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NOTE

Resilience to ocean acidification: decreased carbonic anhydrase activity in sea anemones under high pCO_2 conditions

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ABSTRACT: Non-calcifying photosynthetic anthozoans have emerged as a group that may thrive under high carbon dioxide partial pressure (pCO_2) conditions via increased productivity. However, the physiological mechanisms underlying this potential success are unclear. Here we investigated the impact of high pCO_2 on the dissolved inorganic carbon (DIC) use in the temperate sea anemone *Anemonia viridis*. We assessed the impacts of long-term exposure to high pCO_2, i.e. sampling *in situ* natural CO_2 vents (Vulcano, Italy), and short-term exposure, i.e. during a 3 wk controlled laboratory experiment. We focused on photo-physiological parameters (net photosynthesis rates, chlorophyll *a* content and *Symbiodinium* density) and on carbonic anhydrase (CA) activity, an enzyme involved in the energy-demanding process of DIC absorption. Long-term exposure to high pCO_2 had no impact on *Symbiodinium* density and chlorophyll *a* content. In contrst, short-term exposure to high pCO_2 induced a significant reduction in *Symbiodinium* density, which together with unchanged net photosynthesis resulted in the increase of *Symbiodinium* productivity per cell. Finally, in both *in situ* long-term and laboratory short-term exposure to high pCO_2, we observed a significant decrease in the CA activity of sea anemones, suggesting a change in DIC use (i.e. from an HCO_3^- to a CO_2 user). This change could enable a shift in the energy budget that may increase the ability of non-calcifying photosynthetic anthozoans to cope with ocean acidification.

KEY WORDS: Dissolved inorganic carbon uptake · Carbonic anhydrase · Ocean acidification · Plasticity · CO_2 vent · *Anemonia viridis*

INTRODUCTION

Increasing anthropogenic CO_2 emissions are causing a reduction in ocean pH and shift in the relative proportion of dissolved inorganic carbon (DIC) species, a process called ocean acidification (OA; Doney et al. 2009). Under future lower pH conditions, the concentration of bicarbonate ions (HCO_3^-, most abundant DIC species) and dissolved CO_2 will be significantly higher, while the concentration of carbonate ions (CO_3^{2-}) will be lower (Doney et al. 2009). Reduced ocean pH and CO_3^{2-} are expected to nega-

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tively impact a wide range of calcifying species (Hofmann et al. 2010). In contrast, non-calcifying photosynthetic species may benefit from the increase in [CO_2] associated with OA, through increased levels of productivity and growth (Koch et al. 2013).

Mutualistic symbioses between non-calcifying anthozoans and dinoflagellates of the genus *Symbiodinium* are widespread in the marine environment, and play key ecological and biogeochemical roles (Muller-Parker & Davy 2001). The host supplies the *Symbiodinium* with mainly HCO_3^- as a metabolic substrate and in return uses the photosynthate products to support its own metabolism, growth and reproduction (Furla et al. 2005). To take advantage of the large HCO_3^- pool available at current ocean pH, non-calcifying photosynthetic anthozoans, like many marine photosynthetic organisms, have evolved energy-consuming CO_2-concentrating mechanisms (CCMs; Giordano et al. 2005). Initial DIC uptake by the host involves the secretion of H^+ by an H^+-ATPase, which acidifies the external medium at the level of the boundary layer, resulting in the protonation of HCO_3^- to carbonic acid (Furla et al. 2000). DIC incorporation is then facilitated by a key CCM enzyme, carbonic anhydrase (CA). CA accelerates the conversion of carbonic acid into CO_2, which then diffuses into the host (Furla et al. 2000). A number of studies have recently demonstrated that *Symbiodinium* productivity in non-calcifying anthozoans is enhanced under high carbon dioxide partial pressure (pCO_2; Suggett et al. 2012, Towanda & Thuesen 2012, Jarrold et al. 2013), suggesting that the DIC uptake mechanism is somehow affected. Increased photosynthetic activity is also closely linked with the ability of the host cells to maintain intracellular pH under acidified conditions (Gibbin et al. 2014). How future OA scenarios will affect the host's physiological mechanisms facilitating the process of photosynthesis is much less known.

