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Robustness of *Adamussium colbecki* shell to ocean acidification in a short-term exposure

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Author Contributions

The authors substantially contributed to the study's conception and data acquisition. All authors have given approval to the final version of the manuscript. In particular, Dr. Dell'Acqua carried out the experiment, performed the data collection and all the analyses and wrote the manuscript; Dr. Trębala strongly contributed to the mechanical analyses; Prof. Chiantore planned the experimental design and revised the manuscript; Prof. Hannula supervised all the analyses and revised the manuscript, approving the final version.

Abstract

Atmospheric pCO_2 has increased since the industrial revolution leading to a lowering of the ocean surface water pH, a phenomenon called ocean acidification (OA). OA is claimed to be a major threat for marine organisms and ecosystems and, particularly, for Polar regions. We explored the impact of OA on the shell mechanical properties of the Antarctic scallop *Adamussium colbecki* exposed for one month to acidified (pH 7.6) and natural conditions (unmanipulated littoral water), by performing Scanning Electron Microscopy, nanoindentation and Vickers indentation on the scallop shell. No effect of pH could be detected either in crystal deposition or in the mechanical properties. *A. colbecki* shell was found to be resistant to OA, which suggests this species to be able to face a climate change scenario that may threat the persistence of the endemic Antarctic species. Further investigation should be carried out in order to elucidate the destiny of this key species in light of global change.

Keywords: Ocean Acidification, pH, benthos, shell, SEM, nanoindentation, Vickers, *Adamussium colbecki*, Antarctica

Introduction

Atmospheric $p\mathrm{CO}_2$ has increased since the industrial revolution leading to a lowering of the ocean surface water pH of 0.1 units since pre-industrial time and a further reduction of 0.4 units is expected for the end of the century, a phenomenon called ocean acidification (OA, Caldeira and Wickett 2003; Orr *et al.* 2005 and references therein). OA is expected to be a major threat to calcifying marine invertebrates either because it decreases the availability of carbonate ions (Hahn *et al.* 2012 and reference therein) or because it may lead to carbonate dissolution (Roleda *et al*. 2012). The calcium carbonate saturation state (Ω) is a function of CO_3^{2-} and calcium ion concentrations ($[Ca^{2+}]$); seawater Ω describes the $CaCO_3$ precipitation and dissolution thermodynamic potential: when $\Omega > 1$, $CaCO_3$ will be maintained in the solid state, when $\Omega < 1$, the mineral will tend to dissolve (Cyronak *et al*. 2015). Many marine invertebrates are known to use bicarbonate instead of carbonate ions and many species so far investigated have shown to be able to control Ω at the calcification site, so that seawater Ω may not affect their ability to biomineralize (Cyronak *et al*. 2015 and references therein; Roleda *et al.* 2012 and references therein). However, calcification mechanisms are still far from being understood and further research is required in order to elucidate the response of the different species.

The Antarctic marine environment is supposed to be one of the most threatened by rising atmospheric CO_2, because of the peculiar seawater physico-chemical parameters (Fabry *et al*. 2008; McNeil and Matear 2008). At the same time, Antarctic species adaptive capacity is suggested to be poor due to extremely slow development (Pearse *et al.* 1991; Peck *et al.* 2002) and because their evolution took place in a relatively physically stable environment (Kapsenberg and Hofmann 2014; Matson *et al.* 2014). Most of the studies investigating OA effects on Antarctic species dealt with the sea urchin *Sterechinus neumayeri*, assessing different physiological aspects (e.g. Collard *et al.* 2015; Morley *et al*. 2016), fertilization (e.g. Suckling *et al*. 2015), larval development (e.g. Kapsenberg and Hofmann 2014) and genetic expression (Dilly *et al.* 2015; Foo *et al.* 2016). Only few works investigated the response of other Antarctic key benthic species to OA. Gonzalez-Bernat *et al.* (2013) found significant effects of low pH on larval survival and morphology of the sea star *Odontaster validus.* Cummings *et al.* (2011) reported a genetic shell adaptation in the clam *Laternula elliptica*, as a response to OA. Benedetti *et al.* (2016) found a significant effect of the interaction among OA, temperature and cadmium exposure in digestive gland and gills of the Antarctic scallop *Adamussium colbecki*. Recent investigations show that the low pH exposure affects the reproductive system of adult *S. neumayeri* and *A. colbecki*, conversely to *O. validus*, that is able to cope with OA with no negative effects on the gonads (Dell'Acqua *et al*. 2019).

Adamussium colbecki is a key benthic species that can be found patchily aggregated all around the Antarctic continent (Schiaparelli and Linse 2006). In the littoral area of Terra Nova Bay (Victoria Land, Ross Sea) the scallop is very abundant from -20 m to -80 m (Chiantore *et al.* 2001) and plays a key role in the benthic-pelagic coupling (Chiantore *et al.* 1998). This species is also acknowledged as an ecosystem engineer (Cerrano *et al*. 2006, 2009), sentinel organism (Bonacci *et al*. 2004; Regoli *et al*. 2002) and as a relevant food source for higher trophic levels (Dell'Acqua *et al*. 2017; Vacchi *et al*. 2000). For all these reasons, the Antarctic scallop is listed in the Vulnerable Marine Ecosystem species (CCAMLR 2009). Due to the relatively low levels of mineral incorporation and the low amount of organic matrix, its shell is extremely thin and fragile (Berkman *et al*. 2004; Halloran and Donachy 1995; Meng et al. 2019 and reference therein). This kind of shell is a result of an evolution *in situ* in the high Antarctic areas, related to low-energy hydrodynamic environments (mostly areas with extensive sea-ice coverage) where the species can still be found (Schiaparelli and Linse 2006). This fact, in addition to the absence of durophagous predators over the Antarctic fauna evolution (Aronson and Blake 2001; Watson *et al*. 2012) has allowed the evolution of the thin shell of the scallop.

