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From reproductive behaviour to responses to predators: Ocean acidification does not impact the behaviour of an herbivorous marine gastropod

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Abstract :

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

Graphical abstract

Highlights

► OA does not have any significant effect on the behavioural repertoire of adult abalone. ► No change in the expression of genes involved in sensory performance and behaviour was detected. ► The ecological niche of this species probably allows it to be relatively resilient to OA.

Keywords : ocean acidification, behaviour, predation, diurnal rhythm, reproduction, abalone, Haliotis tuberculata

1. Introduction

Rising atmospheric carbon dioxide (CO_2) reduces ocean pH and causes a shift in seawater carbonate chemistry (Orr et al., 2005; Doney et al., 2009). The average ocean surface water pH is expected to decrease at least by 0.3 pH unit at the end of the century if annual CO_2 emissions continue to increase and are not stabilised rapidly, as in scenarios with high and very high greenhouse gas concentrations, SSP3 -7.0 and SSP5 – 8.5 (IPCC, 2021). These changes will take place at an unprecedented rate for marine organisms (Gattuso et al., 2015) threatening calcifying species such as corals and molluscs (Hofmann et al., 2010; Kroeker et al., 2013).

One way animals may respond to a changing environment is through modifications of their behaviour (Nagelkerken and Munday, 2016). Ocean acidification (OA) affects a plethora of marine animal behavioural traits. A majority of the studies have been done on fish behaviour, focusing specifically on predator-prey interactions: larval, juveniles or adult fishes are reported to become less risk averse, and are even attracted to the odour of predators. In addition, a reduction of the ability to find settlement sites and to find food or prey was reported (reviews by Briffa et al. 2012; Nagelkerken and Munday 2016; Ashur et al. 2017).

Results are much less consistent in marine invertebrates, with contrasting results depending on taxon, stage, pCO_2 exposure and the behaviour studied (Clements and Comeau, 2019). Some experiments have reported negative effects on predator avoidance (Manriquez et al., 2014; Jellison et al., 2016; Manriquez et al., 2016), predation rate (Dodd et al., 2015), foraging performance (Leung et al., 2015; Horwitz et al., 2020; Park et al., 2020), shelter use (Park et al., 2020), swimming behaviour (Gravinese et al., 2019), response to flow (Cohen-

Rengifo et al., 2019), righting time (Manriquez et al., 2013) or decision-making (de la Haye et al., 2011). However, others found no detectable effects on the ability to right (Schram et al., 2014; McCarthy et al., 2020), capacity to detect the position of the prey/food (Manriquez et al., 2014), escape ability (Schram et al., 2014) or motility (Jellison et al., 2016). Some even observed increased avoidance behaviour to predators (Bibby et al., 2007), increased activity (Watson et al., 2017) or righting ability (Manriquez et al., 2016).

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

Haliotis spp. are calcifying gastropods in the order Vetigastropoda, and some are among the largest marine herbivores in the world. Most species live on rocky shores, in narrow crevices or under boulders, in low-tidal zone and shallow subtidal environments. On the Atlantic coast of France, *H. tuberculata* is naturally exposed to variable seawater pH for several hours during spring low tides in rock pools at the low tide zone level. In rock pools of the mid-tidal zone, pH on the total scale (pH_T) can decrease down to 7.5 at night and increase up to 10 during day-light periods (Legrand et al., 2018). However, most of the individuals are found in subtidal zones where the pH is much less variable. For instance, in shallow subtidal habitats of the Bay of Brest, where the species is naturally present, pH_T ranges from 7.9 in autumn to 8.2 during winter and spring periods (Qui-Minet et al., 2018).

This study aimed to elucidate the effects of the OA expected under a high greenhouse gas emission scenario for 2100 (IPCC, 2021) on a wide range of key behavioural parameters that determine the persistence of the species in coastal areas. The experimental approach involved various time-scales and extensive behavioural measurements. Tests were performed one week and up to five months after the start of OA exposure. In addition to stress responses under different pH conditions, unstressed abalone behaviour was studied in the experimental aquariums, in the absence of any handling stress. Because reproductive behaviour directly impacts the fitness of a species, spawning behaviour was also measured under ambient and decreased pH.

Furthermore, because the effects of OA on behaviour might be a consequence of chemosensory impairment, altered central processing impairment, or both (Ashur et al., 2017), we measured the expression profile of two genes involved in neurosensory transmission (the GABA A-like receptor and the serotonin receptor). The consequences of

OA on behaviour of marine species has recently been quite controversial (Clark et al., 2020). To take into account the "decline effect" (i.e. the decrease in significant negative effects found in recent studies of OA effects on fish behaviour compared to initial studies), the estimated size of the effect was calculated for each test performed in the present study (Clements et al., 2022) to highlight the magnitude of the difference, if any was present.

2. Materials and methods

2.1. Abalone collection and experimental set-up

The abalone were produced by systematic mating in each generation between wild and farmed broodstock (either males or females were wild broodstock) to prevent any inbreeding. Juveniles were reared in tanks on plates covered with *Ulvella lens* (Daume et al., 2004) for feeding until 10 months of age before to be transferred into offshore sea-cage structures at the France Haliotis abalone farm (48°36'30N, 4°36'3W; Plouguerneau, Brittany, France). Sea-cage structures (1 m x 0.5 m x 0.5 m) were immerged 5-m to 10-m deep, with a mesh allowing a good flow of water. During the sea-rearing procedure, fresh algae, mainly composed of a mixture of *Palmaria palmata*, *Laminaria digitata* and *Saccharina latissima*, were collected on the shore and provided *ad libitum* to each sea-cage once a month.

In January 2017, 3.5-year-old *H. tuberculata* were collected from a sea-cage containing 600 individuals (48.5 ± 4.2 mm shell length, 16.2 ± 4.4 g shell weight). The abalone were brought to the France Haliotis land-based facilities in the sea-cage in less than one hour, ensuring minimum stress during transport and minimum handling. After gently detaching the abalone from their support with a spatula, each abalone was measured and weighed. Because abalone are mostly nocturnal foragers (Roussel et al., 2020), a phosphorescent tag was glued to the

shell with cyanoacrylate gel to record their night-time behaviour. In addition, numbered Hallprint® tags were glued to the shell to identify the abalone individually in tanks.

The individuals were randomly distributed into ten 45 L experimental rearing tanks (l x w x h, 50 x 30 x 35 cm) equipped with baked clay hiding places (n = 26 abalone per tank) and the abalone were fed twice every week with macroalgae. A baked clay ring weight was used to hold the algae on the bottom of the tank, opposite the hiding place (Figure 1). The daily light versus dark regime was adjusted following the seasonal cycle (9 : 15 hr in February, 10 : 14 in March, 11 : 13 in April, 12 : 12 in May, and 13 : 11 in June). Temperatures were adjusted monthly from 12.5°C in January to 18.5°C in June to follow the natural seasonal change. To avoid stressful conditions during light/dark changes, a transition of light level was programmed over 30 min during dawn and dusk using a dimmer (Gold Star, Besser Elektronik, Italy). Abalone were conditioned in the laboratory for three weeks before the start of the experiment under ambient pH/pCO_2 conditions.

