</sub> 8.0)  
 394 or to low pH (pH<sub>T</sub> 7.7) (chi-square or mixed model F test unless otherwise stated). Results are lsmeans ± s.e.m if mixed model analysis, otherwise  
 395 counts or means ± s.e.

396

Tests	W1				M3			
	pH 8.0	pH 7.7	F/ $\chi^2$ /W	p	pH 8.0	pH 7.7	F/ $\chi^2$ / W	p
<i>Foot contact test</i>								
Number of abalone doing a swivelling movement <sup>δ</sup>	3 out of 105	3 out of 105	0 <sup>δ</sup>	1 <sup>δ</sup>	14 out of 83	7 out of 86	2.96	0.086
Number of abalone changing of position after touching	63 out of 105	61 out of 105	0.078	0.779	54 out of 83	54 out of 86	0.094	0.759
	42 out of 105	38 out of 105	0.389	0.533	49 out of 83	48 out of 86	0.179	0.672
Number of abalone returning to their initial position								
<i>Righting test</i>								
Time to right (s)	75.2 ± 15.21	91.9 ± 15.21	0.965	0.332 <sup>β</sup>	62.5 ± 20.92	108.3 ± 20.65	1.61	0.239 <sup>β</sup>
Number of attempts to turn over	1.65 ± 0.42	1.85 ± 0.32	17	0.396 <sup>γ</sup>	2.2 ± 0.79	3.4 ± 0.53	20.50	0.115 <sup>γ</sup>
<i>Hiding test</i>								

Time until the first movement (s)	131.3 ± 34.96	47.1 ± 34.96	1.24	0.297 <sup>β</sup>	28.4 ± 4.81	23.2 ± 4.71	0.34	0.564 <sup>β</sup>
Time before complete hiding (s)	406.0 ± 81.86	370.6 ± 80.56	16	0.548 <sup>γ</sup>	172.5 ± 39.74	156.5 ± 38.74	0.61	0.439 <sup>β</sup>
<i>Predator test</i>								
Number of abalone performing the four escape behaviours	2 out of 20 45.9 ± 5.61	3 out of 20 31.3 ± 5.61	0.23 3.95	0.633 <sup>δ</sup> 0.082 <sup>β</sup>	10 out of 18 45.0 ± 29.39	6 out of 20 30.0 ± 5.00	2.54 7	0.111 0.309 <sup>γ</sup>
Time until the first movement (s)	216.0 ± 16.20	223.9 ± 8.15	14	0.841 <sup>γ</sup>	192.5 ± 13.30	190.1 ± 14.04	11	0.841 <sup>γ</sup>
Time spent moving (s)	4.9 ± 0.79	6.5 ± 0.79	1.92	0.173	7.6 ± 1.13	7.0 ± 1.07	0.026	0.872 <sup>β</sup>
Number of squares crossed	197.7 ± 19.57	171.2 ± 19.57	1.47	0.233 <sup>β</sup>	160.6 ± 45.72	133.4 ± 39.51	9	0.548 <sup>γ</sup>
Time to reach 20 cm far from the predator (s)								
	M5							
<i>Spawning behaviour</i>								
Number of abalone spawning	31 out of 37	30 out of 34	0.29	0.737 <sup>δ</sup>				
Time of preparation (min)	187.6 ± 18.49	178.1 ± 18.79	0.12	0.735				
Time of spawning (min)	245.1 ± 8.00	232.0 ± 10.46	0.96	0.360 <sup>β</sup>				

397 <sup>δ</sup> Fisher's exact test    <sup>γ</sup> Wilcoxon rank sum test with continuity correction    <sup>β</sup> mixed model with log, inverse or square root transformations