Here we investigated how the symbiotic sea anemone *Anemonia viridis* (Forskål, 1775) regulates its DIC use under high pCO_2 conditions. We tested the photo-physiology (net photosynthetic rates, chlorophyll *a* [chl *a*] content and *Symbiodinium* density) and CA activity of *A. viridis* following exposures to present and future predicted CO_2 conditions *in situ* and in the laboratory. To assess the responses to long-term exposure to OA, we collected specimens of *A. viridis* living near natural CO_2 vents found at the volcanic island of Vulcano (Italy). Here, sessile benthic organisms are continuously exposed to high pCO_2 levels, similar to levels expected for the year 2100 (Boatta et al. 2013). In addition, to investigate the responses of the symbiotic system under controlled conditions, we carried out a 3 wk laboratory experiment exposing anemones collected from the intertidal zone on the SW coast of the UK. We hypothesised that the increase in seawater pCO_2 would cause a decrease in CA activity, as anemones would need to rely less on HCO_3^- as their primary DIC source.

MATERIALS AND METHODS

Long-term *in situ* exposure to high pCO_2

Individuals of *Anemonia viridis* were collected in May 2011 from a maximum depth of 2.0 m at Vulcano Island (38° 25′ N, 14° 57′ E), NE coast of Sicily (Italy). We selected 2 sites, each representing a different environmental condition (Table 1), according to Suggett et al. (2012): one site at ambient pCO_2 (482 µatm, control pCO_2, n = 12), 300 m away from the vents, and a second site closer to the vents, at high and fluc-

Table 1. Seawater physico-chemical parameters measured at Vulcano (*in situ*) at both control and high pCO_2 sites (n = 7) and during the 21 d laboratory experiment: pH (NBS scale), temperature, total alkalinity (TA), carbon dioxide partial pressure (pCO_2), bicarbonate and carbonate ion concentration ([HCO_3^-] and [CO_3^{2-}], respectively) and aragonite saturation states (Ω_{ara}). Data are means (min.–max.)

	Long-term *in situ*		Laboratory	
	Control	High pCO_2	Control	High pCO_2
pH	8.13 (8.35–8.06)	7.71 (8.24–6.80)	8.01 (7.99–8.07)	7.59 (7.56–7.67)
Temp (°C)	21.0	21.0	15.59 (15.1–16.2)	16.40 (15.9–16.6)
TA (µequiv kg^{-1})	2513.50	2535.36	2301.8 (2275.1–2340.9)	2276.1 (2201.1–2299.8)
pCO_2 (µatm)	482 (257–584)	1461 (358–12839)	520.5 (472.41–580.1)	1841.4 (1404.0–1955.1)
[HCO_3^-] (µmol kg^{-1})	2015 (1782–2076)	2319 (1926–2319)	1920.6 (1887.5–1944.0)	2141.7 (2041.0–2168.9)
[CO_3^{2-}] (µmol kg^{-1})	201 (177–290)	88 (11–248)	154.7 (139.9–164.6)	54.5 (51.7–64.5)
Ω_{ara}	3.1 (2.7–4.5)	1.3 (0.2–3.8)	2.4 (2.2–2.6)	0.8 (0.8–1.0)

tuating pCO_2 (1461 µatm, high pCO_2, n = 11). Samples were frozen and kept at −80 °C.

Laboratory exposure to high pCO_2

On 12 April 2014, we collected 24 *A. viridis* individuals from the intertidal zone of Burgh Island, Bigbury, UK (50° 16′ N, 3° 54′ W). Sea anemones were maintained individually during 4 wk in small pond plant baskets, to allow them to adjust to laboratory conditions (temperature = 15.0 ± 0.5°C, salinity = 37 ± 2‰, pH = 8.10 ± 0.05 [NBS scale] and 200 µmol photons m^{-2} s^{-1} using LED strips [Reef White Aquabeam 600 Ultra Strips, Tropical Marine Centre]) with a 14:10 h (L:D) photoperiod. Sea anemones were fed *ad libitum* once a week on frozen *Mysis* shrimp.

Laboratory experimental design

Twelve sea anemones were exposed for 3 wk to either control (450 µatm) or high (2000 µatm) pCO_2 conditions. The high pCO_2 condition was chosen because previous experiments, both *in situ* (Suggett et al. 2012) and in the laboratory (Jarrold et al. 2013), have shown that *A. viridis* is able to tolerate such high levels. Photo-physiological parameters and CA activity were measured at the beginning of the experiment (Day 0) and then again after 5 and 21 d of exposure. To ensure that feeding did not influence measurements, sae anemones were always fed after the measurements.