A detailed description of the experimental set-up and carbonate chemistry parameters in the experimental pH treatments can be found in Avignon et al. (2020) and Supplementary Table S1. Briefly, five tanks were randomly assigned to each of two pH treatments: a control condition corresponding to the local seawater pH (pH_T of 8.0, corresponding to a pCO_2 of ~460 µatm) and a lower pH value (pH_T of 7.7, pCO_2 of ~1000 µatm) corresponding to the projected decrease of -0.3 pH units under climate change scenario SSP3-7.0 (IPCC, 2021). Experimental design recommendations in OA research (Cornwall et Hurd, 2016) were followed by using header tanks (Figure 1) with independent CO_2 bubbling and pH control as well as conversion to pH on the total scale (pH_T) after calibration with Tris/HCl and 2-aminopyridine/HCl buffers (Dickson, 2010).

2.2. Control and monitoring of pH and carbonate parameters

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

2.3. Behavioural test procedures

A trained observer carried out behavioural analyses for the diurnal rhythm, hiding, righting, predator and foot contact tests described below, using the Observer program (Observer©XT, Noldus). It was not possible to do blinded analyses as the abalone were identified with individual tags.

The diurnal rhythm, feed intake and foot contact tests, involving no or mild stress, were done in the experimental tanks, using all the abalone in the tank. Tests were performed just after reaching the target pH to measure acute stress responses to pH decrease (week 1, W1), and after three months of exposure (M3) to measure chronic stress effects (see below for details).

Responses to acute stress situations such as the righting, hiding and predator tests were done in an aquarium or a raceway in the laboratory next to the experimental facility. The aquarium or raceway was cleaned 3 times and renewed with clean water before testing each individual abalone. It was filled with seawater taken from the buffer tank for the same treatment. For each acute stress, four abalone from each tank were tested using different abalone to avoid handling bias. The same individuals were tested one week after reaching the target pH (W1) and again after 3 months of pH exposure (M3). A 3-month period is sufficiently long to allow a recovery from the experimental procedure for abalone (Hooper et al., 2011). The four abalone from the same tank were tested consecutively and returned to their tank after all were tested to avoid confronting other abalone with stress cues. The pH treatments were tested alternatively but in a random sequence of the five replicate tanks.

2.3.1. Diurnal rhythm

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

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

2.3.2. Feed intake

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

2.3.3. Foot contact test

This test was performed 4 days after reaching the target pH (W1, n = 105 abalone per treatment) and after 3 months of exposure (M3, n = minimum 83 abalone per treatment). The test involved gently touching an abalone when it was resting in the tank (relaxed foot with epipodium visible) with a finger on the outer mantle edge border, and running the finger clockwise around the abalone in 10 s while the abalone were in their tanks. The test stopped once the abalone returned to their initial resting position (or after 90 s if they did not return). The following variables were measured: number of abalones doing a swivelling movement

after touching (swivelling movement of the abalone shell from one side to the other, often observed to break the predator grip), number of abalones changing position after touching (epipodium i.e. appendages along the foot with sensory organs or / and foot contraction) and number of abalone changing of position and returning to their initial position before the end of the observation period.

2.3.4. Righting test

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

2.3.5. Hiding test

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

2.3.6. Starfish predator test

Starfish *Marthasterias glacialis* (20 cm width) were collected from the offshore abalone sea cages of France Haliotis, placed in a 30 L aquarium and fed with dead, non-experimental

abalone twice a week during the experimental test period. A raceway (l × w × h, 2.5 × 0.4 × 0.15 m) filled with 50 l of seawater was used, with a 10 × 10 cm square grid printed on the bottom and on the side of the raceway. An abalone was placed in the centre of the raceway on its foot. When the abalone had a semi-relaxed or relaxed foot attached to the bottom of the tank, the starfish was held in contact with the abalone foot for 10 s. While the starfish touched the abalone, any protective swivelling movement of the abalone shell was recorded as well as any mucus release, any turn-around behaviour and any movement directly away from the starfish. The number of abalone that performed these four escape behaviours was recorded. The time until the first movement was measured. In addition, the time spent moving, the number of squares crossed as well as the time to reach the edge of a circle of radius 20 cm away from the predator were recorded during the 5 min period of the test.

2.3.7. Spawning behaviour

The spawning behaviour was measured in the spawning room of France Haliotis, after 5 months of exposure (M5) when abalone were mature enough to spawn. A LED light covered with a red filter (Medium red, Rosco Supergel) allowed us to observe abalone behaviour while not disturbing them during the spawning process. On the day of spawning induction, a maximum of 4 female and 4 male abalone were selected per tank out of the 12 abalone available (i.e. from the abalone used for the righting, hiding and predator response tests). Sex-ratios were equalised as far as possible. In total, 34 abalone for pH_T 7.7 and 37 abalone for pH_T 8.0 were studied. Abalone were placed individually in 5 L buckets with continuous water renewal. Spawning induction was performed by shining ultraviolet (UV) light and simultaneously heating the filtered seawater fed into the buckets from 17°C to 21°C over the course of 1h. Two buffer tanks connected to the 5 L buckets were used: one tank at pH_T 8.0 and one tank at pH_T 7.7 to match the treatment applied to the abalone. The circulation of UV

irradiated water was stopped as the abalone started to spawn. Abalone were allowed to spawn for 5.5 h from the start of the experiment. The time of active preparation movement (active movement in the bucket, crawling to the top of the bucket or a swivelling behaviour) as well as the duration of spawning (ejection of the gametes into the water column) and the number of abalone spawning was recorded. The effects of pH on other reproduction parameters are presented in Avignon et al. (2020).

2.4. Gene expression analysis

The expression profiles of selected genes were analysed in the head of one to two individual abalone per aquarium to target most of the neural ganglia including the cerebral ganglion system (n = 9 for pH_T 8.0 and n = 10 for pH_T 7.7) after four months (M4) of exposure to ambient and low pH. The GABA A-like receptor and the serotonin (5-HT) receptor genes were selected because of their putative functions in the neuroendocrine regulation of behaviour (notably locomotion, memory and learning) in gastropods. The sampling procedure and methods of gene expression analysis are described in Avignon et al. (2020).

As a predicted sequence for the serotonin receptor of *Haliotis tuberculata* we used the sequence TR104080_c0_g1_i2 from the transcriptome of this species (Harney et al., 2016) as this sequence was homologous to the *Haliotis rubra* 5-HT receptor mRNA (GenBank accession number AY237917.1). We carried out a tblastx search of the *Haliotis asinina* GABA A receptor sequence GenBank (accession no. EF222254) against the unfiltered transcriptome of *Haliotis tuberculata*. This found 38 unigenes with hits, of which 13 had E-values < 1E-3. We carried out blastx against the non-redundant database of *Haliotis tuberculata* with all 38 candidate sequences. Based on sequence similarities to *Aplysia californica* GABA A receptor sequences (Moroz et al., 2006), we selected TR57267_c2_g1_i1 as the best predicted sequence candidate for the *H. tuberculata* GABA A-

like receptor gene. The PCR products obtained with the primers shown in supplementary Table S2 were sequenced to verify potential errors in the predicted sequences and primers. Afterward, the same primers were used in RT-qPCR to target the specific genes of the *Haliotis tuberculata* serotonin receptor and GABA A-like receptor. 18S and EF1 were used as reference genes to normalise the values of expression levels.