Adamussium colbecki has an entirely calcitic shell, except for a negligible amount of aragonite in the lower valve. (Barrera *et al.* 1990; Denny and Miller 2006; Taviani and Zhang 1998). In particular, the peripheral margin (the edge opposite to the umbo, where growth occurs) is composed of calcite only (Berkman 1994). From the inner face of each valve, the shell is made by a foliated layer composed by calcite laths, that continues, toward the external shell face, developing in a prismatic calcitic layer. For a full and detailed description of the crystallographic structure of the scallop shell, see Barrera *et al*. (1990). In light of the particular traits of *A. colbecki* shell and the needs to deepen understanding of OA effects on Antarctic species, we assessed the response of structure and mechanical properties of the *A. colbecki* shell to short term (37 days) experimental low pH, investigating if and to what extent this exposure may alter shell structure, hardness and elasticity.

Material and Methods

Experiment set-up

We performed an acidification experiment at the Italian Mario Zucchelli Station (Terra Nova Bay, Ross Sea), during austral summer 2014-2015. Scuba divers collected adult specimens of *A. colbecki* at around 15 m depth in Tethys Bay (74° 41.407’ S; 164° 06.311’ E), close to the Italian Station. After 2 weeks of acclimation, we started the experiment in running seawater tanks. Seawater was continuously pumped from -6 m from the littoral area, in front of the Italian base, to a first tank, where the water was cooled. From here, the water was pumped into two 50 l header tanks (polyethylene), where pH levels were adjusted by bubbling pure-CO_2 using a continuous pH-stat system (IKS Aquastar, Karlsbad, Germany). Two pH levels were nominally chosen: pH 7.6 and control condition (unmanipulated water at 8.12). Each header tank supplied 2 polyethylene tanks (*i.e.* 2 experimental units or replicates for each pH level, 20 x 25 x 20 cm), each through an individual pipe. Four animals were placed in each experimental unit, for a total of 16 individuals (2 pH x 2 replicates x 4 individuals). We chose adult individuals with 76 ± 3 mm shell height (SH, the distance from the umbo to the opposite side of the shell) in order to detect similar ontogenetic performance.

We ran the experiment in flow through, in order to avoid uncontrolled pH oscillations due to organism respiration. After filling the experimental units at a rate of 150 ml/min, the water was discharged through a relief hole. All the tanks were covered with transparent lids (made by the same material of the replicate tanks) to avoid gas exchange and escapes. The cover was only removed every 2-3 days for about 5 minutes, in order to clean the tank bottom through siphoning. The animals were not fed, but, since the water intake pumped unfiltered seawater, diatoms and other detrital material were available in the water. pH electrodes from the pH-stat system were inter-calibrated every 3-4 days on total scale (pH_T) using TRIS buffer solutions with a salinity of 35 psu (Dickson *et al*. 2007). Concurrently, pH on total scale was also measured, together with salinity and temperature, with two different multiprobes, previously calibrated on

Antarctic littoral surface waters: Ocean Seven 310 CTD (Idronaut, Brugherio, Italy) and C6 Multi-sensor Platform (Turner Design, San Jose, CA, USA). pH values were also periodically measured in the littoral waters at the collection site. Triplicate seawater samples for total alkalinity (TA) measurements were collected once a week, poisoned with $HgCl_2$ and stored at +4°C. In Italy, TA was determined at the Polytechnic University of Ancona (Italy) using an open cell potentiomentric titration, according to Dickson *et al.* (2007) procedures and standards. Temperature, salinity, pH_T and total alkalinity were input in the software SWCO2_V2 (http://neon-old.otago.ac.nz/research/kah/software/swco2/index.html) in order to calculate pCO_2 and calcite and aragonite saturation state of the two pH levels. We used the equilibrium constants of Millero *et al.* (2006), since the lowest value of their temperature range (0 – 40°C) is close to Antarctic waters. The experiment lasted 37 days and, at the end of the experiment, *A. colbecki* specimens were frozen, carefully stored in order to avoid shell damages, and shipped to Italy.

Samples preparation

We investigated the outermost portion of the shell peripheral margin (at maximum 200 μm from the edge), where longitudinal growth occurs (Wheeler and Wilbur 1977). Unfortunately, we could not assess growth of the experimental specimens because the scallops were not marked before the experiment (with chemical mark, such as calcein) nor measured before and after because common caliper has an error (around 100 to 200 μm) that may lead to misled growth measurements in such a slow growing species (1 to 3 $mm*y^{-1}$; Heilmayer *et al*. 2003; Trevisiol *et al*. 2010). Therefore, our results need to be considered as encompassing the dissolution of the pre-existing, recently deposited shell and the response of the shell potentially grown during the experiment and. This portion is thinner and less dense than the rest of the shell (Lu *et al*. 2015, Meng *et al*. 2018, 2019) and, consequently, may be more susceptible to dissolution. The *A. colbecki* shell peripheral margin is displayed in Fig. 1.

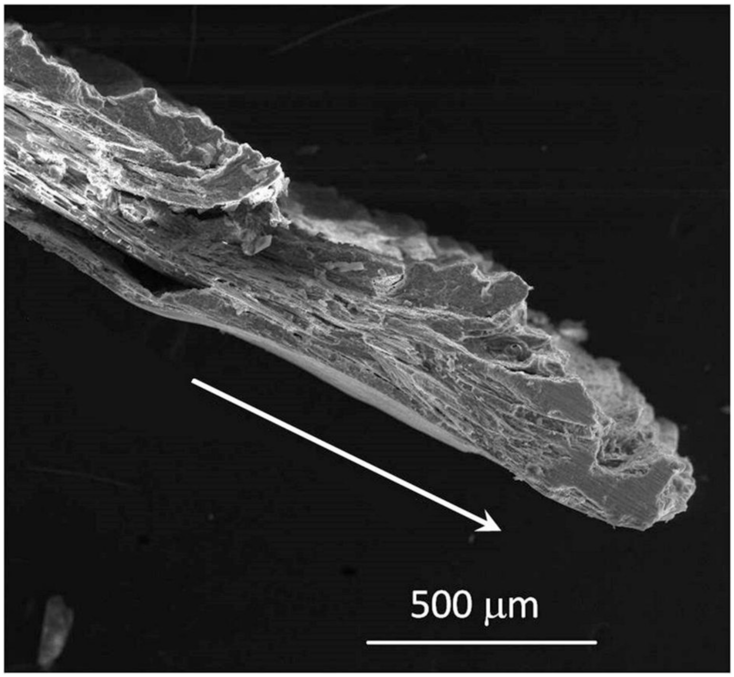

Figure 1. SEM picture of a manually fractured *A. colbecki* shell edge section. The arrow indicates the growth direction.

