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

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Abstract :

CO2 absorption is leading to ocean acidification (OA), which is a matter of major concern for marine calcifying species. This study investigated the effects of simulated OA on the reproduction of European abalone Haliotis tuberculata and the survival of its offspring. Four-year-old abalone were exposed during reproductive season to two relevant OA scenarios, ambient pH (8.0) and low pH (7.7). After five months of exposure, abalone were induced to spawn. The gametes, larvae and juveniles were then exposed for five months to the same pH conditions as their parents. Several biological parameters involved in adult reproduction as well as in larval, post-larval and juvenile fitness were measured. No effects on gametes, fertilisation or larval oxidative stress response were detected. However, developmental abnormalities and significant decreases in shell length and calcification were observed at veliger stages. The expression profile of a GABA A receptor-like gene appeared to be regulated by pH, depending on larval stage. Larval and post-larval survival was not affected by low pH. However, a lower survival and a reduction of growth were recorded in juveniles at pH 7.7. Our results confirm that OA negatively impacts larval and juvenile fitness and suggest the absence of carry-over effects on abalone offspring. This may compromise the survival of abalone populations in the near future.

Highlights

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

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

1. Introduction

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

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

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

2020; Wessel et al. 2018). As with most marine molluscs, abalone have a pelago-benthic life cycle with a larval planktonic stage followed by a critical metamorphosis into benthic juveniles, making them highly sensitive to environmental changes (Byrne et al., 2011). Several studies have also focused on early life stages of abalone, especially larvae, demonstrating adverse effects of elevated CO_2, such as reduced survival, developmental delay, body and shell abnormalities and reduced mineralization (Byrne et al., 2011; Crim et al., 2011; Guo et al., 2015; Kimura et al., 2011; Onitsuka et al., 2018; Wessel et al., 2018; Zippay and Hofmann 2010). More recently, Kavousi et al. (2021) investigated the combined effects of OA and ocean warming on *H. tuberculata* larvae in a full factorial experiment. Despite no interaction between elevated temperature and low pH being observed, the results provided additional evidence for the sensitivity of abalone larvae to OA (Kavousi et al., 2021). Only one study has yet reared abalone embryos in acidified conditions from the fertilisation stage (Byrne et al., 2011); this demonstrated that resulting larvae were more severely affected by OA than those exposed at a later stage. In all the above-mentioned papers, experiments were carried out on a single life-history stage using short-term or acute exposure to pH decrease, that provided information on the vulnerabilities of embryos and larvae (Parker et al., 2015; Ross et al., 2016). More recently, a multigenerational experiment conducted on the red abalone *Haliotis rufescens* (Swezey et al., 2020) demonstrated that larval mortality under OA was strongly correlated with differences in lipid provisioning and metabolism. The differences were linked to population-specific variation in the maternal provisioning of lipids with a positive correlation between lipid concentrations and survival under lower pH. This relationship also persisted in experiments on second-generation animals (Swezey et al., 2020), confirming the importance of parental exposure when studying the effects of OA.

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

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

2. Material and Methods

2.1. Abalone collection and conditioning

Adult *H. tuberculata* (n = 260, 48.5 ± 4.2 mm shell length) were selected at random from an offshore sea-cage structure at the France Haliotis abalone farm (48°36'50N, 4°36'3W; Plouguerneau, Brittany, France) in January 2017, as previously described in Avignon et al. (2020). These abalone were distributed without any selection among ten 45-L open-circuit aquaria (n = 26 abalone per aquarium) supplied with a minimum of 15 L/h of 3 µm filtered seawater at ambient temperature. They were conditioned for three weeks in the laboratory under ambient pCO_2/pH conditions and fed *ad libitum* with the macroalgae *Palmaria palmata*.

2.2. Experimental set-up

The experimental system used to test the two pH treatments (ambient pH 8.0 and reduced pH 7.7) on adult abalone was previously described in detail in Avignon et al. (2020). Briefly, each experimental aquarium (n = 10) was supplied from a separate header tank where pCO_2 was adjusted by bubbling CO_2 (Air liquid, France) through electro-valves controlled by a pH-stat system (IKS Aquastar, Germany). Five replicate aquaria, were used per pH condition. The adult experiment was conducted for five months between January and June 2017, during reproductive conditioning.

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

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

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

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

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

2.3. pH and carbonate parameters

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

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

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

The seawater carbonate chemistry analysis included dissolved carbonate (CO_3^{2-}), bicarbonate (HCO_3^-), dissolved inorganic carbon (DIC), pCO_2, aragonite saturation state ($\Omega_{aragonite}$) and calcite saturation state ($\Omega_{calcite}$). These values were determined by entering the values of pH_T, AT, temperature and salinity into CO_2SYS software (Pierrot et al., 2006) using constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

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

2.4. Number of gametes released and spermatozoon shape

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

2.5. Spermatozoon motility

Aliquots of 5 µL of spermatozoon suspension were diluted in 500 µL of activating solution (1-µm fresh seawater, 0.3% pluronic acid, 15 mM Tris, pH 8.0., Boulais et al., 2018). Then, 12 µL of this diluted suspension were transferred to a Fast-Read 102 cell (Biosigma, Italy), and spermatozoon movement was triggered under a microscope (Olympus BX51, X200 magnification) connected to a camera (Qicam Fasr 1394) as described in Boulais et al. (2015). Two aliquots per male were analysed, with an average of 200 spermatozoa tracked (minimum of 56 spermatozoa observed per male).