2.5. Ethical notes

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

2.6. Statistical analysis

All statistical analyses were performed with R software (R Core Team, 2015). Where continuous data from individual abalone were recorded (Righting test, hiding test, starfish predator test, spawning behaviour, gene expression), differences between treatments were tested using linear mixed models with the lmerTest package (Kuznetsova et al., 2017) based on the methods described by Winter (2013). This model used the pH as a fixed factor and aquarium as a random factor. In addition, the abalone length was added as covariate for behavioural tests. For the diurnal rhythm, a mixed model was used with pH and days as a fixed factor, and the aquarium as a random factor. Statistical analysis was performed separately for the data obtained after reaching the target pH (W1) and after several months of low pH exposure (M3, M4 or M5 tests). The denominator degrees of freedom and F statistic were computed using Satterthwaite's method. The normality of the residuals was verified with Shapiro's test and homogeneity of variance with Levene's test. When assumptions of homogeneity of variance and normal distribution of residuals were not confirmed, the data

were log, inverse or square root transformed. If normality of residuals was not verified after data transformations, values from individual abalone were averaged by aquarium, and aquariums were used as the replicates (n = 5 per treatment) in a Mann-Whitney U test (also called Wilcoxon rank sum test). If homogeneity of variance could not be verified after data transformations, a Welch's test was applied using averaged values per tank (Day and Quinn, 1989). Differences were considered significant at $p < 0.05$, and a trend at $p < 0.10$. Data are presented as means of treatments ± standard error unless otherwise indicated. Where the data recorded were counts (i.e. foot contact test), contingency tables were used and a Pearson's chi-square test was performed or a Fisher's exact test if there were less than 5 expected counts per cell. No aquarium effect could be included when using contingency table analysis.

In order to evaluate how large the difference between the two treatments (low pH and ambient pH) was, effect size and effect size variances were estimated for the continuous and ratio-type behavioural variables using the methodology of Clements et al. (2022). A logarithmic transformed response ratio was calculated using the formula:

$$\ln RR = \ln\left(\frac{X_{pH7.7}}{X_{pH8}}\right)$$

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

Effect size variance was calculated as

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

where S and n are the standard deviation and sample size for $pH_{7.7}$ and pH_8 treatment, and $X_{pH7.7}$ and X_{pH8} are the average measured response in each experimental and control treatment.

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

3. Results

3.1. Behavioural tests

Diurnal rhythm and feed intake

No significant effect of decreased pH was observed at either W1 or M3 for the time spent moving (W1: $F_{1,8} = 0.259$, p = 0.624; M3: $F_{1,8} = 2.133$, p = 0.178, mixed model analysis), the time spent feeding (W1: $F_{1,8} = 3.416$, p = 0.102; M3: $F_{1,8} = 0.966$, p = 0.354, mixed model analysis), and the time spent in the open area of the tank floor (W1: $F_{1,8} = 0.564$, p = 0.474; M3: $F_{1,8} = 2.504$, p = 0.152, mixed model analysis) (Table 1). No significant effect of the covariate abalone length was found. Similarly, the feed intake was not significantly affected by the pH during the first 3-months ($W_{1,8} = 0.083$, p = 0.780, Welch's test, Table 1).

Foot contact test

There was no difference between the numbers of abalone swivelling after touching under the two pH condition at W1 ($\chi^2 = 0$, df = 1, p = 1, Fisher's exact test), although a trend was observed at M3, with less abalone swivelling in the pH 7.7 treatment compared to those exposed to pH 8.0 at M3 ($\chi^2 = 2.96$, df = 1, p = 0.086, Pearson's chi-square test). The number of abalone changing position after touching did not differ significantly between the treatments (W1: $\chi^2 = 0.078$, df = 1, p = 0.779; M3: $\chi^2 = 0.094$, df = 1, p = 0.759, Pearson's chi-square

test), nor did the number of abalone changing position and returning to their initial position before the end of the observation period (W1: $\chi^2 = 0.389$, df = 1, p = 0.533; M3: $\chi^2 = 0.179$, df = 1, p = 0.672, Pearson's chi-square test, Table 2).

Righting test

No significant difference was observed for the time to right (W1: $F_{1, 38} = 0.965$, p = 0.332, mixed model analysis with inverse transformation; M3: $F_{1, 8.2} = 1.61$, p = 0.239, mixed model analysis with log transformation), nor for the number of attempts to turn over (W1: W = 17, p = 0.396; M3: W = 20.5, p = 0.115, Wilcoxon rank sum test, Table 2) between the two pH treatment.

Hiding test

The time until the first movement did not differ significantly between pH treatments (W1: $F_{1, 8} = 1.24$, p = 0.297, mixed model analysis with log transformation; M3: $F_{1, 37} = 0.34$, p = 0.564, mixed model analysis with square root transformation), nor did the time before complete hiding (W1: W = 16, p = 0.548, Wilcoxon rank sum test; M3: $F_{1, 37} = 0.61$, p = 0.439, mixed model analysis with inverse transformation, Table 2).

Starfish predator test

After the first week, there was a trend towards faster response in the lower pH treatment: these abalone moved faster after the contact with the starfish (W1: $F_{1, 8} = 3.95$, p = 0.082, mixed model analysis with inverse transformation) but there was no significant difference at M3 (W = 7, p = 0.309, Wilcoxon rank sum test). Similarly, there were no significant differences in the time spent moving (W1: W = 14, p = 0.841, Wilcoxon rank sum test; M3: W = 11, p = 0.841, Wilcoxon rank sum test), the numbers of squares crossed (W1: $F_{1, 38} =$

1.92, p = 0.173, mixed model analysis; M3: $F_{1, 36}$ = 0.026, p = 0.872, mixed model analysis with square root transformation), the times to reach 20 cm away from the predator (W1 : $F_{1, 38}$ = 1.47, p = 0.233, mixed model analysis with log transformation; M3: W = 9, p = 0.548, Wilcoxon rank sum test) and the number of abalone performing the four escape behaviours (W1: χ^2 = 0.23, df = 1, p = 0.633, Fisher's exact test; M3: χ^2 = 2.54, df = 1, p = 0.111, Pearson's chi-square test) (Table 2).

Spawning behaviour

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

3.2. Gene expression

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

3.3. Estimated effect size

Calculation of estimated effect sizes showed that, if any difference was present for the behavioural tests, these differences would be small (Supplementary Fig. S1): 33 estimated effect sizes were below 0.5 out of the 36 variables measured in total for the different periods. In addition, estimated effect sizes were inconsistently positive or negative in different tests and periods of the pH treatment.