Before analyses, we cleaned the shells in a bath of NaOH 5% for 5 to 10 minutes and, afterwards, we gently brushed the whole edge of the shells to remove most epibionts and, at the end, we rinsed them in alcohol, at increasing percentage, from 70% to 100%. This procedure allows to remove the water that can be a problem when performing mechanical tests at the nanoscale. With a small circular saw we cut the upper valve of the shell in two halves along the maximum growth line, the imaginary line that goes from the umbo to the middle point of the external edge. For SEM and nanoindentation, we used the two sections at the external shell of each half, in order to avoid any bias due to not homogeneous growth (either previous or new) along the edge of the shell. All the analyses were performed at the Department of Chemistry and Materials Science at Aalto University (Espoo, Finland).

Scanning Electron Microscopy

Firstly, we performed Scanning Electron Microscopy (SEM) on the shell edge in order to investigate the mineralized structure, but also to check for potential damages due to freezing storage. After manually fracturing the shell peripheral margin edge of the 16 specimens and obtaining small (about 5 mm x 5 mm) pieces, we mounted them on aluminum stubs and performed carbon coating. SEM images were taken with a Mira 3 and a Vega 3 (Tescan Brno s.r.o., Czech Republic).

Nanoindentation

Nanoindentation tests were performed on the outermost 100 μm of shell edge of each of the 16 experimental specimens using a Triboindenter TI950 (Hysitron/Brooker, Minneapolis, MN, USA) on 5 x 5 mm pieces. We first polished the sections with sandpaper from 800 to 1200 (nominal number) and, then, with diamond paste from 6 μm to 0.1 μm grain size. Differently to pre-existing literature (*e.g.* Fitzer *et al*. 2015, 2016), we did not embed the samples in resin in order to avoid its effects and potential biases (Presser *et al.* 2010 and references therein). We mounted the shell samples, with the section of interest side up, perpendicularly on aluminum supports. In order to avoid bending, we framed the samples between two small iron blocks in a sandwich-like arrangement, leaving only 200 μm of the sample height exposed from the blocks. We investigated 3 to 6 areas (20 x 20 μm each) per each shell sample and, in each area, we made 5 to 10 indentations, using a standard quasi-static method with a loading, holding and unloading times of 10, 1 and 10 seconds, respectively. We chose a low force, *i.e*. 1000 μN, since the lower is the force, the higher is the chance to detect treatment differences (Presser *et al*. 2010 and references therein). For nanoindentation we used a conical tip with 1 μm radius and 90° cone angle. Despite less common than the Berkovich tip, the conical one has already been used for nanoindentation on mollusk shells (Li and Ortiz 2014; Yao *et al.* 2010). Indentation curves of conical tip are less dependent on surface shape and damage (Schwiedrzik and Zysset 2015) and, having a larger contact area than the Berkovich one, this kind of tip averages the material properties in the contact area. This provides more consistent results in case of anisotropic material and complex structures (such as biologically deposited calcium carbonate) and in case of potential holes caused by water evaporation and organic matter deterioration due to storage (Hirvonen *et al*. 1994). TriboScan software was used to analyze the loading and unloading curves of the indentation test to yield the nanohardness (H) and the reduced modulus of elasticity (E_r) (Oliver and Pharr 1992).

Part of the nanoindentation curves revealed presence of pop-in (see Fig. 4), probably due to surface cracking or inflections (Bruet *et al.* 2005; Meyers *et al.* 2009). In order to understand if these anomalies biased our E_r results, we performed additional mechanical test at 300 μN on some randomly chosen specimens, either from control pH or 7.6. This lower force corresponds to a lower indentation depth, where the pop-in did not occur. Afterwards, we compared the results from the two forces: 1000 and 300 μN, by means of *t*-test or Wilcoxon-Mann-Withney (depending from data distribution shape). Null hypothesis was that E_r resulted by using different forces are statistically equivalent, which would mean that surface cracking or inflections did not bias our results. As far as H, differences were actually expected, since the amount of force applied determines, at least in part, the value of the resulting hardness, a phenomenon known as 'indentation size effect' (Morris *et al.* 2011 and references therein).

Vickers indentation

We investigated the Vickers microhardness (HV) using the Micro Hardness Tester (1000 Plus, Jaten Precision Instrument, Dongguan, China), indenting the surface of the shell edge, since the pyramidal tip of the Vickers is too large to indent the shell section which thickness ranges from 100 to 200 μm only. We used 3 x 3 mm pieces of the shell external edge that were chosen next to the pieces we used for the previously described analyses. We selected the smallest sample length to minimize the shell arching effect. To determine the right force that allows to get well defined prints without disrupting the shell, we chose force and dwelling time ranges from existing literature dealing with Vickers tests on bivalves (e.g. Beniash *et al*. 2010; Jiao *et al*. 2015; Lee *et al*. 2009). After trial tests on expendable samples, we chose 100 g force and 10 s dwelling time. 7 to 11 indentations were made for each of the 16 experimental specimens, with a variation coefficient from 2% to 17%. A high variability is expected for such a complex biological material made by different matrices (organic and mineral) and it is consistent with the variability found in literature (e.g. Fitzer *et al*. 2014; Fleischli *et al.* 2008; Ji *et al*. 2014). After the test, we took SEM pictures of the Vickers indentation marks in order to detect potential visual differences in the material plasticity.

