



Effect of CO₂-induced ocean acidification on the early development and shell mineralization of the European abalone (*Haliotis tuberculata*)

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ARTICLE INFO

Keywords:

Ocean acidification
Abalone
Larval development
Shell mineralization

ABSTRACT

Ocean acidification is a major global stressor that leads to substantial changes in seawater carbonate chemistry, with potentially significant consequences for calcifying organisms. Marine shelled mollusks are ecologically and economically important species providing essential ecosystem services and food sources for other species. Because they use calcium carbonate (CaCO₃) to produce their shells, mollusks are among the most vulnerable invertebrates to ocean acidification, with early developmental stages being particularly sensitive to pH changes. This study investigated the effects of CO₂-induced ocean acidification on larval development of the European abalone *Haliotis tuberculata*, a commercially important gastropod species. Abalone larvae were exposed to a range of reduced pHs (8.0, 7.7 and 7.6) over the course of their development cycle, from early-hatched trochophore to pre-metamorphic veliger. Biological responses were evaluated by measuring the survival rate, morphology and development, growth rate and shell calcification. Larval survival was significantly lower in acidified conditions than in control conditions. Similarly, larval size was consistently smaller under low pH conditions. Larval development was also affected, with evidence of a developmental delay and an increase in the proportion of malformed or unshelled larvae. In shelled larvae, the intensity of birefringence decreased under low pH conditions, suggesting a reduction in shell mineralization. Since these biological effects were observed for pH values expected by 2100, ocean acidification may have potentially negative consequences for larval recruitment and persistence of abalone populations in the near future.

1. Introduction

Ocean acidification and warming are major concerns for marine ecosystems. By the end of the 21st century, global mean surface temperatures are expected to increase by 1 to 3 °C, while surface ocean pH is likely to decrease by 0.1 to 0.3 units (Gattuso et al., 2015). These changes will lead to alterations in seawater carbonate chemistry and a reduction in the degree of saturation with respect to calcium carbonate

(Gattuso et al., 2015; IPCC, 2014). Since marine benthic ecosystems constitute a reservoir for biodiversity, several studies have focused on the evaluation of changing ocean conditions on marine biodiversity via the measurement of key physiological and ecological processes in marine organisms (for a review, see Widdicombe and Spicer, 2008).

Changing ocean conditions are considered as major threats to marine species, affecting early development, skeletal growth and key physiological functions, which can ultimately impact animal behaviour

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<https://doi.org/10.1016/j.jembe.2018.08.005>

Received 31 July 2017; Received in revised form 7 August 2018; Accepted 20 August 2018

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and species distribution (Kroeker et al., 2010; Widdicombe and Spicer, 2008). Reduced oceanic pH has been shown to impact a variety of calcifying species, such as corals, mollusks and echinoderms, leading to contrasting biological responses (Hendriks et al., 2010; Hofmann et al., 2010; Wittmann and Pörtner, 2013). Because calcium carbonate (CaCO_3) is necessary for shell production, mollusks are among the most vulnerable invertebrates to ocean acidification, with larval and juvenile stages being particularly vulnerable (Gazeau et al., 2013; Orr et al., 2005; Przeslawski et al., 2015; Talmage and Gobler, 2010). Indeed, it is during larval development that marine mollusks initiate the deposition of calcium carbonate (CaCO_3) to build their shell (Kurihara, 2008).

A number of studies have reported delays in development, reduced growth rate and/or shell abnormalities in larval mollusks that could potentially affect larval survival, metamorphosis and recruitment into adult populations (Parker et al., 2013; Ross et al., 2011). The impacts of ocean acidification may be particularly severe for bivalves and gastropods, which start to calcify at early developmental stages (Parker et al., 2013). Since many mollusk species are commercially important food sources, negative impacts of ocean acidification may also result in significant economic loss (Ekstrom et al., 2015; Gazeau et al., 2007). Several studies have recently focused on the impacts of elevated partial pressure of CO_2 ($p\text{CO}_2$) on embryonic and larval stages of shelled mollusks, especially cultivated bivalves (see reviews by Gazeau et al., 2013; Parker et al., 2013). In comparison to marine bivalves, our knowledge of the impacts of elevated $p\text{CO}_2$ on early developmental stages in gastropods is based on fewer studies, covering only about four genera (*Cavolinia*, *Crepidula*, *Littorina* and *Haliotis*). In the gastropods studied to date, ocean acidification was shown to reduce larval survival, increase development time, alter morphology and/or impair shell formation and calcification (Byrne et al., 2011; Comeau et al., 2010; Crim et al., 2011; Ellis et al., 2009; Guo et al., 2015; Kimura et al., 2011; Noisette et al., 2014; Zippay and Hofmann, 2010).

Among the species that have been considered in acidification studies, abalone (*Haliotis* spp.) are ecologically and economically important, acting as grazers in the marine ecosystem and as a food source for humans (Cook, 2014; Huchette and Clavier, 2004). Many abalone species worldwide have experienced severe population declines due to both overfishing and the combined effects of environmental stressors such as elevated CO_2 , global warming and pathogen occurrence (Crim et al., 2011; Travers et al., 2009; Morash and Alter, 2015). Early-life-history stages of abalone appear to be negatively affected by elevated CO_2 , with a higher percentage of deformed larvae under low pH conditions than other intertidal marine mollusks such as oysters (Byrne et al., 2011; Crim et al., 2011; Guo et al., 2015; Kimura et al., 2011; Zippay and Hofmann, 2010). For example, elevated seawater CO_2 concentrations impaired larval development and reduced larval survival in the northern abalone *Haliotis kamschatkana* (Crim et al., 2011). In the abalone *H. coccordiata*, embryos were dramatically affected by a combination of warm (+2 to +4 °C) and acidified conditions (−0.4 to −0.6 pH units) with only a small percentage surviving, and with those embryos that did survive producing unshelled larvae (Byrne et al., 2011).

The European abalone *Haliotis tuberculata* is a commercially important mollusk species, for which the whole life cycle is completed under anthropogenic control (Courtois de Viçose et al., 2007). As for most marine invertebrates, abalone display a pelago-benthic life cycle with a larval planktonic stage followed by a critical metamorphosis into the benthic juvenile, making them highly sensitive to environmental changes (Byrne et al., 2011). Larval development and shell formation have been extensively studied in *H. tuberculata*, with deposition of amorphous calcium carbonate (ACC) in the early larval shell followed by deposition of aragonite in the juvenile and adult shell (Auzoux-Bordenave et al., 2010). Since ACC and aragonite are relatively soluble forms of CaCO_3 (compared to calcite), the abalone shell is a relevant model for investigating the effects of ocean acidification. The controlled production of *Haliotis tuberculata* embryos and larvae provides a unique

opportunity to study the impact of acidification on the early development of a marine calcifying species.

Here we investigated the effects of CO_2 -induced ocean acidification on survival, early development, growth and shell mineralization during the entire larval development of the European abalone *Haliotis tuberculata*. Abalone larvae, obtained from a controlled fertilization carried out at the ‘France-Haliotis’ hatchery, were exposed to three experimental pHs (8.0, 7.7 and 7.6) throughout their larval development. Biological responses of larvae were evaluated by measuring the survival rate, morphology and development, growth and shell calcification. Optical and SEM microscopy analyses were performed to assess whether reduced pH had an influence on larval shell morphology and microstructure.

2. Materials and methods

2.1. Production of abalone larvae

Haliotis tuberculata parental stock were collected from northwest Brittany (Roscoff, France) and conditioned in flowing seawater at the ‘France-Haliotis’ commercial abalone hatchery (Plouguerneau, France). Larvae were obtained from controlled fertilizations carried out in September 2013 at a water temperature of 17.0 ± 0.5 °C, which led to approximately 6×10^6 larvae. Following fertilization, egg density was evaluated under a binocular microscope and the embryos were transferred to experimental tanks for acidification experiments.