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

2.6. Fertilisation percentage

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

2.7. Larval survival

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

Larval survival = (total number of swimming larvae at t+1) / 100 x (total number of swimming larvae at t)

2.8. Slide preparation for larval morphometry and birefringence

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

Approximately 40 larvae per tank (n = 200 larvae per pH condition), regardless of their orientation, shape and development were photographed with an Olympus binocular microscope (Olympus, Hamburg, Germany) under phase contrast and polarized light. The same microscope was equipped with polarizing filters for the birefringence analysis. Microscopic observations were fully blinded to prevent bias arising from any a priori knowledge of larval treatment. All images were taken with a digital camera (DS-Ri1, Nikon, Japan) at 20X magnification and 40-ms exposure. Images were acquired with NIS-element and analysed using ImageJ software (v 1.52a).

2.9. Morphometric analysis

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

- *Normal tissue development/ normal shell = normal phenotype*
- *Abnormal or partially developed tissues/ partial or no shell = altered phenotype*

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

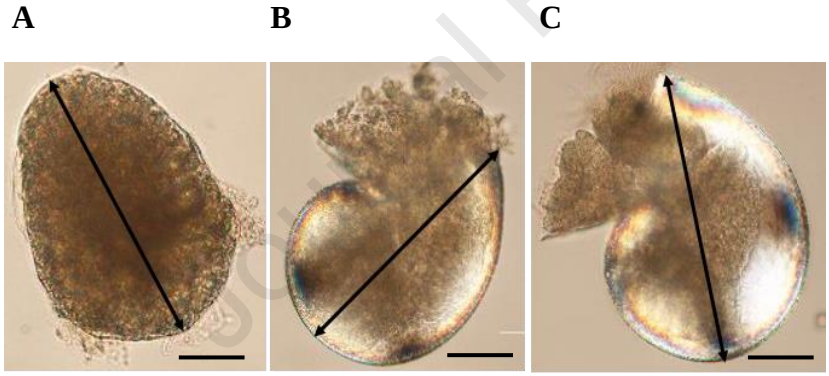


Fig. 1. Measurements of larval length at 20 hpf (A), and shell length at 48 hpf (B) and 96 hpf (C). Scale bar: 50µm

2.10. Birefringence analysis

Birefringence intensity has been previously used as a proxy for the assessment of $CaCO_3$ mineralisation within the larval abalone shell (Kavousi et al., 2021; Wessel et al., 2018). Birefringence was measured on cross-polarized images taken of 48 and 96 hpf larval stages (n = 200 per pH treatment). The younger 20 hpf larvae were not considered because the shell at this stage lacks sufficient crystallized $CaCO_3$ to calculate birefringence (Wessel et al., 2018). The mean greyscale level (0–255 pixels) was determined for each area of the larval shell showing

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

2.11. Oxidative stress

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

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

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

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

USA) following the manufacturer's instructions. Results are expressed in units per mg protein (U/mg), where 1 U of SOD is the amount of enzyme necessary to inhibit the formation of xanthine/XO complex by 50%. Catalase activity was quantified in lysates at room temperature following the method of Curd et al. (2019). Briefly, 10 µL of pure samples were added to 195 µL of hydrogen peroxide solution (10 mM) to initiate the reaction. Absorbance was immediately recorded every 15 s for 4 min using a Synergy HT microplate reader (BioTek, Winooski VT, USA). CAT activity is expressed in mU/mg proteins, where 1 U is the amount of enzyme necessary to catalyse 1 µmole of H_2O_2 per minute (using εH_2O_2, 39.4 mM^{-1} cm^{-1}).

2.12. Gene expression analysis

The expression profiles of selected genes were analysed in the abalone larvae at 48 and 96 hpf (pooled per treatment from 5 tanks and corresponding to about 50 000 larvae/sample). The genes were chosen with respect to their putative functions in shell biomineralization (lustrin A; carbonic anhydrase 1, CA1; carbonic anhydrase 2, CA2) and neurosensory transmission (GABA A receptor-like; serotonin receptor, 5HTR; **Table 1**). 18S-rRNA (18S) and elongation factor 1 (EF1) were chosen as reference genes. Specific primers for each gene were designed as detailed in **Table 1**. To search for potential candidate hits for the GABA A receptor in *Haliotis tuberculata*, tblastx (Basic Local Alignment Search Tool, NCBI) research was carried out using the GABA A receptor sequence of *Haliotis asinina* (EF222254.1) as a query sequence against the unfiltered transcriptome of *H. tuberculata* published by Harney et al. (2016). Then, blastx (Basic Local Alignment Search Tool, NCBI) research against the non-redundant protein sequences database was performed with all 38 candidate sequences previously obtained. The sequence TR57267_c2_g1 (GenBank accession number GEAU01240864.1) was chosen as the GABA A receptor-like sequence of *H. tuberculata* based on sequence similarities to the GABA A receptor sequence of *Aplysia californica* (XP005111291.2). The *H. tuberculata* serotonin receptor sequence (GEAU01264085.1) was identified by using serotonin receptor sequences available in *Haliotis* against the unfiltered transcriptome of *H. tuberculata* (Harney et al., 2016).