398 *Foot contact test*

399 There was no difference between the numbers of abalone swivelling after touching under the  
400 two pH condition at W1 ( $\chi^2 = 0$ ,  $df = 1$ ,  $p = 1$ , Fisher's exact test), although a trend was observed  
401 at M3, with less abalone swivelling in the pH 7.7 treatment compared to those exposed to pH  
402 8.0 at M3 ( $\chi^2 = 2.96$ ,  $df = 1$ ,  $p = 0.086$ , Pearson's chi-square test). The number of abalone  
403 changing position after touching did not differ significantly between the treatments (W1:  $\chi^2 =$   
404  $0.078$ ,  $df = 1$ ,  $p = 0.779$ ; M3:  $\chi^2 = 0.094$ ,  $df = 1$ ,  $p = 0.759$ , Pearson's chi-square test), nor did  
405 the number of abalone changing position and returning to their initial position before the end  
406 of the observation period (W1:  $\chi^2 = 0.389$ ,  $df = 1$ ,  $p = 0.533$ ; M3:  $\chi^2 = 0.179$ ,  $df = 1$ ,  $p = 0.672$ ,  
407 Pearson's chi-square test, Table 3).

408

409 *Righting test*

410 No significant difference was observed for the time to right (W1:  $F_{1,38} = 0.965$ ,  $p = 0.332$ , mixed  
411 model analysis with inverse transformation; M3:  $F_{1,8.2} = 1.61$ ,  $p = 0.239$ , mixed model analysis  
412 with log transformation), nor for the number of attempts to turn over (W1:  $W = 17$ ,  $p = 0.396$ ;  
413 M3:  $W = 20.5$ ,  $p = 0.115$ , Mann-Whitney U test - Wilcoxon rank sum test, Table 3) between  
414 the two pH treatment.

415

416 *Hiding test*

417 The time until the first movement did not differ significantly between pH treatments (W1:  $F_{1,8}$   
418  $= 1.24$ ,  $p = 0.297$ , mixed model analysis with log transformation; M3:  $F_{1,37} = 0.34$ ,  $p = 0.564$ ,  
419 mixed model analysis with square root transformation), nor did the time before complete hiding  
420 (W1:  $W = 16$ ,  $p = 0.548$ , Mann-Whitney U test - Wilcoxon rank sum test; M3:  $F_{1,37} = 0.61$ ,  $p$   
421  $= 0.439$ , mixed model analysis with inverse transformation, Table 3).

422

423 *Starfish predator test*

424 After the first week, there was a trend towards faster response in the lower pH treatment: these  
425 abalone moved faster after the contact with the starfish (W1:  $F_{1,8} = 3.95$ ,  $p = 0.082$ , mixed  
426 model analysis with inverse transformation) but there was no significant difference at M3 (W  
427 = 7,  $p = 0.309$ , Wilcoxon rank sum test). Similarly, there were no significant differences in the  
428 time spent moving (W1:  $W = 14$ ,  $p = 0.841$ , Wilcoxon rank sum test; M3:  $W = 11$ ,  $p = 0.841$ ,  
429 Wilcoxon rank sum test), the numbers of squares crossed (W1:  $F_{1,38} = 1.92$ ,  $p = 0.173$ , mixed  
430 model analysis; M3:  $F_{1,36} = 0.026$ ,  $p = 0.872$ , mixed model analysis with square root  
431 transformation), the times to reach 20 cm away from the predator (W1 :  $F_{1,38} = 1.47$ ,  $p = 0.233$ ,  
432 mixed model analysis with log transformation; M3:  $W = 9$ ,  $p = 0.548$ , Wilcoxon rank sum test)  
433 and the number of abalone performing the four escape behaviours (W1:  $\chi^2 = 0.23$ ,  $df = 1$ ,  $p =$   
434  $0.633$ , Fisher's exact test; M3:  $\chi^2 = 2.54$ ,  $df = 1$ ,  $p = 0.111$ , Pearson's chi-square test).

435

436 *Spawning behaviour*

437 The number of abalone spawning ( $\chi^2 = 0.24$ ,  $df = 1$ ,  $p = 0.623$ , Pearson's chi-square test), the  
438 time of active preparation movement ( $F_{1,7.5} = 0.29$ ,  $p = 0.603$ , mixed model analysis) and the  
439 time before spawning ( $F_{1,7.1} = 1.26$ ,  $p = 0.298$ , mixed model analysis with inverse  
440 transformation) were all similar between the two treatments (Table 3).