2.2. Experimental design

The effects of lowered pH were investigated by exposing abalone larvae to three pH conditions, including one present-day control pH 8.0 ($p\text{CO}_2 \approx 400$ μatm) and two levels of pH predicted under varying climate change scenarios: 7.7 ($p\text{CO}_2 \approx 1000$ μatm) and 7.6 ($p\text{CO}_2 \approx 1400$ μatm), as outlined by Riebesell et al. (2010). Experiments were carried out in September 2013 at the ‘France-Haliotis’ hatchery, according to an experimental design adapted from Martin et al. (2011). Fertilized embryos were grown in 300 L tanks of flowing seawater at a density of 5×10^5 per tank, under the three $p\text{CO}_2$ conditions, and with two replicate tanks per condition. The effects of seawater acidification were investigated over the total duration of larval development (5 days), from early-hatched trochophore larvae to the pre-metamorphic stage. *Haliotis* larvae are lecithotrophic and were not fed during the experiment, avoiding any influence of diet on the biological responses.

2.2.1. pH control and carbonate chemistry

Larval tanks were kept in a temperature-controlled room and supplied with natural filtered seawater that was continuously aerated with ambient air. The temperature and salinity were measured daily using a conductivity meter (3110, WTW, Germany). Low seawater pH was obtained by bubbling CO_2 (Air liquide, France) into the tanks through electrovalves regulated by a pH-stat system (Aquastar, IKS Computer System, Germany). Seawater pH (pH_T , expressed on the total hydrogen ion concentration scale, Dickson, 2010) was adjusted to the desired level from ambient pH_T (8.0) to low pH_T (7.7 and 7.6) to within ± 0.05 pH units. pH values on the National Bureau of Standards (NBS) scale obtained with the pH-stat system were adjusted daily with measurements of pH_T for each tank using a pH meter (Metrohm 826 pH mobile, Metrohm, Switzerland) with a glass electrode (Metrohm electrode plus) calibrated on the total scale using Tris/HCl and 2-aminopyridine/HCl buffer (Dickson et al., 2007). pH (NBS) was recorded every 15 min in each tank by the pH-stat system and converted to pH_T by using daily measurements.

Total alkalinity (A_T) of seawater was measured twice on 100 mL samples taken from incoming water and from each experimental tank. Seawater samples were filtered through 0.7 μm Whatman GF/F membranes, immediately poisoned with mercuric chloride and stored in a

cool dark place pending analyses. A_T was determined potentiometrically using an automatic titrator (Titroline alpha, Schott SI Analytics, Germany) calibrated with the National Bureau of Standards scale. A_T was calculated using a Gran function applied to pH values ranging from 3.5 to 3.0 as described by Dickson et al. (2007) and corrected by comparison with standard reference material provided by Andrew G. Dickson (CRM Batch 111). pCO_2 and other parameters of carbonate chemistry were determined from pH_T , A_T , temperature and salinity by using the CO_2SYS software (Lewis and Wallace, 1998) using constants from Mehrbach et al. (1973) refitted by Dickson and Millerro (1987).

2.2.2. Larval sampling and measurements

Larval development was monitored by daily observations with a binocular microscope. Larvae were sampled at four different development stages: trochophore (approximately 20 h post fertilization, hpf), early veliger (30 hpf), post-torsional veliger (48 hpf) and pre-metamorphic stage (96 hpf). At each sampling time point, 10–12 L of seawater containing larvae were collected from each tank. Larval viability was calculated at each stage as the proportion of live larvae divided by the total number of larvae ($n \approx 200$). Larvae were filtered on a 40 μm -sieve and aliquoted into 15 mL tubes. Larval samples were then fixed in either 70% ethanol for transmitted and polarized light microscopy or in a 3% glutaraldehyde solution in Sørensen-sucrose buffer, adjusted to 1100 mOsm for SEM analysis.

2.3. Light and polarized microscopy

2.3.1. Slide preparation and observation

Microscopic slides were prepared with ethanol-fixed larvae from the different pH conditions (two slides per condition). Approximately 100 larvae were whole-mounted in a drop of glycerol, with the amount of ethanol transferred kept to a minimum. Slides were kept at room temperature for 5 to 10 min, allowing ethanol to evaporate and larvae to settle. Four spots of vacuum gel were deposited on the corners of a squared cover slip to prevent the larvae being crushed. After placing the cover slip over the glycerol, the slides were gently sealed with varnish. About 200 larvae per condition were observed and photographed under phase contrast and polarized light with an Olympus binocular microscope (Olympus, Hamburg, Germany) equipped with polarizing filters. All images were acquired with a digital camera (DS-Ri1, Nikon) at 100 \times magnification and 40 ms light exposition. Images were analyzed with NIS-element and Image-J software.

2.3.2. Morphometrical analysis

Larval development stage, shell presence and size were determined under light microscopy ($n = 100$ larvae per tank, 200 larvae per pH condition). Maximal larval length and width of normal larvae were measured in specimens lying on the lateral side, as shown by the dotted

black arrows in Fig. 1. The product of length * width was calculated for each larva and the mean of these values per tank was computed. A growth index per tank was calculated as the square root of each of these means.

Trochophore larvae were scored as one of four possible morphological groups, according to the presence/absence of larval shell, the occurrence of body abnormalities and/or delayed development:

- 1- normal shelled larvae (Fig. 1A),
- 2- shelled larvae with abnormalities or delayed development,
- 3- unshelled larvae with normal body (Fig. 1B),
- 4- unshelled larvae with abnormalities or delayed development,

For veliger stages, the presence/absence of shell, body abnormalities and/or delayed development were also recorded. Additional attributes like mantle formation, appearance of eyes or tentacles, and shell abnormalities were included for the assessment of morphological status.

According to these parameters, veliger larvae were scored as one of the following four morphological groups:

- 1- normal shelled larvae (Fig. 2A, D, G)
- 2- larvae with shell malformation (Fig. 2B, E)
- 3- larvae presenting body abnormalities or delayed development (Fig. 2H)
- 4- larvae with both shell and body abnormalities (Fig. 2C, F, I)

2.3.3. Birefringence analysis

The degree of $CaCO_3$ mineralization within the larval shell was evaluated by measuring the intensity of birefringence under cross-polarized light using an Olympus microscope, according to the method described by Jardillier et al. (2008). Cross-polarized light passing through calcium carbonate (an anisotropic material) is double refracted. As shells with higher calcium carbonate content double refract more light, the intensity of birefringence can be used as a proxy for the evaluation of shell mineralization (Noiset et al., 2014). Measurements were obtained for 30, 48 and 96 hpf larvae. Earlier larval stages were not considered because their shell lacked sufficient crystallized $CaCO_3$ to calculate birefringence. Birefringence was determined for 40 larvae per treatment using Image J software. The mean of grey-scale level (in pixels) was determined for each area of the larval shell showing birefringence (1 to 3 areas per larval shell). The values for each area were averaged into a global mean grey-scale value, providing the birefringence intensity (in %) for each larval shell (Fig. S1, electronic supplementary material). Larvae were categorized as one of three types: fully mineralized (birefringence > 90%); partially mineralized (70% < birefringence < 90%) and less mineralized (birefringence < 70%). Finally, the birefringence intensity of larval shells grown under

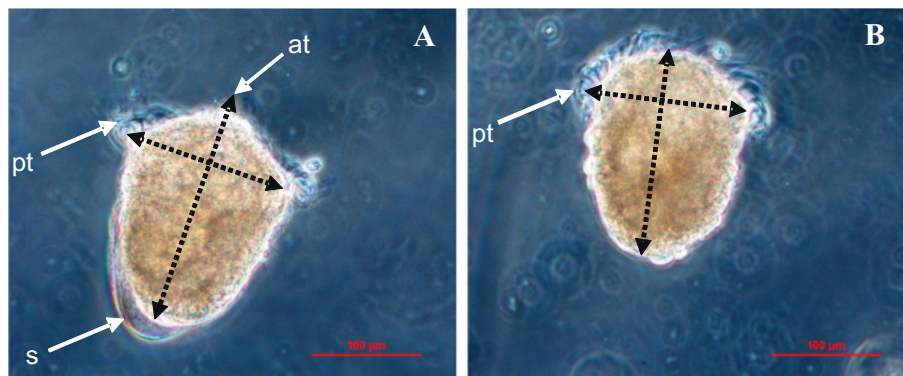


Fig. 1. Trochophore larvae grown in control condition (A) and at pH 7.6 (B). These larvae were respectively classified in the morphological groups 1 (normal shelled) and 3 (unshelled larval phenotype). s: shell, pt.: prototrochal ciliary band, at: apical tuft. Dotted black arrows indicate the length and width measurements.