Laboratory experimental setup

A re-circulating seawater CO_2 system was used to monitor and provide seawater at control or high pCO_2 conditions (for details, see the Supplement at www.int-res.com/articles/suppl/m559p257_supp.pdf). During the experiment, pH, temperature, salinity, total alkalinity and carbonate system parameters were measured and calculated in each experimental tray as detailed by Jarrold et al. (2013). Values for all seawater parameters are presented in Table 1.

Determination of photosynthetic rates

Photosynthetic rates were measured on 2 tentacles detached from each individual as the rate of net O_2 production, using closed-system respirometry, and at the incubation light intensity of ~200 µmol photons m^{-2} s^{-1}. Repeated cutting of tentacles throughout the experiment had no significant effect on sea anemone performance (for details see Table S1 in the Supplement). Tentacles were placed for 1 h on the bottom of a 5 ml chamber, with filtered (0.22 µm), autoclaved seawater at the corresponding pCO_2 treatment and a small magnetic flea to ensure water mixing. Oxygen levels in the chambers were first measured after 20 min of stabilisation, using a calibrated optical oxygen meter (OxySense 5250i) and again after 60 min. All photosynthetic rate measurements were carried out in a controlled temperature room, at 15 ± 0.6°C. Oxygen consumption (MO_2) was calculated as the change in pO_2 h^{-1} from the linear least-squares regression of pO_2 (mbar) plotted against time (min). This was multiplied by the solubility coefficient for oxygen, adjusted for salinity and temperature, and the volume of water within each respirometer. MO_2 values were expressed as mmol O_2 min^{-1} g^{-1} protein.

Determination of chl *a* content, *Symbiodinium* density and protein content

Chl *a* was extracted from 2 tentacles, in cold absolute ethanol at 4°C in the dark, for 24 h according to Ritchie (2006). Chl *a* concentration was determined using a spectrophotometer reader (SAFAS Xenius XM), calculated following the equation parameters of Ritchie (2006) and standardised per *Symbiodinium* cell numbers.

Symbiodinium density was determined according to Zamoum & Furla (2012). Chlorophyll-free tentacles were immersed in 1 M NaOH and incubated at 37°C for 30 to 45 min to dissolve all animal tissue. To determine *Symbiodinium* density, 3 replicates $sample^{-1}$ were counted using a Neubauer haemocytometer. The remaining extract was used to determine protein content from which we normalised *Symbiodinium* density.

Determination of CA activity

CA activity was measured using animal extracts. Animal cytosoluble proteins were extracted from 4 tentacles in ice-cold extraction medium (50 mM potassium phosphate, pH 7.8 and protease inhibitor cocktail P-8340, Sigma), and kept at 4°C throughout the extraction procedure. Animal CA activity was determined according to Weis et al. (1989). Briefly, animal CA activity was measured using CO_2 as a sub-

strate and following the reduction of pH due to the hydration of CO_2 into HCO_3^- and H^+. Assays were performed by adding Veronal buffer (25 mM Na Veronal, 5 mM EDTA, 5 mM DTT and 10 mM $MgSO_4$; pH 8.2) into a measuring chamber and adding 100 µg proteins of animal extracts. The CA activity was normalised per animal protein content.

Statistical analyses

For the *in situ* experiment, we used Mann-Whitney *U*-tests to compare all measured parameters in sea anemone tissues between control and high pCO_2 sites. For the laboratory experiment, the comparison of all measured parameters (except chl *a*) between control and high pCO_2 conditions was investigated with repeated-measures ANOVA since the same individuals were sampled over the 3 wk. Post hoc tests were performed with Tukey's HSD tests. When assumptions failed, appropriate transformations were used. For chl *a*, no transformations met the assumptions, so the non-parametric Friedman test was used. All statistical tests were carried out using STATISTICA 10.0.

RESULTS

Long-term *in situ* exposure to high pCO_2

Symbiodinium density was not affected by exposure to high pCO_2. No differences between control and high pCO_2 sites were found (U = 143, p = 0.707; Fig. 1a). The same result was observed for chl *a* concentration, where values of chl *a* were similar for control and high pCO_2 sites (U = 156, p = 0.751; Fig. 1b). CA activity was approximately 30% lower in anemones exposed to higher pCO_2 conditions (U = 81, p = 0.042; Fig. 1c).