441

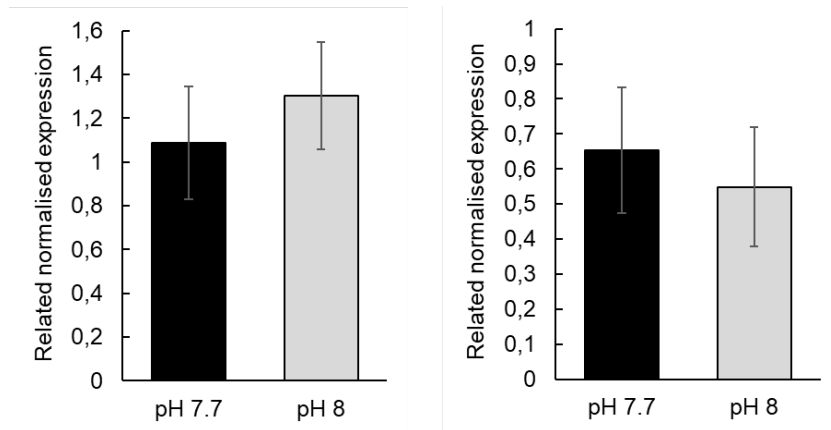
442 *3.2. Gene expression*

443 The gene expressions of the serotonin receptors and GABA A-like receptors were not  
444 significantly different between the two treatments (serotonin receptor:  $F_{1,17} = 0.032$ ,  $p = 0.860$   
445 mixed model analysis with log transformation; GABA A-like receptor:  $F_{1,17} = 0.366$ ,  $p = 0.553$ ,  
446 mixed model analysis) (Figure 2).

447

(A)

(B)



448

449 **Fig. 2:** Gene expression of the (A) GABA A-like and (B) serotonin receptors of adult *Haliotis*  
 450 *tuberculata* exposed for 4 months to ambient pH (pH<sub>T</sub> 8.0) or to low pH (pH<sub>T</sub> 7.7). Lsmeans ±  
 451 S.E.M.