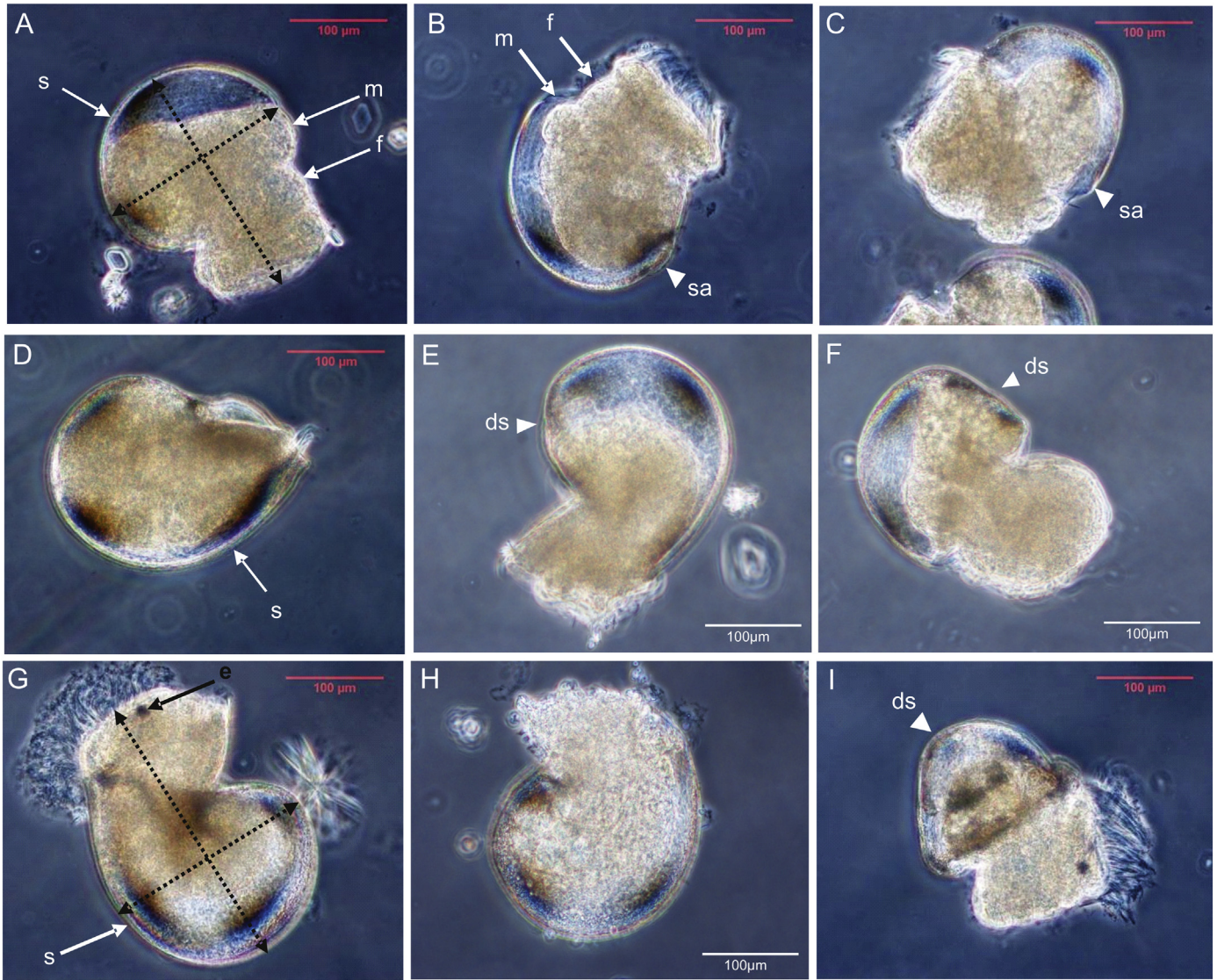


Fig. 2. Morphological variables measured in abalone veliger larvae grown in various pH conditions; the group in brackets refers to morphological groups described in section 2.3.2. A: 30 hpf at pH 8.0 (group 1), B: 30 hpf at pH 7.6 (group 2), C: 30 hpf at pH 7.6 (group 4). D: 48 hpf at pH 8.0 (group 1), E: 48 hpf at pH 7.6 (group 2), F: 48 hpf at pH 7.6 (group 4). G: 96 hpf at pH 8.0 (group 1), H: 96 hpf at pH 7.6 (group 3), I: 96 hpf at pH 7.6 (group 4). Arrows indicate attributes: eyes (e), foot (f), mantle (m), shell (s); arrowheads indicate shell abnormalities (sa) or deformed shell (ds); dotted arrows indicate the length and width measurements.

Table 1

Mean parameters of seawater carbonate chemistry during the experiment. Seawater pH on the total scale (pH_T), temperature (17.0 ± 0.5 °C), salinity (37.0 ± 0.1) and total alkalinity (mean 2344 µEq.kg⁻¹) were used to calculate CO₂ partial pressure (pCO₂; µatm), Dissolved Inorganic Carbon (DIC; µmol.kg SW), HCO₃⁻, CO₃²⁻, aragonite saturation state (Ω_{ar}) and calcite saturation state (Ω_{calc}) by using the CO₂SYS software. pH_T is the average value logged throughout the 5 days of experiment (every 15 mn) in the two tanks (n = 96/tank). Temperature and salinity were measured daily (n = 5); total alkalinity was measured twice (n = 2). Results are expressed as mean ± SD.

Nominal pH	pH _T	pCO ₂ (µatm)	DIC (µmol/kg - ¹)	HCO ₃ ⁻ (µmol/kg - ¹)	CO ₃ ²⁻ (µmol/kg - ¹)	Ω _{ar}	Ω _{calc}
8.1	8.00 ± 0.002	460 ± 3	2119 ± 2	1940 ± 3	163 ± 1	2,47 ± 0,01	3,8 ± 0,02
7.7	7.68 ± 0,001	1055 ± 3	2254 ± 1	2132 ± 0,05	85,4 ± 0,3	1,30 ± 0,01	2 ± 0,01
7.6	7.58 ± 0,003	1331 ± 10	2286 ± 2	2170 ± 2	70,1 ± 0,6	1,06 ± 0,01	1,6 ± 0,01

the different pH conditions was expressed as a percentage of larvae distributed in the three categories for each developmental stage.

2.4. Scanning electron microscopy (SEM)

Four individuals per pH condition were used for SEM analysis. Larval samples were fixed in a 3% glutaraldehyde solution and then washed in Sørensen-sucrose buffer. Samples were subsequently

dehydrated in a series of increasingly concentrated ethanol solutions and were critical point dried with liquid carbon dioxide. Finally, samples were carbon-coated and observed at the “Plateforme d’Imagerie et de Mesures en Microscopie” (PIMM, Université de Bretagne Occidentale, Brest, France) with a scanning electron microscope operating at 5 kV (Hitachi S-3200 N).

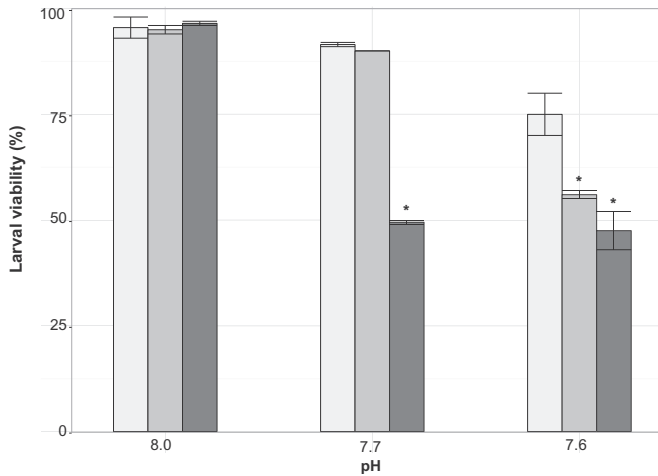


Fig. 3. Viability of abalone larvae exposed to different pH conditions at 30 hpf (light grey), 48 hpf (grey) and 96 hpf (dark grey). Errors bars represent standard deviations. Asterisks denote significant difference between control and low-pH condition (unpaired Student's *t*-test, $p < 0.05$).