Short-term laboratory exposure to high pCO_2

Incubation during 5 and 21 d at high pCO_2 conditions resulted in a significant reduction of *Symbiodinium* density ($F_{2,44}$ = 16.015, p = 0.00001; 0 vs. 5 d, p = 0.003; 0 vs. 21 d, p = 0.008; Fig. 2a, Table S2 in the Supplement). Conversely, concentration of chl *a* was not affected by exposure to high pCO_2, with values being unchanged during the 21 d of exposure (χ^2_2 = 0.750, p = 0.687; Fig. 2b, Table S2).

Net photosynthetic rates of individuals incubated during 5 and 21 d at high pCO_2 (p = 0.6677) were not affected by pCO_2 exposure ($F_{2,38}$ = 0.684, p = 0.510; Fig. 2c, Table S2).

CA activity of anemones decreased during the first 5 d of exposure to high pCO_2, but it was only significantly strongly down-regulated after 21 d of exposure ($F_{6,38}$ = 3.975, p = 0.003; 0 vs. 21 d, p = 0.0003). Indeed, we found that CA activity decreased by 32% after 1 wk of exposure to high pCO_2 and by 78% at the end of the 21 d experiment (Fig. 2d, Table S2).

DISCUSSION

Contrary to hard corals, symbiotic sea anemones may thrive under future high pCO_2 levels (Suggett et al. 2012, Towanda & Thuesen 2012). To understand the mechanisms that underpin the ability of symbiotic sea anemones to be successful under high pCO_2

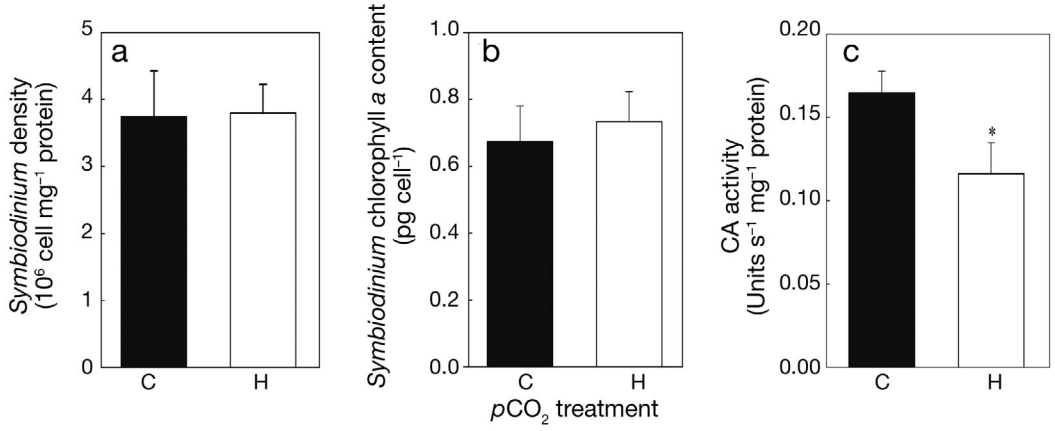

Fig. 1. Effect of *in situ* long-term exposure to 'control' (C, black) and 'high' (H, white) pCO_2 conditions on (a) mean *Symbiodinium* density, (b) mean *Symbiodinium* chlorophyll *a* content and (c) mean carbonic anhydrase (CA) activity in *Anemonia viridis* at the Vulcano CO_2 vent. *p < 0.05. Error bars indicate SE, n = 12