Statistical analyses

Statistical analyses on E_r, H and HV, were run using the statistical software R (R Core Team 2013). We used Linear Mixed Model (LMM) included in the 'lme4' package (Bates *et al*. 2015) for normally distributed data, and Generalized Linear Mixed Model (GLMM) included in the package 'nlme' (Pinheiro *et al.* 2018) for not normally distributed observations. Both models account for random effect which is, in our case, represented by the individuals in the same tank (pseudo-replicates) and for nested design (the tank level nested in the pH level). ANOVA analysis was not considered since it does not allow for pseudoreplicates.

Results

The experimental set up, including the acidification system, provided stable seawater parameters for the whole experimental duration (Table 1), while Fig. 2 shows the pH variability both in the field and in the two reservoirs that fed the replicates. Data from the field stopped on 2nd January, when the fragility of the sea ice did not allow to perform seawater sampling; measures started again on 23rd January.

Table. 1. Mean and standard deviation of the sea water variables for the two pH treatments, measured throughout the experiment. pH_T = pH on total scale; TA = total Alkalinity; Ω = saturation state of calcite (Ω_C) and aragonite (Ω_A).

Nominal treatment	**Temperature (°C)**	**Salinity (psu)**	**pH_T**	**TA (µmol/kg_{SW})**	**pCO_2 (ppm)**	**Ω_C**	**Ω_A**
control pH	-0.395 ± 0.12	33.06 ± 0.02	8.12 ± 0.05	2208.5 ± 75.7	381.7 ± 31.2	2.63 ± 0.33	1.65 ± 0.21
7.6	-0.369 ± 0.12	33.09 ± 0.02	7.625 ± 0.02	2254.6 ± 89.2	1091.4 ± 55.9	0.89 ± 0.03	0.56 ± 0.02

Fig. 2. Trend of pH values measured in the littoral at the collection site of the animals (rhombus) and in the two reservoir tanks: ctrl (empty circles) and pH 7.6 (filled circles)

Scanning Electron Microscopy

Representative sections of the shell edge of *A. colbecki* individuals maintained under control pH (a, c and e) and under pH 7.6 (b, d and f) are displayed in Fig. 2. SEM images allowed to exclude the presence of cracks due to the freezing storage, assuring reliability of the results in the mechanical analyses at the microscale. Crystal grains in the two treatments account for similar shape, dimension and deposition pattern (Fig. 3).

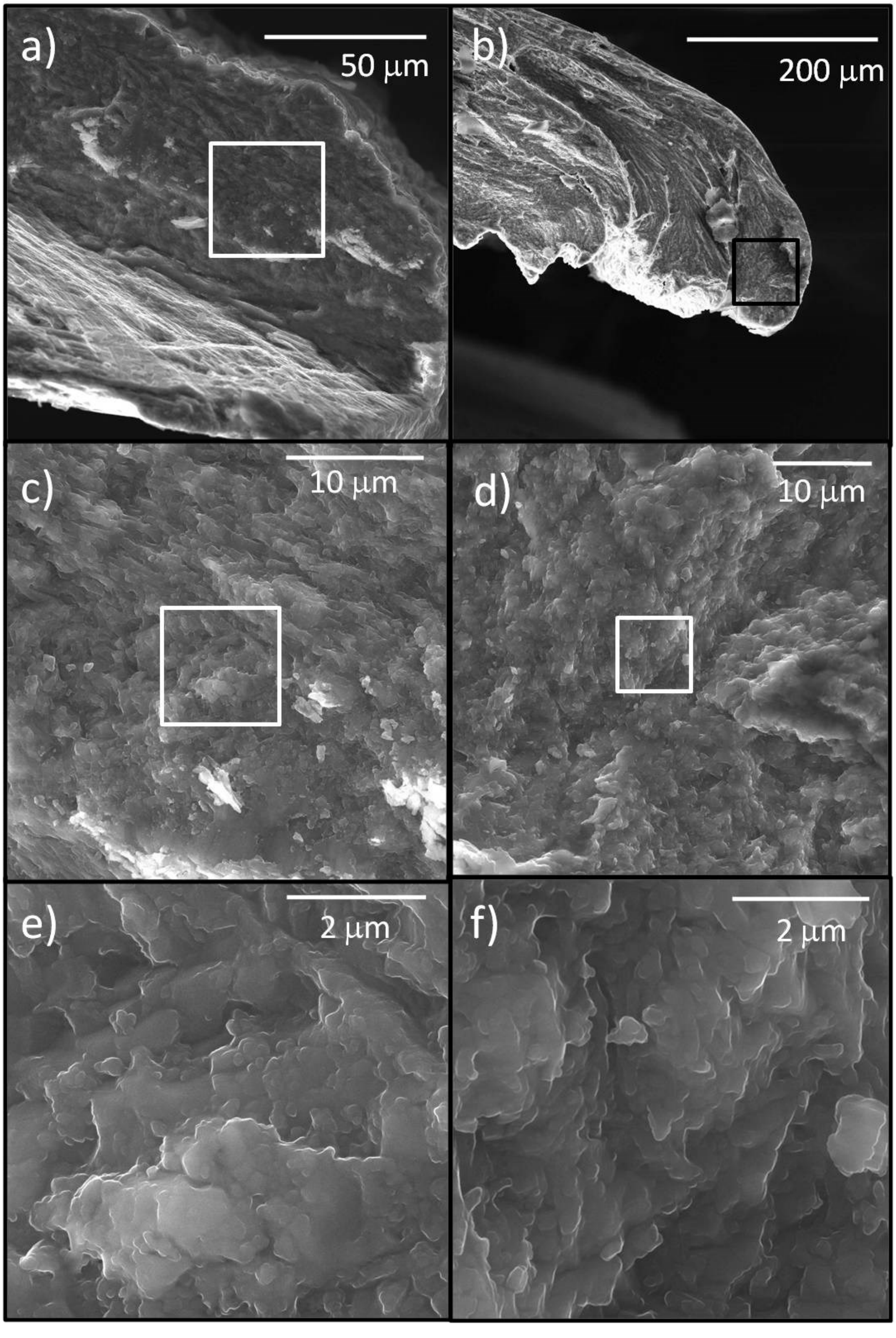

Figure 3. Representative SEM images of manually fractured *A. colbecki* shell sections in correspondence of the outermost portion of the peripheral margin; (a), (c) and (e): specimens from control pH; (b), (d) and (f): specimens from pH 7.6. In each panel, a box indicates the portion displayed below. Scale bars are indicated in each panel.