2.5. Statistical analyses

All statistical analyses were performed with R software (R Development Core Team, 2014) or Systat 12. In order to determine if larval viability was significantly different between pH treatments, an unpaired Student's *t*-test with Welch correction was performed. For morphological and developmental data, a homogeneity χ^2 test was performed in order to evaluate the effect of elevated pCO_2 on larval phenotypes. Correlation between larval length and width across pH

treatments was assessed using a Spearman's correlation test, allowing length and width to be combined as one parameter to estimate growth. Differences in larval growth across pH treatments were assessed by repeated measures ANOVA using the growth index per tank (pH: fixed crossed factor, time: fixed repeated factor) followed by post-hoc Tukey tests for multiple comparisons using the appropriate mean square error (Doncaster and Davey, 2007). To test the effect of pH on shell birefringence, a homogeneity χ^2 test was performed followed by a pairwise Wilcoxon rank sum. For all tests, differences were considered significant at $p < 0.05$.

3. Results

3.1. Seawater parameters

Mean values of seawater carbonate chemistry parameters are reported in Table 1. The pH_T of experimental aquaria was maintained closed to nominal values throughout the experiment, respectively at a $pH_T = 8.0$ (pCO_2 of $460 \mu atm \pm 3 \mu atm$), $pH_T = 7.68$ (pCO_2 of $1055 \pm 3 \mu atm$) and $pH_T = 7.58$ (pCO_2 of $1331 \pm 10 \mu atm$). In the following sections, rounded mean pH_T values are used namely pH 8.0, 7.7, and 7.6). The temperature was maintained at $17.0 \pm 0.5 \text{ }^\circ C$ ($n = 5$) and salinity at 37.0 ± 0.1 ($n = 5$) in all tanks and there were no significant differences across treatments. Total alkalinity (A_T) measured from incoming water and from experimental tanks differed only slightly and remained stable over the experiment (mean = $2344 \mu Eq.kg^{-1}$).

3.2. Larval viability

Larval viability was measured for the three development stages in the different pH conditions (Fig. 3). In control seawater (pH 8.0) larval

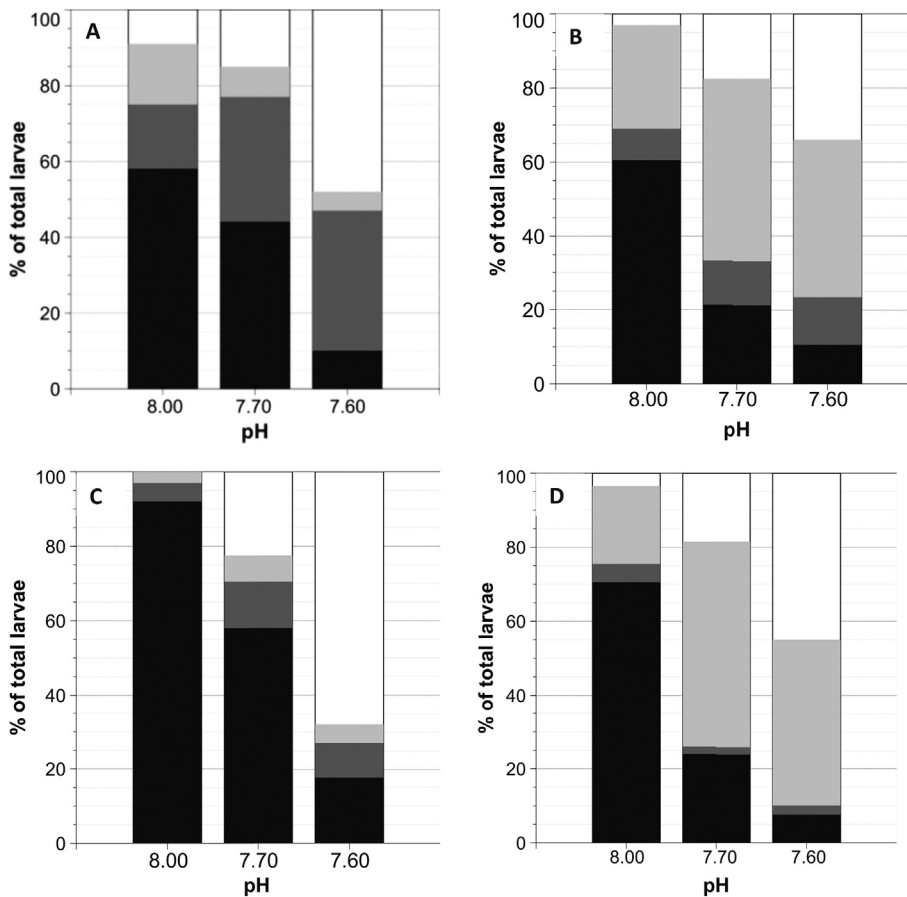


Fig. 4. Morphology and development of abalone larvae exposed to different pH conditions at 20 hpf (A), 30 hpf (B), 48 hpf (C), and 96 hpf (D). Grey levels represent the morphological groups, according to the developmental stage. A (trochophore larvae): black: normal shelled larvae; dark grey: shelled larvae with abnormalities or delayed development; light grey: unshelled larvae with normal body; white: unshelled larvae with abnormalities or delayed development; B, C, D (veliger larvae): black: normal shelled larvae; dark grey larvae with shell malformation(s)/ normal body; light grey: shelled larvae with body abnormalities or delayed development; white: larvae with both shell and body abnormalities.

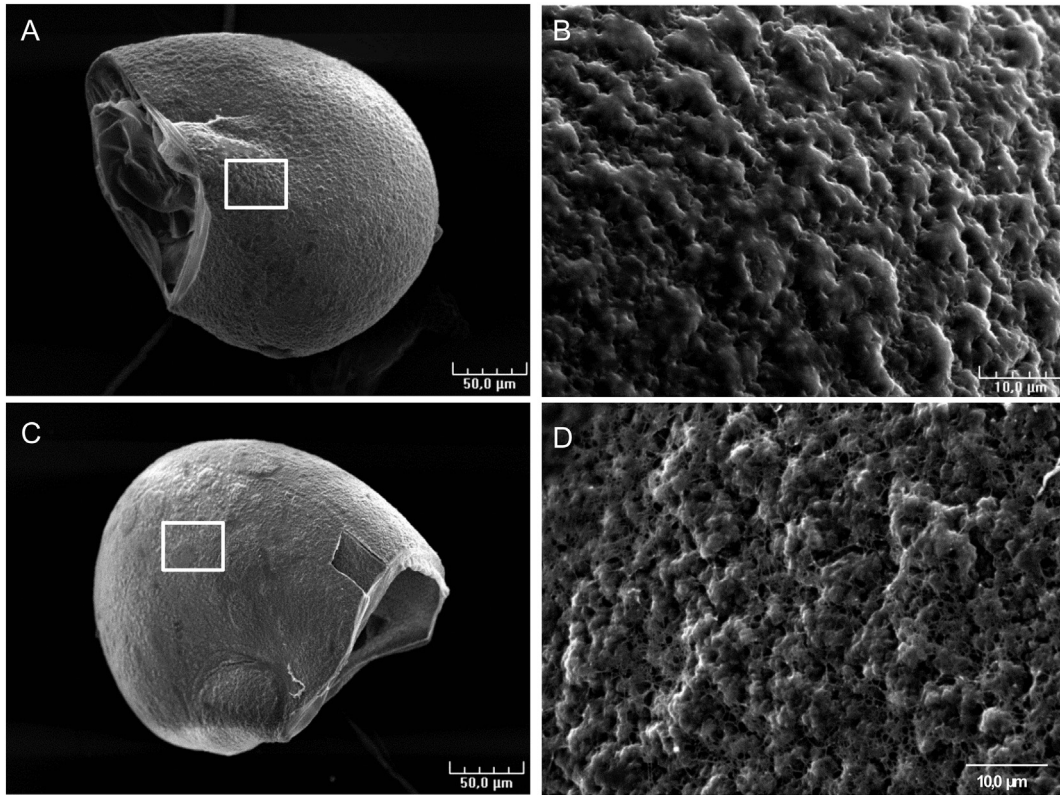


Fig. 5. Scanning electron microscopy (SEM) images of abalone larvae grown under control (A, B) and low pH condition (C, D). Shell surfaces of 48 hpf veliger are shown. A: lateral view of a 48 h-old veliger under control pH (8.0); the protoconch is well developed and covers almost completely the larval body; B: detail of the shell surface boxed in 5A showing a uniform granular texture covered by a very thin organic coating; C: lateral view of a 48 h-old veliger exposed to low pH (7.6); the protoconch appears well developed but exhibits a heterogeneous surface; D: detail of the protoconch surface boxed in 5C showing numerous small holes interspaced between the biominerals and remnants of the organic coating.