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

2.13. Evaluation of post-larvae and juveniles

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

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

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

2.14. Statistical analysis

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

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

3. Results

3.1. Seawater parameters

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

During the reproductive conditioning (January to June 2017), seawater temperature followed natural variations and ranged from 12.6°C ± 0.7°C at the start of the experimental period to 19.2°C ± 0.2°C at the end. Salinity was 34.6 ± 0.6 in all experimental aquaria and remained stable over the experiment. Total alkalinity (AT) measured in the experimental tanks was 2355 ± 9 µEq. kg^{-1} and remained stable throughout the experiment and among all aquaria. The seawater pH_T was maintained close to the nominal value throughout the experiment, with 8.01 ± 0.05 (pCO_2 439 ± 57 µatm) in the ambient pH aquaria and 7.71 ± 0.06 (pCO_2 951 ± 138 µatm) in the low pH aquaria. In pH 8.0 and pH 7.7 conditions, $\Omega_{aragonite}$ was 2.30 ± 0.31 and 1.25 ± 0.19 and $\Omega_{calcite}$ was 3.59 ± 0.46 and 1.95 ± 0.28, respectively (**Table 2**).

During the larval and juvenile period (July to December 2017), the seawater temperature ranged from 19.2°C ± 0.2°C at the start to 10.5°C ± 0.05°C at the end of the experiment. Salinity over the larval/juvenile period was 35.2 ± 0.2 in all experimental aquaria and remained stable throughout the experiment. Total alkalinity (AT) was 2323 ± 19 µEq. kg^{-1} and remained stable throughout the experiment and among all aquaria. The mean pH_T was 8.10 ± 0.06 (pCO_2: 351 ±

54 µatm) in ambient pH aquaria and 7.71 ± 0.03 (pCO_2: 951 ± 138 µatm) in low pH aquaria. In pH 8.0 and pH 7.7, $\Omega_{aragonite}$ was 2.78 ± 0.41 and 1.26 ± 0.13 and $\Omega_{calcite}$ was 4.32 ± 0.63 and 1.97 ± 0.19 conditions, respectively (**Table 2**).

Table 2. Seawater pH_T temperature and parameters of the carbonate system in each pH treatment (i.e., pH 8.0. and pH 7.7, n = 25 per pH treatment). Seawater pH on the total scale (pH_T), temperature, salinity and total alkalinity (AT) were used to calculate CO_2 partial pressure (pCO_2; µatm), dissolved inorganic carbon (DIC; µmol/kg), HCO_3^- (µmol/kg), CO_3^{2-} (µmol/kg), aragonite saturation state ($\Omega_{aragonite}$) and calcite saturation state ($\Omega_{calcite}$) by using the CO_2SYS program. Values are means ± s.d.

Experimental period	Nominal pH	pH_T	Temperature (°C)	AT (µmol·kg^{-1})	pCO_2 (µatm)	DIC (µmol·kg^{-1})	HCO_3^- (µmol·kg^{-1})	CO_3^{2-} (µmol·kg^{-1})	$\Omega_{aragonite}$	$\Omega_{calcite}$
Reproductive period[1] (5 months)	8.0	8.01 ± 0.05	14.4 ± 1.4	2355 ± 9	439 ± 57	2150 ± 28	1984 ± 48	151 ± 20	2.30 ± 0.31	3.59 ± 0.46
	7.7	7.71 ± 0.06	14.4 ± 1.5	2355 ± 9	951 ± 138	2277 ± 25	2154 ± 30	82 ± 12	1.25 ± 0.19	1.95 ± 0.28
Larval and juvenile period[2] (5.5 months)	8.0	8.10 ± 0.06	15.4 ± 2.2	2323 ± 19	351 ± 54	2069 ± 41	1875 ± 64	181 ± 26	2.78 ± 0.41	4.32 ± 0.63
	7.7	7.71 ± 0.03	15.3 ± 2.1	2323 ± 19	966 ± 69	2237 ± 15	2119 ± 20	83 ± 8	1.26 ± 0.13	1.97 ± 0.19

[1]Data from Avignon *et al.* 2020; [2] 5 time points per month (n = 25 per treatment)

3.2. Gametes and fertilisation

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

3.3. Larval survival

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

385 at 96 h for pH 8.0 vs pH 7.7: 78.8 ± 17.83% vs 56.2 ± 11.74%, t = -1.057, df = 8, p = 0.321,

386 Student t-test, mean ± s.e.).

387

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

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

388 ***3.4. Larval morphology***

389 At 20 hpf, larvae exposed to pH 7.7 showed less alteration of tissue organogenesis than those

390 exposed to pH 8.0 (25% for pH 7.7 vs 43% for pH 8.0, $\chi^2 = 15.2$, $p < 0.001$, Pearson Chi-squared

391 test). On the contrary, 20 hpf-larvae showed a significant increase in shell abnormalities at pH

392 7.7 compared with larvae reared in ambient conditions (69% for pH 7.7 vs 59% for pH 8.0, χ^2 =

393 4.31, p = 0.038, Pearson Chi-squared test, **Fig. 2A**).