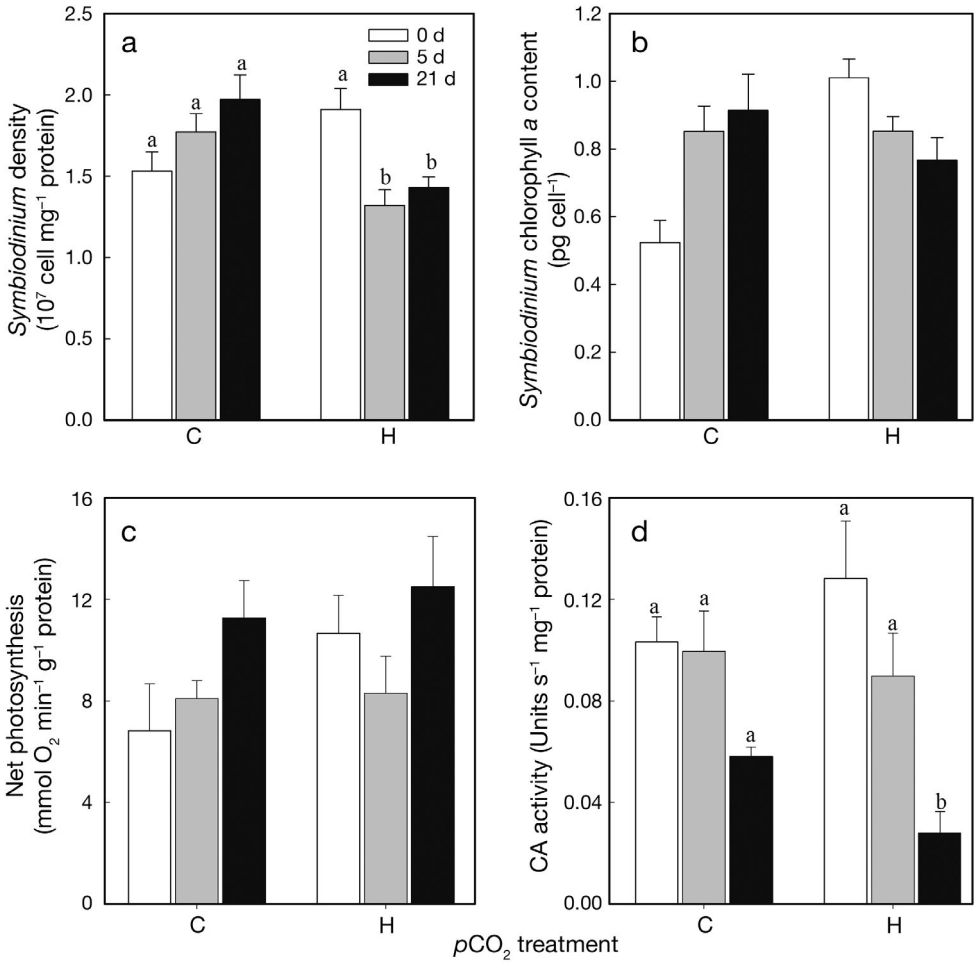

Fig. 2. Effect of laboratory short-term exposure to control (C) and high (H) pCO_2 on (a) mean *Symbiodinium* density, (b) mean *Symbiodinium* chlorophyll *a* content, (c) net photosynthesis rates and (d) CA activity in *Anemonia viridis*. Data are shown at the beginning of the exposure (0 d), after 5 d and after 21 d. Error bars indicate SE, n = 12. Means with different letters differ significantly from 0 d ($p < 0.05$)

conditions, we performed both an *in situ* (long-term) and a laboratory (short-term) exposure experiment. In particular, we wanted to define how future OA conditions would affect the use of DIC by the sea anemone *Anemonia viridis.*

Long-term *in situ* exposure to high pCO_2

Several studies have demonstrated the capacity of *A. viridis* to colonize the pCO_2-rich environment found near the CO_2 vents around Vulcano Island (Suggett et al. 2012, Borell et al. 2014, Horwitz et al. 2015). Even without significant differences in *Symbiodinium* density between *A. viridis* from the control and high pCO_2 sites (Borell et al. 2014, Horwitz et al. 2015, this study), increases in photosynthetic productivity at high pCO_2 sites with an enhanced autotroph:heterotroph ratio have been observed (Horwitz et al. 2015). However, an increase in *Symbiodinium* density at high pCO_2 sites compared with control sites was reported by Suggett et al. (2012). This result was potentially related to the normalisation procedure used, i.e. surface area instead of milligrams of protein, an issue already raised by Horwitz et al. (2015).

One of the hypotheses proposed to explain the increase in productiveness is the modification of DIC use. In a 'normal' pCO_2 environment, HCO_3^- is the preferential DIC species removed from sea water (Furla et al. 2005). In a high pCO_2 environment, however, CO_2 could replace HCO_3^- as the main carbon source for photosynthesis, leading to a decrease in energy investment for CCMs. If the proportion of HCO_3^- uptake decreases, favouring the less costly diffusion of CO_2, the role of CA could then be reduced. Indeed, we found a significant 30% decrease in total animal