Nanoindentation

Means and standard deviations of the E_r and H are reported in Table 2, along with the indentation depth.

Table 2. Means and standard deviations of the nanoindentation results, obtained through the tests performed at 1000 μN loading force, on *A. colbecki* shell edge sections of specimens from control (ctrl) and acidified (7.6) pH condition. E_r = reduced modulus and H = nanohardness.

Nominal pH	**N. specimens**	**N. obs**	**E_r (GPa)**	**H (GPa)**	**Indentation depth (nm)**
Ctrl	8	173	66 ± 7	3.1 ± 0.5	68.9 ± 8.0
7.6	8	166	68 ± 5	3.3 ± 0.4	65.5 ± 5.6
Overall mean	16	339	67 ± 6	3.2 ± 0.5	67.2 ± 7.2

Since E_r and H observations resulted to be at the limit of normal distribution (Kolmogorov-Smirnov for E_r: N = 339, *p*-value = 0.051 and H: N = 339, *p*-value = 0.054), we ran both LMM and GLMM. Both models indicated that low pH did not affect shell mechanical properties (LMM: $F_{1,2}$ = 2.937, N = ~~339~~ 8, *p*-value = 0.289 and GLMM: *t*-value$_{311}$ = 60.998, N = ~~339~~ 8, *p*-value = 0.245 for E_r; LMM: $F_{1,2}$= 0.673, N = ~~339~~ 8, *p*-value = 0.441 and GLMM: *t*-value$_{311}$ = 29.695, N = ~~339~~ 8, *p*-value = 0.358 for H). No difference between treatments were present in the indentation depth as well (Kolmogorov-Smirnov, N = 339, p-value = 0.297; LMM: $F_{1,2}$ = 1.933, N = ~~339~~ 8, *p*-value = 0.299).

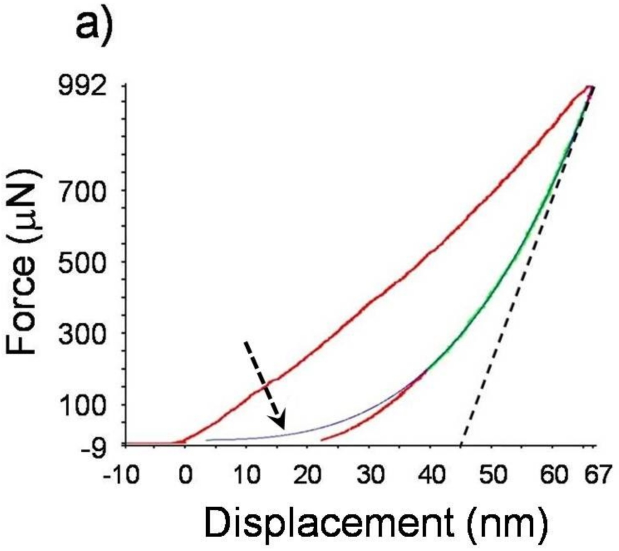

Figure 4. Nanoindentation curves, performed at 1000 μN on *A. colbecki* shell sections. (a) regular curve with no pop-in; (b) a curve characterized by the presence of pop-in events (black arrows). Dashed lines represent the initial unloading stiffness, while dashed arrow (in 'a' only) points to the ideal unloading behavior in a perfectly elastic material (see Oliver and Pharr 1992, for details).

While some of the curves resulted to be fully regular (such as in Fig. 4a), others displayed pop-in behavior, in coincidence with one or more indentation depth points (Fig. 4b). This happened in all the samples, without differences between treatments. As mentioned in M&M section, we performed additional tests at 300 μN on some individuals (n = 5): results are displayed in Table 3. Pop-in behavior was still present in the curves obtained using 300 μN (Fig. 5), indicating that it did not depend on indentation force or depth. For each individual, not significant differences were observed in E_r (at 300 vs 1000 μN; *p*-value ranging from 0.792 to 1). Pooling together the samples according to pH level, the E_r values were comparable (*p*-value = 0.490 for the control samples and 0.892 for the samples in pH 7.6), as well as pooling all the samples together (irrespective of pH level; *p*-value = 0.663). Conversely, H resulted to be significantly lower at the lower force, the *p*-values ranging from $2.2*10^{-16}$ to $7.36*10^{-6}$. Usually, the hardness should have increased along with the decrease in the indentation depth, following what is called indentation size effect. This is obviously not the case: the lower value of hardness closer to the surface is likely to arise from the surface roughness and/or a larger portion of softer organic material close to the surface. The similar behavior of H, in the first 100 nm, is reported for indentation performed on other mollusk shells (Romana *et al.* 2013; Song *et al.* 2015).

Table 3. Means and standard deviation of the nanoindentation results, obtained through the tests performed at 300 μN loading force, on *A. colbecki* shell sections experimentally exposed to control (ctrl) and acidified (7.6) pH condition. E_r = reduced modulus and H = nanohardness (calculated by the TriboScan software).