394 At 48 hpf, larvae exposed to pH 7.7 showed more tissue abnormalities than larvae exposed to

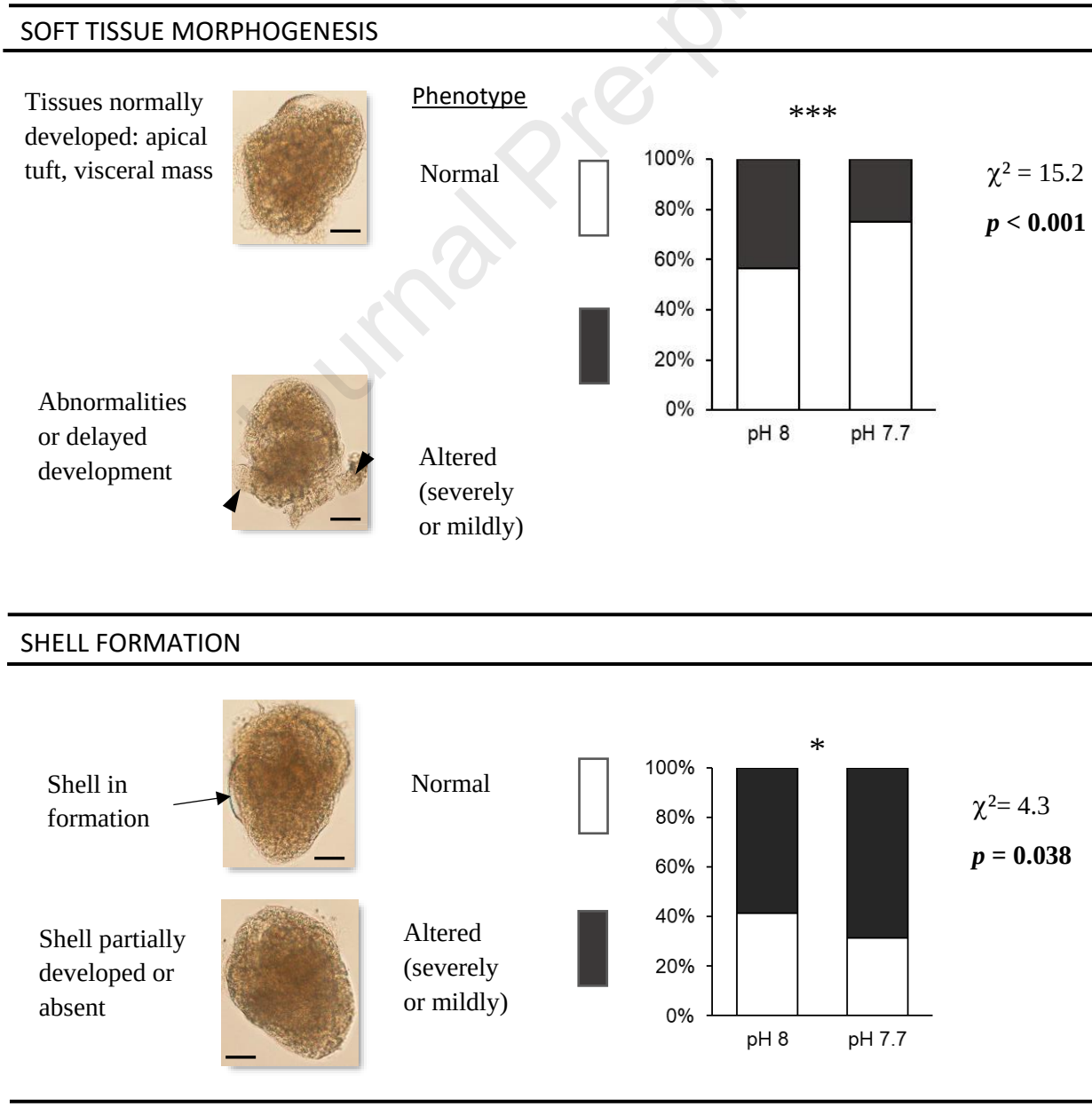
395 pH 8.0 (75% for pH 7.7 vs 64% for pH 8.0, $\chi^2 = 6.27$, p = 0.012, Pearson Chi-squared test). In

396 addition, larvae exposed to pH 7.7 showed a significant increase in shell abnormalities compared

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

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

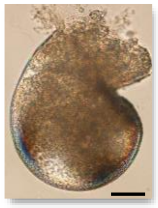
A. 20 hpf larvae



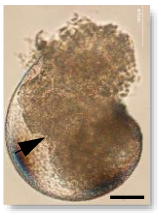
B. 48 hpf larvae

SOFT TISSUES

Tissues normally developed (velum, foot, operculum) and full larval body



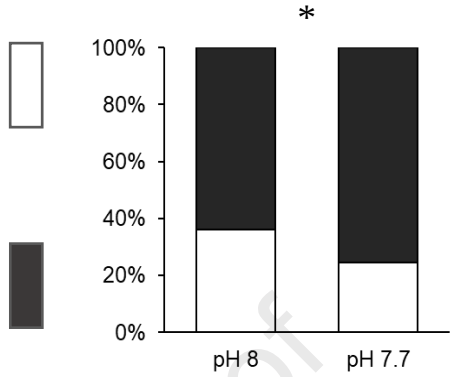
Larval body contracted



Phenotype

Normal

Altered (severely or mildly)

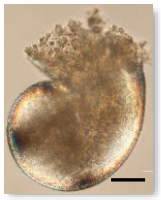


$\chi^2 = 6.27$

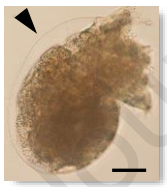
$p = 0.012$

SHELL

Shell surrounding the entire larval body

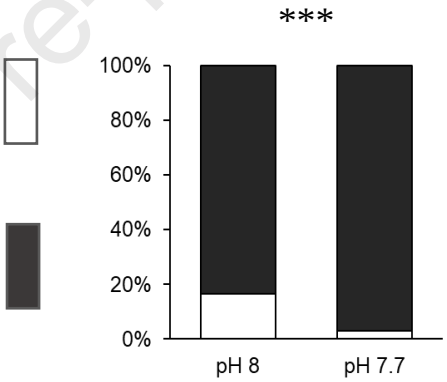


Shell residual or absent



Normal

Altered (severely or mildly)