4. Discussion

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

Effects on behaviour

Because interactions between the prey and their predators constitute an integral part of the ecology and evolution of marine organisms, as well as the structure and function of communities, we focused first on prey-predator responses, with a simulation of an attack in the foot contact test and direct exposure to a predator. When exposed to a starfish, *H. tuberculata* responds with stereotyped behaviours: it does a swivelling movement, releases some mucus, turns around and flees thereafter (Roussel et al., 2019b). Similar behavioural patterns were reported for other species such as *Haliotis rubra* (Day et al., 1995). The responses to the predator were similar between the pH treatments, indicating little or no effect of a lower pH on predator detection and escape responses. These results differ strongly from

those obtained in marine fishes, in which an impairment of prey-predator responses due to OA was reported (reviews by Briffa et al., 2012; Nagelkerken and Munday, 2016; Ashur et al. 2017). In other gastropods, the effects of decreased pH on prey-predator responses are variable (Clements and Comeau, 2019). Low pH had a negative effect on the response to a predator in a rocky shore species, *Concholepas concholepas*, by disrupting predator-avoidance behaviour in juveniles reared at pH_T 7.7 for five months (Manriquez et al., 2014) and affecting predator-escape response in juveniles exposed to pH_T 7.5 for 3 months in comparison to juveniles exposed to current-day conditions at pH_T 7.7-7.85 (Manriquez et al., 2016). In contrast, other studies found an increased avoidance in the gastropod *Littorina littorea* exposed to predator cues when kept at very low pH_{NBS} (6.6 for low pH versus 8.0 for ambient pH) (Bibby et al., 2007), and no effect of 6-week exposure to pH_T 7.8 on the maximum escape speed of the limpet *Nacella concinna* when exposed to starfish (Schram et al., 2014).

Due to its habitat preference, in subtidal and intertidal areas, *H. tuberculata* can be exposed to waves and surges (Clavier and Chardy, 1989). For many benthic invertebrates, the ability to right after detachment by strong wave action reduces vulnerability to predation or unwanted spatial transport. Hiding is another key behaviour to avoid predation that is crucial for individual survival, especially for juveniles, for which higher mortalities are reported in the natural environment (Shepherd and Breen, 1992). In the present study, adult abalone exposed to ambient pH and low pH took similar times to find a hiding place, to right themselves and to escape when facing a starfish. As for prey-predator effects, the absence of effects of simulated OA on the hiding response of the abalone contrasts with the significant effects found on other gastropods (Manriquez et al., 2013; Schram et al., 2014). In abalone, the righting time is a reliable indirect indicator of its energetic status and later survival (Lachambre et al., 2017). Metabolic rates and immune function measured on other abalone from the same experiment

were not significantly affected by lowered pH in spite of significant effects on shell growth and calcification (see Avignon et al. 2020 for physiological and calcification measures). This lack of effects on overall metabolism is consistent with the absence of effect on the righting behaviour.

We found no evidence that OA modified the quantity of algae ingested during the 3-month exposure of abalone to a reduction of 0.3 pH unit. In addition, there was no detectable difference in diurnal rhythm, including feeding duration, during 48h observations of a large number of individuals, and on spawning behaviour after 5-months of low pH exposure. Few experiments have measured the long-term effect of acidification on feed intake of herbivores. Results in the literature are contradictory for OA, even for the same taxon (Nagelkerken and Munday, 2016). Our results suggest that short term (one week) as well as long term exposure (3 months) to seawater acidification of 0.3 of a pH unit does not modify the foraging capacity or the feed intake of *H. tuberculata* as well as reproductive behaviour even if a lower gonad was reported on other individuals from the same experiment (Avignon et al., 2020). Our measurement of diurnal rhythm in a "non-stressful" environment, adapted to the biology of the animals (the presence of a hiding place, access to algae and the potential for active foraging behaviour) would be expected to provide valuable information about daily behaviours that would likely occur in a natural context. In addition, the lack of effects did not result from a stress bias due to a new environment, because abalone had at least 3 weeks of acclimation to the novel environment before the measurements.

Effect on gene expression

Gene expression profiling has already been used in a range of organisms exposed to near-future pH scenarios. The hypothesis was that marine organisms exposed to acidified seawater would present compensatory changes in behavioural processes that would be reflected by

gene expression changes. In this context we aimed to detect gene expression changes in targeted genes involved in the processes of neurotransmission and central regulation of behavior when the abalone *H. tuberculata* was exposed to OA conditions. Recent evidence shows that GABA and serotonin signaling play major roles in the nervous systems of gastropod mollusks, notably in the modulation of motor control and cognitive processes such as memory and learning (Miller, 2019; Aonuma et al., 2020). The GABA A receptor has often been described as responsible for the sensory impairment at a central level observed in fish exposed to acidification (Nilsson et al., 2012). However, no modification of the expression of the GABA A receptor or the serotonin receptor in the head of these adult abalone was observed. This is in contrast to the effects of low pH_{NBS} 7.85 compared to pH_{NBS} 8.17 for 2-5 days on a caenogastropod, the stromb *Gibberulus gibbosus*, which produced disrupted predator escape function (Watson et al., 2014). This effect appeared to be due to malfunction of GABA A-type receptors in the stromb, as the gabazine blocker of these receptors restored the predator escape behaviour. Our results in this abalone species indicate that long term exposure to decreased pH predicted by the "business as usual" scenario for the end of the century does not significantly impact the expression of the two main signalling receptors or the function of these receptors as shown by the lack of effects of acidification on the tested behaviour (notably the diurnal rhythms, predator responses and hiding activity). Interestingly, GABA and serotonin signalling are implied in a wide range of cognitive functions as the central transmission of sensorial signals and locomotion. A further and complete study of the expression profile of the other key players of the GABAergic and serotoninergic signalling would be necessary to exclude any effects of elevated pCO2 on neurosecretory system of European abalone at transcriptional level.

Why no effects?

The most plausible hypothesis to explain the lack of significant effects on adult abalone behaviour is the evolution of resistance to variable environmental pH. This might be due to the ecological niche of abalone, in intertidal and/or subtidal areas, where diel and seasonal variation in coastal pCO_2 can often far exceed near-future projections, as observed in other invertebrates living under variable seawater pH (Miller et al., 2009; Ramajo et al., 2019). In Brittany, *H. tuberculata* populations in the subtidal zone can experience pH variation from 7.9 up to 8.2 seasonally (Qui-Minet et al., 2018). In mid-intertidal pools, the fluctuations are even more extreme, with daily pH variations from 7.5 up to 10 in a few hours. These variations are due to community respiration with CO_2 release at night and photosynthesis with CO_2 uptake during daylight in pools that are isolated from the adjacent ocean during low tides, with supplementary variations according to the season and period of the day (Legrand et al., 2018). The degree of variability a species encounters is an important consideration to understand biological responses to global changes (Waldbusser and Salisbury, 2014; Ziegler et al., 2021). In this experiment, *H. tuberculata* were reared prior the experiment during 10 months in tanks on plates covered by *Ulvella lens* as described in Daume et al. (2004). The photosynthetic activity of this macro-algae raised pH during daytime largely above the variation applied during the experiment (we observed variation from pH_T 7.8 to pH_T 8.8 during day time in tanks containing *Ulvella lens*), similar to variation observed in mid-intertidal pools (Legrand et al., 2018). This diel fluctuation experienced during ontogeny by juvenile abalone may well condition the physiology and behaviour of the individuals at later stages, even if juveniles were transferred after 10 months of age into more stable pH condition over the next 2.5 years, with pH variation similar to the one experienced in subtidal environments (diel pH variation lower than 0.1 unit, Qui-Minet et al. 2018). Organisms already facing pH variation in its

natural environment, especially during ontogeny, might be more robust to pH fluctuations due to ocean acidification