Table 2

Results of the Spearman's correlation performed to evaluate the nullity of correlation between larval length and width for each pH treatment in the course of the experiment.

Treatment (pH)	Rho	p-value
8.0	0.2842896	< 0.001
7.7	0.1919181	< 0.001
7.6	0.4593034	< 0.001

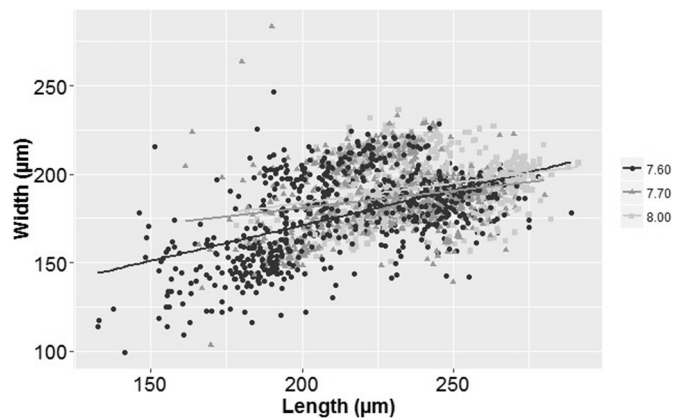


Fig. 6. Correlation between length and width for each pH treatment. Light grey represents the control pH (8.0), dark grey represents the pH 7.7, and black represents the pH 7.6. Results of the statistical analyses are reported in Table 2.

viability remained > 95% until the pre-metamorphic stage. A significant decrease in larval viability was observed in reduced pH treatments. At pH 7.7 reduced larval viability was observed in 30 and 48 hpf larvae, although the decrease was not significant ($p = 0.257$ and $p = 0.125$ respectively), but a 50% reduction in viability at 96 hpf was significant (unpaired Student's *t*-test, $p < 0.001$). At pH 7.6, viability slightly decreased to 75% at 30 hpf ($p = 0.067$) and dropped to 56% and 47% at 48 and 96 hpf respectively (unpaired Student's *t*-test, $p = 0.001$ and $p = 0.008$ respectively). Abnormalities in larval morphology and hyperactive movements were observed in the two low pH conditions for all four larval stages (S. Auzoux-Bordenave, pers. observations).

3.3. Morphology and development

Larval developmental stage and shell presence were determined at each time point and compared between the different treatments, according to the four morphological groups defined in section 2.3.2. Larval distribution within these groups across the different pH treatments is shown for the trochophore stage (Fig. 4A) and later veliger stages (Figs. 4B–D). At each developmental stage, conformity χ^2 tests showed that the distribution within the groups was dependent on the pH ($p < 0.001$).

At the first developmental stage (20 hpf trochophore), larval distribution into the different morphological groups was pH dependent (Fig. 4A; $\chi^2 = 83.31$, $df = 6$, $p < 0.001$). Normal shelled larvae represented 60% of the total amount of larvae in the control group, while this proportion was < 10% at the lowest pH ($\chi^2 = 83.31$, $df = 6$, $p < 0.001$). The percentage of shelled larvae, including those with normal and abnormal bodies, was significantly lower at pH 7.6 (47%) compared to controls (75%) $\chi^2 = 25.19$, $df = 2$, $p < 0.001$). In

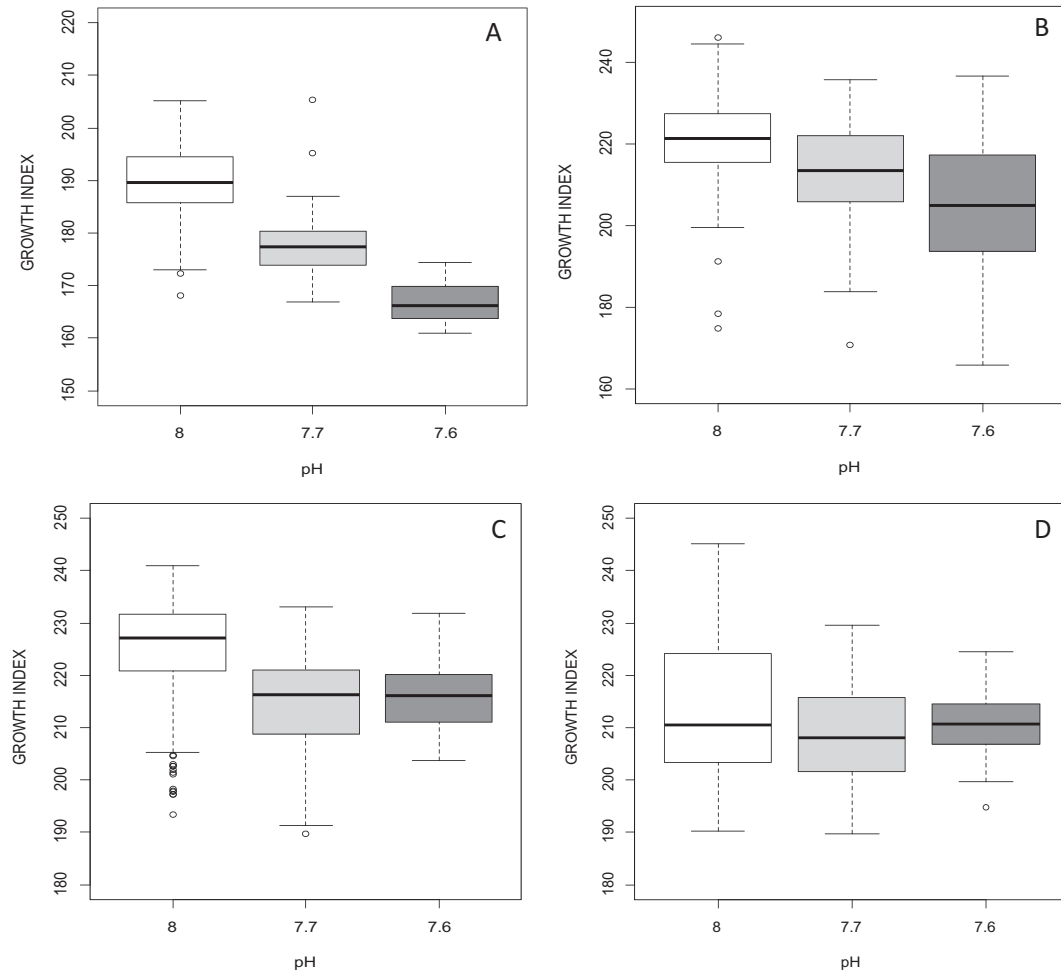


Fig. 7. Effect of decreased pH on abalone larval growth, expressed as square roots of (length x width) at 20 hpf (A), 30 hpf (B), 48 hpf (C), and 96 hpf (D). Grey level of the boxes denote the different pH conditions. Centre lines of box plots show the medians; box limits indicate the first and third quartile respectively with lines encompassing data within 1.5 times the spread from the median ($p < 0.05$). Results of the statistical analyses are reported in Table 3.

parallel, the percentage of unshelled larvae was significantly greater at pH 7.6 (53%) compared to controls (10%) ($\chi^2 = 25.19$, $df = 2$, $p < 0.001$). The presence of unshelled larvae with normal bodies in the control group probably occurred due to differences in the timing of shell morphogenesis among individuals.