$\chi^2 = 20.7$

$p < 0.001$

C. 96 hpf larvae

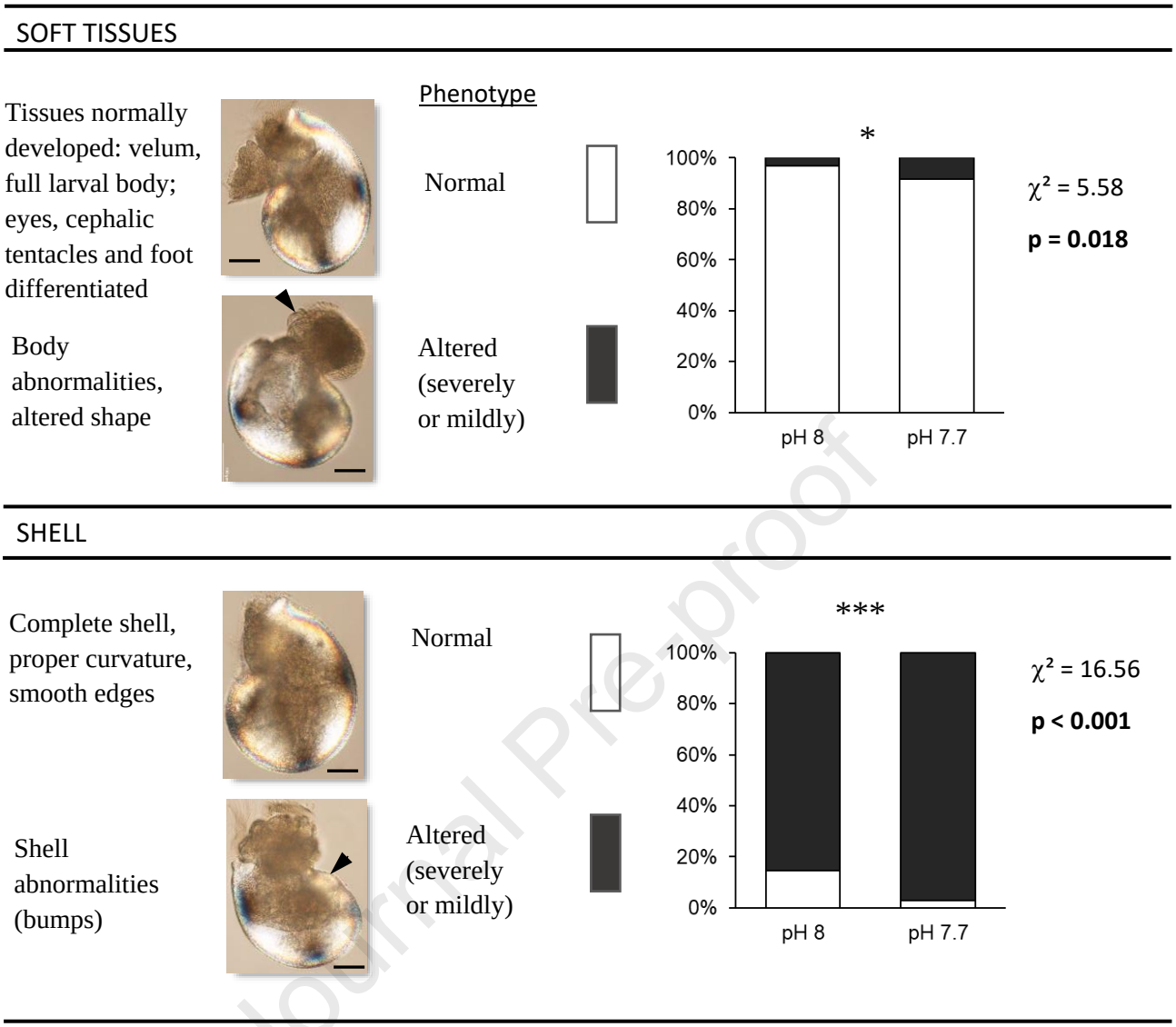


Fig. 2. **Percentage of larvae (%) presenting either normal (white squares) or altered phenotypes (black squares) based on the degree of soft tissue morphogenesis and shell formation after exposure to two pH conditions (8.0 and 7.7).** (A) 20 hpf, (B) 48 hpf and (C) 96 hpf (n = 200 larvae per pH treatment,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