452

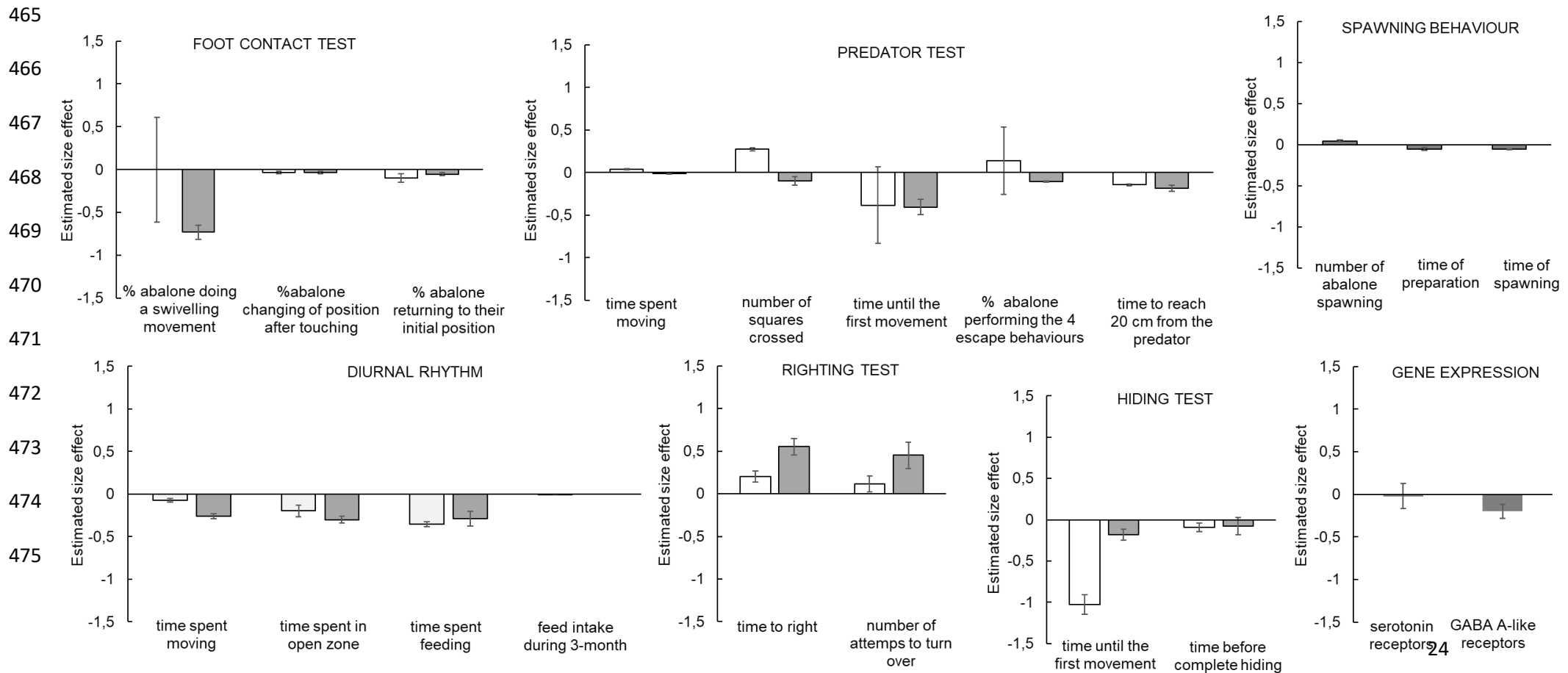
### 453 3.3. Estimated effect size

454 Calculation of estimated effect sizes showed that, if any difference was present for the  
 455 behavioural tests, these differences would be small (Figure. 3): 33 estimated effect sizes were  
 456 below 0.5 out of the 36 variables measured in total for the different periods. In addition,  
 457 estimated effect sizes were inconsistently positive or negative depending of the test and period  
 458 of measure.

459



460 **Fig. 3.** Estimated size effect and variances of *Haliotis tuberculata* studied during a foot contact test, a righting test, a hiding test, a predator test, a  
 461 48-h diurnal rhythm measured in housing tank, stimulated for spawning, or sampled for serotonin and GABA A-like gene expression. Abalone  
 462 were tested 1 week (white column) and several months (three, four or five months, grey column) after exposure to ambient pH (pH<sub>T</sub> 8.0) or low  
 463 pH (pH<sub>T</sub> 7.7). Estimated size effects and variances were calculated following Clements et al (2022) 's methodology, using the number of tanks (n  
 464 = 5 per treatment) as sample size.



#### 476 4. Discussion

477

478 The effect of OA was studied on a large range of behaviour traits that could impact survival  
479 and fitness of the abalone *H. tuberculata*. The reduction by 0.3 pH unit from ambient pH did  
480 not significantly modify responses to predators, righting and hiding abilities, spawning  
481 behaviour, feeding behaviour or any measured diurnal activity pattern, after one week of  
482 exposure as well as after several months of exposure. These tests have already been used for  
483 testing other factors in previous studies, and were sensitive measures for evaluating stress of  
484 abalone (Lachambre et al., 2017a; Lachambre et al., 2017b; Roussel et al., 2019b). In addition,  
485 no change in relative expression was reported for the two genes involved in the neuroendocrine  
486 control of behaviour. Calculation of the estimated size effect showed that almost 90% of the  
487 behavioural variables measured had an estimated effect size (lnRR) between 0.5 and -0.5. Even  
488 if there were some effects of the pH treatments, they were small and non-consistent, with  
489 abalone performing better in pH 8.0 or in pH 7.7 depending on the tests.

490

#### 491 *Effects on behaviour*

492 Because interactions between the prey and their predators constitute an integral part of the  
493 ecology and evolution of marine organisms, as well as the structure and function of  
494 communities, we focused first on prey-predator responses, with a simulation of an attack with  
495 the foot contact test and a direct exposure to a predator. When exposed to a starfish, *H.*  
496 *tuberculata* responds with stereotyped behaviours: it does a swivelling movement, releases  
497 some mucus, turns around and flees thereafter (Roussel et al., 2019b). Similar behavioural  
498 patterns were reported for other species such as *Haliotis rubra* (Day et al., 1995). The responses  
499 to the predator were similar between the pH treatments, indicating little effect of a lower pH on  
500 predator detection and escape responses. These results strongly differ from those obtained in