The distribution of veliger stages into the different morphological groups was also pH dependent, and similar to that observed in trochophore larvae (Fig. 4B–D). At 30 hpf, a strong decrease in the percentage of normal larvae was observed, from 60% in the control group to only 10% at the lowest pH (Fig. 4B; $\chi^2 = 75.25$, $df = 6$, $p < 0.001$). Meanwhile, the proportion of malformed or delayed larvae increased from 25 to 45%. An important impact of acidification was observed at 48 hpf, where the proportion of normal larvae decreased from 90% in the control condition to about 58% at pH 7.7 and < 20% at pH 7.6 (Fig. 4C; $\chi^2 = 133.77$, $df = 6$, $p < 0.001$). The proportion of larvae showing both shell and body abnormalities (cumulative effects) strongly increased when pH was reduced, from a negligible percentage in the control group, to above 65% at pH 7.6 ($\chi^2 = 133.77$, $df = 6$, $p < 0.001$). At 96 hpf, the percentage of normal larvae also drastically decreased in the reduced pH treatments, from 70% in the control group to 25% at pH 7.7 and to 12% at pH 7.6 (Fig. 4D) ($\chi^2 = 119.14$, $df = 6$, $p < 0.001$). As a result, the amount of larvae with body malformations or delayed development increased significantly with seawater acidification, from 20% in control conditions to 45% at pH 7.6. For all three veliger stages, the proportion of larvae that showed both shell and body abnormalities (cumulative effects) strongly increased when lowering

the pH, while the proportion of larvae displaying only shell malformation remained < 15% and did not differ significantly among treatments.

Larval shells (48 hpf) grown at lower pH exhibited differences in the texture and porosity of the surface layer (Fig. 5). In control larvae, the mineralized protoconch almost completely covered the larval body (Fig. 5A). At higher magnification, the shell surface showed a uniform granular texture covered by a very thin homogeneous layer (Fig. 5B). In 48 hpf veliger larvae exposed to decreased pH (7.6), the protoconch exhibited an irregular surface, with differences in thickness, and the homogeneous outer layer was not as distinct as that of control larvae (Fig. 5C). The outer surface had a porous appearance with numerous small holes interspersed between the biominerals and remnants of the organic coating (Fig. 5D).

3.4. Larval growth

Length and width of abalone larvae reared in the three pH conditions were measured. Only normal larvae were used in order to determine if growth rate differed according to pH. For each treatment, a Spearman's correlation was carried out to evaluate the relationship between larval length and width during the course of the experiment (Table 2). The results showed a linear relationship between the two measures for each pH group (Fig. 6), allowing length and width to be combined as one parameter to estimate larval growth. The growth index (calculated as the square root of length * width) of larvae exposed

Table 3

Summary of statistics used to test the differences in larval growth. A. Results of the repeated measures ANOVA on larval growth index (square root of length * width); B. Multiple comparison Tukey tests testing the effects of pH (fixed crossed factor) according to time (fixed repeated factor). Significant results in bold ($p < 0.05$).

A. Repeated measures ANOVA				
Effect between subjects	df	MS	F-ratio	p-value
pH	2	357.3	33.1	0.009
Error	3	10.8		
Effect within subjects				
Effect within subjects	df	MS	F-ratio	p-value
Time	3	1998	141.6	< 0.001
Time * pH	6	38.0	2.7	0.088
Error	9	14.1		
B. Multiple comparisons				
on factor time:				p-value
20 h vs 30 h				0.002
20 h vs 48 h				< 0.001
20 h vs 96 h				< 0.001
30 h vs 48 h				0.159
30 h vs 96 h				0.418
48 h vs 96 h				0.009
On factor pH:				p-value
7.6 vs 7.7				0.105
7.6 vs 8.0				0.008
7.7 vs 8.0				0.031

to the three pH conditions and according to time is presented in Fig. 7.

Both pH and time significantly affected the growth index (Table 3A; $p = 0.009$ and $p < 0.001$, respectively), while the interaction term was not significant (Table 3A; $p = 0.088$). Larval size significantly increased between 20 hpf and 30 hpf and between 48 hpf and 96 hpf (Table 3B; Tukey, $p = 0.002$ and $p = 0.009$, respectively). Larval size was significantly lower at pH 7.7 and 7.6 vs 8.0 (Tukey, $p = 0.008$ and $p = 0.031$, respectively) but did not differ significantly between the two low pH treatments (Table 3B; Tukey, $p = 0.105$).

3.5. Shell calcification

Under control pH conditions, larvae clearly exhibited the characteristic dark cross indicating a radial arrangement of the aragonite crystals within 2 to 3 areas of birefringence (Fig. 8B). Larvae grown at lower pH (7.6) typically showed a decrease in shell birefringence indicating reduced calcification (Fig. 8D). At 30 hpf, the distribution of larvae into the three categories (i.e. fully mineralized, partially mineralized and less mineralized) was not significantly different between the three pH treatments (Fig. 9A; Table 4A, $\chi^2 = 9.28$, $df = 4$, $p = 0.055$). At 48 hpf, the number of fully mineralized larvae was strongly reduced at low pH conditions, while the number of partially and less mineralized larvae was significantly higher (Fig. 9B; Table 4B, $p < 0.001$). Similar results were observed in 96 hpf larvae, with significant differences in larval birefringence between the control group and the low pH group 7.7. (Fig. 9C; Table 4B, $p < 0.001$). At pH 7.6, larval distribution within the three categories was close to that observed in the control group (Table 4B, $p = 0.073$).

4. Discussion

This study provides the first evidence that decreased pH negatively

impacts survival, early larval development and shell calcification in the European abalone *Haliotis tuberculata*. Biological responses were measured over the total duration of larval development (from early-hatched trochophore to the pre-metamorphic stage), using near-future oceanic CO₂ levels. Our results are consistent with previous studies on mollusk larvae, in which near-future CO₂ concentrations reduced survival and impaired early development, suggesting potential negative consequences for larval recruitment and the persistence of abalone populations (see reviews by Gazeau et al., 2013; Ross et al., 2011).

Our results showed that *H. tuberculata* experienced a significant decrease in larval survival at pH 7.7 and 7.6 with a maximum decrease of 47% at pH 7.6 relative to the control. Furthermore, low pH conditions negatively affected larval morphology, inducing a developmental delay and a decrease in the proportion of shelled larvae. In addition to developmental delays and body abnormalities, seawater acidification resulted in decreased growth rate (length and width). These results are consistent with previous studies on other abalone species, such as those of Byrne et al. (2011), Crim et al. (2011) and Kimura et al. (2011) that found reduced survival in, respectively, *H. coccordiata*, *H. kamschatkana* and *H. discus hannai*. Moreover, these studies showed similar results with respect to increasing numbers of unshelled larvae at lower pHs (Byrne et al., 2011; Crim et al., 2011), as well as reduction in larval size (Crim et al., 2011; Kimura et al., 2011). Abalone appears to be particularly sensitive to ocean acidification at early life stages, which may lead to lower recruitment in these commercially and ecologically important species. Similarly, a recent comparison of two abalone species (*H. discus hannai* and *H. diversicolor*) together with the oyster *Crassostrea angulata* found reduced size to be a common response to reduced pH (Guo et al., 2015). Among other species, deformities have been observed in the gastropod *Crepidula fornicata* under reduced pH (Noisette et al., 2014), and the oysters *Crassostrea gigas* and *Saccostrea glomerata* displayed reduced development rates and increasing levels of abnormalities in low pH treatments (Kurihara et al., 2007; Kurihara, 2008; Parker et al., 2009; Parker et al., 2010; Timmins-Schiffman et al., 2013; Watson et al., 2009). More generally, it seems that reduced survival, abnormal or prolonged development and reduced size represent a common response of many marine mollusk larvae exposed to elevated CO₂ (Gazeau et al., 2013; Ross et al., 2011).

In addition to reduced survival, development abnormalities and decreased growth rate, seawater acidification also resulted in reduced shell calcification of abalone larvae, as shown by the decrease in birefringence intensity under cross-polarized light. The reduction in shell calcification in larvae grown at lower pH treatments was highly significant at later stages (48 and 96 hpf). However, only a slight, but not significant, increase in the percentage of partially mineralized shells was observed at 30 hpf under low pH 7.7, and no significant differences were observed between control and pH 7.6. This could be related to low amounts of calcium carbonate in the earliest larval shell, which in combination with heterogeneous crystalline orientation, might have resulted in higher variability in the grey levels. In the future, attempts to calibrate grey scale levels with measures of shell mass may allow improvements in the use of birefringence as a proxy for determining shell mineralization.

Cross-polarized light and SEM observations of shelled larvae provided evidence of a lack of calcification under lower pH, suggesting that larvae deposited less calcium carbonate and produced a thinner shell. The impacts of ocean acidification on shell calcification have been well studied in juvenile and adult mollusks, showing that their calcified shell is highly sensitive to elevated pCO₂ (Gazeau et al., 2013). For example, in the marine bivalves *Mytilus edulis* and *C. gigas*, the calcification rate was negatively impacted by a short-term exposure to low pH (−0.2 units from ambient, Gazeau et al., 2007). Previous studies have reported a decrease in shell size and shell thickness as well as changes in morphology and shape of the larval shell under elevated pCO₂ (Gazeau et al., 2010; Kurihara et al., 2007, 2008; Miller et al., 2009; Talmage and Gobler, 2009). Few studies have reported changes in

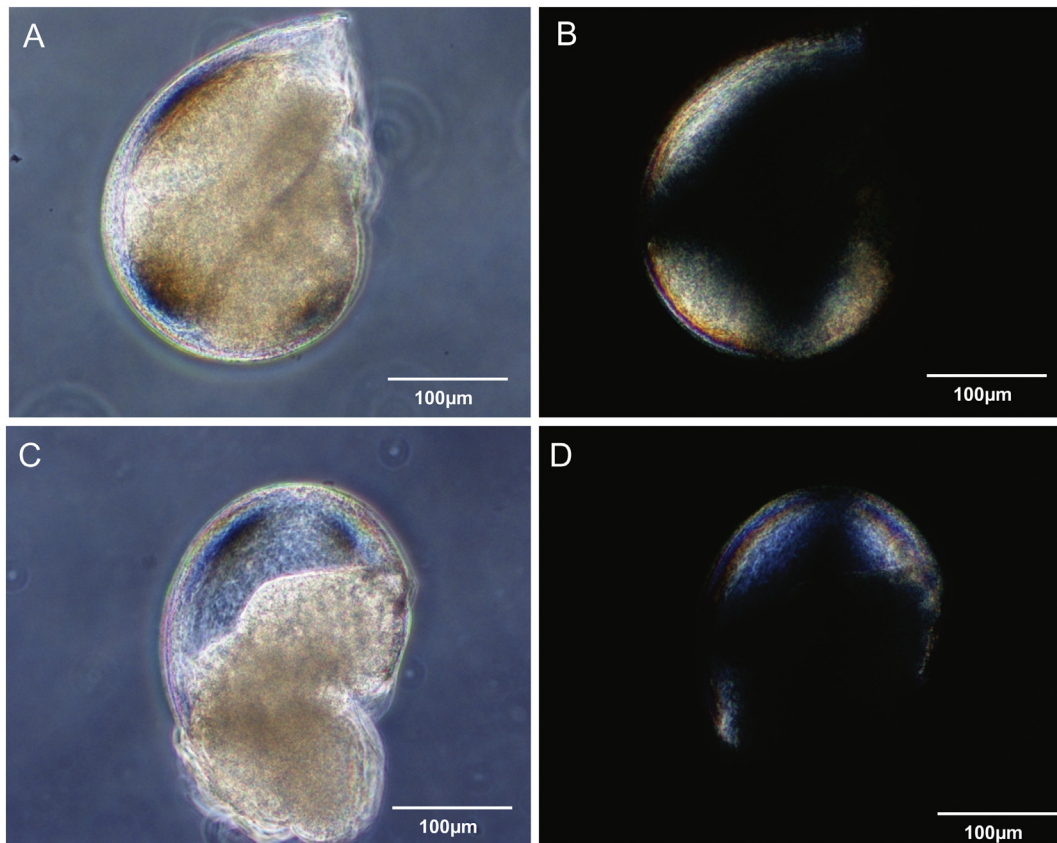


Fig. 8. Morphology and shell birefringence of 48 hpf veliger larvae grown under control (A, B) and low pH conditions (C, D). Larvae were observed under phase contrast (A, C) and polarized microscopy (B, D). Normal larvae under control pH (8.0) showing the characteristic black cross of birefringence (A, B). Shell abnormalities in larvae exposed to low pH (7.7) result in a significant decrease of calcified areas (C, D).

larval shell calcification under OA stress by measuring birefringence intensity; however, our results on larval abalone shell are consistent with those of [Kurihara et al. \(2007\)](#) and [Noisette et al. \(2014\)](#), obtained respectively in *Crassostrea gigas* and *Crepidula fornicata*, showing a reduction in larval shell calcification when seawater pH is lowered.

Mollusk shell formation is a complex process starting at the trochophore stage in abalone. The larval shell is formed by a specialized group of ectodermal cells that form the shell gland and the organic periostracum. Primary mineralization takes place between the shell gland and the periostracum, producing the so-called protoconch in gastropods ([Eyster, 1986](#)). In abalone, the first deposition of CaCO_3 occurs at an early veliger stage with the deposition of amorphous calcium carbonate (ACC), which is rapidly transformed into crystalline aragonite ([Auzoux-Bordenave et al., 2010](#)). Since ACC and aragonite are more soluble forms of CaCO_3 than calcite in lower pH conditions, the larval shell is likely to be more susceptible to dissolution than juvenile or adult shell. In the present study, SEM images of larval shells reared at pH 7.6 revealed numerous small holes at the shell surface, suggesting that CaCO_3 dissolution is likely to be a factor explaining reduced calcification in abalone larvae experiencing acidified conditions. Shell dissolution processes may arise as a result of a lower availability of carbonate ions at the site of calcification (a direct effect of the carbonate chemistry) or through indirect physiological changes in ionic composition, matrix protein formation or enzymatic activities ([Hüning et al., 2012](#)).

Recent studies have suggested that lowered aragonite saturation state may be one of the key parameters controlling whether shell development in larval mollusks occurs normally ([Thomsen et al., 2015](#); [Waldbusser et al., 2015](#)). In the oyster *C. gigas*, developmental success and growth rates were not significantly altered as long as carbonate ion concentrations were above aragonite saturation levels, but they

strongly decreased when carbonate ion concentrations dropped below aragonite saturation levels ([Gazeau et al., 2011](#)). The authors suggested that the mechanisms used by oyster larvae to regulate calcification rates were not efficient enough to compensate for the low availability of carbonate ions under acidified conditions. Interestingly, recent studies suggest that initial shell formation in larvae can occur even when aragonite is reduced below saturation levels ([Frieder et al., 2016](#)), and that dissolution of the initial shell, at least in species such as *M. edulis*, only occurs at very high levels of OA ([Ramesh et al., 2017](#)). However, it is likely that resource allocation trade-offs between biomineralization and other vital biological processes will begin to occur as larval development progresses, as shown in sea urchins ([Pan et al., 2015](#)). This could help to explain why reduced birefringence of *H. tuberculata* larvae under elevated $p\text{CO}_2$ was only observed at 48 and 96 hpf, and not at 30 hpf (this study).

As well as directly causing shell dissolution, a number of other biological processes responsible for larval shell calcification, such as matrix protein production, chitin synthesis and enzymatic control are influenced by changes in seawater $p\text{CO}_2$ ([Weiss et al., 2013](#)). For example, the activity of carbonic anhydrase, an enzyme that catalyzes the reversible hydration of CO_2 to HCO_3^- and H^+ , reaches its maximum activity at the end of each developmental stage and has been correlated with larval shell biomineralization ([Gaume et al., 2011](#); [Medakovic, 2000](#)). In the mussel *M. edulis*, six months of incubation at 750 μatm $p\text{CO}_2$ (pH 7.5) significantly reduced carbonic anhydrase activity within the mantle tissue, explaining shell growth reduction ([Fitzer et al., 2014b](#)). More recently, evidence from proteomic studies suggests that elevated $p\text{CO}_2$ influences a wide range of molecular pathways, including several associated with biomineralization, metabolism and the cytoskeleton, and which may correlate with calcification ([Dineshram et al., 2015](#); [Harney et al., 2016](#)).

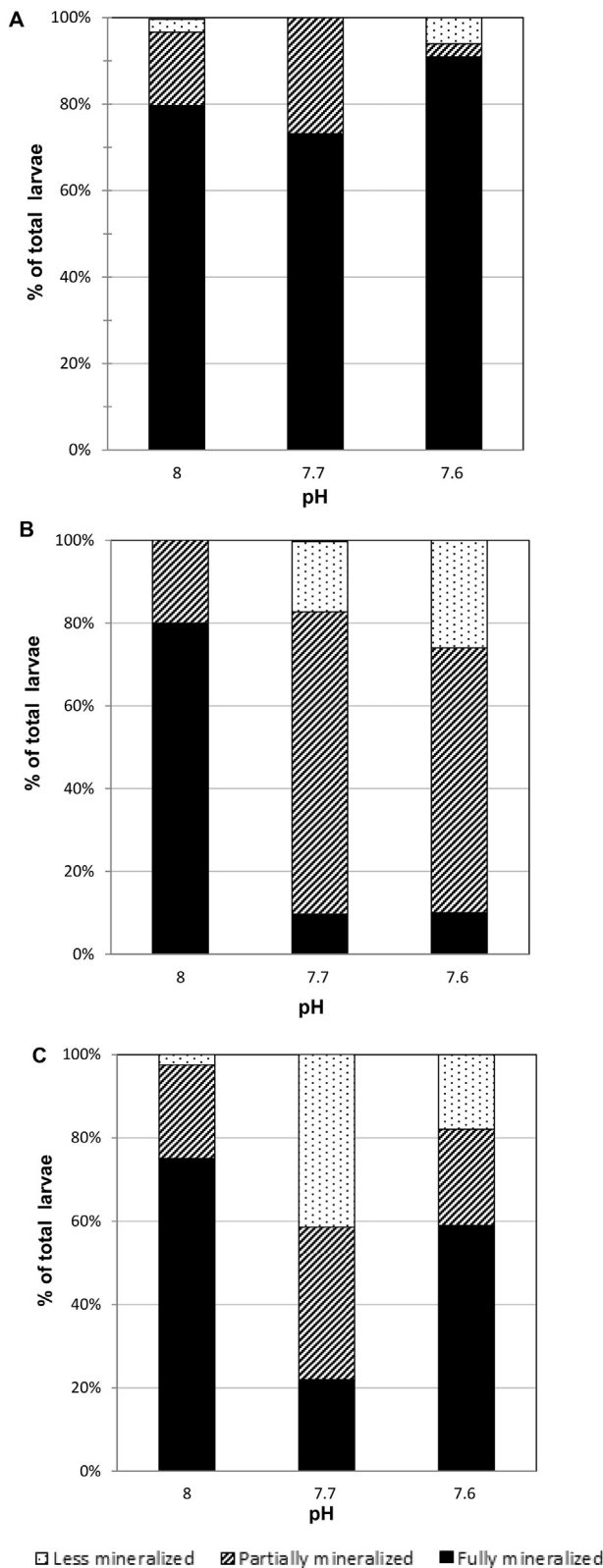


Fig. 9. Shell mineralization of larval abalone determined by polarized light microscopy for each development stage (A: 30 hpf, B: 48 hpf, C: 96 hpf). Grey levels represent the three categories of mineralization: black bars for fully mineralized (birefringence > 90%); stripe bars for partially mineralized (70% < birefringence < 90%) and dotted bars for less mineralized shells (birefringence < 70%). Differences in larval distribution across pH treatments were tested using a homogeneity χ^2 test by treating birefringence as a categorical factor ($n = 40$ larvae per pH condition). Results of the statistical analyses are reported in Table 4.

Table 4

Summary of statistics used to test the differences in shell birefringence. A. Homogeneity χ^2 test used to test the effect of pH on shell birefringence, for each development stage. B. Pair-wise Wilcoxon rank sum test showing differences between pH groups; Bonferroni adjusted p -values. Significant results in bold ($p < 0,05$).

A. Homogeneity χ^2 test			
Development Time	Chi-sq	df	p -value
30 h	9.28	4	0.055
48 h	67.53	4	< 0.001
96 h	28.48	4	< 0.001

B. Pair wise Wilcoxon rank sum test		
	48 h	96 h
pH factor	p -value	p -value
7.7 vs 8.0	< 0.001	< 0.001
7.6 vs 8.0	< 0.001	0.218
7.6 vs 7.7	1	0.0032

In order to discriminate between the effect of the aragonite saturation state and the complex physiological effects of pH decrease on mollusk shell formation, future studies should explicitly consider CaCO_3 saturation state in experimental seawater and the ability of the species to maintain internal pH at the site of calcification. Failure to properly biomineralise at this early stage not only results in reduced survival and developmental problems for larvae (Byrne, 2012), but can also have carry-over effects later in life (Hettinger et al., 2013; Rühl et al., 2017), which can explain why larval stages represent such a major bottleneck for population persistence under changing environmental conditions (Przeslawski et al., 2015). In this study, fertilization was carried out under ambient conditions, and resulting embryos were transferred into experimental tanks with different $p\text{CO}_2$ conditions, as is the case of most studies assessing the impacts of ocean acidification on mollusk larvae. It is now well established that exposure of adults to elevated CO_2 during reproductive conditioning can result in positive or negative carry-over effects being transmitted from adults to their offspring, influencing the resilience of mollusks to ocean acidification (Fitzer et al., 2014; Parker et al., 2013). In the oyster *S. glomerata*, Parker et al. (2012) found that larvae from parents exposed to elevated CO_2 during reproductive conditioning were larger and developed faster when they also experienced reduced pH, as compared to larvae from parents conditioned under ambient pH. These results highlight the importance of assessing carry-over effects for determining species responses to ocean acidification. To overcome this problem, future projects should conduct long-term transgenerational experiments. Biological responses measured during long-term exposure to elevated $p\text{CO}_2$, from reproducing adults to larval and juvenile stages, will provide valuable information regarding acclimation and adaptation of abalone to changing ocean conditions. In addition, the exploration of multiple stressors known to interact with pH (e.g. temperature, salinity, pathogens and pollutants) will result in more ecologically realistic simulations of the impact of global environmental change, providing a greater understanding of ecological relationships (e.g. Ko et al., 2014). Indeed, consideration of multiple stressors is also crucial in fisheries and aquaculture to identify optimal conditions and adapt culturing practices for sustainable shellfish production.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jembe.2018.08.005>.

Acknowledgements

N. Wessel was supported by a post-doctoral fellowship from the MNHN (Ministère de l'Enseignement Supérieur et de la Recherche, Paris, France). This work was financed in part by the ATM program "Biomineralization" of the MNHN funded by the Ministère délégué à l'Enseignement Supérieur et à la Recherche (Paris, France). V. Julia, F. Nunes and E. Harney were supported by a grant from the Regional Council of Brittany, from the European Funds (ERDF) and supported by the "Laboratoire d'Excellence" LabexMER (ANR-10-LABX-19) and co-funded by a grant from the French government under the program "Investissements d'Avenir". We thank Gérard Sinquin for his assistance in scanning electron microscopy (Plateforme d'Imagerie et de Mesures en Microscopie, Université de Bretagne Occidentale, Brest). P. Dubois is a Research Director of the National Fund for Scientific Research (Belgium).

The experiments complied with the current French laws.

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