# Acid–base balance in the hæmolymph of European abalone (*Haliotis tuberculata*) exposed to $CO_2$-induced ocean acidification

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## Abstract

Ocean acidification (OA) and the associated changes in seawater carbonate chemistry ~~that~~ pose a threat to calcifying organisms. This is particularly serious for shelled molluscs, in which shell growth and microstructure has been shown to be highly sensitive to OA. To improve our understanding of the responses of abalone to OA, this study investigated the effects of $CO_2$-induced ocean acidification on extra-cellular acid–base parameters in the European abalone *Haliotis tuberculata*. Three-year-old adult abalone were exposed for 15 days to three different pH levels (7.9, 7.7, 7.4) representing current and predicted near-future conditions. Hæmolymph pH and total alkalinity were measured at different time points during exposure and used to calculate the carbonate parameters of the extracellular fluid. Total protein content was also measured to determine whether seawater acidification influences the composition and buffer capacity of hæmolymph. Extracellular pH was maintained at seawater pH 7.7 indicating that abalones are able to buffer moderate acidification (-0.2 pH units). This was not due to an accumulation of $HCO_3^-$ ions but rather to a high hæmolymph protein concentration. By contrast, hæmolymph pH was significantly decreased after 5 days of exposure to pH 7.4, indicating that abalone do not compensate for higher decreases in seawater pH. Total alkalinity and dissolved inorganic carbon were also significantly decreased after 15 days of low pH exposure. It is concluded that changes in the acid-base

balance of the hæmolymph might be involved in deleterious effects recorded in adult *H. tuberculata* facing severe OA stress. This would impact both the ecology and aquaculture of this commercially important species.

**Keywords:** ocean acidification; abalone; acid–base balance; hæmolymph pH and alkalinity; buffer capacity

**Abbreviations**: $A_T$: Total alkalinity; DIC: Dissolved Inorganic Carbon; $pH_{NBS:}$ pH of the National Bureau of Standard; $pH_{T:}$ pH on the Total scale; $pH_{SW:}$ seawater pH (total scale); $pH_{HL}$-: hæmolymph pH (total scale); RCP 8.5: Representative Concentration Pathway 8.5

## 1. Introduction

The increase of anthropogenic $CO_2$ emissions over the past 200 years and its subsequent absorption (about ¼) by the ocean is responsible for seawater pH decrease and substantial changes in carbonate chemistry, a process known as ocean acidification (OA) (Caldeira and Wickett, 2003; Fabry et al., 2008; Gattuso et al., 2015). According to the most pessimistic scenario for future greenhouse gas emissions (Representative Concentration Pathway 8.5: RCP 8.5), surface ocean pH should decrease by 0.33 pH units by the year 2100 (IPCC, 2014), resulting in deleterious effects on the biology and ecology of numerous marine organisms (Widdicombe and Spicer, 2008; Kroeker et al., 2010; Wittmann and Pörtner, 2013). Changes in ocean chemistry may be more pronounced in coastal areas where numerous species of economic importance (such as molluscs) can be found and such changes may be exacerbated by diurnal and seasonal variations in shallow and intertidal zones (Truchot, 1988; Byrne et al., 2011; Legrand et al., 2018). OA is also predicted to affect aquaculture activities around the globe through its effects on species physiology, and behaviour in relation to farming practices (Cochrane et al., 2009; Clements and Chopin, 2016; Weatherdon et al., 2016).

Among its reported biological effects, increased seawater $pCO_2$ has been shown to induce hypercapnia and acidosis in organisms, both of which are energetically costly processes that can affect vital functions such as growth and calcification (Pörtner et al., 2004; Fabry et al., 2008; Melzner et al., 2009; Orr et al., 2005; Hofmann et al., 2010). Due to a low capacity to regulate

66 their acid–base balance and the highly calcified shells they build, marine molluscs are
67 considered to be among the most vulnerable species with regard to OA (Fabry et al., 2008;
68 Gazeau et al., 2013; Parker et al., 2013). Abalones (Mollusca, Vetigastropoda) are
69 ecologically important herbivore species, providing key ecosystem services through their role
70 in nutrient and mineral cycling, and are economically important as a food source (Cook,
71 2016; Huchette and Clavier, 2004). Many abalone species have experienced severe
72 population declines worldwide due to both overfishing (Rogers-Bennett et al., 2002) and
73 environmental disruptions, such as global warming and wasting disease (Cook, 2016; Moore
74 et al., 2002; Morales-Bojórquez et al., 2008; Nicolas et al., 2002; Travers et al., 2009). In the
75 context of a worldwide expansion in their aquaculture, understanding the effects of
76 environmental stresses on abalone physiology is an important issue for the management of
77 wild populations as well as for the optimization of fisheries and aquaculture practices
78 (Morash and Alter, 2015).

79 *Haliotis tuberculata* is the only abalone species present in Europe, living in low intertidal
80 and subtidal rocky coastal areas where it offers a high potential for fishery and aquaculture
81 (Huchette and Clavier, 2004; Courtois de Viçose et al., 2007). Several studies focusing on
82 early life-history stages of abalone, especially larvae, have demonstrated adverse effects of
83 elevated $pCO_2$, such as reduced survival, developmental delay, body and shell abnormalities
84 and reduced mineralization (Byrne et al., 2011; Crim et al., 2011; Guo et al., 2015; Kimura et
85 al., 2011; Onitsuka et al., 2018; Wessel et al., 2018; Zippay and Hofmann, 2010). Impacts of
86 OA on shell calcification and integrity have been well studied on various stages of *H.*
87 *tuberculata*, from larvae to juveniles and adults (Wessel et al., 2018; Auzoux-Bordenave et
88 al., 2020; Avignon et al., 2020). Reduced growth in shell length and alterations of the shell
89 microstructure were observed in juvenile abalone exposed to $pH_T$ 7.6, which correspond to
90 projected pH values expected for 2100 (IPCC, 2014; Gattuso et al., 2015). Since these pH
91 conditions corresponded to an aragonite saturation state below 1, it was concluded that the
92 effects on shell growth and integrity were principally caused by direct aragonite dissolution
93 within the abalone shell (Auzoux-Bordenave et al., 2020). Additional effects on the organic
94 periostracum and nacre structure suggested that other processes involved in shell

biomineralization, such as matrix protein production and enzymatic activities, would also be influenced by changes in seawater pH. Avignon et al. (2020) demonstrated significant effects on growth in shell length, microstructure and resistance in adult *H. tuberculata* exposed to $pH_T$ 7.7. Although the aragonite saturation was above 1 (1.24) here, the effects on shell integrity were partly attributed to direct effects on aragonite dissolution. The study also found that the expression of genes involved in either stress responses or biomineralization processes was not significantly modified in response to decreased pH. These observations suggested that other indirect effects, due to extracellular acid–base balance modification, could lead to metabolic disturbances affecting growth, calcification and, ultimately, fitness (Pörtner et al., 2004; Fabry et al., 2008; Melzner et al., 2009; Michaelidis et al., 2005). According to Cyronak et al. (2016), elevated $H^+$ concentration and subsequent problems of homeostasis would be more likely than carbonate ion concentration to induce the reduction of calcification in marine organisms facing OA.

Metazoans use two buffer systems in response to potential fluctuations of their extracellular pH (Heisler, 1989). The most commonly used is a bicarbonate-based buffer system which is a relatively low-cost system energetically depending on the number of protons ($H^+$) to be eliminated and the capacity of the species to accumulate bicarbonate ions in their extracellular fluids (Heisler, 1989; Melzner et al., 2009). The second buffer system is non-bicarbonate based and relies on organic molecules (protein and peptides) present in the hæmolymph that are able to capture $H^+$ with their polypeptide side chains.

The ability to prevent $CO_2$-induced changes of the hæmolymph pH ($pH_{HL}$) is believed to be a key determinant of an organisms' ability to tolerate near future OA (Collard et al., 2013; Wittmann and Pörtner, 2013; Melzner et al., 2009). Marine molluscs are usually considered to be poor acid–base regulators compared with other taxa and their ability to buffer their extracellular fluid when experiencing OA stress is thus very limited (Melzner et al., 2009; Gazeau et al., 2013, Parker et al., 2013). However, some intertidal molluscs are able to compensate the decrease of their extracellular pH to some extent (Widdicombe and Spicer, 2008; Marchant et al., 2010; Scanes et al., 2017). In adult bivalves, long-term hypercapnia was seen to cause a reduction in extra-cellular $pH_{HL}$, which was partly prevented by an increase of

bicarbonate hæmolymph concentration, supposedly coming from shell dissolution (Michaelidis et al., 2005; Thomsen et al., 2010; Heinemann et al., 2012). Cao-Pham et al. (2019) reported an $Na^+/H^+$ exchanger-like in the apical membrane of the epithelium facing the sea water in the inner mantle giant clam *Tridacna squamosa* which hosts zooxanthellae. Based on the localization of the exchanger and the upregulation of the expression level of this transporter by light, these authors suggested that the exchanger could be involved in the elimination of protons produced during light-enhanced calcification. This indicates a possible pH compensation mechanism. In cephalopods, the cuttlefish *Sepia officinalis* was shown to partially compensate its $pH_{HL}$ by accumulating bicarbonate ions in its extracellular fluid (Gutowska et al., 2010). To our knowledge, the acid–base physiology has been studied in only two genera of gastropods: in abalone and in patellid limpets. The limpet *Patella vulgata* showed an ability to fully compensate its $pH_{HL}$ after 5 days of exposure to low pH ($pH_{NBS}$ 7.5) by increasing its hæmolymph bicarbonate concentration, supposedly from shell dissolution (Marchant et al., 2010). Apart from patellid limpets *Patella* sp. which are distantly related to other gastropods), there are only two studies on the acid–base regulation abilities of abalone exposed to environmental stress. Cheng et al. (2004) reported that a decrease in dissolved $O_2$ disrupted the acid-base balance as well as anaerobic metabolism of *H. diversicolor supertexta*. On the other hand, when exposed to elevated $pCO_2$, juvenile *Haliotis fulgens* showed no significant changes in the concentration of products from the anaerobic metabolism, compared with control conditions (Tripp-Valdez et al., 2017).

As an herbivorous gastropod, abalone is considered as a low trophic species exhibiting a low metabolism (compared with more active carnivorous molluscs). Low rates of metabolism typically correlate with lower concentrations of ion transport proteins (such as $Na^+/K^+$ and $H^+$-ATPases, Gibbs and Somero, 1990), suggesting reduced capacities of acid–base balance and a poor ability to compensate for changes in acid–base status (Fabry et al., 2008; Pörtner et al., 2004; Melzner et al., 2009; Parker et al., 2013). In adult *H. tuberculata* under OA stress, the hæmolymph extracellular pH was reported to be 0.1 pH unit lower in individuals subjected to $pH_T$ 7.7, compared with those maintained in $pH_T$ 8.0, suggesting that abalone only poorly compensate for the pH decrease of their extracellular fluid if at all (Avignon et al., 2020).

Interestingly, compared with abalones, patellid limpets were only moderately affected by OA (Marchant et al., 2010; Duquette et al., 2017). In other taxa, such as crustaceans, sipunculids or echinoderms, the capacity to tolerate a moderate sea water pH decrease has been linked with ~~to~~ the ability to regulate the extracellular acid–base balance ~~or not~~ (Pörtner et al., 2004; Di Giglio et al., 2020). Consequently, we hypothesize that abalone have a limited ability to compensate $pH_{HL}$.

To better understand how abalone respond to $CO_2$-induced OA, the present study investigated, for the first time, the extra-cellular acid–base parameters in the hæmolymph of adult *Haliotis tuberculata* exposed to acute OA stress. Three $pH_T$ levels (7.9, 7.7, 7.4) were compared, to which the animals were exposed for 15 days. Extracellular pH and total alkalinity of the hæmolymph were measured at different time points through the experiment and were used to calculate the carbonate parameters of the extracellular fluid (e.g. $pCO_2$, carbonate and bicarbonate ions, dissolved inorganic carbon: DIC, aragonite and calcite saturation state). Total protein content was also measured in hæmolymph samples to determine whether lowering the seawater pH influenced the composition or buffer capacity of extracellular fluid in adult abalone.

## 2. Material and Methods

### 2.1 Abalone collection and acclimation

Three-year-old adult abalone *Haliotis tuberculata* were picked up without any selection from an offshore sea-cage structure containing 600 individuals per cage, at the France Haliotis abalone farm (48°36'50N, 4°36'3W; Plouguerneau, Brittany, France). Abalone were transported to the laboratory ensuring minimum stress and minimum handling during transport. They were conditioned before experiments under ambient seawater $pCO_2$/pH and temperature conditions and fed *ad libitum* with the macroalga *Palmaria palmata*.