Another hypothesis explaining the lack of significant effect on behaviour could have been the effect of domestication process due to captivity on the abalone used for this experiment, modifying the level of stimulus necessary to induce a change in behaviour (Price, 1984). Indeed, a deficit in anti-predator responses was reported in hatchery-reared abalone *Haliotis kamtschatkana* compared to wild counterparts (Hansen and Gosselin, 2016). In this experiment, individuals resulted from systematic breeding between wild and farmed broodstock to reduce inbreeding, with no selection. Genetic modification due to domestication had probably no effect on the abalone behaviour as observed in recently domesticated juveniles (Chauvaud et al., 2021) or only subtle changes in responses to predators as observed in adult selected on growth for one generation (Roussel et al., 2019b). If any modification occurred, they would more likely have resulted from the environment experienced during ontogeny in captivity than genetic modification due to domestication process, and be moderate after one generation of hatchery-reared individuals.

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

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

Behavioural impairment might also be observed in animals that cannot avoid low pH. The ability to cope with environmental changes will be different depending on the animal mobility. Adult abalone, in contrast to fish, have limited mobility. They rely on strong attachment to the substratum by the muscular foot to avoid most predation. Because of this reduced mobility and the pH variability in their ecological niche, abalone have probably been selected for resilience to variations in pH, at least for a few hours per day. However, the shell integrity of marine molluscs is essential to protect their soft body from predators and other external stressors (Shepherd and Breen, 1992). In juvenile and adult *H. tuberculata*, shell mechanical properties as well as biomineral architecture were greatly impacted by a pH

reduction of 0.3 unit after several months of exposure (Auzoux-Bordenave et al., 2020; Avignon et al., 2020), suggesting that OA might reduce protection from predators and resistance to hydrodynamic forces, potentially impacting wild abalone populations. Even if abalone did not change their behavioural responses when facing OA, a more fragile shell might potentially jeopardize wild populations already threatened by overfishing and environmental perturbations.

5. Conclusion

Biological responses to OA are thought to depend on a number of physiological and life-history traits at larval, juvenile and adult stages. The results of the present study demonstrated that the behaviour of adult *H. tuberculata* is not impacted by an experimental seawater pH decrease of 0.3 pH unit, suggesting that the adult stage is a robust stage from a behavioural point of view in comparison to other parameters such as survival, development or calcification at larval or juvenile stages in the same species (Auzoux-Bordenave et al., 2020; 2022; Wessel et al. 2018). Because abalone naturally experience pH variations in their low-intertidal or high-subtidal environment, the species may well have evolved a relative resilience to this environmental stress. However, the net fitness outcome and the capacity of organisms to survive and persist under OA is a complex trade-off between behaviours and other biological processes and should be monitored over a long term period (i.e at least a representative duration of the species cycle). A multi-criteria approach should be used to study the full range of effects of ocean acidification on the biology of a species Although no behavioural impairment occurred in adult *H. tuberculata* from this OA experiment, a reduction of 0.3 pH unit impacted other physiological functions such as calcification and reproduction in the same experiment (Avignon et al., 2020). Together, these results highlight

that a multicriteria approach should be applied when studying the effect of acidification on marine species to fully evaluate the long term consequences, and ultimately, the effect of ocean acidification on the dynamics of a population.

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AUTHOR CONTRIBUTIONS STATEMENT

Sabine Roussel : conceptualization, formal analysis, investigation, writing – original draft, visualization, co-supervision, project administration; Manon Coheleach : investigation, data curation; Sophie Martin: conceptualization, investigation, writing-review & editing, supervision, project administration; Rob Day : conceptualization, writing-review & editing, formal analysis; Aicha Badou : investigation, writing-review & editing; Sylvain Huchette : resources, investigation; Philippe Dubois : writing-review & editing; Arianna Servili : resources, investigation, writing-review & editing, funding acquisition; Fanny Gaillard : investigation, writing-review & editing; Stéphanie Anzeux-Bordenave : conceptualization, investigation, writing-review & editing, co-supervision, project administration, funding acquisition.

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:

Stephanie Bordenave reports financial support was provided by Foundation for Research on Biodiversity. Stephanie Bordenave reports financial support was provided by Ministère de la Transition Ecologique et Solidaire (MTES), France. Sabine Roussel reports financial support was provided by French LabexMER program (OASYS project).

However, they are financial support by public bodies (Ministry, University grant...)