Nominal pH	N. specimens	N. obs	E_r (GPa)	H (GPa)	Indentation depth (nm)
Ctrl	2	50	66 ± 6	2.3 ± 0.5	26.4 ± 4.6
7.6	3	59	69 ± 6	2.9 ± 0.5	43.2 ± 21.1
Overall mean	5	109	68 ± 6	2.6 ± 0.6	35.5 ± 17.9

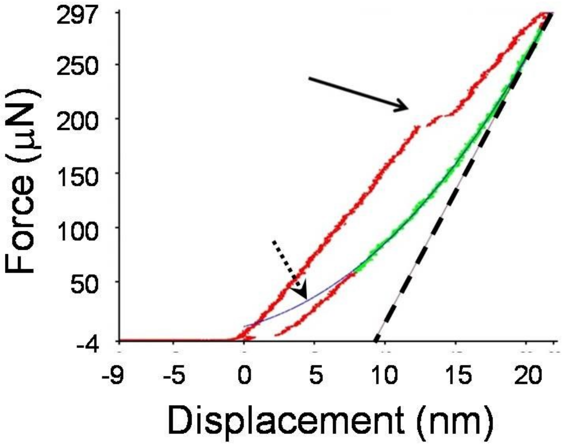

Figure 5. Nanoindentation curves, performed at 300 μN on *A. colbecki* shell section. Pop-in is indicated by black arrow. Dashed line represents the initial unloading stiffness, while dashed arrow points to the ideal unloading behavior in a perfectly elastic material (see Oliver and Pharr 1992, for details).

Vickers indentation

For Vickers indentations, we found an overall value of 204 ± 35 HV, which corresponds to 2.0 ± 0.3 GPa; mean HV for control pH was 197 ± 35, while for pH 7.6 was 209 ± 34, corresponding to 1.9 ± 0.3 and 2.1 ± 0.3 GPa, respectively. Observations from Vickers tests resulted to be normally distributed (Kolmogorov-Smirnov, N = 8, *p*-value = 0.596), so we used LMM, which provided a lack of significance between control and pH 7.6 ($F_{1,2}$ = 0.143, N = 134, *p*-value = 0.586). The Vickers indentation marks are shown in Fig. 6. The shape and sharpness of the indentation are similar between treatments; none of the samples (either from control or pH 7.6) displayed any fracture propagations.

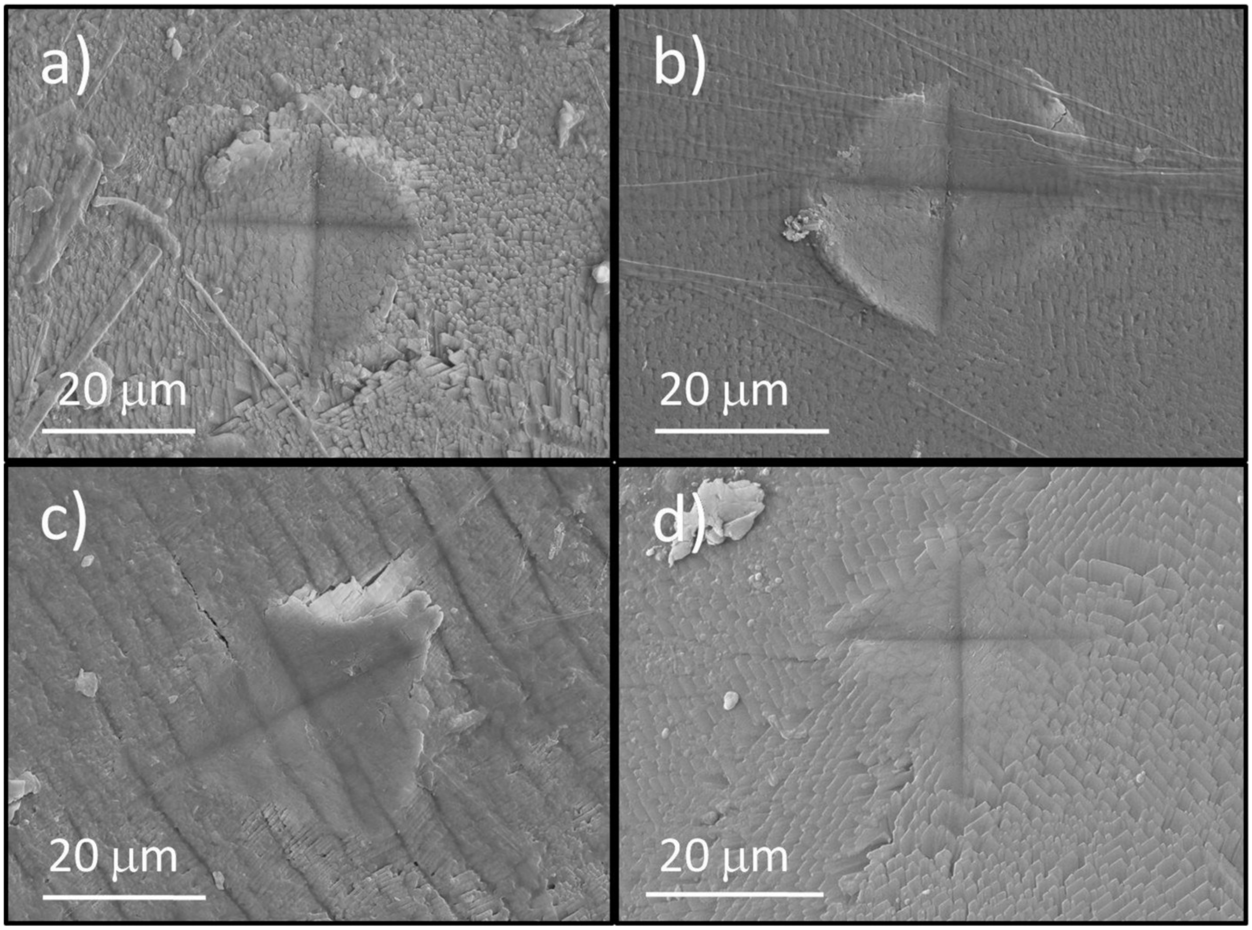

Figure 6. Representative SEM images of Vickers indentation marks on the surface of *A. colbecki* shell edge; (a) and (c): specimens from control pH; (b) and (d): specimens from pH 7.6. Scale bars are indicated in each figure.