## 2.2 Experimental set-up

Two experiments were carried out in two different laboratories. A first ~~pilot~~ experiment (Exp.1), carried out in February 2015 at the Marine Biology Laboratory (ULB, Brussels), was done to assess acid–base parameters and how to measure them in adult abalones conditioned in ambient conditions of $pCO_2$. A second experiment (Exp.2), carried out in November 2015 at the MNHN Concarneau marine station (Brittany, France), was then done to assess whether the decrease of seawater pH had an impact on acid–base parameters of abalone.

### *2.2.1. Experiment 1: ambient conditions*

Adult abalones (63 ± 5 mm in shell length, n = 48) were ~~randomly~~ distributed without any selection among three 45-L open-circuit experimental aquaria (n = 16 abalone per aquarium) supplied with filtered seawater renewed at a rate of 60–65 $L.h^{-1}$ and continuously aerated with ambient air. Animals were conditioned for 10 days in the laboratory in ambient conditions of temperature and $pCO_2$ and were fed *ad libitum* with *P. palmata*.

### *2.2.2. Experiment 2: OA experiment*

Adult abalone (61 ± 3 mm in shell length, n = 180) were ~~randomly~~ distributed without any selection among nine 45-L open-circuit experimental aquaria (n = 20 abalone per aquarium) supplied with through-flowing 3-µm filtered natural field seawater renewed at a rate of 60–65 $L.h^{-1}$ and continuously aerated with ambient air. The aquaria were cleaned twice a week using a siphoning hose and the water filters were changed daily. Following the three weeks of conditioning, aquaria housing abalone were assigned to three pH treatments for 15 days. The pH treatments were as follows: present-day field conditions $pH_T$ 7.9 ($pCO_2 \approx 600$ µatm); $pH_T$ 7.7 ($pCO_2 \approx 1000$ µatm), predicted to occur in 2100 according to the RCP 8.5 scenario (IPCC, 2014; Gattuso et al., 2015); and an extreme level of $pH_T$ 7.4 ($pCO_2 \approx 2000$ µatm). Three replicate 45-L aquaria were set up for each pH treatment, in which seawater $pCO_2$

concentrations were adjusted by bubbling $CO_2$ (Air Liquide, France). $pCO_2$ in each tank was controlled through electro-valves regulated by a pH-stat system (IKS Aquastar, Germany). pH values of the IKS system were adjusted from daily measurements of the electromotive force in the aquaria using a pH meter (Metrohm 826 pH mobile, Metrohm, Switzerland) with a glass electrode (Metrohm electrode plus) converted into pH units on the total scale ($pH_T$) using Tris/HCl and 2-aminopyridine/HCl buffers (Dickson, 2010). Before the start of the experiment, pH was gradually decreased by 0.1 pH unit per day until the different target pH levels were reached.

### 2.3. pH and carbonate parameters monitoring

Seawater parameters were monitored throughout the 10 days of control conditions in Exp.1 and the 15 days of exposures in Exp.2. Temperature and $pH_T$ were recorded daily in each experimental aquarium using a pH meter as described above, and salinity was measured twice a week using a conductivity meter (3110, WTW, Germany). Total alkalinity ($A_T$) of the seawater was measured weekly on 100 mL samples taken from each experimental aquarium. Seawater samples were filtered through 0.7 µm Whatman GF/F membranes, immediately poisoned with mercury chloride and stored at 4°C until 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). Seawater carbonate chemistry, i.e. bicarbonate ($HCO_3^-$), carbonate ($CO_3^{--}$) and dissolved inorganic carbon (DIC) concentrations, $pCO_2$ and the saturation states of aragonite ($\Omega_{aragonite}$) and calcite ($\Omega_{calcite}$) were calculated from $pH_T$, $A_T$, temperature and salinity using the CO2SYS program (Pierrot et al., 2006) set with the constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987).

## 2.4. Abalone survival and sampling

Abalone survival was assessed every day throughout the experiments and any dead individuals were removed from the aquaria immediately. Survival (%) was calculated as the proportion of living individuals at the end of the experiment relative to the total number of abalones per aquarium at the beginning of the experiment. Abalones were randomly sampled for hæmolymph analysis. In Exp. 1, 3 to 5 individuals were removed at each sampling time. The haemolymph was pooled from these individuals and the data were averaged over the 3 sampling times (n = 3). In Exp.2, abalones were sampled after 5, 10 and 15 days of pH exposure (D5, D10 and D15, respectively). Hæmolymph was pooled from 3 to 5 individuals collected from the same aquarium and the data were averaged over the 3 aquaria per pH treatment (n = 3 per sampling time and pH treatment). Hæmolymph was sampled immediately from these animals and shell length and width were measured to the nearest 0.5 mm using Vernier callipers. Animals were replaced into their initial aquaria, with a tag to avoid re-sampling of the same individual.

## 2.5. Hæmolymph sampling and analysis

Hæmolymph was withdrawn carefully from the pedal sinus using a refrigerated 2-mL syringe and 25 G x ½ needles. Hæmolymph from the 3 to 5 individuals was pooled in a 15-mL vial on ice and the electromotive force was measured using a glass micro-electrode (Biotrode, Metrohm, Germany). This value was converted into pH units of the total scale ($pH_T$) as described above for seawater $pH_T$ determination.

Pooled samples were pelleted in a centrifuge (250 g, 10 min, 6°C) and the supernatant was distributed immediately into 96-well microplates for protein analysis. Protein concentration was determined spectrophotometrically by BCA assay (Pierce, SIGMA) using bovine serum albumin (BSA) as a standard (Smith et al., 1985). A calibration curve was obtained by measuring a dilution series of standard BSA solution, 25 to 500 $\mu g.mL^{-1}$ (n = 6 replicate wells in a 96-well microplate). Absorption of each sample was determined using a microplate reader (BioTek plate reader, Winooski, VT, USA) operating at 570 nm, and protein content

was calculated from the average absorbance of the six replicate wells according to the calibration curve.

$A_T$ of the hæmolymph was determined by a potentiometric titration method adapted to small volumes (Gran, 1952; Collard et al., 2013, 2014), using a 3-mm diameter glass microelectrode (Biotrode, Metrohm, Germany). $A_T$ was tentatively measured on several samples of whole hæmolymph and the supernatant of the 250-g centrifugation (also used for protein determination). Because these measurements turned out to be unfeasible (see Results section), the supernatant of the 250-g centrifugation (7 mL) was ultra-filtrated using a centrifugal filter unit with a molecular cut-off of 3 kDa (Amicon Ultra 2 mL, Millipore, USA 4000g, 45min, 6°C). The ultra-filtrated fraction was then transferred into a 1.5-mL tube for $A_T$ measurement. Hæmolymph $pCO_2$, bicarbonate, carbonate and DIC concentrations were calculated from $pH_T$, $A_T$, temperature and salinity using the CO2SYS program as described in section 2.2 for the determination of seawater carbonate parameters. The methods of hæmolymph sampling and analysis apply to both experiment 1 and 2.

### 2.6. Statistical analyses

All statistical analyses were performed with Rstudio software (R Core Team, 2015). Differences in abalone survival, shell length and hæmolymph parameters (i.e. $pH_{HL}$, total alkalinity, $pCO_2$, bicarbonate ion concentration, saturation states and protein content) across $pH_{SW}$ treatments were analysed with general linear model ANOVAs ($pH_{SW}$: fixed factor) using the mean value per aquarium (n=3 replicates per pH condition, Exp.2). The normality of the residuals and homogeneity of variances were verified using respectively Shapiro-Wilk and Bartlett tests. Statistical analyses were performed separately for each time point on these data. Post-hoc HSD Tukey tests were used to test the differences between the group means. Data given in the text and figures are presented as mean ± standard deviation (SD), unless otherwise indicated. Differences were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Experiment 1: ambient conditions

Mean values of seawater parameters over the whole period (n = 6) are given in Table 1A. Seawater temperature was 14.5 ± 0.3°C, salinity was 34.1 ± 0.1, $pH_T$ 8.03 ± 0.01 and $pCO_2$ 576 ± 30 µatm. Extra-cellular acid-base parameters measured in adult abalones kept in ambient conditions are reported in Table 1B. Mean hæmolymph $pH_T$ ($pH_{HL}$) was 7.32, *ca.* 0.7 units lower than seawater $pH_T$ (Table 1B). The titration carried out on freshly collected hæmolymph and on the supernatant of hæmolymph centrifuged at 250 g did not allow total alkalinity to be determined because the titration curve obtained did not fit a Gran function (Suppl. material S1). After ultrafiltration of the hæmolymph through a membrane with a molecular cut-off of 3kDa, the typical titration curve was obtained and used to calculate the total alkalinity according to the Gran method (Suppl. material S1). Average value of the latter for abalones kept in ambient conditions was 3796 ± 72 $\mu E.kg^{-1}$. pH and $A_T$ of the haemolymph were used to calculate other parameters of the carbonate system (Table 1B). $pCO_2$ reached 4331µatm, bicarbonate 3664µmol.$kg^{-1}$, $\Omega_{aragonite}$ and $\Omega_{calcite}$ were respectively lower and higher than 1.

### 3.2. Experiment 2: Acidified conditions

Mean values of seawater parameters are presented in Table 2A. Temperature followed the natural variations found in the Bay of Concarneau, from 15.1° ± 0.1°C at the start of the experiment (T0, early November) to 13.4°C ± 0.3°C at the end of the experiment (D15). The $pH_T$ levels of the experimental aquaria were maintained close to the nominal values throughout the experiment, at means of $pH_T$ = 7.91 ($pCO_2$ = 567 ± 61 µatm), $pH_T$ = 7.69 ($pCO_2$ = 997 ± 126 µatm), and $pH_T$ = 7.40 ($pCO_2$ of 2035 ± 114 µatm) (Table 2A). Total alkalinity ($A_T$) measured in the nine experimental aquaria averaged 2295 ± 11 $\mu Eq.kg^{-1}$ during the experiment. Mean salinity was 34.6 ± 0.2 in all experimental aquaria and remained

315 stable over the experiment (n = 30). $\Omega_{aragonite}$ was higher than 1 in pH treatments 7.9 and 7.7
316 and below 1 in the lowest pH treatment (7.4).
317
318 3.2.1. Survival and growth
319
320 The mortality of adult abalones during the experiment was very low, with a survival
321 percentage higher than 90% at the end of the experiment (Day 15). There were no significant
322 differences in survival between the three pH treatments (ANOVA, $F$ (2,6) = 0.6, p = 0.58).
323 Mean shell length of adult abalone at the start of the experiment (T0, Exp. 2) was 61 ± 3mm
324 and did not differ significantly across the four time points (ANOVA, $F$ (3,120) = 0.75, p = 0.52).
325 There were no significant differences in total length between the three pH treatments
326 (ANOVA, $F$ (2,121) = 0.90, p = 0.41).
327
328 3.2.2. Hæmolymph acid–base status
329
330 *Hæmolymph $pH_T$*
331 Mean hæmolymph pH ($pH_{HL}$) measured in control abalones ranged between 7.29 and 7.36
332 *i.e.* 0.54 to 0.61 lower than seawater $pH_T$ (Table 2B). A significant effect of decreased
333 seawater pH was observed on $pH_{HL}$ of abalone at any time point (Fig.1A, Table 3A). At D5
334 and D10, $pH_{HL}$ of abalone exposed to $pH_{SW}$ 7.4 was significantl'y lower than that of control
335 individuals exposed to $pH_{SW}$ 7.9 (Table 3B). At D15, $pH_{HL}$ of abalone exposed to $pH_{SW}$ 7.4
336 was significantly lower than that of individuals exposed to 7.7 and 7.9 (Table 3B). There were
337 no significant differences in $pH_{HL}$ between abalone exposed to $pH_{SW}$ 7.7 and 7.9 at any time
338 point (Table 3B).
339
340 *Total alkalinity ($A_T$)*
341 Total alkalinity ($A_T$) measured in the hæmolymph of control abalones was 3177 ± 103 $\mu E.kg^{-1}$
342 (Table 2B). $A_T$ of the hæmolymph was significantly different in abalones exposed to lower
343 seawater pH (Fig. 1B, Table 3A). After 15 days of exposure, hæmolymph $A_T$ of abalones

exposed to $pH_{SW}$ 7.4 was significantly lower than that of control individuals exposed to 7.7 and 7.9 (Table 3B). There were no significant differences in hæmolymph $A_T$ between abalones exposed to $pH_{SW}$ 7.7 and 7.9 at any time point (Table 3B).