CA activity of *A. viridis* specimens from the high pCO_2 sites. A significant down-regulation in CA activity under OA conditions was also observed in the calcifying coral *Acropora millepora* (Moya et al. 2012). Taking into account the role of CA in pH homeostasis, this CA reduction could reflect an adaptive mechanism that enables the anemone to deal with low pH conditions. This result is in agreement with the behaviour of free-living unicellular eukaryotic organisms, which modify the relative contributions of CO_2 and HCO_3^- uptake as a function of environmental pH, and become CO_2 users at low pH as demonstrated in diatoms (Wu et al. 2015) and coccolithophorids (Lohbeck et al. 2014). Reduction of CCM energy demands under high pCO_2 conditions could result in increased energy availability to other functions (i.e. reserve production or reproduction). Indeed, Suggett et al. (2012) reported an increase in the abundance of *A. viridis* around Vulcano at the high pCO_2 sites. Similarly, high pCO_2 exposure coincided with significantly greater asexual budding rates in the sea anemone *Exaiptasia pallida* (Hoadley et al. 2015).

Laboratory exposure

In this study, we further explored the ability of *A. viridis* to tolerate OA by investigating its phenotypic plasticity response under controlled laboratory conditions. We exposed *A. viridis* specimens from the English Channel, which are genetically distant from those of the Vulcano population (D. Forcioli pers. comm.), to high pCO_2 conditions for 3 wk. We measured the same photosynthetic rate with 23% less *Symbiodinium*, resulting in increased productivity per *Symbiodinium* at high pCO_2, which was also observed in Vulcano anemones (Horwitz et al. 2015). These results are in agreement with previous short-term experiments performed on other symbiotic cnidarians, which show that exposure to OA conditions induces a decrease in *Symbiodinium* density concomitantly with an increase in photosynthetic activity of the symbiotic cnidarian (Langdon & Atkinson 2005, Krief et al. 2010).

The depletion of *Symbiodinium* load in *A. viridis* could be linked to a shuffling of intra-individual *Symbiodinium* populations causing a modification of *Symbiodinium* productivity under high pCO_2 conditions. Brading et al. (2011) showed that the response of several tropical *Symbiodinium* strains to high pCO_2 levels was phylotype-specific, ranging from unaffected to highly sensitive strains with changes in growth rate and photosynthetic capacity. In temperate symbiotic cnidarians, most of the *Symbiodinium* populations belong to phylotype 'temperate clade A', which nevertheless show high intra-clade variability (Forcioli et al. 2011). Unfortunately, no data are available concerning different physiological properties or pCO_2-specific sensitivity of these intra-clade populations. Future work will need to address this important aspect of the biology of the *A. viridis*–*Symbiodinium* system, in order to more deeply understand the contribution of the resilience property of the symbiont.

Symbiodinium are thought to be CO_2-limited at current ocean pH conditions (Davy & Cook 2001, Jarrold et al. 2013), despite the presence of host CCMs. Consequently, our observed increase in *Symbiodinium* productivity suggests that the DIC uptake mechanism is in some way affected. Indeed, similarly to the *in situ* experiment, we found a decline of anemone CA activities after 21 d, which corroborates the hypothesis of a similar change in DIC use in non-preconditioned organisms (compared to Vulcano) and on the short-term scale. However, host CA activities remained unchanged after the first week of exposure. A similar result was observed in *E. pallida*, where no significant difference was detected between normal and high pCO_2 treatments (400–1000 ppm) within 7 d (Siddiqui & Bielmyer-Fraser 2015), demonstrating that shifting DIC use is not immediate.

The similarity of responses observed in our *in situ* and laboratory experiments suggests that the physiological plasticity observed in DIC use is an intrinsic property of *A. viridis* that does not appear to be the result of selection *in situ* at the Vulcano CO_2 vents. However, we cannot completely discard the idea that specimens of *A. viridis* from the UK population may have evolved the capacity to tolerate pH and pCO_2 fluctuations as a consequence of inhabiting intertidal environments. The physiological plasticity we observed could also be linked to circadian intracellular pH variation (from pH 7.4 at night to 8.9 during the day; Furla et al. 2000) experienced by anemones during photosynthetic activity of *Symbiodinium*. This confers symbiotic cnidarians a high buffering capacity (Gibbin et al. 2014, Laurent et al. 2014), which could increase resilience to OA.

In conclusion, this study showed that being able to shift the utilisation of different inorganic carbon species toward energetically most favourable ones (i.e. from HCO_3^- to CO_2) is a key feature enabling non-calcifying photosynthetic anthozoans to tolerate and thrive under long-term exposure to high pCO_2 levels, but also to allow rapid (3 wk, from this study) acclimation to future predicted CO_2 conditions.

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