Discussion

Adamussium colbecki is one of the key benthic species in the Antarctic littoral area, including Terra Nova Bay and investigations on how this scallop will cope with future climate change that foresees calcium carbonate undersaturation in the Antarctic marine environment McNeil and Matear 2008) are necessary. The bulk of papers dealing with effects of OA on polar organisms point out the problem of aragonite undersaturation foreseen for incoming decades (by 2030; e.g. McNeil and Matear 2008). Yet, since *A. colbecki* shell is made by calcite, for our purpose we should consider calcite undersaturation, that occurred throughout the experiment in the treatment at pH 7.6 (Table 1). Calcite undersaturation is also foreseen for Antarctic waters for 2100 (McNeil and Matear 2008).

Potential effects of ocean acidification (OA) on mechanical properties are linked to both impaired calcification and dissolution that may affect the integrity of the mineral and/or the organic matter that binds calcite crystals. While we cannot state if and to what extent new shell has been deposited during our experiment, we cannot exclude that growth occurred either. Micro- and nanoindentation encompass the response of both dissolution of the pre-existing shell and the impaired calcification in potential new deposited shell. In fact, dissolution caused by corrosive seawater has been reported to occur even in short term experiments (Beniash *et al*. 2013; Green *et al*. 2004, 2009; Melzner *et al.* 2011; Welladsen *et al*. 2010; Zhao *et al*. 2017) and it may alter shell mechanical response at the micro and the nano-scale (e.g. Beniash *et al*. 2010; Liu *et al*. 2017; Meng *et al*. 2018).

Measurements of pH in the littoral area showed a remarkable variation during the short summer season, starting with the lowest lower value (7.78) at the beginning of December. The increase of pH value is consistent with the sympagic bloom, a process that was also visible in the aquaria water (per. obs.). Unfortunately, the fragility of the sea ice (but still present, precluding the chance to use the boat) did not allow the continuity of the field measurements. Most probably, pH kept increasing until the primary production peak, as shown by measurements performed during summer in other sites of the Ross Sea (Kapsenberg *et al*. 2015; McNeil *et al*. 2010; Roden *et al*. 2013). The field pH variability is probably due to the absence of gas exchange plus the respiration processes under the sea ice when this is still present, and the following sea ice melting that allows gas exchange and the sympagic algae bloom (Schram *et al*. 2015).

In our samples, no difference in the crystals texture was observed in the SEM images; the SEM images also excluded any damage due to freezing and thawing storage procedure. Mechanical tests, both at the nano- and microscale, also indicate that *A. colbecki* shell mechanical properties were not affected by a short-term acidification. Actually, E_r and H measured at pH 7.6 were slightly higher, both at 1000 and 300 μN. This is similar to what Fitzer *et al.* (2015) found in the shell portion that grew under experimental exposure in *Mytilus edulis*. Yet, since statistical analyses indicated no differences between treatments, we cannot argue any relation with the pH level and the differences in the means between treatments could be due to natural inter-individual variability.

Variability was to some extent observed in the Vickers indentation marks, that revealed some levels of asymmetry. A possible reason is the high variability of the biological material, also characterized by a remarkable plasticity, as shown by calcite crystal sliding under load compression (similar to what is shown in Lee *et al*. 2009, despite being a polished surface). Another reason may be a slight ‘arc effect’, still present despite choosing the smallest possible sample size. However, these characteristics are present in the ~~ctrl~~ control as well as in the low pH group, so that it is probably an intrinsic behavior of the material; in fact, similar asymmetry is present in some of the Vickers prints in literature (e.g. Chen *et al*. 2013). However, our results are consistent with literature reporting microhardness value of bivalves shell in general (e.g. Jiao *et al*. 2015; Liang *et al*. 2016; Yang *et al*. 2010; Yang *et al*. 2011) and, in particular, with Fleischli *et al*. (2008) who reported similar microhardness for another Pectinidae, *Pecten maximus*.

Calcite E_r and H may be slightly affected by the orientation of the crystals with respect to the nanoindenter tip, but we positioned the samples in such an arrangement that assured the full perpendicularity of the surface with respect to the nanoindenter tip. Our results are consistent with the E_r and H values that other authors found for biogenic calcite (Bignardi *et al*. 2010; Fleischli *et al*. 2008; Presser *et al.* 2010; Zhang *et al*. 2011). This consistency provides further evidence that the choice of not embed the samples in resin did not affect the tests, rather, it avoided potential biases (Presser *et al*. 2010 and references therein). Conversely, a more necessary step in the sample preparation is represented by a perfect polishing of the surface: in fact, preliminary trials performed on less polished samples provided not reliable measurements, suggesting that a finely flat surface is a much more important requirement for this kind of procedure. Similarly, the choice of the tip is fundamental: preliminary trials with Berkovich tip did not provide reliable and consistent E_r or H values (in the range of 10 ÷ 20 and 0.2 ÷ 1 GPa, respectively) while the conical tip was the best solution for this kind of material.

The presence of minor pop-in along the loading curve is a behavior already observed in shells (Bruet *et al.* 2005; Li and Ortiz 2014; Romana *et al.* 2013) and can be due to nanocracking in the surface that comes into contact with the nanoindenter tip. Meyers *et al.* (2009) showed that nanocracks can happen in dried organic samples because of the age hardening of the organic matter. The organic material in the shell is originally soft and ductile, but as it dries, it becomes a high-toughness ceramic-organic composite (Meyers *et al.* 2008). Qian *et al.* (2005) and Zhu *et al.* (2008) state that generation of cracking can largely reduce the elastic modulus values in the tests, but our additional tests at 300 μN confirmed those at 1000 μN. Lower applied force (300 μN) corresponds to a lower indentation depth (down to 30 nm, in our case) and the hardness should be higher, following the indentation size effect (Morris *et al.* 2011 and references therein). Instead, the hardness we measured using lower force resulted to be lower. Rather than a reverse size indentation effect that can be due to artifacts occurring during indentation (Pharr *et al.* 2010), our case seems to be a typical behavior of hardness at very small depths, such as in our range (down to ~ 80 nm, at 1000 μN). In fact, the same behavior is reported for the shell nanoindentation of other invertebrates (*e.g.* Romana *et al.* 2013; Song *et al.* 2015) and for other totally different crystalline materials (*e.g.* Wei *et al.* 2004).