501 marine fishes, in which an impairment of prey-predator responses due to OA was reported  
502 (reviews by Briffa et al., 2012; Nagelkerken and Munday, 2016; Ashur et al. 2017). In other  
503 gastropods, the effects of pH on prey-predator responses are variable (Clements et Comeau,  
504 2019). Low pH had a negative effect on the response to a predator in a rocky shore species,  
505 *Concholepas concholepas*, by disrupting predator-avoidance behaviour in juveniles reared at  
506 pH<sub>T</sub> 7.7 during five months (Manriquez et al., 2014) and affecting predator-escape response in  
507 juveniles exposed to pH<sub>T</sub> 7.5 during 3 months in comparison to juveniles exposed to current-  
508 day levels at pH<sub>T</sub> 7.7-7.85 (Manriquez et al., 2016). In contrast, some studies found an increased  
509 avoidance in *Littorina littorea* exposed to predator cues when kept at very low pH<sub>NBS</sub> (6.6 for  
510 low pH vs 8.0 for ambient pH) (Bibby et al., 2007), and no effect of 6-week exposure to pH<sub>T</sub>  
511 7.8 on the maximum escape speed of the limpet *Nacella concinna* when exposed to starfish  
512 (Schram et al., 2014).

513 Due to its habitat preference, in subtidal and intertidal areas, *H. tuberculata* can be exposed  
514 to waves and surges (Clavier et Chardy, 1989). For many benthic invertebrates, the ability to  
515 right after detachment by strong wave action reduces vulnerability to predation or unwanted  
516 spatial transport. Hiding is another key behaviour to avoid predation that is crucial for  
517 individual survival, especially for juveniles, for which higher mortalities are reported in the  
518 natural environment (Shepherd et Breen, 1992). In the present study, adult abalone exposed to  
519 ambient pH and low pH took similar times to find a hiding place, to right themselves and to  
520 escape when facing a starfish. As for prey-predator effects, the absence of effects of OA on the  
521 hiding response of the abalone contrasts with the significant effects found on other gastropods.  
522 An experiment performed on the caenogastropod *Concholepas concholepas* found that  
523 juveniles exposed for 83 days to lower pH values (pH<sub>T</sub> 7.8 and 7.6) were faster to right  
524 themselves compared to juveniles exposed to ambient pH<sub>T</sub> of 8.0 (Manriquez et al., 2013). A  
525 full-factorial experiment combining pH and temperature stress on the common limpet *Nacella*

526 *concinna* and the vetigastropod snail *Margarella antarctica* found a non-significant difference  
527 in the proportions of limpets displaying the capacity to right, but a significant temperature-pH  
528 interaction effect on the mean righting times of snails (Schram et al., 2014). In abalone, the  
529 righting time is a reliable indirect indicator of its energetic status and later survival (Lachambre  
530 et al., 2017b). Metabolic rates and immune function measured on other abalone from the same  
531 experiment were not significantly affected by lowered pH in spite of significant effects on shell  
532 growth and calcification (see Avignon et al. 2020 for physiological and calcification measures).  
533 This lack of effects on overall metabolism corresponds with the absence of effect on the righting  
534 behaviour.

535 We found no evidence that OA modified the quantity of algae ingested during the 3-month  
536 exposure of abalone to a reduction of 0.3 pH unit. In addition, there was no detectable difference  
537 in diurnal rhythm, including feeding duration, during 48h observations of a large number of  
538 individuals (130 abalone per treatment for W1 and at least 83 abalone for M3). Few experiments  
539 have measured the long-term effect of acidification on feed intake of herbivores. Results in the  
540 literature are contradictory for OA, even for the same taxon (Nagelkerken et Munday, 2016).  
541 Our result suggests that short term (one week) as well as long term exposure (3 months) to  
542 seawater acidification of 0.3 pH unit does not modify the foraging capacity or the feed intake  
543 of *H. tuberculata*. Our measurement of diurnal rhythm in a “non-stressful” environment,  
544 adapted to the biology of the animals (the presence of a hiding place, access to algae and the  
545 potential for active foraging behaviour) would be expected to provide valuable information  
546 about daily behaviours that would likely occur in a natural context. In addition, the lack of  
547 effects did not result from a stress bias due to a new environment, because abalone had at least  
548 3 weeks of acclimation to the novel environment before the measurements.