Figure 1

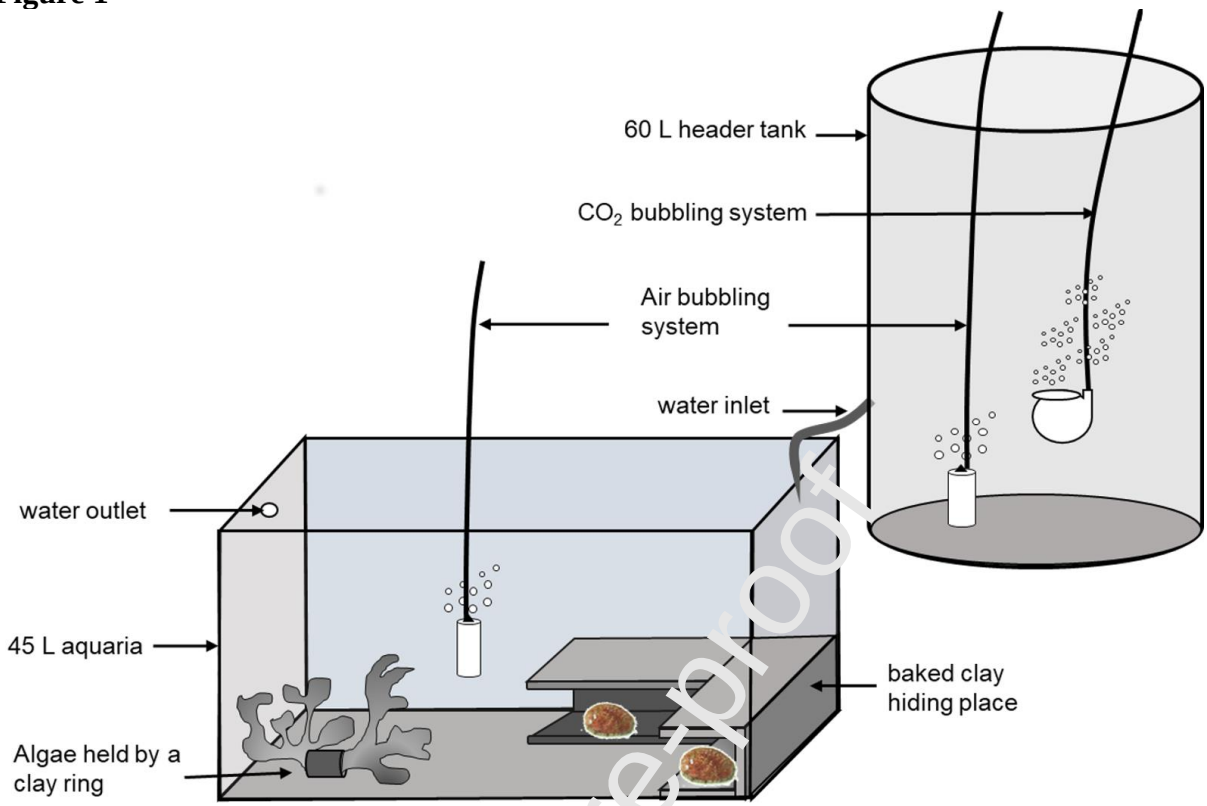


Figure 2

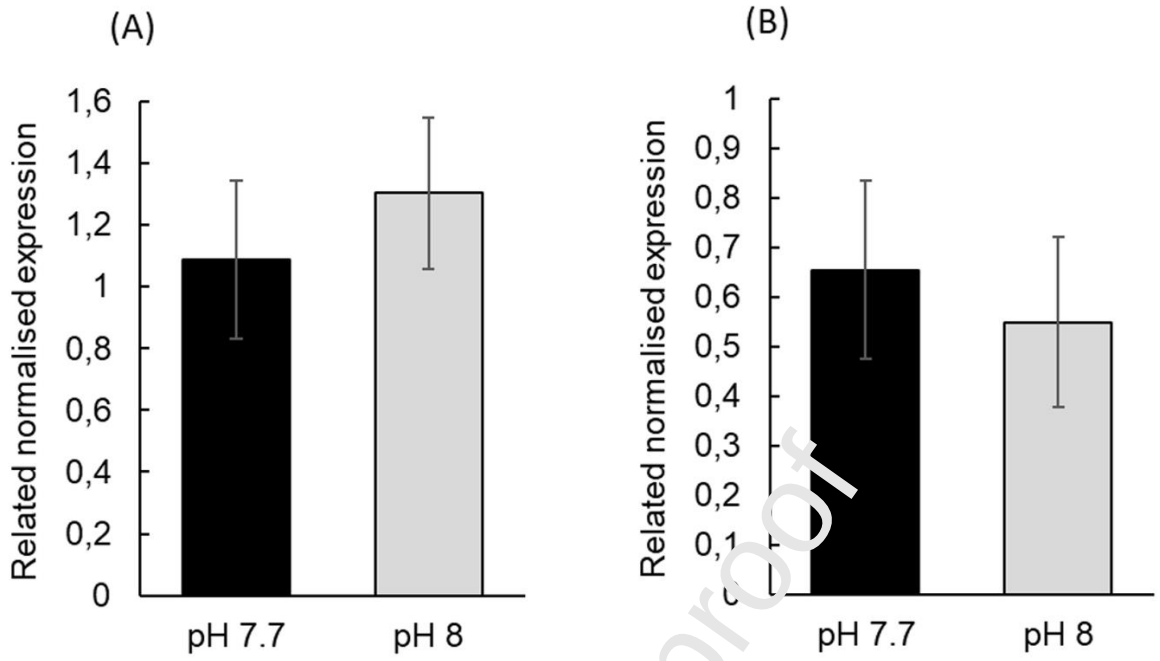


Fig. 1: Experimental apparatus showing the experimental rearing tank and header tank where pCO_2 was adjusted to the target pH

Fig. 2: Gene expression of the (A) GABA A-like and (B) serotonin receptors of adult *Haliotis tuberculata* exposed for 4 months to ambient pH (pH_T 8.0) or to low pH (pH_T 7.7). Lsmeans ± s.e.m.

Table 1

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

pH	pH_T 8.0		pH_T 7.7		$F_{1,8}$ / F_w	P
W1 diurnal behaviour (min. abalone^{-1}.day^{-1})						
Time spent moving	60.0	±	55.7	±	0.259	0.624
Time spent feeding	6.09		6.09		3.416	0.102
Time spent in open zone	19.6	±	13.8	±	0.564	0.474
	2.22		2.22			
	56.1	±	46.0	±		
	9.49		9.49			
M3 diurnal behaviour (min. abalone^{-1}.day^{-1})						
Time spent moving	49.3	±	37.9	±	2.183	0.178
Time spent feeding	5.44		5.44		0.966	0.354
Time spent in open zone	37.0	±	27.6	±	2.504	0.152
	6.71		6.71			
	54.1	±	40.1	±		
	6.28		6.28			
Feed intake during a 3-month period						
Quantity of algae ingested per gram of abalone (mg	31.0 ± 0.		30.7	±	0.083	0.780

algae. g^{-1} abalone. day^{-1}) $^{\delta}$	0	.70

$^{\delta}$ Welch's test, mean ± s.e.