***$pCO_2$:***

Mean $pCO_2$ in control abalones ranged between 3141 and 3675 $\mu mol.kg^{-1}$ (Table 2B). Significant differences in hæmolymph $pCO_2$ were observed in abalones exposed to lower seawater pH (Table 3A). At D10, extracellular $pCO_2$ in abalones exposed to $pH_{SW}$ 7.4 was significantly lower than that of control individuals exposed to 7.7 and 7.9 (Table 3B). There were no significant differences in hæmolymph $pCO_2$ between abalones exposed to $pH_{SW}$ 7.7 and 7.9 at any time point (Table 3B).

*$HCO_3^-$*

Mean hæmolymph $[HCO_3^-]$ in control abalones ranged between 3059 and 3077 $\mu mol.kg^{-1}$ (Table 2B). In abalones exposed to decreased pH, hæmolymph $[HCO_3^-]$ was significantly different after 15 days of exposure (Table 3A). At this time point, $[HCO_3^-]$ in abalones exposed to $pH_{SW}$ 7.4 was significantly lower than that of individuals exposed to $pH_{SW}$ 7.7 and 7.9 (Table 3B). There were no significant differences in hæmolymph $[HCO_3^-]$ between abalones exposed to $pH_{SW}$ 7.7 and 7.9 at any time point (Table 3B).

*Saturation state ($\Omega$)*

$\Omega_{aragonite}$ and $\Omega_{calcite}$ in control abalones were respectively 0.71 ± 0.08 and 1.11 ± 0.12 (Table 2B). A significant effect of decreased seawater pH was observed on $\Omega_{aragonite}$ and $\Omega_{calcite}$ at any time point (Table 3A). At D5, $\Omega_{aragonite}$ in abalone exposed to $pH_{SW}$ 7.4 and 7.7 was significantly lower than that of control individuals exposed to $pH_{SW}$ 7.9 (Table 3B). At D10 $\Omega_{aragonite}$ in abalone exposed to $pH_{SW}$ 7.4 was significantly lower than that of control

individuals exposed to $pH_{SW}$ 7.9 (Table 3B). At D15, $\Omega_{aragonite}$ in abalone exposed to $pH_{SW}$ 7.4 was significantly lower than that of individuals exposed to $pH_{SW}$ 7.9 and 7.7 (Table 3B).

3.2.3. Protein content

The average protein content in the hæmolymph of control abalones was 16.7 ± 5 $g.L^{-1}$ (Table 2B) and did not differ significantly between pH treatments at any time point (Table 3A).

## 4. Discussion

This paper reports the first investigation of extra-cellular acid–base parameters and buffer capacity in the adult abalone *H. tuberculata* exposed to $CO_2$-induced ocean acidification. In control *H. tuberculata*, $pH_{HL}$ was 7.4, which is close to the range for extracellular pH measured in the Taiwan abalone *H. diversicolor supertexta* (between 7.23 and 7.28, Cheng et al., 2004). In adult *H. tuberculata* facing OA stress, all the extra-cellular acid–base parameters measured or calculated (except $pCO_2$) were significantly reduced in the $pH_{SW}$ 7.4 treatment (0.5 units below control pH), while $pH_{SW}$ 7.7 did not affect the variables significantly.

A pH-bicarbonate diagram (Davenport diagram, Fig. 2) further indicates that adult *H. tuberculata* are able to maintain their hæmolymph pH with moderate acidification (-0.2 $pH_{SW}$ units) but not at a more severe level (-0.5 $pH_{SW}$ units). This is consistent with our previous results showing that adult abalones exposed to a -0.3 pH decrease for two months were unable to maintain their $pH_{HL}$ (Avignon et al., 2020). It is noteworthy that the $pH_{HL}$ homeostasis at $pH_{SW}$ 7.7 is not due to an accumulation of bicarbonate ions, contrary to the limpet *Patella vulgata*, which was reported to increase its hæmolymph bicarbonate concentration to compensate its $pH_{HL}$ (Marchant et al., 2010). Furthermore, it appears that abalone subjected to $pH_{SW}$ 7.4 suffered metabolic acidosis (reduced hæmolymph pH and bicarbonate concentration). This suggests the induction of anaerobic metabolism by OA at

$pH_{SW}$ 7.4. This finding does not agree with the results obtained by Tripp-Valdez et al. (2017) in *Haliotis fulgens*. When subjected to $pH_T$ 7.3–7.4 (recalculated from their $pCO_2$ and $A_T$ data using $CO_2$SYS; -0.4 to -0.5 $pH_T$ units compared with the control), *H. fulgens* juveniles, maintained at control temperature in normoxic conditions, showed no significant changes in the concentration of products from the anaerobic metabolism, compared with control conditions. However, the abalone in the present study are large adults, which probably develop anaerobic conditions more easily, particularly in the foot (see Venter et al., 2018).

In addition to $pH_{HL}$, total alkalinity ($A_T$) of the hæmolymph was also measured in *H. tuberculata* using a potentiometric titration method adapted to small volumes (Gran, 1952; Collard et al., 2013, 2014). The titrations carried out on either freshly collected hæmolymph or on the 250-g centrifugation supernatant differed from the classical titration of a bicarbonate/carbonate buffered marine solution and did not allow the determination of $A_T$. A classical titration curve allowing $A_T$ calculation was, however, obtained on the ultra-filtrated fraction after removing organic molecules of MW > 3kDa. As the large hemocyanin protein molecule in abalone is carried in the hæmolymph, this may have interfered with the titration of $A_T$. These results suggested that abalone hæmolymph contained a significant concentration of proteins and/or peptides (MW > 3kDa). Indeed, the protein concentration measured in the hæmolymph of *H. tuberculata* (mean 15–22 $g.L^{-1}$) is much higher than that found in other gastropods (e.g. 0.5 to 1.5 $mg.L^{-1}$ in the limpet *Patella sp*, Brown et al., 2004) or in the range of concentrations measured in the Australian abalone *H. rubra* (around 10 $g.L^{-1}$, Hooper et al., 2014). We suggest that these proteins/peptides scavenge protons of respiratory origin when an abalone is subjected to moderate acidification ($pH_{SW}$ 7.7). However, they appeared incapable of buffering the extra protons when the $pH_{SW}$ was lowered to 7.4. The protein content of the hæmolymph appeared to be not much influenced by hypercapnia, but this conclusion should be carefully considered in view of the high variability in protein concentration between individuals.

In summary, adult abalone *H. tuberculata* appeared able to buffer moderate (-0.2 $pH_{SW}$ units) acidification, probably due to its high hæmolymph protein concentration but was not

able to compensate for greater decreases in $pH_{SW}$. These results are consistent with previous studies on the mussel *Mytilus edulis* (Thomsen et al., 2010, 2013). In this bivalve species, these authors clearly demonstrated the absence of $HCO_3^-$ accumulation and suggested that buffering by extracellular proteins is the sole mechanism acting to stabilize hæmolymph pH (Thomsen et al., 2010). However, this strategy contrasts with that of the more active cephalopod *Sepia officinalis*, which greatly increases extracellular [$HCO_3^-$] in order to stabilize its extracellular $pH_{HL}$ upon exposure to OA (Gutowska et al., 2010). Generally, active invertebrates tend to show a stronger $HCO_3^-$ buffering capacity, while less active invertebrates may experience metabolic suppression associated with a decline in pH (Melzner et al. 2009; Pörtner, 2008).

Mean $pCO_2$ values in abalone hæmolymph were higher for the $pH_{SW}$ 7.4 treatment, but the differences were significant only at day 10 of the experiment. As previously shown in marine molluscs an increase in extra-cellular $pCO_2$ may cause hypercapnia and acidosis, two energetically processes that can negatively affect vital processes, such as somatic growth and calcification (Pörtner et al., 2004; Fabry et al., 2008; Melzner et al., 2009). The saturation state of abalone haemolymph ($\Omega$) towards aragonite was below 1 in all conditions including the control treatment. Nevertheless, *H. tuberculata* calcifies efficiently aragonite in control conditions, indicating that conditions in the calcifying site strongly differ from those in the hæmolymph and that acidosis of the hæmolymph is not directly responsible for the effects on shell calcification reported in previous OA experiments (Wessel et al., 2018; Auzoux-Bordenave et al., 2020; Avignon et al., 2020).

The results of the present study support the hypothesis that changes in extracellular acid-base balance might be involved in deleterious effects recorded in adult *H. tuberculata* facing severe OA stress, as previously reported in marine bivalves (Michaelidis et al., 2005; Melzner et al., 2009; Waldbusser et al., 2011). As previously emphasized, very little information is available on the acid–base homeostasis abilities of marine gastropods. *P. vulgata* lives in the higher intertidal zone where important local variations in physico-chemical conditions might occur inducing hypoxic, hypercapnic and desiccation stresses (Marchant et al. 2010). When facing

severe acidification (-0.7 $pH_{SW}$ units) of its environment, the limpet was able to compensate its $pH_{HL}$ by increasing its $HCO_3^-$ buffering capacity (Marchant et al., 2010). *H. tuberculata*, in contrast, inhabits the subtidal and low intertidal zone where physico-chemical stresses are much lower. For instance, $pH_T$ in the Bay of Brest, one of this specie' natural habitat, ranges between 8.2 and 7.9 (Qui-Minet et al., 2018). This suggests that *H. tuberculata* has not developed the biochemical machinery to ensure a strong acid–base homeostasis. This weakness, together with the effects reported on growth and shell calcification (Wessel et al., 2018; Auzoux-Bordenave et al., 2020; Avignon et al., 2020), would impact both the ecology and aquaculture of this commercially important species. Understanding how different abalone life stages respond to OA will make it possible to reveal the capacity of abalone to adapt genetically to $pCO_2$ increases of their environment and to identify bottlenecks for population persistence under near-future pH conditions.

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**Compliance with ethical standards**

The authors declare that they have no conflicts of interest or competing financial interests.

The experiments complied with the current French laws. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

## Figures and tables

**Figure 1**. Hæmolymph $pH_T$ (A) and Total alkalinity (B) in adult abalone exposed to three pH levels (7.9, 7.7 and 7.4) after 15 days of exposure (n = 3 per pH treatment). Means of bars with different letters are significantly different ($P < 0.05$).

**Figure 2**. $pH_{HL}$ bicarbonate concentration (Davenport) diagram showing the time course of acid–base compensation (mean ± SD) in the hæmolymph of abalone *H. tuberculata* over 15 days of exposure to elevated $pCO_2$. The solid curved lines represent $pCO_2$ isopleths. Symbols represent the exposure time (respectively 5, 10 and 15 days), while the grey levels correspond to the seawater pH value.

**Table 1**. **A**. Seawater temperature and carbonate chemistry parameters of control Experiment 1 (mean ± SD). pH on the total scale ($pH_T$), temperature (°C) and total alkalinity ($A_T$;$\mu Eq.kg^{-1}$) were used to calculate $CO_2$ partial pressure ($pCO_2$; µatm), dissolved inorganic carbon (DIC; $\mu mol.kg^{-1}$), $HCO_3^-$ and $CO_3^{2-}$concentrations (µmol. $kg^{-1}$), aragonite saturation state ($\Omega_{aragonite}$) and calcite saturation state ($\Omega_{calcite}$) using the CO2SYS program. $pH_T$ and temperature are the average values of those logged daily in the aquaria throughout the experiment (n = 3).

**B**. Acid–base parameters in the hæmolymph of control abalone in Exp.1 (mean ± SD). Hæmolymph pH ($pH_{HL}$), temperature (°C) and total alkalinity ($A_T$; $\mu Eq.kg^{-1}$) were used to calculate carbonate chemistry parameters using the CO2SYS program (n = 3).

**Table 2. A.** Seawater temperature and carbonate chemistry parameters at each time point of Experiment 2 after 5 (D5), 10 (D10) and 15 (D15) days of exposure to the experimental pH (mean ± SD). pH on the total scale ($pH_T$), temperature (°C) and total alkalinity ($A_T$;$\mu Eq.kg^{-1}$) were used to calculate $CO_2$ partial pressure ($pCO_2$; µatm), dissolved inorganic carbon (DIC; $\mu mol.kg^{-1}$), $HCO_3^-$ and $CO_3^{2-}$concentrations (µmol. $kg^{-1}$), aragonite saturation state ($\Omega_{aragonite}$) and calcite saturation state ($\Omega_{calcite}$) using the CO2SYS program. $pH_T$ and temperature are the

515 average values of those logged daily in the aquaria throughout the experiment (n = 3 per pH
516 treatment and sampling time).
517 **B.** Acid–base parameters and total protein content (mean ± SD) in the hæmolymph of
518 abalone *H. tuberculata* exposed to three pH treatments after 5 (D5), 10 (D10) and 15 (D15)
519 days of exposure (Exp.2). Hæmolymph pH ($pH_{HL}$), temperature (°C) and total alkalinity ($A_T$;
520 $\mu Eq.kg^{-1}$) were used to calculate carbonate chemistry parameters using the CO2SYS
521 program. Total protein content ($g.L^{-1}$) was determined spectrophotometrically by BCA assay
522 using BSA as standard (n = 3 per pH treatment and sampling time).