Exposure to acidified conditions in bivalves provides a high variability in the response of shell structure and mechanical properties. Fitzer *et al*. (2014) found a significant effect of low pH exposure in the crystal deposition in the shell of adult *Mytilus edulis*, following a six months exposure. Milano *et al*. (2016) found a strong dissolution pattern in the calcitic shell of *Cerastoderma edulis* specimens maintained at low pH for about three months, but this alteration was not reflected at the nanoscale, where the hardness was not statistically different among pH levels. Beniash *et al*. (2010) reported changes in the mineralization of the foliated layer (calcite) of *Crassostrea virginica* at the microscale, but only in juveniles exposed to high $p\mathrm{CO}_2$ for 20 weeks, while adults did not report alteration (although after only 2 weeks of exposure). Stemmer *et al*. (2013) did not detect changes at the microscale in the bivalve *Arctica islandica* after 90 days of exposure to different $p\mathrm{CO}_2$ concentrations, indicating that this bivalve shows an adaptation to a wide range of $p\mathrm{CO}_2$. Dickinson *et al*. (2012) and Ivanina *et al*. (2013) found significant effects of OA in the microhardness, but only in combination with temperature and/or salinity, while

Dickinson *et al*. (2013) reported a significant effect of low pH alone on Vickers hardness. While the Dickinson *et al*. (2013) experiment lasted 21 weeks, Timmins-Schiffman *et al*. (2014) reported the same effect after only 4 weeks (a duration comparable to our experiment).

The robustness of *A. colbecki* shell and other bivalves in literature may result from a trade-off of the energy re-allocation among body parts and functions. In fact, shell deposition can demand up to 75% of the assimilated energy and up to four times the energy required for reproduction (Sokolova *et al*. 2012). Cummings *et al*. (2011) showed that, in *L. elliptica*, the expression of chitin synthase increases as pH decreases. If *A. colbecki* activates resilience mechanisms as well and their additional energy demand would affect other functions is not known, but this would be in agreement with Dell'Acqua *et al*. (2019) who reported an effect of OA in the reproductive tissue of the scallop at pH 7.6.

Moreover, since the most expensive process in the shell deposition and maintenance is the organic matrix production (Digby 1968; Mount *et al.* 2004; Palmer 1992; Wheeler 1992), the low level of organic content in the shell of *A. colbecki* (Halloran and Donachy 1995) may help the scallop in maintain the shell integrity during low pH exposure. It is also worth to notice that our study was carried out during the summer season, when the coastal phytoplankton bloom was regularly occurring, clearly detected in the unfiltered aquaria water (pers. obs.). Many studies already showed that food availability strongly determines the response of bivalves to OA (Ramajo *et al.* 2016a, 2016b; Sanders *et al*. 2013; Thomsen *et al*. 2013) and *A. colbecki*, being able to feed on phytoplankton entering the aquaria, probably gained enough energy to face the stress given by low pH exposure. Nothing can be inferred in case of food low availability, such as during winter time, when pH lowering is supposed to be exacerbated (McNeil and Matear, 2008).

Despite High Antarctic waters display less variability than temperate and tropical regions (e.g. Cornwall *et al*. 2013; Hofmann *et al*. 2011; Wotton *et al*. 2008), they still account for a notable seasonal pH variability (Kapsenberg *et al*. 2015; McNeil *et al*. 2010; Roden *et al*. 2013) which may have had a role in littoral species preadaptation, as already recognized in the resilience ability of *S. neumayeri* (Collard *et al*. 2013; Kapsenberg and Hofmann 2014; Morley *et al*. 2016). This kind of preadaptation may not mean a complete robustness to OA, but, rather the ability to reallocate energy where most needed, as seen in *S. neumayeri* and *A. colbecki* (Dell'Acqua *et al*. 2019). Local preadaptation may have a genetic fingerprint (Sunday *et al*. 2014) and could differ among populations of the same species, especially when they have low or no connection (Guidetti *et al*. 2006). However, OA could exacerbate littoral pH oscillation (Kapsenberg *et al*. 2015; McNeil and Matear 2008) and the consequences of a prolonged natural low pH exposure needs to be further investigated.

Being our experiment a short-term exposure, we cannot infer an *A. colbecki* response after a long-term exposure. Interestingly, short-term experiments are not supposed to account for adult acclimation, especially in a slow growing species, in opposite with long-term exposures (Suckling *et al*. 2015): still, we found no effects of OA in our short-term experiment that should have not allowed for acclimation.

Conclusions

Our study shows that *A. colbecki* shell structure and mechanical properties are resistant to a short-term OA exposure, both at the micro- and at the nanoscale, suggesting potential robustness of this benthic key species in light of future calcium carbonate undersaturation.

The scallop shell accounts for a very low amount of organic matrix, whose production is the most expensive process in shell deposition. *A. colbecki* shell robustness may reflect either a lower energy requirement for shell repair and maintenance, or the ability to reallocate energies among body functions/organs, thanks to a preadaptation history: this ability can be enhanced by conspicuous levels of food availability. As *A. colbecki* is strongly patchily distributed, with low or no connection between populations, further studies should consider to test the response of different populations, possibly also exposed to different food level conditions. Additionally, longer term experiments, performed at low levels of food availability (to mimic winter time) are necessary.

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