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

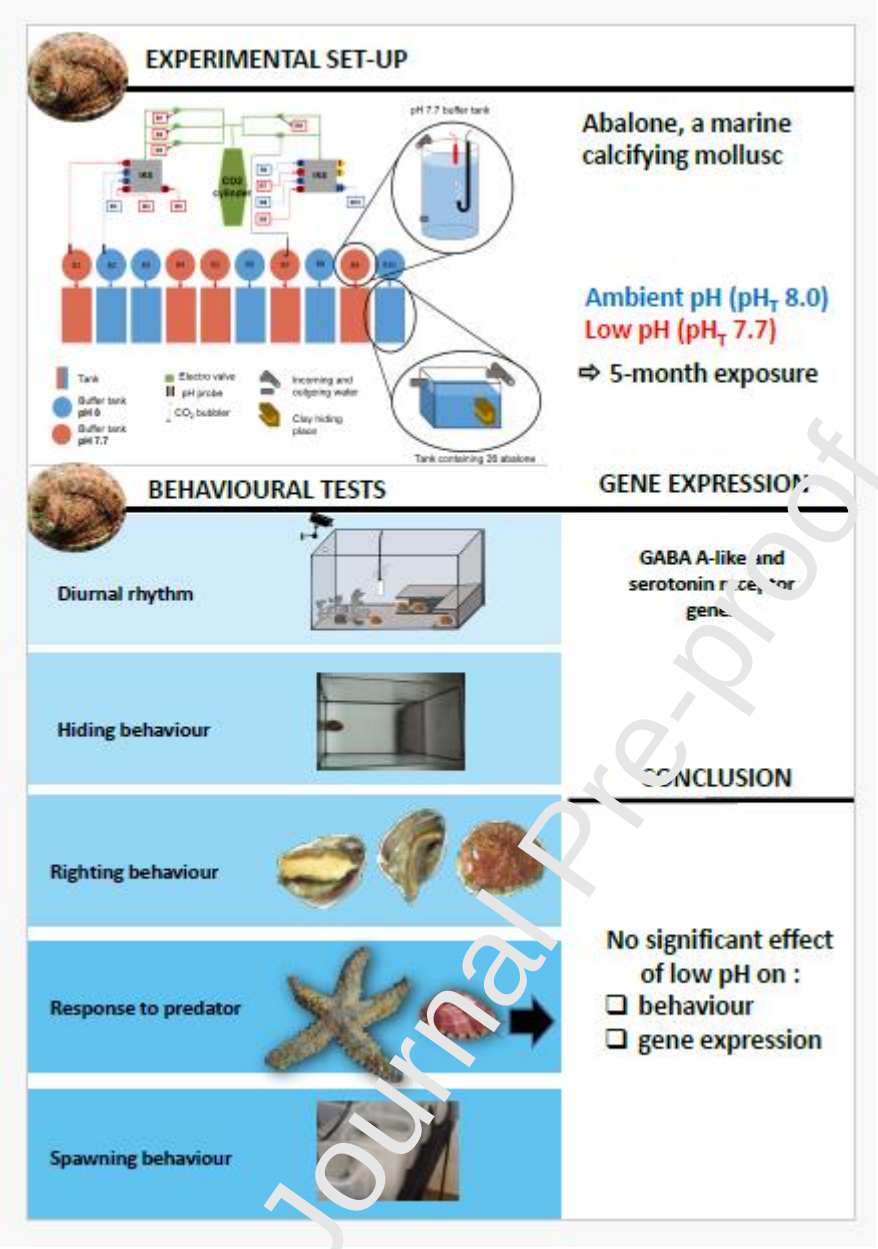
Tests	W1				M3			
	pH_T 8.0	pH_T 7.7	F/χ^2/W	p	pH_T 8.0	pH_T 7.7	F/χ^2/ W	p
Foot contact test								
Number of abalone doing a swivelling movement[δ]	3 out of 105	3 out of 105	0.[illegible]	1 [δ]	14 out of 83	7 out of 86	2.96	0.086
Number of abalone changing of position after touching	63 out of 105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

Hiding test								
Time until the first movement (s)	131.3 ±	47.1 ±	1.24	0.297 β	28.4 ±	23.2 ±	0.34	0.564 β
Time before complete hiding (s)	34.96	34.96	16	0.548	4.81	4.71	0.61	0.439 β
	406.0 ±	370.6 ±		ϒ	172.5 ±	156.5 ±		
	81.86	80.56			39.74	38.74		
Predator test								
Number of abalone performing the four escape	2 out of 20	3 out of 20	0.23	0.633 δ	10 out of 18	6 out of 20	2.54	0.111
behaviours	45.9 ±	31.3 ±	3.95	0.082 β	45.0 ±	30.0 ±	7	0.309
Time until the first movement (s)	5.61	5.61	14	0.841	29.39	5.00	11	ϒ
Time spent moving (s)	216.0 ±	223.9 ±	1.92	ϒ	192.5 ±	190.1 ±	0.026	0.841
Number of squares crossed	16.20	8.15	1.47	0.173	13.30	14.04	9	ϒ
Time to reach 20 cm far from the predator (s)	4.9 ±	6.5 ±		0.233 β	7.6 ±	7.0 ±		0.872 β
	0.79	0.79			1.13	1.07		0.548
	197.7 ±	171.2 ±			160.6 ±	133.4 ±		ϒ
	19.57	19.57			45.72	39.51		
	M5							

Number of abalone spawning	31 out of 37		30 out of 34		0.29	0.737 [δ]		
Time of preparation (min)	187.6	±	178.1	±	0.12	0.735		
Time of spawning (min)	18.49		18.79		0.96	0.360 [β]		
	245.1	±	232.0	±				
	8.00		10.46					

[δ] Fisher's exact test [ϒ] Wilcoxon rank sum test with continuity correction [β] mixed model with log, inverse or square root transformations

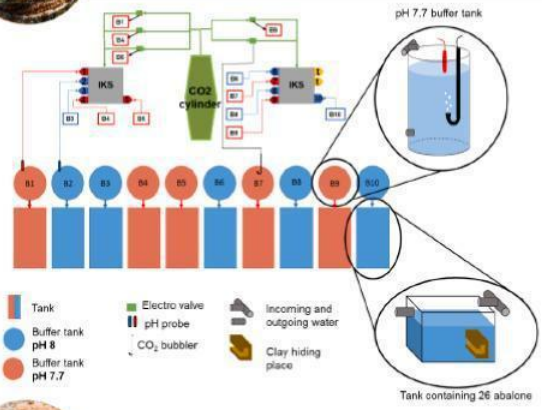
Graphical abstract



HIGHLIGHTS

- OA does not have any significant effect on the behavioural repertoire of adult abalone
- No change in the expression of genes involved in sensory performance and behaviour was detected.
- The ecological niche of this species probably allows it to be relatively resilient to OA

EXPERIMENTAL SET-UP



Abalone, a marine calcifying mollusc

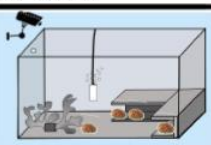
Ambient pH (pH_T 8.0)

Low pH (pH_T 7.7)

⇨ 5-month exposure

BEHAVIOURAL TESTS

Diurnal rhythm



Hiding behaviour

Righting behaviour

Response to predator

Spawning behaviour

GENE EXPRESSION

GABA A-like and serotonin receptor genes

CONCLUSION

No significant effect of low pH on :