3.5. Larval length

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

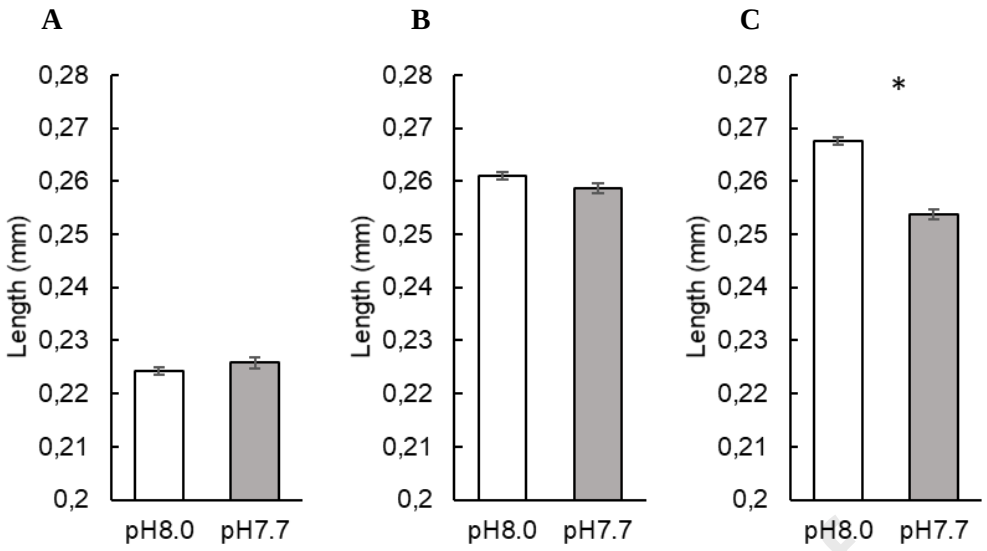


Fig. 3. **Length of *H. tuberculata* larvae reared under ambient (pH 8.0) and low pH (7.7)** at (A) 20 hpf, (B) 48 hpf and (C) 96 hpf (n = 200 larvae per pH treatment, n = 5 tanks per treatment). Larvae were exposed to the same pH as their parents. Ls mean ± s.e.m. * $p < 0.05$ (GLMM analysis with gamma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

3.6. Birefringence analysis

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

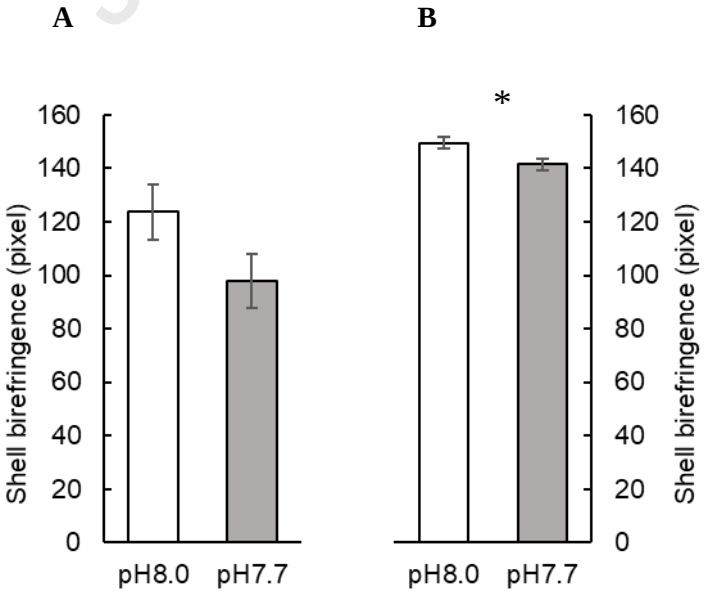


Fig. 4. Mean shell birefringence (number of greyscale pixels) of *H. tuberculata* larvae reared under ambient (pH 8.0) and low pH (7.7) at (A) 48 hpf and (B) 96 hpf. Larvae were exposed to the same pH as their parents. Ls mean ± s.e.m. * $p < 0.05$ (LMM analysis).

3.7. Oxidative stress

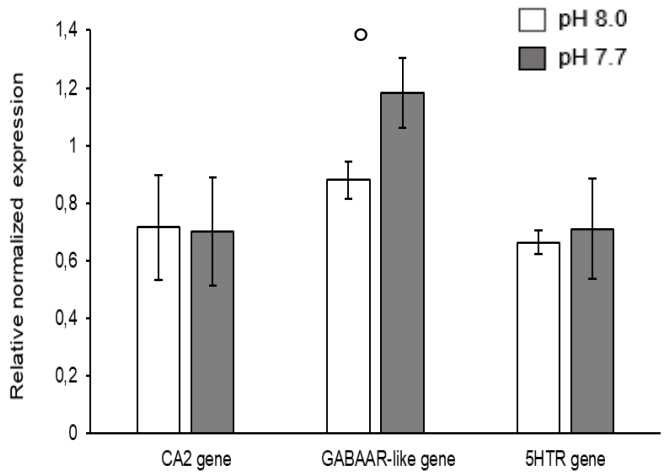
No difference in catalase activity was observed between 96hpf-larvae exposed to pH 8.0 and pH 7.7 (2.25 ± 0.428 vs 2.09 ± 0.408 mU/mg prot, respectively, t = -2.26, df = 8, p = 0.800, Student t-test, mean ± s.e.), MDA (1.14 ± 0.181 vs 1.28 ± 0.152 nmol/mg prot, respectively, t = -0.549, df = 8, p = 0.598,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

