Natural high $pCO_2$ increases autotrophy in *Anemonia viridis* (Anthozoa) as revealed from stable isotope (C, N) analysis

Rael Horwitz$^{1,3}$, Esther M. Borell$^3$, Ruth Yam$^2$, Aldo Shemesh$^2$ & Maoz Fine$^{1,3}$

$^1$The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel, $^2$Department of Earth and Planetary Sciences, Weizmann Institute of Science, Rehovot 7610001, Israel, $^3$The Interuniversity Institute for Marine Sciences, P.O. Box 469, Eilat 8810300, Israel.

Contemporary cnidarian-algae symbioses are challenged by increasing CO$_2$ concentrations (ocean warming and acidification) affecting organisms’ biological performance. We examined the natural variability of carbon and nitrogen isotopes in the symbiotic sea anemone *Anemonia viridis* to investigate dietary shifts (autotrophy/heterotrophy) along a natural $pCO_2$ gradient at the island of Vulcano, Italy. $\delta^{13}C$ values for both algal symbionts (*Symbiodinium*) and host tissue of *A. viridis* became significantly lighter with increasing seawater $pCO_2$. Together with a decrease in the difference between $\delta^{13}C$ values of both fractions at the higher $pCO_2$ sites, these results indicate there is a greater net autotrophic input to the *A. viridis* carbon budget under high $pCO_2$ conditions. $\delta^{15}N$ values and C/N ratios did not change in *Symbiodinium* and host tissue along the $pCO_2$ gradient. Additional physiological parameters revealed anemone protein and *Symbiodinium* chlorophyll a remained unaltered among sites. *Symbiodinium* density was similar among sites yet their mitotic index increased in anemones under elevated $pCO_2$. Overall, our findings show that *A. viridis* is characterized by a higher autotrophic/heterotrophic ratio as $pCO_2$ increases. The unique trophic flexibility of this species may give it a competitive advantage and enable its potential acclimation and ecological success in the future under increased ocean acidification.

Increasing carbon dioxide (CO$_2$) emissions drive ongoing ocean acidification (OA) and place marine ecosystems in a vulnerable state. Predictions warn of a further decrease of 0.3–0.5 pH units in oceanic surface water by the end of this century. Natural CO$_2$ vents at sub-tropical coastal areas and tropical reefs serve as natural laboratory locations to study long-term effects of elevated $pCO_2$ (pH) across many biological and spatial scales. Such a location has been reported in the Levante Bay of Vulcano Island (Italy) in the Mediterranean Sea where many studies have examined physiological adaptations of biota to OA, including seagrass, benthic micro- and macroalgae, sea urchins, and sea anemones. The distinctive characteristics of this location render it a unique environmental setting where the seawater chemistry varies along a $pCO_2$ gradient of several hundred meters moving away from the venting source. The submarine gas emissions in Levante Bay are characterized by high CO$_2$ content volume (>90%) and variable low H$_2$S (ranging 0.8 to 2.5% volume).

A large body of research has focused on the potential impact of OA on reef organisms, particularly scleractinian corals. However, non-calcifying cnidarians such as sea anemones have received less attention. Like many cnidarians, they are mixotrophic organisms, which derive their energy from both photosynthesis translocated from the dinoflagellate symbionts (*Symbiodinium*) and from a variety of external food sources. *Symbiodinium* utilize bicarbonate (HCO$_3^-$) rather than CO$_2(aq)$ as the primary source for photosynthesis. Extrinsic sources of carbon for the host include zooplankton and particulate organic carbon (POC). The two partners that make up the holobiont interact at the basic metabolic level, which includes reciprocal fluxes of energy and nutrient-rich compounds. *Anemonia viridis* Forskål (Cnidaria: Anthozoa), the temperate Mediterranean species chosen for this study, occurs naturally at high densities throughout Levante Bay and harbors the dinoflagellate *Symbiodinium muscatinei* LaJeunesse and Trench (Dinomastigota: Dinophyceae). Hence it is a powerful comparative model to assess the effects of the changing seawater environment along a natural $pCO_2$ gradient.
Other reports on the response of *A. viridis* near CO$_2$ vents discovered changes in their associated microbial communities\(^{29}\), reduced dimethylsulfoniopropionate (DMSP) production\(^{12}\) and enhanced productivity\(^{3,11}\).