- ❑ behaviour
- ❑ gene expression

Graphics Abstract

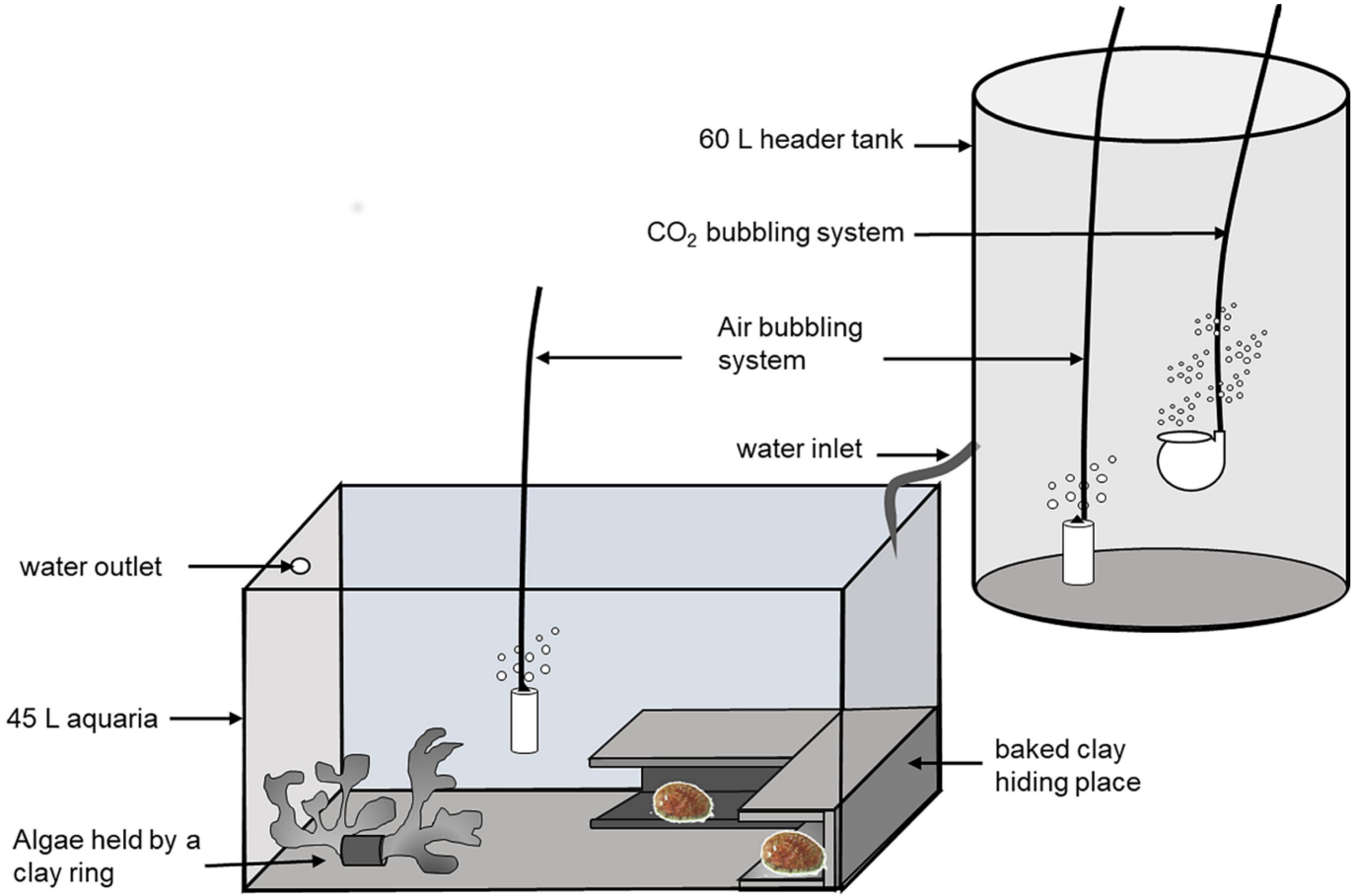

60 L header tank
CO_2 bubbling system
Air bubbling system
water inlet
water outlet
45 L aquaria
baked clay hiding place
Algae held by a clay ring

Figure 1

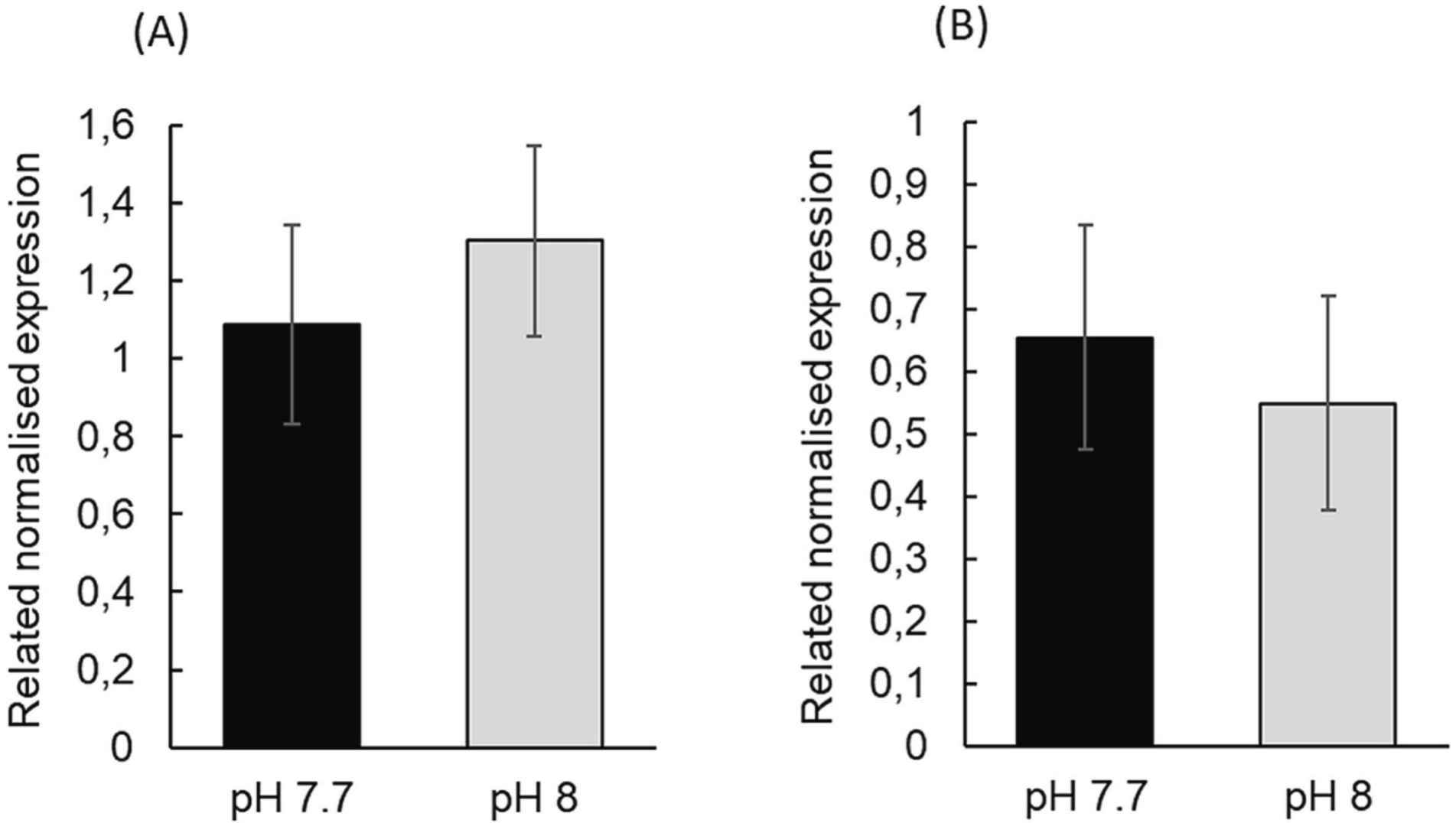

(A)
Related normalised expression
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
pH 7.7
pH 8
(B)
Related normalised expression
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
pH 7.7
pH 8

Figure 2