3.8. Gene expression

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

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

A



B

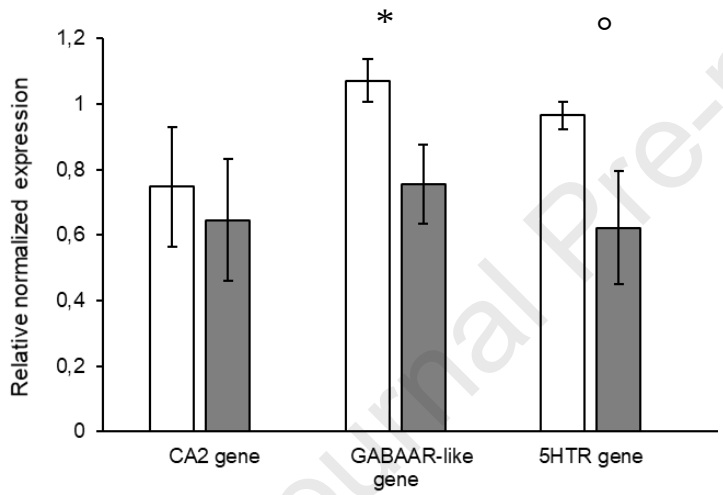


Fig. 5. Expression patterns of CA2, GABAAR-like and 5HTR genes of abalone larvae exposed to ambient (pH 8.0) and low pH (7.7). A: 48 hpf and B: 96 hpf. Larvae were exposed to the same pH as their parents. Mean ± s.e. * $p < 0.05$, ° $p < 0.10$ (Student t-test).

3.9. Post-larval and juvenile evaluation

439 The main characteristics of abalone post-larvae at 9 days after settlement are shown in **Fig. 6**.

440 After metamorphosis, the post-larval shell shows a well-defined transition between protoconch

441 (pr) and newly deposited sculptured, juvenile shell (js).

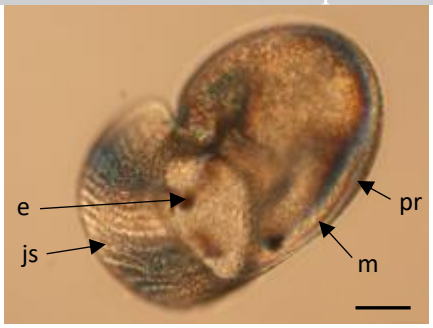


Fig. 6. Microscopic image of post-larval abalone *H. tuberculata* 9 days after settlement. The post-larval shell shows a well-defined transition between the protoconch (pr) and the newly deposited juvenile shell (js). Eyes (e) and mantle (m) are visible through the thin protoconch shell (pr). Scale-bar: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

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

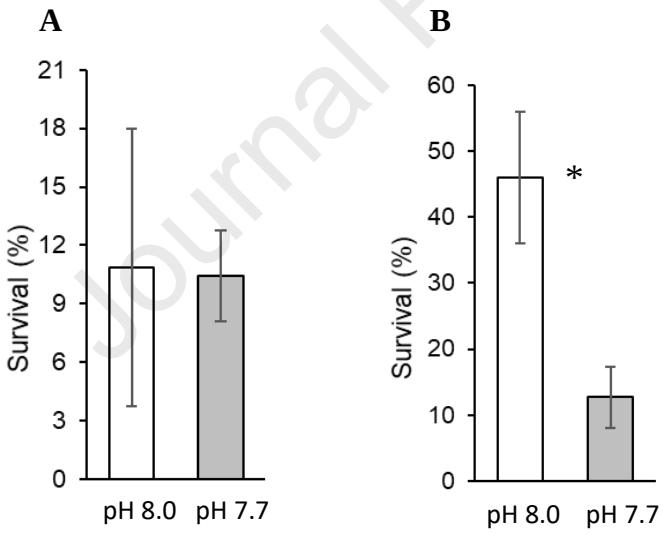
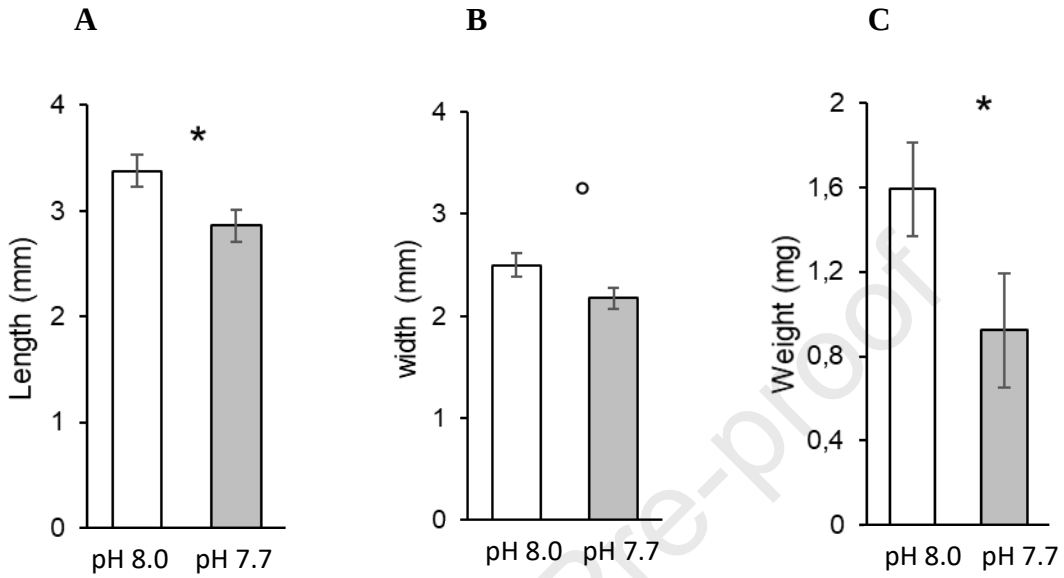


Fig. 7. Survival of abalone post-larvae 9 days (A) and 2 months after settlement (B) in the two pH treatments (pH 8.0 and 7.7). Post-larvae were exposed to the same pH as their parents. Mean ± s.e. * p < 0.05 (Student t-test).