The purpose of this paper is to investigate dietary changes of *A. viridis* using isotopic compositions, particularly carbon source shifts in the anemone metabolism, in response to high pCO$_2$/low pH conditions *in situ*. We measured how the natural variability of carbon and nitrogen isotopes in *Symbiodinium* and host tissues of *A. viridis* varies along a natural pCO$_2$ gradient. This was compared with other key physiological parameters (i.e. total protein concentration; *Symbiodinium* density, mitotic index, and chlorophyll concentration) which were used in the present and in previous studies\(^{11}\). Since the δ$^13$C and δ$^15$N signatures of an organism are related to those of its diet\(^{29,30}\), our main objective was to estimate the relative contribution of photosynthetic compounds *versus* heterotrophically derived food to the anemone energetic budget (autotrophic/heterotrophic ratio) with increasing seawater pCO$_2$. This may facilitate better understanding of the environmental fate of cnidarians in a high CO$_2$ world.

**Results**

Visual observations made during the course of sampling found anemones at all sampling sites attached to hard substratum at high abundances (of ca. 10–40 anemones m$^{-2}$), consistent with previous findings\(^{11}\). Anemones appeared to be healthy with their tentacles fully extended and no visible excess amounts of mucus at the high pCO$_2$ site (Fig. 1b). Data for seawater pH, pCO$_2$, TA, temperature and light intensity at all anemone sampling sites is summarized in Figure 1a.

**Total protein, *Symbiodinium* density, mitotic index and chlorophyll concentration.** There was no significant difference in anemone protein concentration [1-way ANOVA: F (2, 45) = 1.438, P = 0.248] (Fig. 2a). *Symbiodinium* density [1-way ANOVA: F (2, 45) = 0.583, P = 0.562] and cell chlorophyll a concentration [1-way ANOVA: F (2, 45) = 1.125, P = 0.334] between sites (Fig. 2b). Mean protein concentration (mg protein g$^{-1}$ wet wt ± SE) between sites was 37.65 ± 1.12. *Symbiodinium* density (cells mg protein$^{-1}$ ± SE) between sites averaged to 1.06 ± 0.07 × 10$^6$ and mean chlorophyll a content (pg cell$^{-1}$ ± SE) was 4.57 ± 0.27. The number of dividing *Symbiodinium* cells (MI) was progressively greater in anemones inhabiting the higher pCO$_2$ sites [1-way ANOVA: F (2, 21) = 3.722, P = 0.041], increasing from 3.69 ± 0.76% at the control site to 7.12 ± 1.44% and 9.8 ± 0.54% at the intermediate and high pCO$_2$ sites, respectively (Fig. 2c).

**Seawater isotopic signature.** Stable isotope analysis showed constant δ$^{13}$C$_{\text{seawater}}$ between all sites, including the primary vent (Kruskal-Wallis ANOVA: df = 3, P = 0.361), with an average of 0.98 ± 0.01% (Fig. 3; vent site value not shown). δ$^{13}$C$_{\text{DIC}}$S values were similar between sites 1–3, with an average of 1.28 ± 0.05% (Fig. 3), although all were significantly heavier compared to the primary vent site (0.34 ± 0.03%) (Fig. 1a) (Kruskal-Wallis ANOVA: df = 3, P = 0.016).

**δ$^13$C variability.** δ$^13$C values of both animal tissue (δ$^13$C$_T$) and *Symbiodinium* (δ$^13$C$_S$) decreased under high pCO$_2$ conditions (Fig. 4a, b). One-way ANOVA revealed a significant difference in δ$^13$C$_T$ between all sampling sites [F (2, 42) = 42.901, P = 0.000003], with a decrease from −16.66 ± 0.2% at the control site to −17.62 ± 0.19% and −19.12 ± 0.16% at the intermediate and high pCO$_2$ sites, respectively. δ$^13$C$_S$ also differed significantly between all sampling sites [1-way ANOVA: F (2, 42) = 25.606, P = 0.000047], decreasing from −15.1 ± 0.28% at the control site to −16.65 ± 0.37% and −18.21 ± 0.24% at the intermediate and high pCO$_2$ sites, respectively. The difference in δ$^13$C between the anemone tissue (δ$^13$C$_T$) and *Symbiodinium* (δ$^13$C$_S$) at each site was calculated as δ$^13$C$_T$– δ$^13$C$_S$ to evaluate changes in autotrophic/heterotrophic ratios. δ$^13$C$_T$ was considerably lighter than δ$^13$C$_S$ at all sampling sites with δ$^13$C$_T$– δ$^13$C$_S$ reduced with increasing pCO$_2$ (Fig. 4a).