549       Reproduction is one of the crucial functions that ensure the persistence of populations and  
550 communities. Abalone are gonochoric species, with external fertilisation occurring in the

551 seawater column (Shepherd et Laws, 1974; Bilbao et al., 2010). Synchronisation of gamete  
552 releases among individuals within a population is important for fertilisation success. Abalone  
553 submitted to OA over 5 months had the same spawning behaviour as adult abalone reared in  
554 ambient pH: the number of abalone spawning as well as the time to preparatory behaviour and  
555 time before spawning were similar between the pH treatments. However, successful  
556 reproduction will depend not only on the behaviour but also on gonad development and gonad  
557 maturity. Dissection of other individuals from the same experiment after 4 months of exposure  
558 to pH treatments (Avignon et al., 2020) showed that the gonad weight was significantly lower  
559 for abalone exposed to pH<sub>T</sub> 7.7, suggesting some disruption in reproduction. To fully  
560 understand the effects of OA on abalone reproduction, a multi-parameter approach using  
561 reproductive behaviour and physiology as well as larval survival is clearly necessary.

562

### 563 *Effect on gene expression*

564 Gene expression profiling has already been used in a panel of organisms exposed to near-future  
565 pH scenarios. The hypothesis was that marine organisms exposed to acidified seawater would  
566 present compensatory changes in behavioural processes that would be reflected by gene  
567 expression changes. In this context we aimed to detect gene expression changes in targeted  
568 genes involved in the processes of neurotransmission and central regulation of behavior when  
569 the abalone *H. tuberculata* was exposed to OA conditions. Recent evidence shows that GABA  
570 and serotonin signaling play major roles in the nervous systems of gastropod mollusks, notably  
571 in the modulation of motor control and cognitive processes such as memory and learning  
572 (Miller, 2019; Aonuma et al., 2020). The GABA A receptor has often been described as  
573 responsible for the sensory impairment at a central level observed in fish exposed to  
574 acidification (Nilsson et al., 2012). However, no modification of the GABA A receptor or  
575 serotonin receptor in the head of these adult abalone was observed. This is in contrast to the

576 effects of low  $pH_{NBS}$  7.85 compared to  $pH_{NBS}$  8.17 for 2-5 days on a caenogastropod, the stromb  
577 *Gibberulus gibbosus*, which produced disrupted predator escape function (Watson et al., 2014).  
578 This effect appeared to be due to malfunction of GABA A-type receptors in the stromb, as the  
579 gabazine blocker of these receptors restored the predator escape behaviour. Our results in  
580 abalone indicate that long term exposure to decreased pH predicted by the “business as usual”  
581 scenario for the end of the century does not significantly impact the expression of their two  
582 main signalling receptors. Interestingly, GABA and serotonin signalling are implied in a wide  
583 range of cognitive functions as the central transmission of sensorial signals and locomotion.  
584 This is in line with the lack of effects of acidification on the tested behaviour (notably the  
585 diurnal rhythms, predator responses and hiding activity).

586

587 *Why no effects?*

588 The most plausible hypothesis to explain the lack of significant effects on adult abalone  
589 behaviour is the evolution of resistance to variable environmental pH. This might be due to the  
590 ecological niche of abalone, in intertidal and/or subtidal areas, where diel and seasonal variation  
591 in coastal  $pCO_2$  can often far exceed near-future projections. In Brittany, *H. tuberculata*  
592 populations in the subtidal zone can experience pH variation from 7.9 up to 8.2 seasonally (Qui-  
593 Minet et al., 2018). In mid-intertidal pools, the fluctuations are even more extreme, with daily  
594 pH variations from 7.5 up to 10 in a few hours. These variations are due to community  
595 respiration with  $CO_2$  release at night and photosynthesis with  $CO_2$  uptake during daylight in  
596 pools that are isolated from the adjacent ocean during low tides, with supplementary variations  
597 according to the season and period of the day (Legrand et al., 2018). The degree of variability  
598 a species encounters is an important consideration to understand biological responses to OA  
599 (Waldbusser et Salisbury, 2014).