523

524 **Table 3**. Summary of statistics. **A.** Anova results of the effects of seawater pH ($pH_{SW}$) on
525 hæmolymph pH ($pH_{HL}$), total alkalinity ($A_T$), $pCO_2$, bicarbonate ($HCO_3^-$) concentrations,
526 saturation state ($\Omega_{aragonite}$ and $\Omega_{calcite}$) and total proteins in the adult abalone *Haliotis*
527 *tuberculata* after 5 (D5), 10 (D10) and 15 (D15) days ~~(D15)~~ of exposure ($pH_{SW}$: fixed factor).
528 Significant P-values are shown in bold ($P < 0.05$); **B.** Multiple comparison Tukey HSD test
529 testing the influence of seawater pH ($pH_{SW}$) on hæmolymph pH ($pH_{HL}$), total alkalinity, ~~DIC~~
530 ~~and~~ bicarbonate ($HCO_3^-$) concentration~~s~~ and $\Omega_{aragonite}$ at different time points. Significant P-
531 values in bold ($P < 0.05$).

532

533 **Suppl. material S1:** Titration of abalone hæmolymph by 0.1 M HCl **a:** whole hæmolymph,
534 **b**: supernatant after 250-g centrifugation **c**: 3 kDa ultrafiltrated fraction of the hæmolymph.

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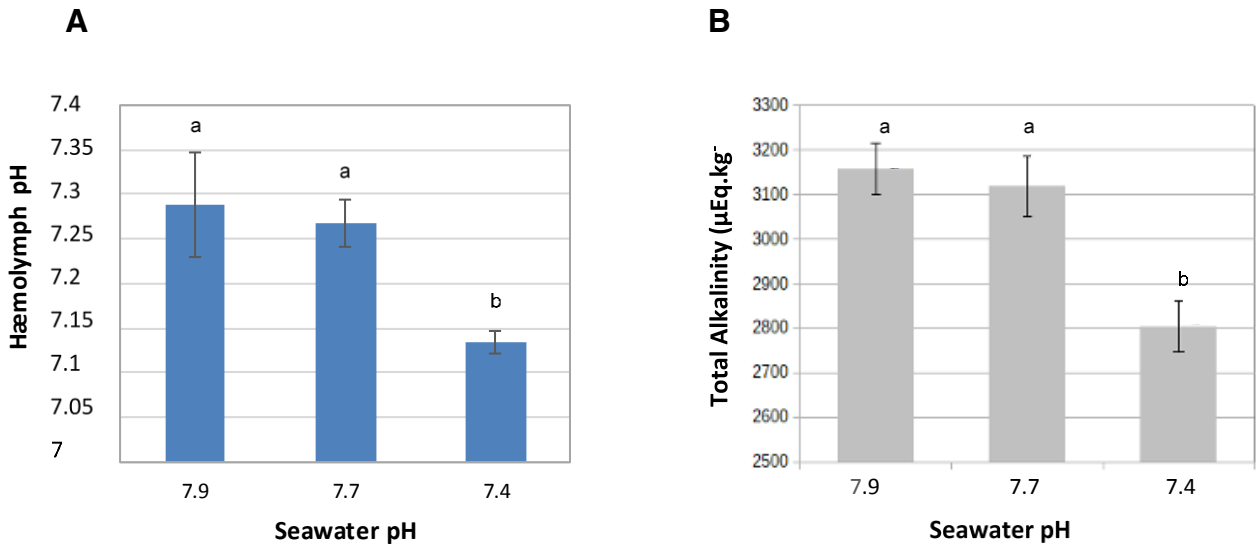
737

Highlights

- The effects of ocean acidification (OA) on extra-cellular acid–base parameters are reported in the European abalone *H. tuberculata*, a commercially and ecologically important gastropod
- Adult abalone were exposed for 15 days to three different pH levels (7.9, 7.7, 7.4) representing current and predicted near-future conditions.
- Abalones are able to buffer a moderate acidification of seawater (-0.2 pH units)
- Haemolymph pH was significantly decreased after 5 days of exposure to pH 7.4 (-0.5 pH units) indicating that abalone do not compensate for higher decreases of in seawater pH.
- OA would impact both the ecology and aquaculture of *H. tuberculata* in the near future

Graphical abstract

Hæmolymph $pH_T$ (**A**) and Total alkalinity (**B**) are significantly decreased in adult abalone *H. tuberculata* exposed to lower seawater pH 7.4. A pH-bicarbonate diagram (**C**) further indicates that adult abalone are able to maintain their extracellular pH with moderate acidification (-0.2 $pH_{SW}$ units) but not at a more severe level (-0.5 $pH_{SW}$ units).



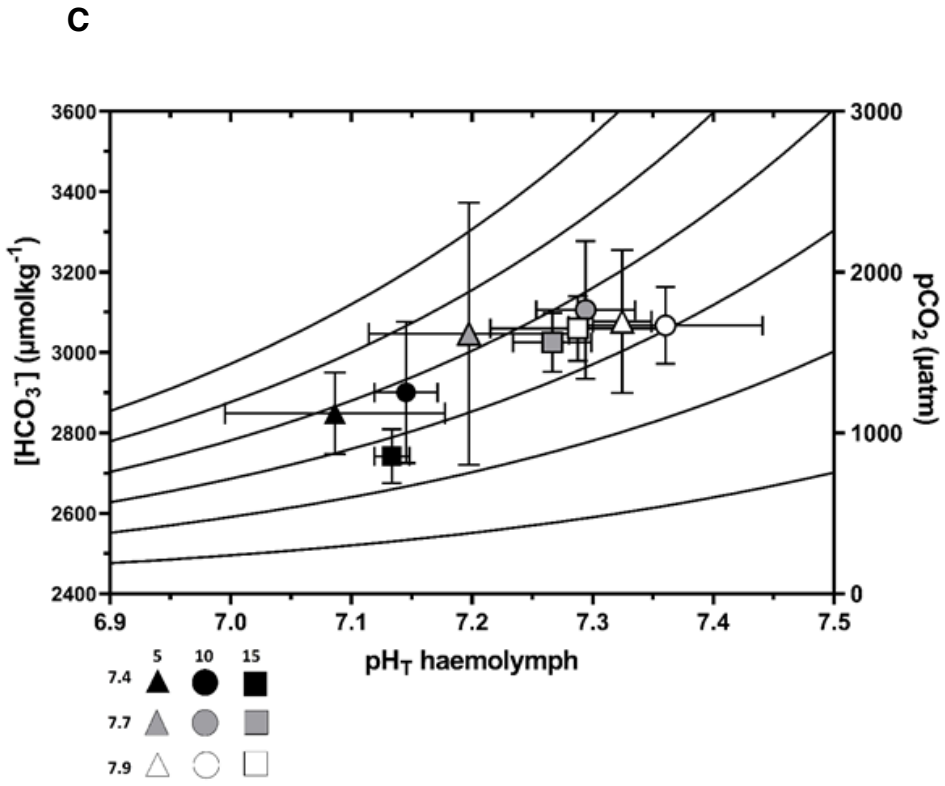



**Figure 1**

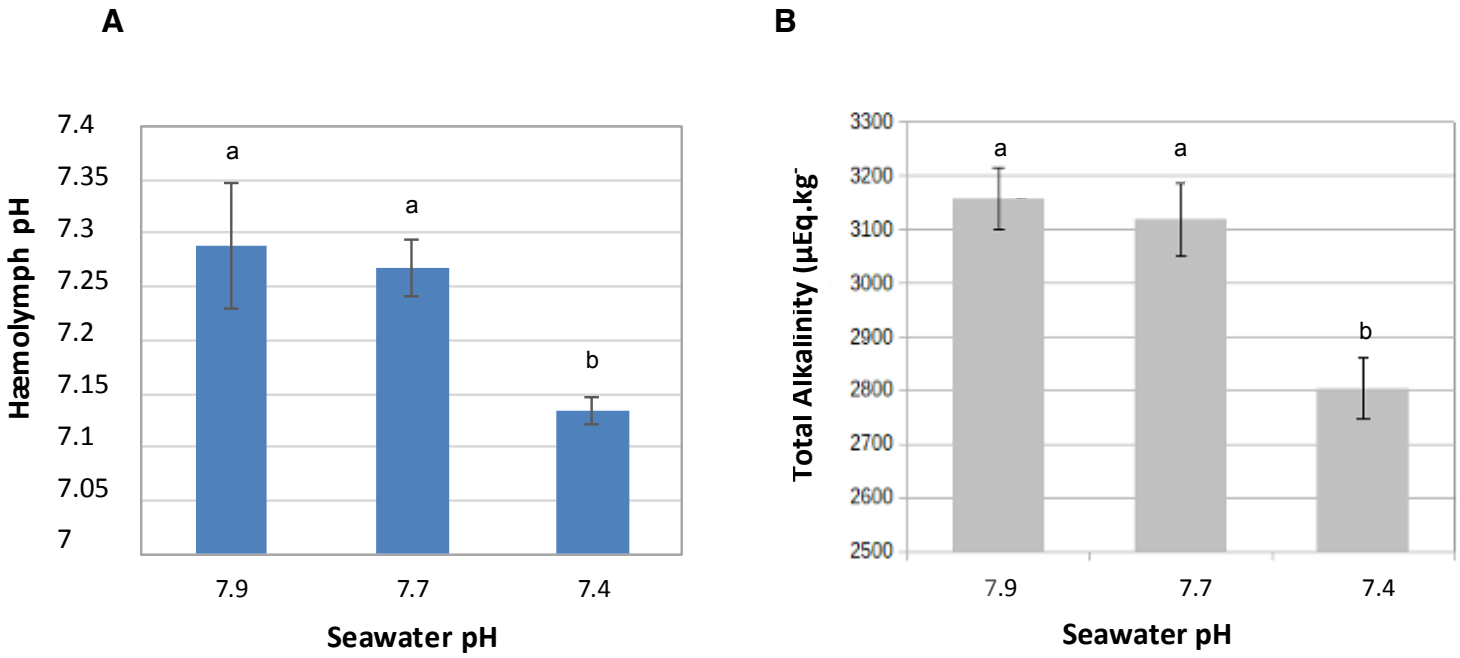


**Figure 2**

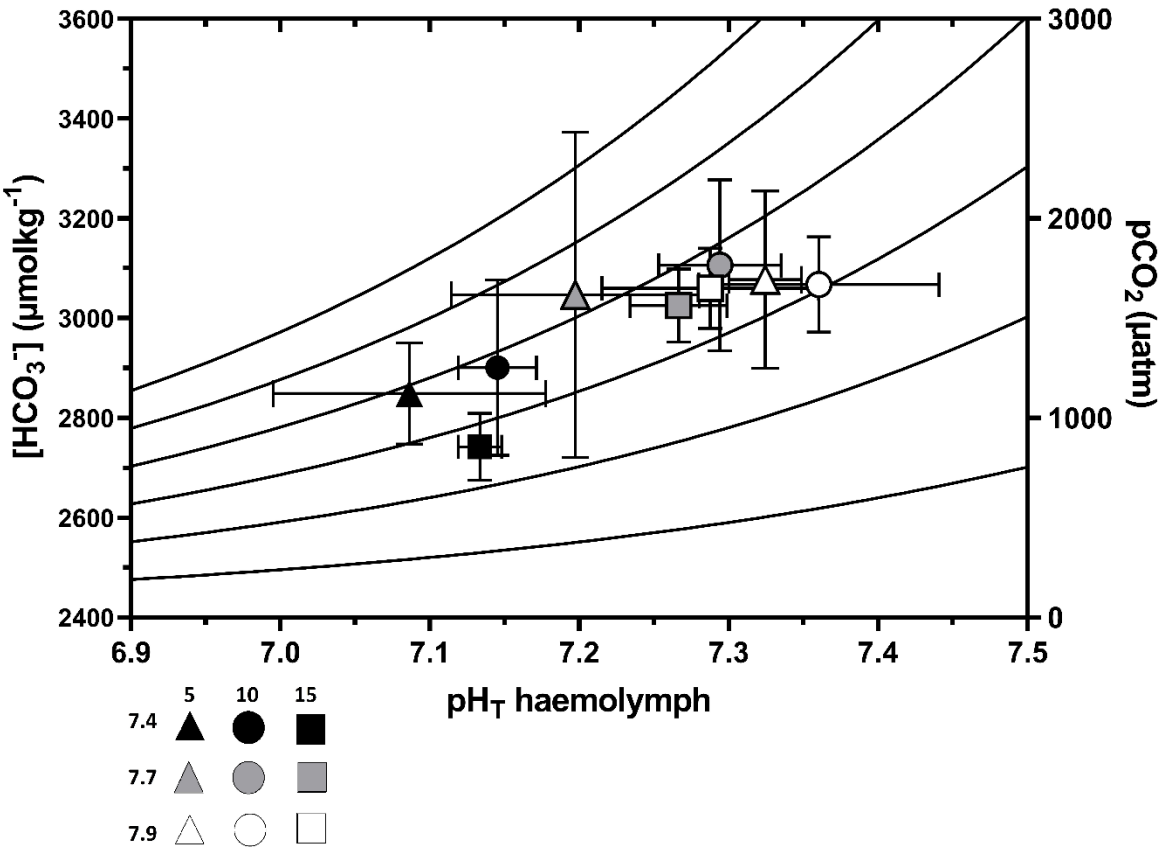


**Table 1**

| | Nominal pH | $pH_T$ | Temperature (°C) | $A_T$ ($\mu Eq.kg^{-1}$) | $p\,CO_2$ ($\mu atm$) | DIC ($\mu mol.kg^{-1}$) | $HCO_3^-$ ($\mu mol.kg^{-1}$) | $CO_3^{2-}$ ($\mu mol.kg^{-1}$) | $\Omega_{aragonite}$ | $\Omega_{calcite}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **A. Seawater** | 8.0 | 8.03 ± 0.01 | 14.5 ± 0.3 | 3105 ± 21 | 576 ± 30 | 2861 ± 18 | 2639 ± 16 | 199 ± 15 | 3.05 ± 0.23 | 4.75 ± 0.19 |
| **B. Hæmolymph** | 8.0 | 7.32 ± 0.16 | 14.5 ± 0.3 | 3796 ± 72 | 4331 ± 1679 | 3887 ± 155 | 3664 ± 109 | 58 ± 16 | 0.89 ± 0.25 | 1.39 ± 0.39 |

**Table 2**

**A. Seawater**

| Time point (days) | Nominal pH | $pH_T$ | Temperature (°C) | $A_T$ ($\mu Eq.kg^{-1}$) | $p\,CO_2$ ($\mu atm$) | DIC ($\mu mol.kg^{-1}$) | $HCO_3^-$ ($\mu mol.kg^{-1}$) | $CO_3^{2-}$ ($\mu mol.kg^{-1}$) | $\Omega_{aragonite}$ | $\Omega_{calcite}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| D5 | 7.9 | 7.87 ± 0.01 | 15.0 ± 0.1 | 2287 ± 3 | 633 ± 14 | 2148 ± 5 | 2014 ± 6 | 110 ± 2 | 1.69 ± 0.03 | 2.63 ± 0.04 |
| | 7.7 | 7.67 ± 0.01 | 15.1 ± 0.0 | 2281 ± 6 | 1056 ± 22 | 2213 ± 2 | 2102 ± 2 | 72 ± 2 | 1.11 ± 0.02 | 1.73 ± 0.04 |
| | 7.4 | 7.43 ± 0.03 | 15.1 ± 0.1 | 2291 ± 1 | 1913 ± 160 | 2298 ± 9 | 2183 ± 7 | 43 ± 3 | 0.66 ± 0.05 | 1.03 ± 0.07 |
| D10 | 7.9 | 7.92 ± 0.01 | 13.9 ± 0.1 | 2297 ± 4 | 555 ± 18 | 2143 ± 8 | 2003 ± 10 | 119 ± 3 | 1.81 ± 0.04 | 2.83 ± 0.07 |
| | 7.7 | 7.66 ± 0.05 | 13.9 ± 0.1 | 2294 ± 5 | 1083 ± 129 | 2234 ± 16 | 2123 ± 18 | 69 ± 7 | 1.06 ± 0.11 | 1.65 ± 0.17 |
| | 7.4 | 7.40 ± 0.02 | 13.9 ± 0.1 | 2306 ± 2 | 2056 ± 119 | 2328 ± 9 | 2209 ± 6 | 39 ± 2 | 0.60 ± 0.03 | 0.93 ± 0.04 |
| D15 | 7.9 | 7.95 ± 0.05 | 13.4 ± 0.3 | 2305 ± 4 | 514 ± 6 | 2141 ± 3 | 1996 ± 3 | 124 ± 1 | 1.90 ± 0.01 | 2.96 ± 0.02 |
| | 7.7 | 7.75 ± 0.04 | 13.4 ± 0.2 | 2303 ± 5 | 852 ± 76 | 2212 ± 10 | 2095 ± 14 | 84 ±7 | 1.27 ± 0.11 | 1,99 ± 0.17 |
| | 7.4 | 7.38 ± 0.07 | 13.4 ± 0.2 | 2305 ± 5 | 2138 ± 390 | 2333 ± 26 | 2211 ± 17 | 38 ± 6 | 0.58 ± 0.09 | 0.90 ± 0.14 |

**B. Hæmolymph**

| Time point (days) | Nominal pH | $pH_{HL}$ | Temperature (°C) | $A_T$ ($\mu Eq.kg^{-1}$) | Total protein (g.L-1) | $p\,CO_2$ ($\mu atm$) | DIC ($\mu mol.kg^{-1}$) | $HCO_3^-$ ($\mu mol.kg^{-1}$) | $CO_3^{2-}$ ($\mu mol.kg^{-1}$) | $\Omega_{aragonite}$ | $\Omega_{calcite}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| D5 | 7.9 | 7.32 ± 0.02 | 15.0 ± 0.1 | 3189 ± 155 | 10.3 ± 3.3 | 3393 ± 5 | 3252 ± 150 | 3077 ±145 | 48 ± 4 | 0.74 ± 0.07 | 1.15 ± 0.11 |
| | 7.7 | 7.20 ± 0.09 | 15.1 ± 0.0 | 3130 ± 260 | 8.3 ± 3.1 | 4617 ± 1036 | 3254 ± 302 | 3047 ± 266 | 36 ± 3 | 0.54 ± 0.04 | 0.85 ± 0.07 |
| | 7.4 | 7.09 ± 0.07 | 15.1 ± 0.1 | 2910 ± 80 | 17.1 ± 4.1 | 5543 ± 1106 | 3082 ± 113 | 2849 ± 83 | 26 ± 3 | 0.40 ± 0.05 | 0.62 ± 0.09 |
| D10 | 7.9 | 7.36 ± 0.07 | 13.9 ± 0.1 | 3185 ± 65 | 18.2 ± 4.6 | 3141 ± 569 | 3239 ± 94 | 3067 ± 78 | 50 ± 6 | 0.76 ± 0.10 | 1.19 ± 0.15 |
| | 7.7 | 7.29 ± 0.03 | 13.9 ± 0.1 | 3208 ± 151 | 15.7 ± 3.1 | 3646 ± 129 | 3290 ± 141 | 3106 ± 140 | 44 ± 5 | 0.67 ± 0.08 | 1.04 ± 0.12 |
| | 7.4 | 7.15 ± 0.02 | 13.9 ± 0.1 | 2969 ± 148 | 20.4 ± 7.5 | 4801 ± 242 | 3115 ± 151 | 2901 ± 143 | 29 ± 2 | 0.44 ± 0.04 | 0.69 ± 0.06 |
| D15 | 7.9 | 7.29 ± 0.06 | 13.4 ± 0.3 | 3157 ± 58 | 21.7 ± 5.1 | 3675 ± 560 | 3246 ± 81 | 3059 ± 66 | 41 ± 5 | 0.63 ± 0.08 | 0.99 ± 0.12 |
| | 7.7 | 7.27 ± 0.03 | 13.4 ± 0.2 | 3117 ± 68 | 20.3 ± 3.4 | 3776 ± 149 | 3212 ± 56 | 3025 ± 60 | 39 ± 3 | 0.59 ± 0.05 | 0.93 ± 0.08 |
| | 7.4 | 7.13 ± 0.01 | 13.4 ± 0.2 | 2804 ± 57 | 18.4 ± 4.6 | 4644 ± 124 | 2950 ± 57 | 2742 ± 55 | 26 ± 1 | 0.40 ± 0.01 | 0.62 ± 0.02 |

**Table 3**

**A.**

| **Parameters** | D5 | D10 | D15 |
|---|---|---|---|
| $pH_{HL}$ | $F_{2,6}$ = 8.472, **p= 0.018** | $F_{2,6}$ = 12.23, **p= 0.007** | $F_{2,6}$ = 9.36, **p= 0.014** |
| Total alkalinity | $F_{2,6}$ = 1.324, p= 0.334 | $F_{2,6}$ = 2.137, p= 0.199 | $F_{2,6}$ = 20.18, **p= 0.002** |
| $pCO_2$ | $F_{2,6}$ = 3.036, p= 0.123 | $F_{2,6}$ = 10.89, **p= 0.01** | $F_{2,6}$ = 4.859, p= 0.056 |
| $HCO_3^-$ | $F_{2,6}$ = 0.933, p = 0.444 | $F_{2,6}$ = 1.547, p= 0.287 | $F_{2,6}$ = 16.83, **p= 0.003** |
| $\Omega_{aragonite}$ | $F_{2,6}$ = 17.11, **p = 0.003** | $F_{2,6}$ = 9.73, **p= 0.013** | $F_{2,6}$ = 10.29, **p= 0.011** |
| $\Omega_{calcite}$ | $F_{2,6}$ = 17.24, **p = 0.003** | $F_{2,6}$ = 9.67, **p= 0.013** | $F_{2,6}$ = 10.67, **p= 0.011** |
| Total Proteins | na | $F_{2,6}$ = 0.372, p= 0.704 | $F_{2,6}$ = 0.372, p= 0.704 |

**B.**

| pH groups | 7.9/7.7 | 7.9/7.4 | 7.7/7.4 |
|---|---|---|---|
| $pH_{HL}$ D5 | 0.149 | **0.015** | 0.215 |
| $pH_{HL}$ D10 | 0.351 | **0.007** | **0.037** |
| $pH_{HL}$ D15 | 0.827 | **0.016** | **0.033** |
| Total alkalinity D15 | 0.795 | **0.003** | **0.005** |
| $pCO_2$ D10 | 0.405 | **0.009** | **0.044** |
| $HCO_3^-$ D15 | 0.840 | **0.004** | **0.008** |
| $\Omega_{aragonite}$ D5 | **0.037** | **0.003** | 0.100 |
| $\Omega_{aragonite}$ D10 | 0.453 | **0.012** | 0.053 |
| $\Omega_{aragonite}$ D15 | 0.792 | **0.013** | **0.028** |

## Conflict of Interest

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.

# Acid–base balance in the hæmolymph of European abalone (*Haliotis tuberculata*) exposed to $CO_2$-induced ocean acidification

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## Abstract

Ocean acidification (OA) and the associated changes in seawater carbonate chemistry pose a threat to calcifying organisms. This is particularly serious for shelled molluscs, in which shell growth and microstructure has been shown to be highly sensitive to OA. To improve our understanding of the responses of abalone to OA, this study investigated the effects of $CO_2$-induced ocean acidification on extra-cellular acid–base parameters in the European abalone *Haliotis tuberculata*. Three-year-old adult abalone were exposed for 15 days to three different pH levels (7.9, 7.7, 7.4) representing current and predicted near-future conditions. Hæmolymph pH and total alkalinity were measured at different time points during exposure and used to calculate the carbonate parameters of the extracellular fluid. Total protein content was also measured to determine whether seawater acidification influences the composition and buffer capacity of hæmolymph. Extracellular pH was maintained at seawater pH 7.7 indicating that abalones are able to buffer moderate acidification (-0.2 pH units). This was not due to an accumulation of $HCO_3^-$ ions but rather to a high hæmolymph protein concentration. By contrast, hæmolymph pH was significantly decreased after 5 days of exposure to pH 7.4, indicating that abalone do not compensate for higher decreases in seawater pH. Total alkalinity and dissolved inorganic carbon were also significantly decreased after 15 days of low pH exposure. It is concluded that changes in the acid–base balance of the hæmolymph might be involved in deleterious effects recorded in adult *H.*

*tuberculata* facing severe OA stress. This would impact both the ecology and aquaculture of this commercially important species.

**Keywords:** ocean acidification; abalone; acid–base balance; hæmolymph pH and alkalinity; buffer capacity

**Abbreviations**: $A_T$: Total alkalinity; DIC: Dissolved Inorganic Carbon; $pH_{NBS:}$ pH of the National Bureau of Standard; $pH_{T:}$ pH on the Total scale; $pH_{SW:}$ seawater pH (total scale); $pH_{HL}$: hæmolymph pH (total scale); RCP 8.5: Representative Concentration Pathway 8.5

## 1. Introduction

The increase of anthropogenic $CO_2$ emissions over the past 200 years and its subsequent absorption (about ¼) by the ocean is responsible for seawater pH decrease and substantial changes in carbonate chemistry, a process known as ocean acidification (OA) (Caldeira and Wickett, 2003; Fabry et al., 2008; Gattuso et al., 2015). According to the most pessimistic scenario for future greenhouse gas emissions (Representative Concentration Pathway 8.5: RCP 8.5), surface ocean pH should decrease by 0.33 pH units by the year 2100 (IPCC, 2014), resulting in deleterious effects on the biology and ecology of numerous marine organisms (Widdicombe and Spicer, 2008; Kroeker et al., 2010; Wittmann and Pörtner, 2013). Changes in ocean chemistry may be more pronounced in coastal areas where numerous species of economic importance (such as molluscs) can be found and such changes may be exacerbated by diurnal and seasonal variations in shallow and intertidal zones (Truchot, 1988; Byrne et al., 2011; Legrand et al., 2018). OA is also predicted to affect aquaculture activities around the globe through its effects on species physiology and behaviour in relation to farming practices (Cochrane et al., 2009; Clements and Chopin, 2016; Weatherdon et al., 2016).

Among its reported biological effects, increased seawater $pCO_2$ has been shown to induce hypercapnia and acidosis in organisms, both of which are energetically costly processes that can affect vital functions such as growth and calcification (Pörtner et al., 2004; Fabry et al., 2008; Melzner et al., 2009; Orr et al., 2005; Hofmann et al., 2010). Due to a low capacity to regulate their acid–base balance and the highly calcified shells they build, marine molluscs are

considered to be among the most vulnerable species with regard to OA (Fabry et al., 2008; Gazeau et al., 2013; Parker et al., 2013). Abalones (Mollusca, Vetigastropoda) are ecologically important herbivore species, providing key ecosystem services through their role in nutrient and mineral cycling, and are economically important as a food source (Cook, 2016; Huchette and Clavier, 2004). Many abalone species have experienced severe population declines worldwide due to both overfishing (Rogers-Bennett et al., 2002) and environmental disruptions, such as global warming and wasting disease (Cook, 2016; Moore et al., 2002; Morales-Bojórquez et al., 2008; Nicolas et al., 2002; Travers et al., 2009). In the context of a worldwide expansion in their aquaculture, understanding the effects of environmental stresses on abalone physiology is an important issue for the management of wild populations as well as for the optimization of fisheries and aquaculture practices (Morash and Alter, 2015).

*Haliotis tuberculata* is the only abalone species present in Europe, living in low intertidal and subtidal rocky coastal areas where it offers a high potential for fishery and aquaculture (Huchette and Clavier, 2004; Courtois de Viçose et al., 2007). Several studies focusing on early life-history stages of abalone, especially larvae, have demonstrated adverse effects of elevated $pCO_2$, 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). Impacts of OA on shell calcification and integrity have been well studied on various stages of *H. tuberculata*, from larvae to juveniles and adults (Wessel et al., 2018; Auzoux-Bordenave et al., 2020; Avignon et al., 2020). Reduced growth in shell length and alterations of the shell microstructure were observed in juvenile abalone exposed to $pH_T$ 7.6, which correspond to projected pH values expected for 2100 (IPCC, 2014; Gattuso et al., 2015). Since these pH conditions corresponded to an aragonite saturation state below 1, it was concluded that the effects on shell growth and integrity were principally caused by direct aragonite dissolution within the abalone shell (Auzoux-Bordenave et al., 2020). Additional effects on the organic periostracum and nacre structure suggested that other processes involved in shell biomineralization, such as matrix protein production and enzymatic activities, would also be

95 influenced by changes in seawater pH. Avignon et al. (2020) demonstrated significant effects
96 on growth in shell length, microstructure and resistance in adult *H. tuberculata* exposed to
97 $pH_T$ 7.7. Although the aragonite saturation was above 1 (1.24) here, the effects on shell
98 integrity were partly attributed to direct effects on aragonite dissolution. The study also found
99 that the expression of genes involved in either stress responses or biomineralization
100 processes was not significantly modified in response to decreased pH. These observations
101 suggested that other indirect effects, due to extracellular acid–base balance modification,
102 could lead to metabolic disturbances affecting growth, calcification and, ultimately, fitness
103 (Pörtner et al., 2004; Fabry et al., 2008; Melzner et al., 2009; Michaelidis et al., 2005).
104 According to Cyronak et al. (2016), elevated $H^+$ concentration and subsequent problems of
105 homeostasis would be more likely than carbonate ion concentration to induce the reduction
106 of calcification in marine organisms facing OA.

107 Metazoans use two buffer systems in response to potential fluctuations of their extracellular
108 pH (Heisler, 1989). The most commonly used is a bicarbonate-based buffer system which is a
109 relatively low-cost system energetically depending on the number of protons ($H^+$) to be
110 eliminated and the capacity of the species to accumulate bicarbonate ions in their extracellular
111 fluids (Heisler, 1989; Melzner et al., 2009). The second buffer system is non-bicarbonate based
112 and relies on organic molecules (protein and peptides) present in the hæmolymph that are able
113 to capture $H^+$ with their polypeptide side chains.

114 The ability to prevent $CO_2$-induced changes of the hæmolymph pH ($pH_{HL}$) is believed to
115 be a key determinant of an organisms' ability to tolerate near future OA (Collard et al., 2013;
116 Wittmann and Pörtner, 2013; Melzner et al., 2009). Marine molluscs are usually considered to
117 be poor acid–base regulators compared with other taxa and their ability to buffer their
118 extracellular fluid when experiencing OA stress is thus very limited (Melzner et al., 2009;
119 Gazeau et al., 2013, Parker et al., 2013). However, some intertidal molluscs are able to
120 compensate the decrease of their extracellular pH to some extent (Widdicombe and Spicer,
121 2008; Marchant et al., 2010; Scanes et al., 2017). In adult bivalves, long-term hypercapnia was
122 seen to cause a reduction in extra-cellular $pH_{HL}$, which was partly prevented by an increase of
123 bicarbonate hæmolymph concentration, supposedly coming from shell dissolution (Michaelidis et

al., 2005; Thomsen et al., 2010; Heinemann et al., 2012). Cao-Pham et al. (2019) reported an $Na^+/H^+$ exchanger-like in the apical membrane of the epithelium facing the sea water in the inner mantle giant clam *Tridacna squamosa* which hosts zooxanthellae. Based on the localization of the exchanger and the upregulation of the expression level of this transporter by light, these authors suggested that the exchanger could be involved in the elimination of protons produced during light-enhanced calcification. This indicates a possible pH compensation mechanism. In cephalopods, the cuttlefish *Sepia officinalis* was shown to partially compensate its $pH_{HL}$ by accumulating bicarbonate ions in its extracellular fluid (Gutowska et al., 2010). To our knowledge, the acid–base physiology has been studied in only two genera of gastropods: in abalone and in patellid limpets. The limpet *Patella vulgata* showed an ability to fully compensate its $pH_{HL}$ after 5 days of exposure to low pH ($pH_{NBS}$ 7.5) by increasing its hæmolymph bicarbonate concentration, supposedly from shell dissolution (Marchant et al., 2010). Apart from patellid limpets *Patella* sp. which are distantly related to other gastropods, there are only two studies on the acid–base regulation abilities of abalone exposed to environmental stress. Cheng et al. (2004) reported that a decrease in dissolved $O_2$ disrupted the acid–base balance as well as anaerobic metabolism of *H. diversicolor supertexta*. On the other hand, when exposed to elevated $pCO_2$, juvenile *Haliotis fulgens* showed no significant changes in the concentration of products from the anaerobic metabolism, compared with control conditions (Tripp-Valdez et al., 2017).

As an herbivorous gastropod, abalone is considered as a low trophic species exhibiting a low metabolism (compared with more active carnivorous molluscs). Low rates of metabolism typically correlate with lower concentrations of ion transport proteins (such as $Na^+/K^+$ and $H^+$-ATPases, Gibbs and Somero, 1990), suggesting reduced capacities of acid–base balance and a poor ability to compensate for changes in acid–base status (Fabry et al., 2008; Pörtner et al., 2004; Melzner et al., 2009; Parker et al., 2013). In adult *H. tuberculata* under OA stress, the hæmolymph extracellular pH was reported to be 0.1 pH unit lower in individuals subjected to $pH_T$ 7.7, compared with those maintained in $pH_T$ 8.0, suggesting that abalone only poorly compensate for the pH decrease of their extracellular fluid if at all (Avignon et al., 2020). Interestingly, compared with abalones, patellid limpets were only moderately affected by OA

(Marchant et al., 2010; Duquette et al., 2017). In other taxa, such as crustaceans, sipunculids or echinoderms, the capacity to tolerate a moderate sea water pH decrease has been linked with the ability to regulate the extracellular acid–base balance (Pörtner et al., 2004; Di Giglio et al., 2020). Consequently, we hypothesize that abalone have a limited ability to compensate $pH_{HL}$.

To better understand how abalone respond to $CO_2$-induced OA, the present study investigated, for the first time, the extra-cellular acid–base parameters in the hæmolymph of adult *Haliotis tuberculata* exposed to acute OA stress. Three $pH_T$ levels (7.9, 7.7, 7.4) were compared, to which the animals were exposed for 15 days. Extracellular pH and total alkalinity of the hæmolymph were measured at different time points through the experiment and were used to calculate the carbonate parameters of the extracellular fluid (e.g. $pCO_2$, carbonate and bicarbonate ions, dissolved inorganic carbon: DIC, aragonite and calcite saturation state). Total protein content was also measured in hæmolymph samples to determine whether lowering the seawater pH influenced the composition or buffer capacity of extracellular fluid in adult abalone.

## 2. Material and Methods

### 2.1 Abalone collection and acclimation

Three-year-old adult abalone *Haliotis tuberculata* were picked up without any selection from an offshore sea-cage structure containing 600 individuals per cage, at the France Haliotis abalone farm (48°36'50N, 4°36'3W; Plouguerneau, Brittany, France). Abalone were transported to the laboratory ensuring minimum stress and minimum handling during transport. They were conditioned before experiments under ambient seawater $pCO_2$/pH and temperature conditions and fed *ad libitum* with the macroalga *Palmaria palmata*.

## 2.2 Experimental set-up

Two experiments were carried out in two different laboratories. A first experiment (Exp.1), carried out in February 2015 at the Marine Biology Laboratory (ULB, Brussels), was done to assess acid–base parameters and how to measure them in adult abalones conditioned in ambient conditions of $pCO_2$. A second experiment (Exp.2), carried out in November 2015 at the MNHN Concarneau marine station (Brittany, France), was then done to assess whether the decrease of seawater pH had an impact on acid–base parameters of abalone.

### *2.2.1. Experiment 1: ambient conditions*

Adult abalones (63 ± 5 mm in shell length, n = 48) were distributed without any selection among three 45-L open-circuit experimental aquaria (n = 16 abalone per aquarium) supplied with filtered seawater renewed at a rate of 60–65 $L.h^{-1}$ and continuously aerated with ambient air. Animals were conditioned for 10 days in the laboratory in ambient conditions of temperature and $pCO_2$ and were fed *ad libitum* with *P. palmata*.

### *2.2.2. Experiment 2: OA experiment*

Adult abalone (61 ± 3 mm in shell length, n = 180) were distributed without any selection among nine 45-L open-circuit experimental aquaria (n = 20 abalone per aquarium) supplied with through-flowing 3-µm filtered natural field seawater renewed at a rate of 60–65 $L.h^{-1}$ and continuously aerated with ambient air. The aquaria were cleaned twice a week using a siphoning hose and the water filters were changed daily. Following the three weeks of conditioning, aquaria housing abalone were assigned to three pH treatments for 15 days. The pH treatments were as follows: present-day field conditions $pH_T$ 7.9 ($pCO_2 \approx 600$ µatm); $pH_T$ 7.7 ($pCO_2 \approx 1000$ µatm), predicted to occur in 2100 according to the RCP 8.5 scenario (IPCC, 2014; Gattuso et al., 2015); and an extreme level of $pH_T$ 7.4 ($pCO_2 \approx 2000$ µatm). Three replicate 45-L aquaria were set up for each pH treatment, in which seawater $pCO_2$ concentrations were adjusted by bubbling $CO_2$ (Air Liquide, France). $pCO_2$ in each tank was

controlled through electro-valves regulated by a pH-stat system (IKS Aquastar, Germany). pH values of the IKS system were adjusted from daily measurements of the electromotive force in the aquaria using a pH meter (Metrohm 826 pH mobile, Metrohm, Switzerland) with a glass electrode (Metrohm electrode plus) converted into pH units on the total scale ($pH_T$) using Tris/HCl and 2-aminopyridine/HCl buffers (Dickson, 2010). Before the start of the experiment, pH was gradually decreased by 0.1 pH unit per day until the different target pH levels were reached.

### 2.3. pH and carbonate parameters monitoring

Seawater parameters were monitored throughout the 10 days of control conditions in Exp.1 and the 15 days of exposures in Exp.2. Temperature and $pH_T$ were recorded daily in each experimental aquarium using a pH meter as described above, and salinity was measured twice a week using a conductivity meter (3110, WTW, Germany). Total alkalinity ($A_T$) of the seawater was measured weekly on 100 mL samples taken from each experimental aquarium. Seawater samples were filtered through 0.7 µm Whatman GF/F membranes, immediately poisoned with mercury chloride and stored at 4°C until 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). Seawater carbonate chemistry, i.e. bicarbonate ($HCO_3^-$), carbonate ($CO_3^{--}$) and dissolved inorganic carbon (DIC) concentrations, $pCO_2$ and the saturation states of aragonite ($\Omega_{aragonite}$) and calcite ($\Omega_{calcite}$) were calculated from $pH_T$, $A_T$, temperature and salinity using the CO2SYS program (Pierrot et al., 2006) set with the constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987).

231 **2.4. Abalone survival and sampling**

232 Abalone survival was assessed every day throughout the experiments and any dead
233 individuals were removed from the aquaria immediately. Survival (%) was calculated as the
234 proportion of living individuals at the end of the experiment relative to the total number of
235 abalones per aquarium at the beginning of the experiment. Abalones were randomly
236 sampled for hæmolymph analysis. In Exp. 1, 3 to 5 individuals were removed at each
237 sampling time. The haemolymph was pooled from these individuals and the data were
238 averaged over the 3 sampling times (n = 3). In Exp.2, abalones were sampled after 5, 10 and
239 15 days of pH exposure (D5, D10 and D15, respectively). Hæmolymph was pooled from 3 to
240 5 individuals collected from the same aquarium and the data were averaged over the 3
241 aquaria per pH treatment (n = 3 per sampling time and pH treatment). Hæmolymph was
242 sampled immediately from these animals and shell length and width were measured to the
243 nearest 0.5 mm using Vernier callipers. Animals were replaced into their initial aquaria, with a
244 tag to avoid re-sampling of the same individual.
245

246 **2.5. Hæmolymph sampling and analysis**

247 Hæmolymph was withdrawn carefully from the pedal sinus using a refrigerated 2-mL syringe
248 and 25 G x ½ needles. Hæmolymph from the 3 to 5 individuals was pooled in a 15-mL vial on
249 ice and the electromotive force was measured using a glass micro-electrode (Biotrode,
250 Metrohm, Germany). This value was converted into pH units of the total scale ($pH_T$) as
251 described above for seawater $pH_T$ determination.

252 Pooled samples were pelleted in a centrifuge (250 g, 10 min, 6°C) and the supernatant was
253 distributed immediately into 96-well microplates for protein analysis. Protein concentration
254 was determined spectrophotometrically by BCA assay (Pierce, SIGMA) using bovine serum
255 albumin (BSA) as a standard (Smith et al., 1985). A calibration curve was obtained by
256 measuring a dilution series of standard BSA solution, 25 to 500 $\mu g.mL^{-1}$ (n = 6 replicate wells
257 in a 96-well microplate). Absorption of each sample was determined using a microplate
258 reader (BioTek plate reader, Winooski, VT, USA) operating at 570 nm, and protein content

was calculated from the average absorbance of the six replicate wells according to the calibration curve.

$A_T$ of the hæmolymph was determined by a potentiometric titration method adapted to small volumes (Gran, 1952; Collard et al., 2013, 2014), using a 3-mm diameter glass microelectrode (Biotrode, Metrohm, Germany). $A_T$ was tentatively measured on several samples of whole hæmolymph and the supernatant of the 250-g centrifugation (also used for protein determination). Because these measurements turned out to be unfeasible (see Results section), the supernatant of the 250-g centrifugation (7 mL) was ultra-filtrated using a centrifugal filter unit with a molecular cut-off of 3 kDa (Amicon Ultra 2 mL, Millipore, USA 4000g, 45min, 6°C). The ultra-filtrated fraction was then transferred into a 1.5-mL tube for $A_T$ measurement. Hæmolymph $pCO_2$, bicarbonate, carbonate and DIC concentrations were calculated from $pH_T$, $A_T$, temperature and salinity using the CO2SYS program as described in section 2.2 for the determination of seawater carbonate parameters. The methods of hæmolymph sampling and analysis apply to both experiment 1 and 2.

### 2.6. Statistical analyses

All statistical analyses were performed with Rstudio software (R Core Team, 2015). Differences in abalone survival, shell length and hæmolymph parameters (i.e. $pH_{HL}$, total alkalinity, $pCO_2$, bicarbonate ion concentration, saturation states and protein content) across $pH_{SW}$ treatments were analysed with general linear model ANOVAs ($pH_{SW}$: fixed factor) using the mean value per aquarium (n=3 replicates per pH condition, Exp.2). The normality of the residuals and homogeneity of variances were verified using respectively Shapiro-Wilk and Bartlett tests. Statistical analyses were performed separately for each time point on these data. Post-hoc HSD Tukey tests were used to test the differences between the group means. Data given in the text and figures are presented as mean ± standard deviation (SD), unless otherwise indicated. Differences were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Experiment 1: ambient conditions

Mean values of seawater parameters over the whole period (n = 6) are given in Table 1A. Seawater temperature was 14.5 ± 0.3°C, salinity was 34.1 ± 0.1, $pH_T$ 8.03 ± 0.01 and $pCO_2$ 576 ± 30 µatm. Extra-cellular acid–base parameters measured in adult abalones kept in ambient conditions are reported in Table 1B. Mean hæmolymph $pH_T$ ($pH_{HL}$) was 7.32, *ca.* 0.7 units lower than seawater $pH_T$ (Table 1B). The titration carried out on freshly collected hæmolymph and on the supernatant of hæmolymph centrifuged at 250 g did not allow total alkalinity to be determined because the titration curve obtained did not fit a Gran function (Suppl. material S1). After ultrafiltration of the hæmolymph through a membrane with a molecular cut-off of 3kDa, the typical titration curve was obtained and used to calculate the total alkalinity according to the Gran method (Suppl. material S1). Average value of the latter for abalones kept in ambient conditions was 3796 ± 72 $\mu E.kg^{-1}$. pH and $A_T$ of the haemolymph were used to calculate other parameters of the carbonate system (Table 1B). $pCO_2$ reached 4331µatm, bicarbonate 3664µmol.$kg^{-1}$, $\Omega_{aragonite}$ and $\Omega_{calcite}$ were respectively lower and higher than 1.

### 3.2. Experiment 2: acidified conditions

Mean values of seawater parameters are presented in Table 2A. Temperature followed the natural variations found in the Bay of Concarneau, from 15.1° ± 0.1°C at the start of the experiment (T0, early November) to 13.4°C ± 0.3°C at the end of the experiment (D15). The $pH_T$ levels of the experimental aquaria were maintained close to the nominal values throughout the experiment, at means of $pH_T$ = 7.91 ($pCO_2$ = 567 ± 61 µatm), $pH_T$ = 7.69 ($pCO_2$ = 997 ± 126 µatm), and $pH_T$ = 7.40 ($pCO_2$ of 2035 ± 114 µatm) (Table 2A). Total alkalinity ($A_T$) measured in the nine experimental aquaria averaged 2295 ± 11 $\mu Eq.kg^{-1}$ during the experiment. Mean salinity was 34.6 ± 0.2 in all experimental aquaria and remained

313 stable over the experiment (n = 30). $\Omega_{aragonite}$ was higher than 1 in pH treatments 7.9 and 7.7
314 and below 1 in the lowest pH treatment (7.4).
315
316 3.2.1. Survival and growth
317
318 The mortality of adult abalones during the experiment was very low, with a survival
319 percentage higher than 90% at the end of the experiment (Day 15). There were no significant
320 differences in survival between the three pH treatments (ANOVA, $F$ (2,6) = 0.6, p = 0.58).
321 Mean shell length of adult abalone at the start of the experiment (T0, Exp. 2) was 61 ± 3mm
322 and did not differ significantly across the four time points (ANOVA, $F$ (3,120) = 0.75, p = 0.52).
323 There were no significant differences in total length between the three pH treatments
324 (ANOVA, $F$ (2,121) = 0.90, p = 0.41).
325
326 3.2.2. Hæmolymph acid–base status
327
328 *Hæmolymph $pH_T$*
329 Mean hæmolymph pH ($pH_{HL}$) measured in control abalones ranged between 7.29 and 7.36
330 *i.e.* 0.54 to 0.61 lower than seawater $pH_T$ (Table 2B). A significant effect of decreased
331 seawater pH was observed on $pH_{HL}$ of abalone at any time point (Fig.1A, Table 3A). At D5
332 and D10, $pH_{HL}$ of abalone exposed to $pH_{SW}$ 7.4 was significantl'y lower than that of control
333 individuals exposed to $pH_{SW}$ 7.9 (Table 3B). At D15, $pH_{HL}$ of abalone exposed to $pH_{SW}$ 7.4
334 was significantly lower than that of individuals exposed to 7.7 and 7.9 (Table 3B). There were
335 no significant differences in $pH_{HL}$ between abalone exposed to $pH_{SW}$ 7.7 and 7.9 at any time
336 point (Table 3B).
337
338 *Total alkalinity ($A_T$)*
339 Total alkalinity ($A_T$) measured in the hæmolymph of control abalones was 3177 ± 103 $\mu E.kg^{-1}$
340 (Table 2B). $A_T$ of the hæmolymph was significantly different in abalones exposed to lower
341 seawater pH (Fig. 1B**,** Table 3A). After 15 days of exposure, hæmolymph $A_T$ of abalones

exposed to $pH_{SW}$ 7.4 was significantly lower than that of control individuals exposed to 7.7 and 7.9 (Table 3B). There were no significant differences in hæmolymph $A_T$ between abalones exposed to $pH_{SW}$ 7.7 and 7.9 at any time point (Table 3B).

*$pCO_2$:*

Mean $pCO_2$ in control abalones ranged between 3141 and 3675 $\mu mol.kg^{-1}$ (Table 2B). Significant differences in hæmolymph $pCO_2$ were observed in abalones exposed to lower seawater pH (Table 3A). At D10, extracellular $pCO_2$ in abalones exposed to $pH_{SW}$ 7.4 was significantly lower than that of control individuals exposed to 7.7 and 7.9 (Table 3B). There were no significant differences in hæmolymph $pCO_2$ between abalones exposed to $pH_{SW}$ 7.7 and 7.9 at any time point (Table 3B).

*$HCO_3^-$*

Mean hæmolymph $[HCO_3^-]$ in control abalones ranged between 3059 and 3077 $\mu mol.kg^{-1}$ (Table 2B). In abalones exposed to decreased pH, hæmolymph $[HCO_3^-]$ was significantly different after 15 days of exposure (Table 3A). At this time point, $[HCO_3^-]$ in abalones exposed to $pH_{SW}$ 7.4 was significantly lower than that of individuals exposed to $pH_{SW}$ 7.7 and 7.9 (Table 3B). There were no significant differences in hæmolymph $[HCO_3^-]$ between abalones exposed to $pH_{SW}$ 7.7 and 7.9 at any time point (Table 3B).

*Saturation state (Ω)*

$\Omega_{aragonite}$ and $\Omega_{calcite}$ in control abalones were respectively 0.71 ± 0.08 and 1.11 ± 0.12 (Table 2B). A significant effect of decreased seawater pH was observed on $\Omega_{aragonite}$ and $\Omega_{calcite}$ at any time point (Table 3A). At D5, $\Omega_{aragonite}$ in abalone exposed to $pH_{SW}$ 7.4 and 7.7 was significantly lower than that of control individuals exposed to $pH_{SW}$ 7.9 (Table 3B). At D10 $\Omega_{aragonite}$ in abalone exposed to $pH_{SW}$ 7.4 was significantly lower than that of control

individuals exposed to $pH_{SW}$ 7.9 (Table 3B). At D15, $\Omega_{aragonite}$ in abalone exposed to $pH_{SW}$ 7.4 was significantly lower than that of individuals exposed to $pH_{SW}$ 7.9 and 7.7 (Table 3B).