In ambient seawater (control) this difference was relatively large (1.56 ± 0.21%), while it decreased significantly at the intermediate and high pCO$_2$ sites (0.96 ± 0.31% and 0.9 ± 0.17%, respectively) [1-way ANOVA: F (2, 12) = 5.036, P = 0.026].

**δ$^{15}$N variability and C/N ratios.** There was no significant difference in δ$^{15}$N values of anemone tissue (δ$^{15}$N$_T$) [1-way ANOVA: F (2, 12) = 0.848, P = 0.452] and *Symbiodinium* (δ$^{15}$N$_S$) [1-way ANOVA: F (2, 12) = 0.266, P = 0.771] with increasing pCO$_2$ (Fig. 5a). δ$^{15}$N$_T$ was lowest at the control site (4.32 ± 0.12%) and increased to 4.55 ± 0.16% and 4.6 ± 0.18% at the intermediate and high pCO$_2$ sites, respectively. δ$^{15}$N$_S$ averaged to 1.34 ± 0.36 at the control site and increased to 1.41 ± 0.42 and 1.82 ± 0.41 at the intermediate and high pCO$_2$ sites, respectively. δ$^{15}$N$_S$ was substantially lighter compared to δ$^{15}$N$_T$ at all sampling sites, with an average difference of 2.5 ± 0.23% (Fig. 5a). The carbon to nitrogen ratios (C/N) of anemone tissue and *Symbiodinium* did not have any significant differences along the pCO$_2$ gradient (1-way ANOVAs; F (2, 12) = 0.301, P = 0.745 for anemone tissue; F (2, 12) = 0.069, P = 0.934 for *Symbiodinium*) (Fig. 5b). The C/N ratio of anemone tissue at the control site was 5.53 ± 0.36 and increased to 5.73 ± 0.21 and 5.91 ± 0.22 at the intermediate and high pCO$_2$ sites, respectively. The C/N ratio of *Symbiodinium* ranged from 7.34 ± 0.7 at the control site to 7.21 ± 0.51 at the high pCO$_2$ site.

**Discussion**

*A. viridis* collected at all pCO$_2$ sites lacked any apparent signs of stress (i.e. no mucus, tentacles fully extended; see Fig. 1b). Their general health was further supported by our results for physiological and algal characteristics. Protein concentrations, which are widely accepted as a sensitive indicator for the health of an organism\(^{35}\), showed no difference between sampling sites, indicating *A. viridis* was in fact well acclimated to the high seawater pCO$_2$ (Fig. 2a). In addition, there were no changes in *Symbiodinium* densities and their chlorophyll a concentrations along the pCO$_2$ gradient (Fig. 2b). This is in agreement with observations of the anemone *Anthopleura elegansissima*, following exposure to elevated pCO$_2$ conditions in a laboratory setting, using the standard algal cell normalization to mg of protein methodology as in the present study\(^{14}\). However, *Symbiodinium* densities in *A. viridis* under high pCO$_2$ conditions nearby the vent at Vulcano have been reported to increase relative to algal densities in anemones at the control site\(^{11}\). This discrepancy may be the result of a different methodology (using surface area as a normalization index in the same study\(^{14}\)) in determining algal cell densities. The handling of anemones greatly influences tentacle contraction, which may have led to inaccuracy in surface area measurement, thereby making the comparison of results difficult.

The substantial increase in dividing algal cells under elevated pCO$_2$ (MI; Fig. 2c) is in accordance with previous studies reporting high MIs in anemones under high pCO$_2$.\(^{11,14}\) It is important to note that there was no variation in algal genotype as the anemones from all three sites were found to harbor *Symbiodinium* type A19$^{28}$, excluding the possibility that genetic makeup of the *Symbiodinium* is responsible for the difference. The marked increase in algal division is most likely a direct result of massive CO$_2$ input, as *Symbiodinium* in anemones remain carbon limited under normal conditions\(^{31,14,36,37}\). Since cnidarians are required to maintain cell-specific densities of their algal symbionts to avoid toxicity from excess oxidative products\(^{28}\), the host may initiate either active expulsion of symbionts and/or chemically-signaled arrest of algal reproduction\(^{28}\). Here, the high MIs but same algal densities, relative to algal densities at the control site, suggest that the anemones were unable to regulate algal repro-
Figure 1 | General information on the study sites and the studied organism. (a) Map of the study area with sampling sites 1 (control), 2 (intermediate $p$CO$_2$) and 3 (high $p$CO$_2$). Boxes show mean values ($\pm$SD) of each site for: pH, $p$CO$_2$, temperature, light and alkalinity. $\delta^{13}$C$_{DIC}$ and $\delta^{18}$O$_{seawater}$ (%) are presented for the primary vent site. The map was created in Adobe Illustrator CS3 (Adobe Systems Inc., San Jose, USA). (b) Image showing A. viridis at sampling site 3 (high $p$CO$_2$). Photo credit: M. F. (b).
Figure 2 | Physiological parameter measurements of A. viridis from sites 1 (control), 2 (intermediate $pCO_2$) and 3 (high $pCO_2$). (a) Protein concentration ($n = 16$). (b) *Symbiodinium* density (bars) and chlorophyll concentration (circles) ($n = 16$). (c) Mitotic index ($n = 8$). Note that the mean $pCO_2$ (μatm; Table 1) is given in parentheses for each site. All data represent the mean ± SEM. Letters indicate significant differences between sites (Tukey, $P < 0.05$).
Production under the elevated pCO2 conditions and therefore densities were likely maintained through Symbiodinium expulsion. Considering that in addition iron (Fe) is the most important trace element for algal growth30, Fe enrichment in the seawater near the vent site13,31 may have also affected algal proliferation to some extent.

The acidification of seawater close to the venting source arises from the constant gas emissions13. In addition to total DIC increasing by 17% at the high pCO2 site as compared to the control, CO2(aq) increased near the venting source (7-fold increase at the high pCO2 site; see Table 1). Although the carbonate system still consists mostly of bicarbonate (94%), CO2(aq) increased from less than 1% at the control site to 4% at the high pCO2 site (Table 1). Nonetheless, the isotopic composition of the inorganic carbon source in this area for the anemones appears to be constant as data shows that δ13CDIC does not change between sites (Fig. 3). Consequently, the pronounced and persistent depletion in 13C in the tissues of A. viridis and its Symbiodinium close to the vent cannot be explained by the assimilation of a 13C-depleted carbon source. The large increase in pCO2 in the seawater (Table 1; Fig. 1a) and its availability for A. viridis most likely account for the decrease in A. viridis δ13C values in both Symbiodinium and host tissue. The values near the vent (Fig. 4a, b) were well below the lower limit of the range reported previously for both tropical and subtropical sea anemones and Symbiodinium12,33.

δ13CT values decreased at the intermediate and high pCO2 sites to −17.62 ± 0.19‰ and −19.12 ± 0.16‰, respectively, as compared to the control site (−16.66 ± 0.2‰) (Fig. 4a), suggesting an increase in photosynthetically fixed carbon relative to heterotrophically acquired carbon in the host20,34,35. Taking seasonal and regional variability into account, average zooplankton and particulate organic carbon (POC) 13C values reported in the area for surface waters range between −21 and −22‰36. We assumed that the availability of these extrinsic carbon sources was constant across all sampling sites in our study, as the relatively short distance between sampling sites (<500 m) and their orientation in Levante Bay towards the open sea renders differences in food availability most unlikely as a factor. Based on mass balance estimation, our calculations show about 5% heterotrophic input to δ13CT at the control site (using δ13CT = −16.66‰ and δ13CS = −15.1‰, assuming

\[ \text{δ13C} \text{ (‰)} = \text{fractionation factor} \times \text{δ13C source} \]

Figure 3 | Isotopic measurements of seawater at the sampling sites. δ13CDIC (circles) and δ18Oseawater (squares) (%o) at sites 1 (control), 2 (intermediate pCO2) and 3 (high pCO2). Note that the mean pCO2 (µatm; Table 1) is given in parentheses for each site. All values represent the mean ± SEM (n = 3).

Figure 4 | δ13C in A. viridis from sites 1 (control), 2 (intermediate pCO2) and 3 (high pCO2). (a) Mean δ13C (‰) values (±SEM; n = 5) of Symbiodinium (white circles) and animal tissue (black circles). (b) δ13CT vs. δ13CS (‰) for individual A. viridis specimens from sites 1 (triangles), 2 (squares) and 3 (circles). Note that the mean pCO2 (µatm; Table 1) is given in parentheses for each sampling site.
This is typical of cnidarian-algae symbioses, in which *Symbiodinium* may contribute up to 95% of their photosynthetically-produced carbon to the host. Based on the same assumptions, at the high $pCO_2$ site the heterotrophic input to $\delta^{13}C$ reduced to about 2.5% (using $\delta^{13}C_T = -19.12\%$ and $\delta^{13}C_S = -18.21\%$, assuming $\delta^{13}C_{zooplankton/POC} = -22\%$), leading to a greater autotrophic input. This observation is also supported by the difference in $\delta^{13}C$ values between host tissue and *Symbiodinium*, which reflects the relative contribution of heterotrophy and photosynthesis to fixed carbon. Cnidarian host tissue and *Symbiodinium* stable carbon isotopic values are usually within 2% of each other. There was a significant reduction in $\delta^{13}C_{zooplankton/POC}$ with increasing $pCO_2$ from 1.56 ± 0.21% at the control site to 0.96 ± 0.31% and 0.9 ± 0.17% at the intermediate $pCO_2$ and high $pCO_2$ sites, respectively (Fig. 4a). This further indicates an increase in the autotrophic/heterotrophic ratio via translocated autotrophic carbon to the host.

Our results suggest that elevated $pCO_2$ near the vent promotes carbon isotope fractionation by *Symbiodinium* during photosynthesis, leading to lighter $\delta^{13}C_S$ values. $\delta^{13}C_S$ showed a substantial decrease from $-15.1 \pm 0.28\%$ at the control site to $-16.65 \pm 0.37\%$ and $-18.21 \pm 0.24\%$ at the intermediate and high $pCO_2$ sites, respectively (Fig. 4a). Many studies have shown that $\delta^{13}C$ is depleted in marine photosynthetic organisms under elevated $pCO_2$. 

---

**Figure 5** | $\delta^{15}N$ and C/N ratios in *A. viridis* from sites 1 (control), 2 (intermediate $pCO_2$) and 3 (high $pCO_2$). Measurements in *Symbiodinium* (white circles) and animal tissue (black circles) of: (a) $\delta^{15}N$ (%), and (b) C/N ratio. Note that the mean $pCO_2$ (μatm; Table 1) is given in parentheses for each site. All values represent the mean ± SEM ($n = 5$).
Table 1 | Carbonate chemistry of seawater at sampling sites 1 (control), 2 (intermediate pCO2) and 3 (high pCO2). Parameters were calculated from pH(NBS), total alkalinity (TA), ambient seawater temperature, and salinity (38%) using the program CO2SYS. All data shown are the mean (± SD). Dissolved inorganic carbon (DIC) was measured in situ using a Seaplex. The log of the carbonate system ([HCO3−] + [CO32−] + [H2CO3]) is shown in μmol kg−1. "13C" values were measured and reported as per mil relative to the Vienna Pee Dee Belemnite (VPDB) standard.

| Site       | pH(NBS) | TA (μeq kg−1) | pCO2 (μatm) | DIC (μmol kg−1) | HCO3− (μmol kg−1) | CO2−3 (μmol kg−1) | CO2aq (μmol kg−1) |
|------------|---------|---------------|-------------|----------------|-------------------|------------------|------------------|
| 1. Control | 8.12(2) | 2554 [47]     | 463 [33]    | 2206 [22]      | 1998 [29]         | 193 [8]          | 15 [1]           |
| 2. Intermediate pCO2 | 7.99 (0.07) | 2486 [9] | 683 [62] | 2287 [46] | 2113 [65] | 152 [23] | 22 [4] |
| 3. High pCO2 | 7.44 (0.26) | 2501 [20] | 3232 [836] | 2585 [123] | 2430 [93] | 50 [25] | 105 [53] |

pCO2. Under normal conditions, the majority of Symbiodinium carbon requirements (~85%) are met via energy-consuming carbon-concentrating mechanisms (CCMs), whilst the remainder diffuses passively from seawater to the Symbiodinium cells. When pCO2 is elevated, CO2 can replace HCO3− as the main carbon source for photosynthesis while energy-consuming CCMs become less important. Form II ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the carboxylating enzyme in Symbiodinium, discriminates against 13C, and is the lack of change in C/N ratio between sites (Fig. 5b). The C/N ratio is considered a good proxy for an organism’s condition since it is the ratio of lipids and carbohydrates to proteins. Generally, animals exposed to high pH have to compensate for acid-base imbalance in intra- and extracellular spaces thereby imposing elevated metabolic costs. A recent study by Laurent et al. (2014) demonstrated the high capacity of A. viridis to regulate against decreases in internal and external pH, thereby maintaining normal cellular metabolism and physiology. Our results indicate the adaptation and potential resilience of A. viridis to acidification conditions, as physiological data (i.e., protein content, Symbiodinium density and chlorophyll a concentration; Fig. 2a, b), along with δ15N and δ18O values and C/N ratios (Fig. 5a, b), remained unaffected among site along the pCO2 gradient. Moreover, the high pCO2 environment probably stimulated cell division of algal symbionts (Fig. 2c).

We have shown that the anemone host relies more on photosynthetically derived carbon under elevated pCO2. We propose that A. viridis optimizes energy utilization under elevated pCO2 through an increased autotrophic input, although isotopic data show that heterotrophy is maintained as an additional source of energy/nutrients. These factors may contribute, at least in part, to the increased size and abundance of the A. viridis population proximate to the vent site as reported in a previous study. In conclusion, increased autotrophic/heterotrophic ratio may enhance the competitive advantage of symbiotic anemones over other invertebrates and improve their ecological success in benthic communities. These are valuable findings that merit further study for predicting the performance of non-calculating symbiotic cnidarians in future high-pCO2 oceans.

Methods

Study sites: This study was conducted along the sublittoral in Levante Bay, Vulcano Island (38° 25’ N, 14° 57’ E), part of the Aeolian Island chain, NE Sicily (Fig. 1a) in May 2012. Shallow-water CO2 vents create a natural pCO2 gradient along the north-easterly side of the bay, ranging from pH 6.05 to 8.29 at ~350 m from the vent site.

Three sites were selected for animal sampling in accordance with previous studies (see Fig. 1a)11,12. Site 1 (control) was an ambient seawater reference station, located outside the vent area (>400 m). Site 2 (intermediate pCO2) was ~300 m away from the vent CO2 vents; Site 3 (high pCO2) was in the proximity of the vent (~260 m). Sampling at the primary vent site (indicated by the star symbol in Fig. 1a) was for collection of seawater samples only.

Carbonate chemistry and physical measurements. Seawater pH (NBS scale) and temperature were measured at all sites several times a day for 4 days using a pH meter (YSI Professional Plus, Handheld Multiparameter Instrument, USA). Water samples for total alkalinity (TA) were collected from each site, cooled and stored in the dark until analysis. TA was quantified with a Metrohm 862 compact titrosirometer. The pCO2 levels were calculated from salinity (~38%), as reported by Johnson et al. (2012)12 and TA and pH(NBS) measurements using the program CO2SYS (Pierrot, D. E., Lewis, E. & Wallace, D. W. R. MS Excel program developed for CO2 system calculations. Carbon dioxide information analysis center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, TN, USA (2006)), selecting the constants of Mehrbach et al. (1973). Carbonate chemistry parameters are shown in Table 1. Light intensity at each site was measured hourly for 3 consecutive days close to the seabed (1–2 m depth) with HORO Pendant® Temperature/Light data loggers (Onset, Pocasset, MA, USA). The logged light data were converted from lux to μmol quanta m−2 s−1 (Fig. 1a).

Sample collection in the field. Anemones, A. viridis, a dominant benthic organism in Levante Bay, was prevalent throughout the study area. Sixteen anemones were collected randomly from each site at a depth of 1–2 m and immediately frozen until further analyses. To minimize any confounding responses due to age and/or size all samples were of similar size (oral disc diameter of 2.5–3.5 cm). Between 5 and 10 tentacles were clipped from each anemone at every site (n = 16). Tentacles were processed for total protein and algal characteristics (i.e., Symbiodinium density, chlorophyll a concentration and mitotic index) at the sampling site. Samples were weighed (CT 1202, Citizen, accuracy 0.01 g) and homogenized in 0.2 μl sterile filtered seawater (FSW) with an electric homogenizer (DIAX 100 homogenizer Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). The homogenate and all anemones were immediately frozen and then transported on dry ice to the Interuniversity Institute for Marine Sciences (IUI), Israel, where they were stored at ~80 °C pending analyses.

Seawater. Seawater samples were collected from the four sites for carbon isotopes of dissolved inorganic carbon (DIC, δ13C(DIC) and oxygen isotopic analysis (δ18Owater)). Triplicate samples for δ13C(DIC) analysis were immediately poisoned upon collection with 60 μl saturated solution of mercuric chloride and stored in 60 ml brown bottles at room temperature until analysis. Triplicate samples for δ18Owater analysis were collected in 50 ml test tubes (Stardest) and stored at room temperature until analysis.

Total protein, Symbiodinium density, mitotic index and chlorophyll concentration. The tissue homogenate of each anemone (n = 16) was further processed and analyzed for measurements of physiological parameters. Total protein analysis was performed by removing 100 μl of the tissue homogenate and sonication on ice with a Branson Sonifier B12 (Branson Sonic Power Co., Danbury, Connecticut, USA) for 20 s. Quantification was done after Bradford (1976) using the Quick Start Bradford Protein Assay Kit and Quick Start Bovine Serum Albumin Standard Set (Bio-Rad Laboratories, Hercules, CA, USA). Optical density was read at 595 nm using an ELISA reader (Multiskan spectrum, Thermo Fisher Scientific Inc., USA). For measurement of algal characteristics, 2 ml of homogenate of each sample (n = 16) was centrifuged (5000 rpm at 4 °C, 15 min) and 2 ml of the supernatant was collected for further analyses. To quantify algal cells, 2 ml of the supernatant was fixed with 1 ml of 1% formaldehyde and kept in the dark until analysis. TA was quantified with a Metrohm 862 compact titrosirometer. The pCO2 levels were calculated from salinity (~38%), as reported by Johnson et al. (2012)12 and TA and pH(NBS) measurements using the program CO2SYS (Pierrot, D. E., Lewis, E. & Wallace, D. W. R. MS Excel program developed for CO2 system calculations. Carbon dioxide information analysis center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, TN, USA (2006)), selecting the constants of Mehrbach et al. (1973). Carbonate chemistry parameters are shown in Table 1. Light intensity at each site was measured hourly for 3 consecutive days close to the seabed (1–2 m depth) with HORO Pendant® Temperature/Light data loggers (Onset, Pocasset, MA, USA). The logged light data were converted from lux to μmol quanta m−2 s−1 (Fig. 1a).
Anemone tissue and Symbiodinium samples were dried with a lyophilizer (VirTis, Sentry 2.0, SP Scientific) to remove any remaining salts. Both the host tissue and Symbiodinium samples were washed with double-distilled water (DDW) to remove remaining tissue. All samples were washed with trifuged for 5 min at 5000 rpm (4K15 centrifuge, Sigma, USA). The procedure was repeated twice in order to remove remaining tissue. The homogenate was centrifuged for 5 min at 5000 rpm (4K15 centrifuge, Sigma, USA), resulting in pelleted host material for analysis. The samples were analyzed on a Gas Bench II and Finigan MAT 252. The carbon to nitrogen ratios (C/N) of anemone tissue and Symbiodinium were calculated from simultaneous %C and %N.

Seawater samples. δ13Cwater was measured by equilibrating 0.5 ml of samples with a mixture of 0.5% CO2 in He at 25 °C for 24 h. The samples were analyzed on a Gas Bench II connected in-line to a Finnigan MAT 252 mass spectrometer. The results are reported relative to VSMOW with 0.005 (±1 SD) long-term precision of the laboratory working standards.

For δ13Coc analysis, 1 ml seawater was injected into vials, flushed with He gas, acidified with 0.15 ml orthophosphoric acid (H3PO4) and left to react for 24 h at 25 °C. The samples were analyzed on a Gas Bench II and Finnigan MAT 252. The results are reported relative to VPDB with 0.008% (±1 SD) long-term precision of the laboratory working standards.

Stable isotope analyses. The isotopic measurements were made at the stable isotopes laboratory in the Department of Earth and Planetary Sciences, the Weizmann Institute of Science, Israel. The oxygen, carbon and nitrogen isotope measurements are reported in the conventional δ notation.

Anemone tissue and Symbiodinium samples. δ13C and δ15N of 240–270 μg of dried tissue and algae were analyzed using an elemental analyzer (CE 1110) interfaced to the MAT 252 mass spectrometer. The standard temperature of precision working standards is δ13C 0.05‰ and for δ15N is 0.1‰ relative to V-PDB and Air respectively (±1 SD). The carbon to nitrogen ratios (C/N) of anemone tissue and Symbiodinium were calculated from simultaneous %C and %N.

Data analyses. All data was checked for normality using the Kolmogorov-Smirnov test and for homogeneity of variance using Cochran’s test. In cases in which homogeneity of variance was achieved, we used one-way ANOVA with a multiple comparison test (Tukey). If homogeneity of variance or normality was not achieved, we used a non-parametric Kruskal-Wallis ANOVA and post-hoc Mann-Whitney U tests for separation of significant factors. Differences between factors were considered significant for a P value < 0.05. Unless otherwise specified, mean values are presented ± standard error of mean (SEM). All data were analyzed using SPSS version 20 (SPSS IBM, New York, USA).
41. Rau, G. H., Takahashi, T., Desmarais, D. J., Repeta, D. J. & Martin, J. H. The relationship between δ13C of organic matter and [CO2]aq in ocean surface water – data from a JGOFS site in the northeast Atlantic Ocean and a model. *Geochem. Cosmochim. Ac.* 56, 1413–1419 (1992).

42. Laws, E. A., Popp, B. N., Bidigare, R. R., Kennicutt, M. C. & Macko, S. A. Dependence of phytoplankton carbon isotopic composition on growth rate and [CO2]aq – theoretical considerations and experimental results. *Geochem. Cosmochim. Ac.* 59, 1131–1138 (1995).

43. Erez, J., Bouevitch, A. & Kaplan, A. Carbon isotope fractionation by photosynthetic aquatic microorganisms: experiments with *Synechococcus PCC7942*, and a simple carbon flux model. *Can. J. Bot.* 76, 1109–1118 (1997).

44. Vizzini, S. et al. Effect of explosive shallow hydrothermal vents on δ13C and growth performance in the seagrass *Posidonia oceanica*. *J. Ecol.* 98, 1284–1291 (2010).

45. Goericke, R. & Fry, B. Variations of marine plankton δ13C with latitude, temperature, and dissolved CO2 in the world ocean. *Global Biogeochem. Cy.* 8, 85–90 (1994).

46. Mayfield, A. B., Hsiao, Y. Y., Chen, H. K. & Chen, C. S. Rubisco expression in the *Synechococcus* strain PCC7942, and a simple carbon flux model. *Can. J. Bot.* 85, 897–907 (1973).

47. Raven, J. A. Inorganic carbon acquisition by marine autotrophs. *Adv. Bot. Res.* 27, 85–209 (1997).

48. Krief, S. et al. Physiological and isotopic responses of scleractinian corals to ocean acidification. *Geochem. Cosmochim. Ac.* 74, 4988–5001 (2010).

49. Bodin, N., Le Loch, F. & Hily, G. Effect of lipid removal on carbon and nitrogen stable isotope ratios in crustacean tissues. *J. Exp. Mar. Biol. Ecol.* 341, 168–175 (2007).

50. Fabry, V. J., Seibel, B. A., Feely, R. A. & Orr, J. C. Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES J. Mar. Sci.* 65, 414–432 (2008).

51. Laurent, J. et al. Regulation of intracellular pH in cnidarians: response to acidosis in *Anemonia viridis*. *J. Exp. Mar. Biol. Ecol.* 281, 683–695 (2001).

52. Cohen, S. Measuring gross and net calcification of a reef coral under ocean acidification conditions: methodological considerations. MSc Thesis. Bar-Ilan University, Israel (2011).

53. Johnson, V. R. A study of marine benthic algae along a natural carbon dioxide gradient. PhD Thesis. University of Plymouth, UK (2012).

54. Mehrbach, C., Culberson, C. H., Hawley, J. E. & Pytkowicz, R. M. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* 18, 897–907 (1973).

55. Thimijan, R. W. & Heins, R. D. Photometric, radiometric, and quantum light units of measure: a review of procedures for interconversion. *HortScience* 18, 818–822 (1983).

56. Perez, S. F., Cook, C. B. & Brooks, W. R. The role of symbiotic dinoflagellates in the temperature-induced bleaching response of the subtropical sea anemone *Aiptasia pallida*. *J. Exp. Mar. Biol. Ecol.* 256, 1–14 (2001).

57. Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254 (1976).

58. Jeffrey, S. W. & Humphrey, G. F. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pfl.* 167, S191–S194 (1975).

59. Wilkerson, F. P., Muller-Parker, G. & Muscatine, L. Temporal patterns of cell division in natural populations of endosymbiotic algae. *Limnol. Oceanogr.* 28, 1009–1014 (1983).

**Acknowledgements**

Thanks to Marco Milazzo (University of Palermo) for essential academic and logistical support. We are grateful to Gabriela Perna for help with the physiological parameter analyses. This study was funded in part by the FP7 ASSEMBLE project no. 227799, the EU MedSeA project, and an Israel Science Foundation grant to M.F.. E.M.B. was funded by the Minerva fellowship program.

**Author contributions**

R.H., E.M.B. and M.F. conceived the overall project. R.H. and E.M.B. conducted the field and laboratory work and analysed data. R.Y. and A.S. carried out stable isotope analyses. All authors reviewed and edited the manuscript.

**Additional information**

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Horwitz, R., Borell, E.M., Yam, R., Shemesh, A. & Fine, M. Natural high pCO2 increases autotrophy in *Anemonia viridis* (Anthozoa) as revealed from stable isotope (C, N) analysis. *Sci. Rep.* 5, 8779; DOI:10.1038/srep08779 (2015).