600 Pollution and OA can influence animal behaviours in three ways (Briffa et al 2012):  
601 disruption of metabolic processes, reduction of the ability to gather information from the  
602 surrounding environment to make subsequent decisions, and limitation of the ability of an  
603 organism to avoid polluted locations. To be able to evaluate the metabolism and energetic  
604 balance, energetic input should be measured by measuring feed intake as well as energy  
605 expenditure. We showed that feed intake measured during 3 months was very similar between  
606 the treatments. In addition, foraging activity and locomotion was similar for hundreds of  
607 individuals video-recorded in their rearing mesocosms, probably indicating no increased  
608 energetic expenditure in otherwise unstressed situations.

609

610 In addition to behavioural measurements, other functions were studied such as basal  
611 metabolism, showing that it was not impacted by decreased pH in adult *H. tuberculata*  
612 (Avignon et al., 2020). The metabolic process can also be disrupted due to elevated costs for  
613 maintaining acid-base balance (Pörtner et al., 2004). In adult *H. tuberculata*, our previous  
614 studies also found that abalone did not compensate for a seawater pH decrease of 0.3 unit during  
615 the first two months of exposure, but started to acclimate after four months, as suggested by the  
616 compensation of their extracellular pH (Avignon et al. 2020). In addition, there was no  
617 detectable disruption of the ability to gather information and then to make decisions in the  
618 abalone. The similar response to starfish predator cues among pH treatments, the similar time  
619 to find a hiding place and the similar response time after stimulation to show preparatory  
620 behaviour for reproduction or before spawning showed that the ability to detect chemical cues  
621 and temperature change was not impaired in those abalone exposed to low pH. These results  
622 were confirmed by the lack of difference in gene expression of the GABA<sub>A</sub> neurotransmitter  
623 receptor, a major inhibitory receptor implicated in various behavioural pathways (Ashur et al  
624 2017).

625 Behavioural impairment might also be observed in animals that cannot avoid low pH. The  
626 ability to cope with environmental changes will be different depending on the animal mobility.  
627 Adult abalone, in contrast to fish, have limited mobility. They rely on strong attachment to the  
628 substratum by the muscular foot to avoid most predation. Because of this reduced mobility and  
629 the pH variability in their ecological niche, abalone have probably been selected for resilience  
630 to variations in pH, at least for a few hours per day. However, the shell integrity of marine  
631 molluscs is essential to protect themselves from predators such as rays and crabs and is involved  
632 in attachment to the substrate in some gastropods (Shepherd et Breen, 1992). In *H. tuberculata*,  
633 adult shell mechanical properties as well as biomineral architecture were greatly impacted by a  
634 pH reduction of 0.3 unit after several months of exposure (Avignon *et al.*, 2020), suggesting  
635 that OA might reduce protection from predators and resistance to hydrodynamic forces,  
636 potentially impacting wild abalone populations. Even if abalone did not change their prey-  
637 predator responses when facing OA, the more fragile shell might potentially jeopardize wild  
638 populations already threatened by overfishing and environmental perturbations.

639

## 640 **5. Conclusion**

641

642 Biological responses to OA are thought to depend on a number of physiological and life-history  
643 traits at larval, juvenile and adult stages. The results of the present study demonstrated that the  
644 behaviour of adult *H tuberculata* is not impacted by an experimental pH decrease of 0.3 pH  
645 unit, suggesting that the adult stage is probably the most robust stage from a behavioural point  
646 of view in comparison to larval or juvenile stages (Auzoux-Bordenave et al., 2020). Because  
647 abalone naturally experience pH variations in their low-intertidal or high-subtidal environment,  
648 the species apparently has developed a relative resilience to this environmental stress. However,  
649 the net fitness outcome and the capacity of organisms to survive and persist under OA is a



650 complex trade-off between behaviours and other biological processes and should be monitored  
651 over a long term period (i.e at least several months). Although no behavioural impairment  
652 occurred, a reduction of 0.3 pH unit impacted other physiological functions such as calcification  
653 and reproduction in adult *H tuberculata* (Avignon et al., 2020). Together, these results highlight  
654 that a multicriteria approach should be applied when studying the effect of acidification on  
655 marine species.

656

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658

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