### 3.2.3. Protein content

The average protein content in the hæmolymph of control abalones was 16.7 ± 5 $g.L^{-1}$ (Table 2B) and did not differ significantly between pH treatments at any time point (Table 3A).

## 4. Discussion

This paper reports the first investigation of extra-cellular acid–base parameters and buffer capacity in the adult abalone *H. tuberculata* exposed to $CO_2$-induced ocean acidification. In control *H. tuberculata*, $pH_{HL}$ was 7.4, which is close to the range for extracellular pH measured in the Taiwan abalone *H. diversicolor supertexta* (between 7.23 and 7.28, Cheng et al., 2004). In adult *H. tuberculata* facing OA stress, all the extra-cellular acid–base parameters measured or calculated (except $pCO_2$) were significantly reduced in the $pH_{SW}$ 7.4 treatment (0.5 units below control pH), while $pH_{SW}$ 7.7 did not affect the variables significantly.

A pH-bicarbonate diagram (Davenport diagram, Fig. 2) further indicates that adult *H. tuberculata* are able to maintain their hæmolymph pH with moderate acidification (-0.2 $pH_{SW}$ units) but not at a more severe level (-0.5 $pH_{SW}$ units). This is consistent with our previous results showing that adult abalones exposed to a -0.3 pH decrease for two months were unable to maintain their $pH_{HL}$ (Avignon et al., 2020). It is noteworthy that the $pH_{HL}$ homeostasis at $pH_{SW}$ 7.7 is not due to an accumulation of bicarbonate ions, contrary to the limpet *Patella vulgata*, which was reported to increase its hæmolymph bicarbonate concentration to compensate its $pH_{HL}$ (Marchant et al., 2010). Furthermore, it appears that abalone subjected to $pH_{SW}$ 7.4 suffered metabolic acidosis (reduced hæmolymph pH and bicarbonate concentration). This suggests the induction of anaerobic metabolism by OA at

395 $pH_{SW}$ 7.4. This finding does not agree with the results obtained by Tripp-Valdez et al. (2017)
396 in *Haliotis fulgens*. When subjected to $pH_T$ 7.3–7.4 (recalculated from their $pCO_2$ and $A_T$ data
397 using $CO_2$SYS; -0.4 to -0.5 $pH_T$ units compared with the control), *H. fulgens* juveniles,
398 maintained at control temperature in normoxic conditions, showed no significant changes in
399 the concentration of products from the anaerobic metabolism, compared with control
400 conditions. However, the abalone in the present study are large adults, which probably
401 develop anaerobic conditions more easily, particularly in the foot (see Venter et al., 2018).
402
403 In addition to $pH_{HL}$, total alkalinity ($A_T$) of the hæmolymph was also measured in *H.*
404 *tuberculata* using a potentiometric titration method adapted to small volumes (Gran, 1952;
405 Collard et al., 2013, 2014). The titrations carried out on either freshly collected hæmolymph
406 or on the 250-g centrifugation supernatant differed from the classical titration of a
407 bicarbonate/carbonate buffered marine solution and did not allow the determination of $A_T$. A
408 classical titration curve allowing $A_T$ calculation was, however, obtained on the ultra-filtrated
409 fraction after removing organic molecules of MW > 3kDa. As the large hemocyanin protein
410 molecule in abalone is carried in the hæmolymph, this may have interfered with the titration
411 of $A_T$. These results suggested that abalone hæmolymph contained a significant
412 concentration of proteins and/or peptides (MW > 3kDa). Indeed, the protein concentration
413 measured in the hæmolymph of *H. tuberculata* (mean 15–22 $g.L^{-1}$) is much higher than that
414 found in other gastropods (e.g. 0.5 to 1.5 $mg.L^{-1}$ in the limpet *Patella sp*, Brown et al., 2004)
415 or in the range of concentrations measured in the Australian abalone *H. rubra* (around 10
416 $g.L^{-1}$, Hooper et al., 2014). We suggest that these proteins/peptides scavenge protons of
417 respiratory origin when an abalone is subjected to moderate acidification ($pH_{SW}$ 7.7).
418 However, they appeared incapable of buffering the extra protons when the $pH_{SW}$ was
419 lowered to 7.4. The protein content of the hæmolymph appeared to be not much influenced
420 by hypercapnia, but this conclusion should be carefully considered in view of the high
421 variability in protein concentration between individuals.
422
423 In summary, adult abalone *H. tuberculata* appeared able to buffer moderate (-0.2 $pH_{SW}$
424 units) acidification, probably due to its high hæmolymph protein concentration but was not

able to compensate for greater decreases in $pH_{SW}$. These results are consistent with previous studies on the mussel *Mytilus edulis* (Thomsen et al., 2010, 2013). In this bivalve species, these authors clearly demonstrated the absence of $HCO_3^-$ accumulation and suggested that buffering by extracellular proteins is the sole mechanism acting to stabilize hæmolymph pH (Thomsen et al., 2010). However, this strategy contrasts with that of the more active cephalopod *Sepia officinalis*, which greatly increases extracellular [$HCO_3^-$] in order to stabilize its extracellular $pH_{HL}$ upon exposure to OA (Gutowska et al., 2010). Generally, active invertebrates tend to show a stronger $HCO_3^-$ buffering capacity, while less active invertebrates may experience metabolic suppression associated with a decline in pH (Melzner et al. 2009; Pörtner, 2008).

Mean $pCO_2$ values in abalone hæmolymph were higher for the $pH_{SW}$ 7.4 treatment, but the differences were significant only at day 10 of the experiment. As previously shown in marine molluscs an increase in extra-cellular $pCO_2$ may cause hypercapnia and acidosis, two energetically processes that can negatively affect vital processes, such as somatic growth and calcification (Pörtner et al., 2004; Fabry et al., 2008; Melzner et al., 2009). The saturation state of abalone haemolymph ($\Omega$) towards aragonite was below 1 in all conditions including the control treatment. Nevertheless, *H. tuberculata* calcifies efficiently aragonite in control conditions, indicating that conditions in the calcifying site strongly differ from those in the hæmolymph and that acidosis of the hæmolymph is not directly responsible for the effects on shell calcification reported in previous OA experiments (Wessel et al., 2018; Auzoux-Bordenave et al., 2020; Avignon et al., 2020).

The results of the present study support the hypothesis that changes in extracellular acid–base balance might be involved in deleterious effects recorded in adult *H. tuberculata* facing severe OA stress, as previously reported in marine bivalves (Michaelidis et al., 2005; Melzner et al., 2009; Waldbusser et al., 2011). As previously emphasized, very little information is available on the acid–base homeostasis abilities of marine gastropods. *P. vulgata* lives in the higher intertidal zone where important local variations in physico-chemical conditions might occur inducing hypoxic, hypercapnic and desiccation stresses (Marchant et al. 2010). When facing

severe acidification (-0.7 $pH_{SW}$ units) of its environment, the limpet was able to compensate its $pH_{HL}$ by increasing its $HCO_3^-$ buffering capacity (Marchant et al., 2010). *H. tuberculata*, in contrast, inhabits the subtidal and low intertidal zone where physico-chemical stresses are much lower. For instance, $pH_T$ in the Bay of Brest, one of this specie natural habitat, ranges between 8.2 and 7.9 (Qui-Minet et al., 2018). This suggests that *H. tuberculata* has not developed the biochemical machinery to ensure a strong acid–base homeostasis. This weakness, together with the effects reported on growth and shell calcification (Wessel et al., 2018; Auzoux-Bordenave et al., 2020; Avignon et al., 2020), would impact both the ecology and aquaculture of this commercially important species. Understanding how different abalone life stages respond to OA will make it possible to reveal the capacity of abalone to adapt genetically to $pCO_2$ increases of their environment and to identify bottlenecks for population persistence under near-future pH conditions.

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**Compliance with ethical standards**

The authors declare that they have no conflicts of interest or competing financial interests.

The experiments complied with the current French laws. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

## Figures and tables

**Figure 1**. Hæmolymph $pH_T$ (A) and Total alkalinity (B) in adult abalone exposed to three pH levels (7.9, 7.7 and 7.4) after 15 days of exposure (n = 3 per pH treatment). Means of bars with different letters are significantly different ($P < 0.05$).

**Figure 2**. $pH_{HL}$ bicarbonate concentration (Davenport) diagram showing the time course of acid–base compensation (mean ± SD) in the hæmolymph of abalone *H. tuberculata* over 15 days of exposure to elevated $pCO_2$. The solid curved lines represent $pCO_2$ isopleths. Symbols represent the exposure time (respectively 5, 10 and 15 days), while the grey levels correspond to the seawater pH value.

**Table 1**. **A**. Seawater temperature and carbonate chemistry parameters of control Experiment 1 (mean ± SD). pH on the total scale ($pH_T$), temperature (°C) and total alkalinity ($A_T$; $\mu Eq.kg^{-1}$) were used to calculate $CO_2$ partial pressure ($pCO_2$; µatm), dissolved inorganic carbon (DIC; $\mu mol.kg^{-1}$), $HCO_3^-$ and $CO_3^{2-}$concentrations ($\mu mol.\ kg^{-1}$), aragonite saturation state ($\Omega_{aragonite}$) and calcite saturation state ($\Omega_{calcite}$) using the CO2SYS program. $pH_T$ and temperature are the average values of those logged daily in the aquaria throughout the experiment (n = 3).

**B**. Acid–base parameters in the hæmolymph of control abalone in Exp.1 (mean ± SD). Hæmolymph pH ($pH_{HL}$), temperature (°C) and total alkalinity ($A_T$; $\mu Eq.kg^{-1}$) were used to calculate carbonate chemistry parameters using the CO2SYS program (n = 3).

**Table 2. A.** Seawater temperature and carbonate chemistry parameters at each time point of Experiment 2 after 5 (D5), 10 (D10) and 15 (D15) days of exposure to the experimental pH (mean ± SD). pH on the total scale ($pH_T$), temperature (°C) and total alkalinity ($A_T$; $\mu Eq.kg^{-1}$) were used to calculate $CO_2$ partial pressure ($pCO_2$; µatm), dissolved inorganic carbon (DIC; $\mu mol.kg^{-1}$), $HCO_3^-$ and $CO_3^{2-}$concentrations ($\mu mol.\ kg^{-1}$), aragonite saturation state ($\Omega_{aragonite}$) and calcite saturation state ($\Omega_{calcite}$) using the CO2SYS program. $pH_T$ and temperature are the

513 average values of those logged daily in the aquaria throughout the experiment (n = 3 per pH
514 treatment and sampling time).
515 **B.** Acid–base parameters and total protein content (mean ± SD) in the hæmolymph of
516 abalone *H. tuberculata* exposed to three pH treatments after 5 (D5), 10 (D10) and 15 (D15)
517 days of exposure (Exp.2). Hæmolymph pH ($pH_{HL}$), temperature (°C) and total alkalinity ($A_T$;
518 $\mu Eq.kg^{-1}$) were used to calculate carbonate chemistry parameters using the CO2SYS
519 program. Total protein content ($g.L^{-1}$) was determined spectrophotometrically by BCA assay
520 using BSA as standard (n = 3 per pH treatment and sampling time).

521

522 **Table 3**. Summary of statistics. **A.** Anova results of the effects of seawater pH ($pH_{SW}$) on
523 hæmolymph pH ($pH_{HL}$), total alkalinity ($A_T$), $pCO_2$, bicarbonate ($HCO_3^-$) concentrations,
524 saturation state ($\Omega_{aragonite}$ and $\Omega_{calcite}$) and total proteins in the adult abalone *Haliotis*
525 *tuberculata* after 5 (D5), 10 (D10) and 15 (D15) days of exposure ($pH_{SW}$: fixed factor).
526 Significant P-values are shown in bold ($P < 0.05$); **B.** Multiple comparison Tukey HSD test
527 testing the influence of seawater pH ($pH_{SW}$) on hæmolymph pH ($pH_{HL}$), total alkalinity,
528 bicarbonate ($HCO_3^-$) concentration and $\Omega_{aragonite}$ at different time points. Significant P-values
529 in bold ($P < 0.05$).

530

531 **Suppl. material S1:** Titration of abalone hæmolymph by 0.1 M HCl **a:** whole hæmolymph,
532 **b**: supernatant after 250-g centrifugation **c**: 3 kDa ultrafiltrated fraction of the hæmolymph.

533

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