At 2.5 months, juveniles exposed to pH 7.7 had a lower length ($F_{1, 9.9}$ = 5.89, p = 0.036, LMM analysis), tended to have a lower width ($F_{1, 9.6}$ = 4.34, p = 0.065, LMM analysis) and were lighter in weight ($F_{1, 5.2}$ = 6.94, p = 0.045, log transformation, LMM analysis) compared with juveniles kept at ambient pH (**Fig. 8 A–C).** At 5.5 months, shell length ($F_{1,21}$ = 16.56, p < 0.001, LMM analysis), width ($F_{1,20}$ = 19,78, p < 0.001, LMM analysis) and weight ($F_{1,3,9}$ = 21.33, p < 0.001,

452 LMM analysis) were also significantly lower in juvenile abalones exposed to pH 7.7 than in those
453 exposed to ambient pH (**Fig. 8 D–F**).
454

2.5 months



5.5 months

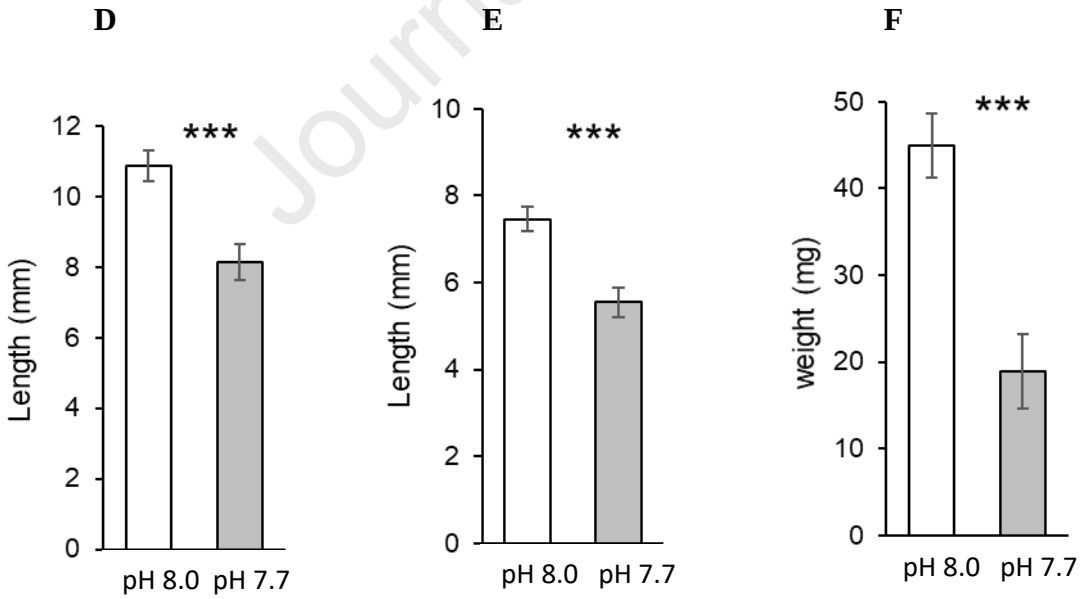


Fig. 8. **Shell length (A, D), width (B, E) and weight (C, F) of 2.5-month-old and 5.5-month-old abalones *H. tuberculata* in the two pH treatments** (pH 8.0 and 7.7). Juveniles were exposed to the same pH as their parents. Ls mean ± s.e.m. * $p < 0.05$; ° $p < 0.10$

4. Discussion

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

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

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

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

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

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

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

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

96 hpf. Interestingly, the expression pattern of the serotonin receptor gene in abalone larvae broadly follows the one described for the GABA A receptor-like gene, at least at 96 hpf. This is in line with the observation that certain invertebrates possess a serotonin-gated chloride channel that may respond to an increase of pCO_2 in the same way as the GABA A receptor (Thomas et al., 2020).

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

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

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

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

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In conclusion, this study demonstrated that OA may have detrimental consequences for abalone populations in natural and aquaculture environments. Because abalone is a slow-growing gastropod with a long transgenerational period, the possibility for this species to adapt to OA is probably lower than that of fast-growing molluscs with rapid life cycles. Consequently, wild abalone populations that are already weakened by anthropogenic pressure (e.g. pollution, overfishing and diseases), will probably be the most at risk over the next decades. In abalone aquaculture, various strategies could be developed to mitigate the effects of OA and help farmers to overcome the challenges posed by climate change. For example, the introduction of macroalgal culture into abalone farming (through IMTA system, Bolton et al., 2009) might be helpful to increase pCO_2 in seawater intake and control the pH in nursery tanks, enabling an upstream protection of the most vulnerable stages. In parallel, the identification of heritable traits involved in resilience to OA, and the subsequent selection of tolerant broodstock may be exploited in the future to ensure the sustainable development of abalone aquaculture.

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610 **Author contributions**

611 Stéphanie Auzoux-Bordenave, Sophie Martin, Sabine Roussel, Sylvain Huchette:
612 Conceptualization, Methodology, Validation;

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

614 Stéphanie Auzoux-Bordenave, Apolline Ledoux, Sophie Martin, Aïcha Badou: Carbonate
615 chemistry analysis, Abalone sampling, Biological measurements;

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

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

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

619 Stéphanie Auzoux-Bordenave, Sabine Roussel: Formal analysis, Visualization, Supervision,
620 Funding, Writing-Original Draft, Reviewing and Editing

621

622 **Declaration of competing interest.** The authors have no conflicts of interest to declare.

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

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: