Effects of CO_2-induced acidification on the intertidal sea anemone *Anthopleura elegantissima* (Cnidaria: Anthozoa) and its algal symbiont *Symbiodinium muscatinei* (Dinomastigota: Dinophyceae)

by

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by

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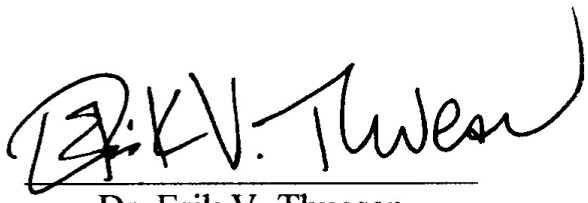

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ABSTRACT

Effects of CO_2-induced acidification on the intertidal sea anemone *Anthopleura elegantissima* (Cnidaria: Anthozoa) and its algal symbiont *Symbiodinium muscatinei* (Dinomastigota: Dinophyceae)

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Oceanic absorption of anthropogenic CO_2 decreases the pH of the ocean and shifts the carbonate equilibrium of seawater, a process known as hypercapnic acidification. Acidification can cause physiological stress and decrease the availability of carbonate ions for the secretion of calcium carbonate; however increased CO_2 may benefit some photosynthetic organisms through increased rates of carbon fixation. This study investigated the impact of hypercapnic acidification on the photosynthetic symbiosis of the non-calcifying anemone, *Anthopleura elegantissima* (Brandt) and it's symbiotic alga *Symbiodinium muscatinei* (zooxanthella). Anemone specimens were maintained in the laboratory for 1 week under current levels of PCO_2 (369 ppmv) and pH (8.1). Clonal pairs of these specimens were then divided into two groups; each group was exposed to one of two hypercapnic conditions for 6 weeks: moderate (PCO_2 = 450 ppmv, pH = 8.1) or high (PCO_2 = 2340 ppmv, pH = 7.3). After 6 weeks, individuals were compared for differences in respiratory rate, photosynthetic rate, and the contribution of zooxanthellae to the animal's respiration (C_{ZAR}). Density of algal cells in anemones, algal cell size, mitotic index and chlorophyll content were measured to compare zooxanthellal characteristics. After 6 weeks of exposure, *A. elegantissima* exhibited higher rates of photosynthesis among anemones at higher PCO_2s (moderate- 3.30, high- 4.20 μmol O_2 g^{-1} h^{-1}) than in anemones at normocapnic levels (1.53 μmol O_2 g^{-1} h^{-1}). Respiration rates were also higher at moderate and high PCO_2 (1.34 and 1.27 μmol O_2 g^{-1} h^{-1} respectively) than at current conditions (0.94 μmol O_2 g^{-1} h^{-1}). Anemones at moderate PCO_2 received more of their respiratory carbon and O_2 (C_{ZAR} = 137.3%) from zooxanthellae than those at current conditions (C_{ZAR} = 66.6%) or at high PCO_2 (C_{ZAR} = 78.2%). Mitotic index and zooxanthellal cell diameter were greater among zooxanthellae in hypercapnic conditions. The response of *Anthopleura elegantissima* to hypercapnic acidification reveals the adaptability of an organism that has evolved a tolerance for high PCO_2.

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Introduction

It is well established that anthropogenic emissions of CO_2 are warming the climate and decreasing the pH of the ocean (Vitousek et al. 1997, Caldeira & Wickett 2005, Feely et al. 2004, Orr et al. 2005). For 800,000 years prior to the industrial revolution, Earth's atmospheric CO_2 ranged from 170-300 parts per million volume (ppmv) (Siegenthaler et al. 2005. Lüthi et al. 2008). At 386 ppmv, the present CO_2 concentration is nearly 40% higher than pre-industrial levels (Houghton 2003) and is expected to increase by approximately 2 ppmv y^{-1} for many decades (IPCC 2007). The projected rate of atmospheric CO_2 increase is approximately 100 times faster than has occurred in the past 650,000 years (Raven et al. 2005, Siegenthaler et al. 2005). Climate models project that this will result in CO_2 concentrations over 500 ppmv by the middle of this century, over 800 ppmv by the turn of the next century (Feely et al. 2004), and as high as 2000 ppmv by the year 2340 (Caldeira & Wickett 2003).

The oceans have absorbed more than a third of the anthropogenic CO_2 released in the last 200 years (Sabine et al. 2004). This increase in the partial pressure of CO_2 gas (PCO_2) is a condition known as hypercapnia. Hypercapnia shifts the carbonate equilibrium of seawater: $[H^+]$ and bicarbonate ions $[HCO^-_3]$ increase, pH and carbonate ions $[CO^{-2}_3]$ decrease (Millero 2007) and hypercapnic acidification (HA) results. The current average pH in the ocean is ~ 8.1 units, a decline of 0.1 pH units from pre-industrial values (Caldeira & Wickett 2003). By the end of this century, pH is predicted to decline by approximately 0.4 pH units (Feely et al. 2001, Caldeira & Wickett 2005), which is equivalent to a 150% increase in H^+. Although the uptake of CO_2 by the oceans has ameliorated the climatic effects of anthropogenic CO_2, HA may adversely affect many marine

organisms and ecosystems (Leclercq et al. 2002, Raven et al. 2005, Hoegh-Guldberg et al. 2007, Doney 2009).

Hypercapnic acidification can suppress marine organisms both by inducing physiological stress and by decreasing the availability of carbonate ions for calcification. Increasing the concentration of protons perturbs acid-base balance by interfering with diffusion processes, ion transport pumps and intracellular buffering capacities (Walsh & Milligan 1989, Seibel & Walsh 2003). Organisms may generate a new steady state to compensate for short-term acidosis but long term effects can include reduced protein synthesis (Hand 1996) and therefore reduced growth and reproduction (Kurihara et al. 2007). Although the physiological impacts of HA have not been well studied at ecologically relevant temporal and pH scales, HA has been demonstrated physiologically harmful to both calcifying and non-calcifying organisms (Grainger et al. 1979, Pörtner et al. 2005, Kurihara et al. 2007, Kurihara 2008, Waters &Thuesen, submitted).

The effects of HA on calcification processes have been more thoroughly researched. Numerous studies have demonstrated the correlation of biogenic calcification on the saturation state of calcium carbonate (Ω $CaCO_3$) (Gattuso et al. 1998, Langdon et al. 2000, Leclercq et al. 2000, Riebesell et al. 2000, Michaelidis et al. 2005, Shirayama & Thornton 2005). Decreasing the concentration of carbonate ions reduces the Ω $CaCO_3$, thereby inhibiting the ability of many calcifying organisms such as calcareous plankton, molluscs and corals to secrete exoskeletons (Kleypas et al. 1999, Caldeira & Wickett 2003, Orr et al. 2005, Schneider & Erez 2006, Gazeau et al. 2007, Fabry et al. 2008). Calcification rates of hermatypic corals and coralline algae are predicted to decrease by 10-50% over the next

century as Ω $CaCO_3$ decreases (Gattuso et al. 1998, Leclercq et al. 2002, Reynaud et al. 2003) and temperatures rise (Marubini et al. 2008).

Because calcification is intertwined with other physiological processes (Furla et al. 2000*b*, Allemand et al. 2004), it is informative to isolate physiological effects of HA on actinians and their photosynthetic symbionts from the calcification process. The mutualist symbiosis between actinians and photosynthetic dinoflagellates, commonly referred to as zooxanthellae (ZX), is characterized by an exchange of nutritional and metabolic goods. ZX produce carbohydrates and O_2 through photosynthesis that contribute to the respiration of the host; ZX receive nitrogen compounds and CO_2 from the host in exchange (Weis 1993, Furla et al. 2005). The interactions of these energetic systems in hypercapnic conditions are difficult to predict. . While the overall metabolism of the whole animal may be suppressed (Seibel and Walsh, 2003), photosynthesis may be enhanced as seen in other CO_2-limited organisms such as eelgrass (Palacios & Zimmerman 2007).

To understand how HA affects metabolic processes in an intertidal, photosynthetic actinian, this study examined the energetic effects of increased PCO_2 (moderate- 450 ppmv and high- 2340 ppmv) on the anemone *Anthopleura elegantissima* Brandt. In the eastern North Pacific Ocean, *A. elegantissima* harbors the dinoflagellate *Symbiodinium muscatinei* (Dinomastigota) (LaJeunesse & Trench 2000) that is congeneric with the symbiotic dinoflagellates found in hermatypic corals. Rates of metabolism, photosynthesis and the contribution of carbon to the needs of animal respiration (CZAR) were compared between *A. elegantissima* at current, moderate and high PCO_2 conditions. In addition, the impacts of HA on

zooxanthellae were studied by comparing cell density, cell size, mitotic index and concentration of chlorophyll *a* within Zx cells.

Materials and Methods

Experimental Design

Anthopleura elegantissima forms aggregations of genetically identical clones through bilateral fission (Ayer & Grosberg 1995). Experiments were designed to examine the effects of hypercapnic acidification through paired comparisons of genetically identical but separated clonal couplets. After the initial acclimation period, clonemates were separated so that each individual (n = 24) was maintained in its own chamber in either a moderate or high PCO_2 condition (Fig. 1). Several respiration chambers without anemones were kept in each experimental aquarium for measurements of background O_2 consumption. Respiratory (MO_2) and photosynthetic (P_g) rates of each specimen were measured after one week in normocapnic conditions and after 6 weeks in experimental conditions. After the initial metabolic rate measurements and after 3 weeks in experimental conditions, 4 tentacles were clipped from each anemone and frozen in liquid nitrogen for zooxanthellal measurements at the mid-point of the experimental course (Saunders & Muller-Parker 1997). At the conclusion of the six-week experiments, anemones were blotted dry and weighed. Oral disks and tentacles of each specimen were weighed and frozen in liquid nitrogen for later measurements of zooxanthellae.

Collection and Maintenance

Zooxanthellate *Anthopleura elegantissima* specimens were collected in April 2008 from Point Grenville, Washington, USA (47° 18.2' N, 124° 16.2' W). This anemone harbors two different types of photosynthetic symbiont: the dinoflagellate *Symbiodinium muscatinei* and a

trebouxiophycean, unicellular green alga (Lewis & Muller-Parker 2004). The intertidal distribution of the symbionts is largely determined by irradiance and temperature (Secord & Muller-Parker 2005, Muller-Parker et al. 2007). The green alga is restricted from depths that are brightly illuminated and subject to warmer temperatures. Because *A. elegantissima* was collected from colonies at ~1.5 - 2.0 m above mean low low water (Secord & Augustine 2000), anemones with the green algal symbiont were excluded. This assumption was verified later by the absence of the green alga during algal cell counts. The largest anemones from each colony were chosen to minimize free space in the respiration chambers. Genetically identical pairs of *A. elegantissima* (hereafter referred to as clonemates) were selected from contiguous colonies within the spatial boundaries that separate genetically distinct clones (Ayre & Grosberg 2005). No clonemate displayed acrorhagial aggression toward its respective clonemate, which indicates that they are genetically identical (Ayre and Grosberg, 1995). Clonemates and individual specimens were collected and transported to the lab at The Evergreen State College, Olympia, Washington in separate plastic bags filled with seawater. In the lab, oral disks and tentacles of individual anemones (n = 12) were weighed and individually frozen in liquid nitrogen for zooxanthellal measurements. Clonemates were cleaned of debris before they were blotted dry and weighed. Each individual clonemate was settled into a labeled, 130-ml glass chamber (with a stir-bar cage adhered to the bottom using silicone aquarium sealer) for the duration of the experimental period to minimize disturbance and the risk of damage prior to respiration measurements. Chambers were covered with a 1-cm mesh screen for the first two weeks to prevent anemones from escaping the chambers. Individuals

obviously damaged and those that failed to adhere to the chamber were not used for experiments.

Anemones were acclimated for 7 days in a 120-L, recirculating aquarium with natural seawater adjusted up to 30 psu with Instant Ocean® synthetic sea salt at 12°C, pH = 8.1, PCO_2 = 368 ppmv. Mean irradiance was adjusted to ~660 μmole m^{-2} s^{-1} and followed a natural spring-summer daily photoperiod (14 h light: 10 h dark) for the acclimation and the two experimental aquaria to maximize potential zooxanthellal photosynthesis without risk of photoinhibition (Fitt et al. 1982, Verde & McCloskey 2002). Every day, each chamber was moved within the aquarium to a different position to ensure that all anemones received equivalent irradiance throughout the experimental period. Each specimen was hand fed twice every week by alternating shrimp and salmon each weighing 5% of the specimen's initial wet weight (Zamer & Shick 1989). Chambers were cleaned 24 h after feeding. Aquaria were cleaned of algal growth and water exchanged twice per week to minimize accumulation of ammonia, nitrate and extracellular proteins.

Experimental aquaria (120 L) were designed to maintain two levels of hypercapnic PCO_2: moderate HA (PCO_2 = 450 ppmv) and high HA (PCO_2 = 2340

ppmv) with pH levels of 8.0 units and 7.3 units, respectively (Table 1). Natural seawater was collected from southern Puget Sound and adjusted up to 30 psu with a combination of Instant Ocean® synthetic seawater and a carbonate-free synthetic seawater (Bidwell & Spotte 1985) to maintain the targeted pH, PCO_2 and alkalinity levels. Carbonate alkalinity (acid neutralizing capacity in mg carbonates L^{-1} seawater) was estimated by

titration with 0.2 N HCl using a Gilmont microburet and Gran Plot analysis with the USGS web-based Alkalinity Calculator, version 2.20 (http://or.water.usgs.gov/alk/). PCO_2 was calculated with the CO2SYS Excel macro (Lewis & Wallace 1998). CO_2 was used to manipulate pH, rather than mineral acids such as HCL or H_2SO_4, due to its ecological relevance and its roles in carbonate chemistry and cellular function (Ishimatsu et al. 2004, Schneider & Erez 2006, Fabry et al. 2008, Marubini et al. 2008). Hypercapnic acidification was generated by bubbling CO_2 through a reactor. The reactor was constructed of PVC pipe and contained 6, pegged plastic balls (Bio-Balls™) to generate turbulence and therefore facilitate dissolution of the gas into the seawater before entering the aquarium. CO_2 was delivered through a solenoid valve with a Milwaukee SMS122 pH controller; pH of each aquaria was monitored daily with an Orion Research 601A digital ionalyzer that was calibrated daily with Markson LabSales National Bureau of Standards (NBS) buffers.

Photosynthesis, Respiration and CZAR

Respiratory and photosynthetic rates were measured following the methods of Thuesen et al. (2005). To minimize microbial O_2 consumption, chambers were cleaned 24 h before rate measurements and 100 mg L^{-1} each of streptomycin and ampicillin were added to the test chambers. Anemones were sealed into their chambers with seawater at their respective level of PCO_2 with a stir-bar to ensure adequate mixing. Chambers were submerged in a circulating water bath at 12°C on stir-plates at 200 rpm. After a 30-minute, lighted acclimation period, O_2 saturation was measured for 30 minutes in the light followed by 30 minutes of measurement in the dark. Oxygen saturation was measured with Microx TX3 temperature-compensated O_2 meters fitted with Type B2 NTH fiber-optic, O_2 micro-

optodes (Precision Sensing). Meters were calibrated to 0% O_2 with a 5% solution of Na_2SO_3 and to 100% O_2 using oxygen-saturated seawater. Optodes were inserted into the respiration chambers through gas-tight septa. Control chambers without anemones were run simultaneously with the same mixtures of antibiotics and seawater at 450 ppmv and 2340 ppmv PCO_2. Background rates of microbial O_2 consumption within chambers at each PCO_2 condition in light and dark were subtracted from corresponding anemone rates.

Ratios of photosynthesis (P_g) to respiration (MO_2) were calculated from the daily gross photosynthetic rate (based on a 14-hour lighted period) relative to the daily respiratory rate. The percent contribution of zooxanthellal carbon to animal respiration (CZAR) was based on the ratio of animal (ß) and algal (1- ß) biomass components (Muscatine et al. 1981) assuming the mean algal biomass ratio of 0.09 = (1- ß) calculated for *A. elegantissima* (McKinney 1978, as reported in Fitt et al. 1982). Because zooxanthellate respiration cannot be measured in the animal in the light, the daytime algal respiratory rate is estimated from the total dark respiration rate as a ratio of biomass. CZAR was estimated with the formula defined by Muscatine et al. (1981) and modified by Verde & McCloskey (1996*b* and 2001):

$$C_{ZAR} = [[(0.375*P_g^{O})(PQ_Z)^{-1}]- [(1-ß)(R_{ae}^{O})(RQ_{ae})] - [C_{\mu}]\cdot 100] / (ß)(0.375* R_{ae}^{O})(RQ_{ae})$$

where daily gross photosynthetic rate (P_g^{O}) is equal to the sum of O_2 production rate in the light and the O_2 consumption rate in the dark for the number of lighted hours per day. The conversion ratio of C to O_2 equivalents

(12:32) equals 0.375 (Verde & McCloskey 2001). Daily MO_2 of the anemone (R_{ae}^{O}) was calculated from the rate of O_2 consumption in the dark and extrapolated to 24 h. The photosnthetic quotient (PQ_z), animal respiratory quotient (RQ_{al}) and zooxanthellal respiratory quotient (RQ_z) were assumed to be 1.1, 0.9 and 1.0, respectively (Kremer et al. 1990, McCloskey et al. 1994, Verde & McCloskey 1996*b*, 2001). The respiratory quotient of the anemone (RQ_{ae}) was determined by:

$$RQ_{ae} = [(1- ß)(RQ_z)^{-1} +((ß)(RQ_{al})^{-1})]^{-1}.$$

Algal-specific growth rates (μ_z) were calculated as described in Verde and McCloskey (1996*b*):

$$\mu_z = (24 \cdot t_d^{-1})\ln(1 + f)$$

with the duration of cytokinesis t_d equal to 28 (Verde & McCloskey et al. 1996*a*) and f equal to the fraction of cells in the division phase as determined from mitotic index. The zooxanthellal carbon-specific growth rates (C_μ) were determined with the formula supplied by Verde & McCloskey (1996*a*):

$$C_\mu = [(SS)(C \cdot cell^{-1})(\mu_z)].$$

Standing stock (SS) was estimated from ZX cell densities, assuming that ~90% of ZX are harbored in the oral disk and tentacles (Shick 1991). Carbon per ZX cell was calculated as reported by Menden-Deuer & Lessard (2000):

pg C·cell^{-1} = 0.760(cell volume$^{0.819}$).

Zooxanthellal Measurements

Symbiodinium muscatinei characteristics were measured to gauge the effects of HA on the photosynthetic symbionts. Previously frozen tentacles and oral disks were thawed on ice and individually ground in hand-held, glass tissue-homogenizers with filtered seawater (0.22 μm, 30 psu) at a ratio of approximately 1 tissue: 10 water. Homogenates were separated into 3 aliquots for protein, chlorophyll and cellular measurements.

Zooxanthellal density was normalized to μg anemone protein. Algal cell counts were performed with a hemocytometer in 10 replicate grid counts per anemone and the number of cells per ml homogenate was converted to the number of cells per μg protein. Algal cell diameters were measured with an ocular micrometer in replicates of 10 per MI anemone. Mitotic index (MI) was measured as an indicator of zooxanthellal growth. was calculated as a percentage from the number of doublets with a complete cleavage furrow observed per 1000 cells.

To prevent saline interference with the protein assay, aliquots of anemone homogenates were desalinated with Millipore Microcon® centrifugal filter units and then diluted to the original concentration with DI water before digestion of the homogenate protein in 5% NaOH. Protein concentrations (mg protein/ml homogenate) were measured with a Thermo Scientific NanoDrop 1000® spectrophotometer against bovine serum albumen standard, and protein density was determined by a modified Lowry Assay (Lowry et al. 1951) according to the NanoDrop protocol on three samples from each anemone.

To measure the concentration of chlorophyll *a*, 3 replicate homogenates per anemone were centrifuged and resuspended 4 times to remove animal fractions (Muller-Parker et al. 2007). Resuspended zooxanthellae were filtered through GF/C Whatman® filters followed by 0.5 mL of 5% $MgCO_3$ (Verde & McCloskey 1996b) to prevent acidification of the samples. Filters were folded and wrapped in foil to freeze, then later submerged in 10 mL 90% acetone and stored for 24 h at 4° C (Augustine & Muller-Parker 1998). Acetone extracts were read for chlorophyll-*a* concentration (mg of chlorophyll/ml acetone) with a Turner Designs® 10 AU Fluorometer and converted to pg per ZX $cell^{-1}$.

Statistical analyses

Data were analyzed with JMP Statistical Discovery Software, version 7.0. Paired t-tests were used to determine if values measured following hypercapnic experimental treatments differed significantly from the values under the initial normocapnic conditions. Linear regression was used to determine if there was a significant relationship between mass-specific metabolism and body mass. ANOVA analyses were performed to identify ZX differences between oral disks because initial OD parameters were measured from individuals that were not clonemates. ANOVA tests were followed with Fisher's LSD post hoc test.

Results

All anemones survived the experimental period. Three individuals in each experimental condition reproduced through bilateral fission during this time and were treated as single individuals. There were no differences in mass of the anemones after 6 weeks of exposure to experimental conditions. The two experimental tanks were maintained within 1% of target PCO_2 and pH levels (448 ppmv and 2342 ppmv, pH 8.08 and 7.35, respectively; Table 1).

Although there was considerable variation of PCO_2 within the moderate tank, t-test analyses were highly significant between the conditions ($p<0.001$). Hereafter, the tank at PCO_2 448 ppmv is referred to as moderate (450 ppmv) and the tank at 2342 ppmv as high (2340 ppmv). Rates of photosynthesis and respiration were measured on 24 individual specimens of *A. elegantissima*; these were 12 pairs of clonemates. Data for one couplet at the current condition (PCO_2 368 ppmv) were discarded due to measurement errors; however the couplet was included for analyses between the two HA conditions. Data from initial measurements at current conditions are combined in the figures as there were no differences between couplet individuals in any parameter at current conditions.

Photosynthesis and respiration

Anthopleura elegantissima exhibited higher rates of mass-specific gross photosynthesis ($\__g$) after 6 weeks of exposure to moderate and high PCO_2 than at current (normocapnic) conditions (Table 2). Anemones at moderate PCO_2 (450 ppmv) had a higher mean $\__g$ than those at the high (2340 ppmv) PCO_2 (paired t-test, $p=0.03$, Fig 2). The $\__g$ of anemones at both moderate PCO_2 (3.30 ± 0.35 μmol O_2 g^{-1} h^{-1}) and high PCO_2 (4.20 ± 0.40 μmol O_2 g^{-1} h^{-1}) were higher than the $\__g$ at current levels (1.53 ± 0.11 μmol O_2 g^{-1} h^{-1}; paired t-test, $p < 0.001$, Fig. 2).

Respiration rates were also higher in the experimental treatments relative to the initial condition. Mean mass-specific respiration ($\__{O2}$) of anemones at high (1.27 ± 0.15 μmol O_2 g^{-1} h^{-1}) and moderate (1.34 ± 0.13 μmol O_2 g^{-1} h^{-1}) PCO_2 were higher than the rates at current conditions (0.94 ± 0.05 μmol O_2 g^{-1} h^{-1}, Paired t-test, $p < 0.03$, Fig. 2). There was no difference in $\_O_2$ between the high and moderate PCO_2 groups (paired t-test,

Fig 4). Mean $_{g}$:$_O_2$ ratios were significantly higher among anemones exposed to HA than among anemones prior to exposure ($p < 0.001$, Fig. 3). Mean $_{g}$:$_O_2$ ratios were greater at moderate PCO_2 than at high PCO_2, however the difference was not significant (paired t-test, $p = 0.1$).

The contribution of oxidizable carbon and O_2 by zooxanthellae to animal respiration (CZAR, Fig. 4) was greatest in the moderate PCO_2 treatment (CZAR = 137.3%) in comparison to the high PCO_2 (CZAR = 78.2%, paired t-test, $p < 0.01$) and at current PCO_2 (CZAR = 66.6%, paired t-test, $p < 0.001$). Over the small size range of specimens used in this study, there was no significant effect of body mass (linear regression analysis) on either $_{g}$ (Fig. 5) or $_O_2$ (Fig. 6).

Zooxanthellal characteristics

Zooxanthellae (ZX) from combined oral disks and tentacles (OD) were compared between *Anthopleura elegantissima* at current PCO_2 (386 ppmv) and between clonemates held in moderate (450 ppmv) or high (2340 ppmv) PCO_2 seawater for 6 weeks. Comparisons were also made between the ZX of anemone tentacles prior to exposure to moderate and high PCO_2 and after 3 weeks of exposure. The characteristics of ZX (cell diameter, cell density, mitotic index, and chlorophyll *a* per cell) were significantly different in tentacles than in OD in all categories (paired t-test, $p \leq 0.01$, Figs. 7-10).

ZX cell diameters were larger in the tentacles than in the OD within each PCO_2 treatment (Fig. 7). Mean ZX cell diameter was larger in the tentacles of anemones at high (12.56 ± 0.09 μm, $p = 0.06$) and moderate PCO_2 (12.31 ± 0.20 μm,) than in the tentacles at current conditions (11.79 ± 0.11 μm, Paired t-test, $p<0.001$). In the OD, there were no significant differences in ZX cell diameters (11.16 ± 0.08 μm) among any of the PCO_2

conditions (ANOVA, $p > 0.05$, Fig. 7). There were no differences in the algal density relative to animal protein between anemones in current, moderate or high PCO_2, (tentacles- 0.48 ± 0.03 and OD- 0.26 ± 0.02 10^6 cells mg protein^{-1}, ANOVA, $p > 0.05$, Fig. 8).

All anemones at moderate and high PCO_2 had increased mitotic index (MI) relative to those from the field (ANOVA, $p < 0.05$, Fig. 8). MI was greater in the OD of anemones at high PCO_2 (0.61 ± 0.08 %) than in those at moderate PCO_2 (0.40 ± 0.04 %) (Paired t-test, $p = 0.03$, Fig. 9). MI was also much higher in tentacles from anemones after 3 weeks of exposure to high PCO_2 (1.20 ± 0.27 %) compared to those in moderate PCO_2 (0.48 ± 0.04 %) (Paired t-test, $p < 0.01$, Fig. 9). Anemones in the high PCO_2 tank released a large amount of mucus-bound zooxanthellea that contained a very high ratio of doublets when examined under a microsocpe (data unreported). Although the concentrations of chlorophyll-*a* per algal cell were higher in tentacles (1.70 ± 0.13 pg per ZX cell) than in OD (3.80 ± 0.25 pg per ZX cell, paired t-test, $p < 0.001$), there were no differences in the concentration of chlorophyll-*a* per algal cell between anemones in current, moderate or high PCO_2 (ANOVA, $p = 0.15$, Fig. 10).

Discussion

This study demonstrated that the non-calcifying actinian *Anthopleura elegantissima* and its photosynthetic dinoflagellate *Symbiodinium muscatinei* can thrive in hypercapnic acidified seawater for a duration of 6 weeks. In photosynthesizing symbiotic organisms, the the percent contribution of zooxanthellae (ZX) to animal respiration (CZAR) is largely determined by the ratio of photosynthetic to respiratory rates (Verde & McCloskey 1998). In this study, higher rates of $_g$ at moderate (450 ppmv, pH=8.1) and high

(2340 ppmv, pH=7.3) PCO_2 levels offset small, corresponding increases in MO_2. Thus the differences in $\dot{M}_g$ and MO_2 were reflected in the CZAR.

Assumptions made in the calculation of CZAR such as the ratio of algal protein to animal protein could affect the outcome of this research (Fitt & Cook 2001). Although the actual ratio of algal protein to animal protein is unknown for this study, the lack of differences in the density of ZX cells and in the concentration of chlorophyll *a* suggests that the ratio is the same in all the of the conditions. Zx growth rates (Verde & McCloskey 2007) are more likely to impact these results due to the differences in MI and may be a source of error in the CZAR comparisons. The differences between the animals at increased PCO_2 and those fresh from the field may also have been affected by the consistent temperature and lighting that were provided in the lab as well as the absence of dessication, UV exposure and wave stress (Shick 1991, Verde & McCloskey 1996*b*). Abundant food in the lab may have contributed to the rise in $\dot{M}_{O2}$ as increasing heterothophic feeding can increase the respiratory rate of *A. elegantissima* (Fitt et al. 1982). In their study, $\dot{M}_{O2}$ in fed *A. elegantissima* was twice the rate of starved animals and CZAR averaged 13% for fed anemones compared to 45% for starved or newly collected anemones. This would suggest that the CZAR reported in the current study may under-estimate the potential increase in CZAR that would occur in the field as PCO_2 increases. However Jensen & Muller-Parker (1994) found high ammonium concentrations in tidepools with high densities of anemones, which suggests that these organisms also have high feeding rates in the field. Taking these factors into consideration, there were still significant increases in metabolic activities that varied with PCO_2 and pH as well as associated changes in ZX cell size and growth rates as indicated by mitotic index (MI). Because the density of ZX cells and

chlorophyll *a* was consistent between conditions, it is likely that the increased availability of CO_2 boosted the capacity of ZX to photosynthesize. This is consistent with the findings of Verde & McCloskey (2007) who posit that ZX-bearing anemones are carbon limited.

The effect of carbon limitation was also exhibited in a recent study by Anthony et al. (2008). This group researched the effects of HA and temperature increase on photosynthetic productivity in three types of calcifying actinians: a crustose coralline alga, a branching coral and amassive coral. As in the current study, they found increased rates of productivity (at temperatures increased by 3° C) at moderately increased levels of CO_2 (520-700 ppmv) in the branching coral *Acropora intermedia* but productivity was diminished in the massive coral *Porites lobata*. However, at the same temperatures, higher concentrations (1000-1300 ppmv) decreased productivity to near zero in both corals. Both moderate and large increases in PCO_2 reduced productivity in the crustose alga *Porolithon onkodes* to the point that respiration rates outpaced photosynthesis (45%-160% reduction in productivity). The authors speculate that the increase in productivity in *P. lobata* may have resulted directly from an increase in CO_2 supply but that at higher concentrations, the effects of a greater CO_2 supply are offset by physiological disruption from HA.

The observed excretion of actively replicating ZX in this study was confirmed by the dramatic increase in the MI of the anemones at 2340 ppmv. McCloskey et al. (1996) found that MI was higher in expelled pellets of ZX than within the host (but suggested that algal division may accelerate after expulsion when released from host restrictions). Several studies have found that the population density of ZX in other actinians is maintained under host control through chemically-signalled arrest of the algal reproduction and

active expulsion of symbionts (Trench 1987, McCloskey et al. 1996, Baghdasarian & Muscatine 2000). If the ZX in *A. elegantissima* are carbon limited, the ready supply of dissolved inorganic carbon (DIC) in a HA environment would necessitate an increase in the rate of expulsion to maintain normal densities of the rapidly reproducing alga in order to avoid toxicity from excess oxidative products (Furla et al. 2005).

ZX depend on the actinian host to transport the majority of DIC needed for photosynthesis (Furla et al. 1998), a process facilitated by the enzyme carbonic anhydrase (Furla et al. 2000*a*). Carbonic anhydrase (CA) is commonly employed by autotrophic symbiontic animals to increase the passage of DIC through host tissues by accelerating the conversion between HCO^-_3 and CO_2 for the consumption by the symbiont (Weis 1993, Goffredi et al. 1999, Furla et al. 2005). In the anemone *Aiptasia pulchella,* photosynthesis increased with the concentration of DIC and also was enhanced by CA (Weis 1993). Furthermore, the expression of CA by the host is induced by the symbiont (Weis & Reynolds 1999). The role of carbonic anhydrase at increased PCO_2 is worthy of additional investigation as increasing the PCO_2 in the environment could reduce the signal for expression of CA by the symbionts and therefore of CA activitiy in the anemone. An increase in the concentration of DIC in the seawater environment also could reduce the energy required from the animal to concentrate CO_2 and result in the increase in CZAR demonstrated by *A. elegantissima* in this study.

In corals, the benefit from increased CO_2 to photosynthesis is eclipsed by the impacts of HA on calcification. The above-mentioned research by Anthony et al. (2008) illustrated that calcification in a corraline crustose alga was extremely sensitive to the effects of HA and that two corals (one

branching, one massive) reduced calcification by 25-40% at PCO_2 of ~1200 ppmv. Another recent study found that the scleractinian coral *Stylophora pistillata* increased $_g$ when seawater was enriched with HCO^-_3 even at decreased pH; however $_g$ was not responsive to decreases in pH nor increases in PCO_2 and calcification rates decreased (Marubini et al. 2008). In contrast, increasing PCO_2 (411ppmv-918 ppmv) increased MO_2 but not $_g$ in a coral community in the Mediterranean (Leclercq et al. 2002) but again calcification decreased. In a third study, Schneider & Erez (2006) independently varied pH, CO^{-2}_3, CO_2, total alkalinity and total dissolved carbon. They found no correlation between any of these carbonate chemistry variables and $_g$ or MO_2 but that calcification diminished in *Acropora eurystoma* with a decrease in the concentration of CO^{-2}_3. The response of $_g$ to carbonate shifts varies in these studies, perhaps due to differences in acclimation and exposure durations, however the effect of acidification on calcification is consistent. Although calcification is tightly linked to photosynthesis (Gattuso et al. 2000), it is clear that the effect of $[CO^{-2}_3]$ on $CaCO_2$ saturation state has a much larger influence on calcification than can be compensated by the increase in photosynthesis as seen in this report.

Reports of reduced calcification and suppressed metabolism are countered by others that found enhanced calcification and metabolic activity with HA. Among photosynthetic organisms, one study of the coccolithophore *Emiliania huxleyi* demonstrated increased primary production and calcification in the presence of high PCO_2, both by experimentation and in geological record with a 40% increase in test mass over the last 220 years (Iglesias-Rodriguez et al. 2008). Gutowska et al. (2008) discovered that the cephalopod *Sepia officinalis* at very high PCO_2 (4000 and 6000 ppmv) had the same capacities for growth and for

calcification of its internal aragonite shell as control animals at ~675 ppmv. Even some corals have demonstrated unexpected resilience to acidification as discovered by Fine & Tchernov (2007) who exposed two Mediterranean coral species to decreased pH for 12 months. Although their aragonite skeletons dissolved, the polyps adopted an anemone-like existence and clung to the rocky substrate until they reformed skeletons after they were returned to normocapnic conditions.

Non-calcifying organisms also exhibit a variety of responses to HA. Studied fishes have shown an elastic response to PCO_2 and display adaptability to HA conditions ranging from acute exposure to CO_2 concentrations that fall within IPCC projections (Pörtner et al 2004, Ishimatsu et al. 2005) to long-term exposure at PCO_2 up to nearly 5800 ppmv (Melzner et al. 2009). Invertebrates tend to be more sensitive to HA; however some species appear able to compensate. The swimming crab *Necora puber* was found to be resistant to acid-base disruption down to a pH of 7.5 (Spicer et al. 2007), as was the shallow-water species *Cancer magister* at pH 7.1 (Pane & Barry 2007). However the deep-sea Tanner crab revealed a limited ability to regulate acid-base ions critical for responding to acidification (Pane et al. 2008). A sipunculid worm likewise was unable to compensate for a decrease in extracellular pH and its metabolic rate decreased with exposure to HA (Pörtner et al. 1998). However, in a study of the effects of HA on burrow structure and sediment nutrient flux of a nereid worm, there were no observed metabolic or behavioral effects of HA on the worm at pH 7.5 (Widdicombe & Needham 2007). The brittle star *Amphiura filiformis* increased metabolic rate when kept in HA; however this was at the expense of muscle wasting in the arms (Wood et al. 2008). The variability

among these investigations points to a need for additional examination of the effects of high PCO_2 on marine organisms, particularly in non-calcifiers.

Organisms such as *A. elegantissima* that are pre-adapted to fluctuations of high CO_2 conditions may be better suited to respond to the challenges of persistent HA. As a rocky intertidal inhabitant, *A. elegantissima* is regularly exposed to fluctuations of PCO_2 and pH due to fresh water input, tidal exchanges and localized, organismal production of metabolic CO_2. Fluctuating conditions are typical in estuaries, where PCO_2 is much more dynamic than in coastal and open ocean areas and may reach concentrations high enough to become a source of atmospheric CO_2 through out-gassing (Frankignoulle et al. 1998). Estuarine pH typically lies between 7.5 to 8.2 units but may be punctuated by occurrences above 9.0 and below 7.0 pH units (Hinga 2002), which is lower than the pH predicted from anthropogenic climate change. Although estuarine PCO_2 usually remains below 2,500 ppmv (Kempe 1982 as reported in Borges 2001), Frankignoulle et al. (1996) found PCO_2 as high as 5700 ppmv. Similar fluctuations in the carbonate system were found in the Palau coral reef lagoon that resulted from nightly respiration and calcification processes (Watanabe et al. 2006).

Although estuarine and intertidal inhabitants may be adapted to periodic acidification, medium to long-term exposure has been shown to be detrimental to both
non-calcifying (Langenbuch & Pörtner 2004) and calcifying organisms, such as bivalves (Michaelidis et al. 2005, Shirayama & Thornton 2005, Berge et al. 2006, Gazeau et al. 2007, Bibby et al. 2008) that inhabit regions of fluctuating pH and PCO_2. Persistent acidification can result in dramatic shifts in marine communities. Ecological studies in a high PCO_2 volcanic vent community off Ischia, Italy have shown that seagrass shoot density was

at its highest at 1827 ppmv PCO_2 and pH 7.6 (along a natural gradient from 8.2 to 7.4) and productivity ~30% greater than in surrounding areas (Hall-Spencer et al. 2008). However epiphytic coralline algae, gastropods and urchins were diminished or completely absent in areas below pH 7.7. Although several species of scleractinian corals are common to the region, photosynthetic anemones were the only cnidarians found in the zones with reduced Ω $CaCO_3$ (Hall-Spencer et al. 2008).

The response of *Anthopleura elegantissima* to hypercapnic acidification reveals the adaptability of organisms that have evolved a tolerance for high internal or external PCO_2. There is much more to be understood about the effects of HA on organisms adapted to high PCO_2, either from the demands of photosynthetic symbioses or in environments such as estuaries, intertidal zones and O_2 minimum zones where high PCO_2 and reduced pH are commonplace. As an intertidal resident, *A. elegantissima* possesses the physiological and behavioral means to thrive in a highly variable environment. The results of this study suggest that *A. elegantissima* can tolerate and even benefit from moderate levels of hypercapnic acidification and indicates the adaptation of *A. elegantissima* to the broad range of PCO_2 and pH characteristic of its intertidal habitat.

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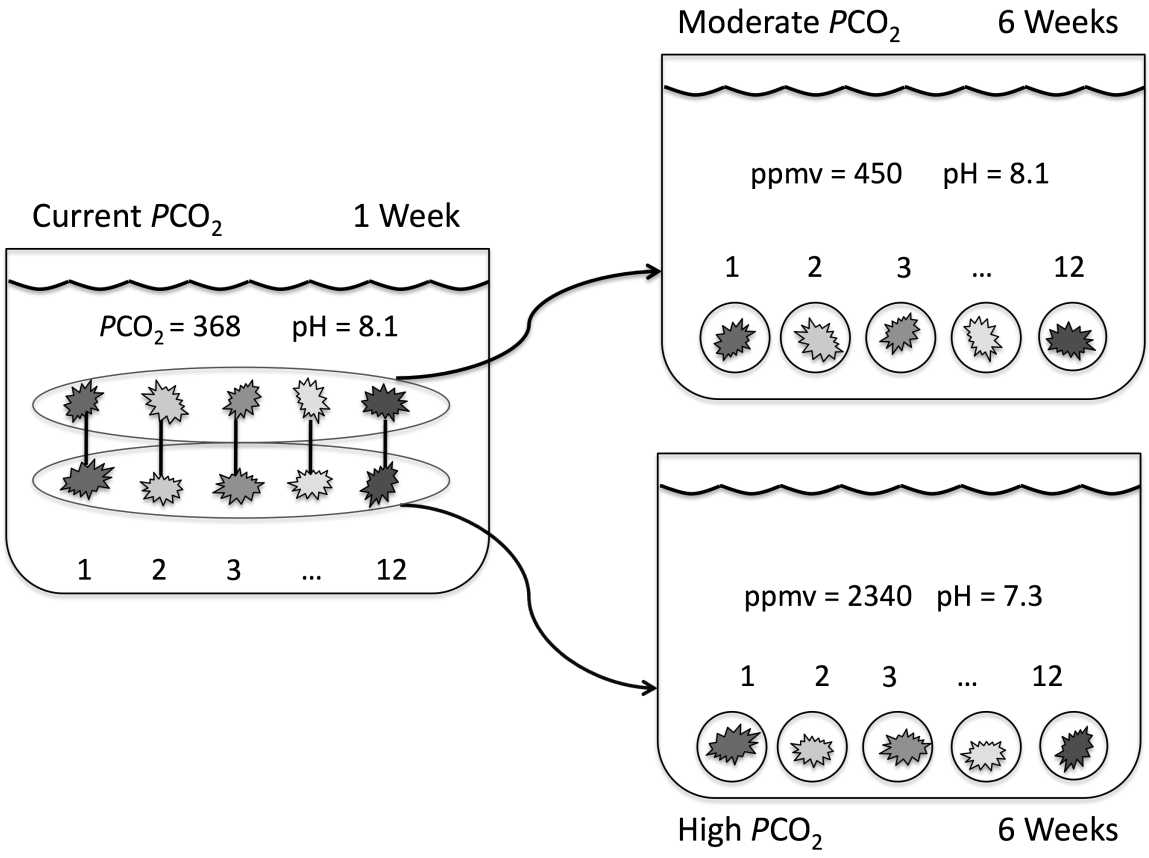

Figure 1. Schematic of experimental set-up. Clonemates were collected from twelve different populations of *Anthopleura elegantissima* from the intertidal zone of the eastern North Pacific Ocean at Point Grenville, Washington. Upon return to the laboratory, specimens were maintained in the same aquarium at PCO_2 conditions of 368 ppmv. After one week, rates of photosynthesis and respiration were measured and clonemates were separated into individual respiration chambers and maintained in aquaria with moderate and high PCO_2 conditions (450 and 2340 ppmv, respectively) for six weeks.

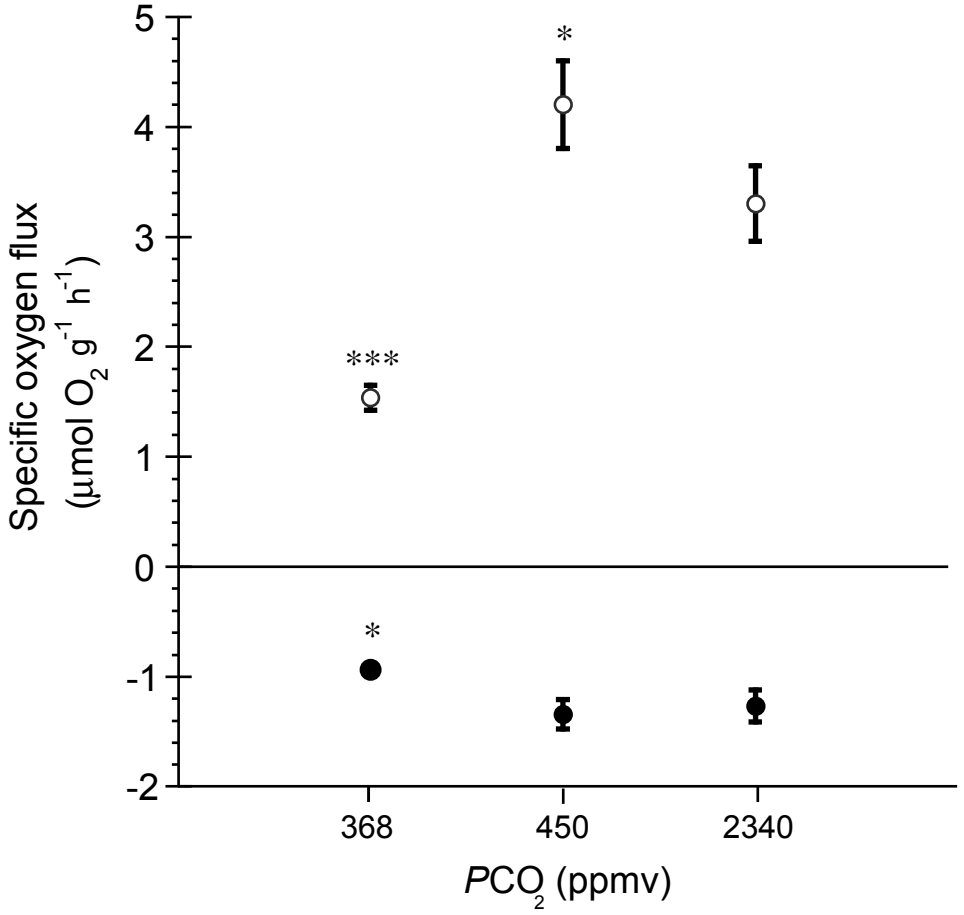

Figure 2. Mean mass-specific rates of gross photosynthesis (positive oxygen flux) and respiration (negative oxygen flux) in *Anthopleura elegantissima* at PCO_2 of 368 ppmv (n = 22), 450 ppmv (n = 12) and 2340 ppmv (n = 12). Error bars represent ± one standard error. Significant differences in the photosynthetic rate between 450 and 2340 ppmv (paired t-test; *: $p < 0.05$). Highly significant differences between the photosynthetic rate at 368 ppmv and the other 2 conditions (paired t-test; ***: $p < 0.001$). Significant differences between the respiratory rate at 368 ppmv and the other 2 conditions (paired t-test; *: $p < 0.05$).

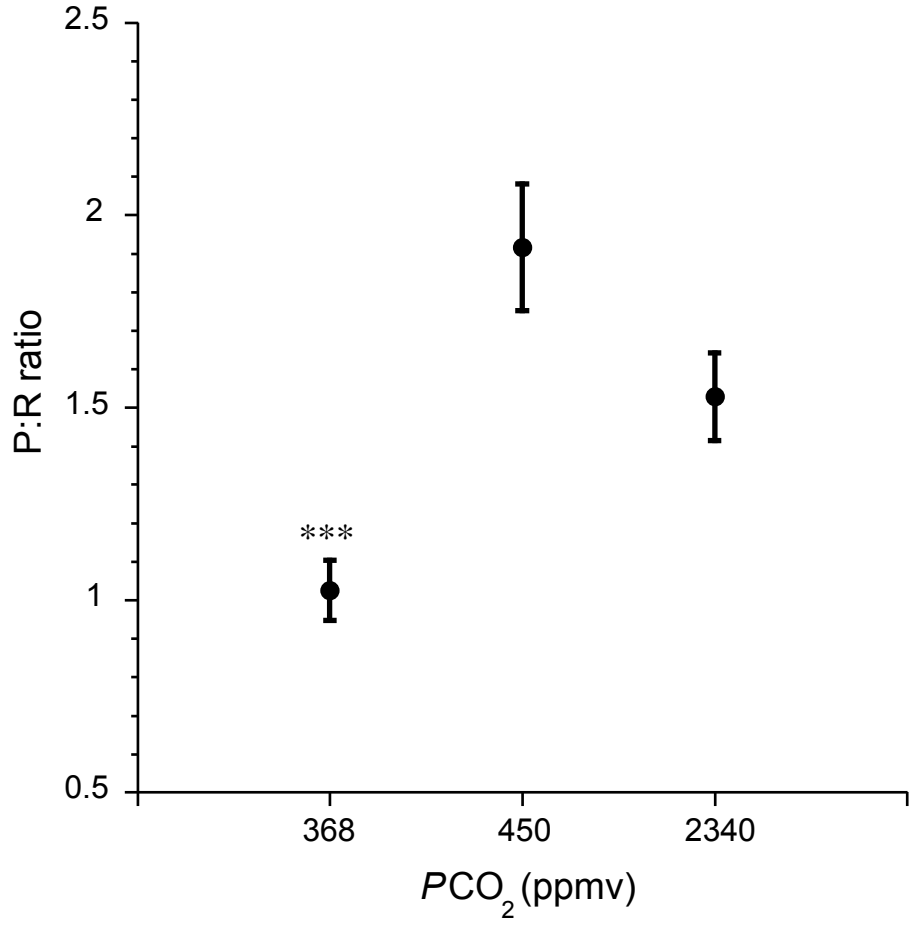

Figure 3. The ratio of photosynthesis to respiration of *Anthopleura elegantissima* at PCO_2 of 368 ppmv (n = 22), 450 ppmv (n = 12), and 2340 ppmv (n = 12). Error bars represent ± one standard error. Highly significant differenes between ratios at PCO_2 368 and the other two conditions (paired t-test; ***: $p<0.001$).

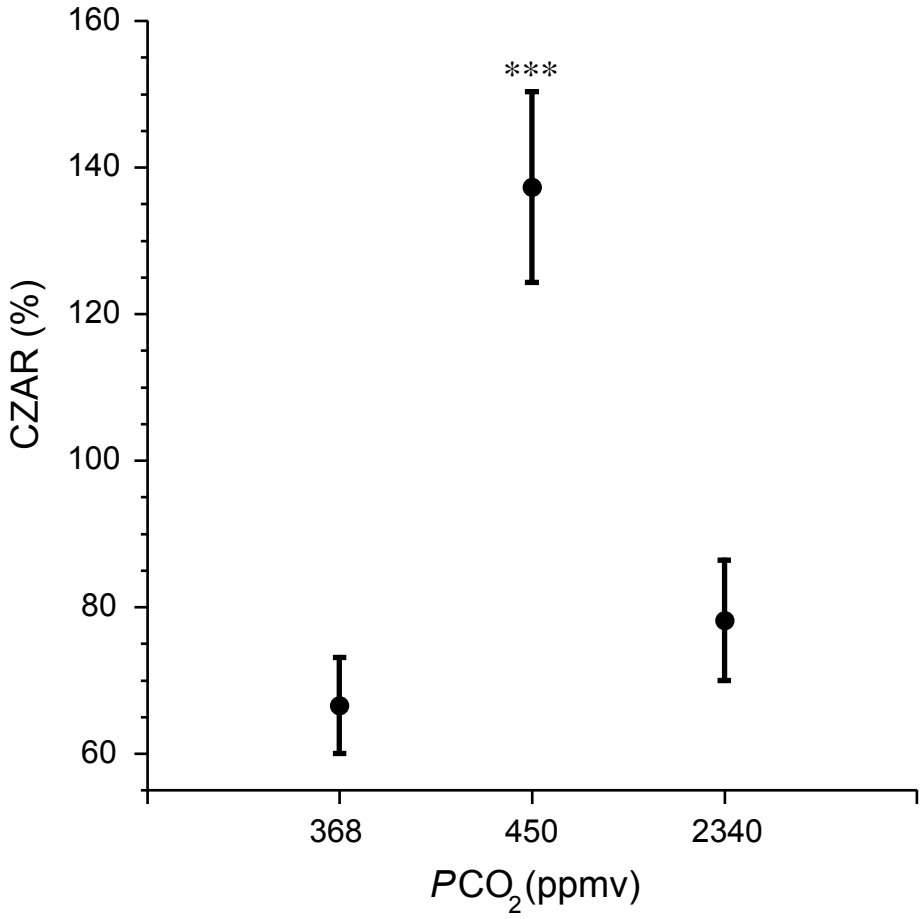

Figure 4. The potential contribution of carbon by zooxanthellae to the animal's respiratory carbon requirements (CZAR) in the *Anthopleura elegantissima* symbiosis at PCO_2 of 368 ppmv (n = 22), 450 ppmv (n = 12) and 2340 ppmv (n = 12). Error bars represent ± one standard error. Highly significant differences between CZAR at PCO_2 450 ppmv and the other two conditions (paired t-test ***: $p<0.001$).

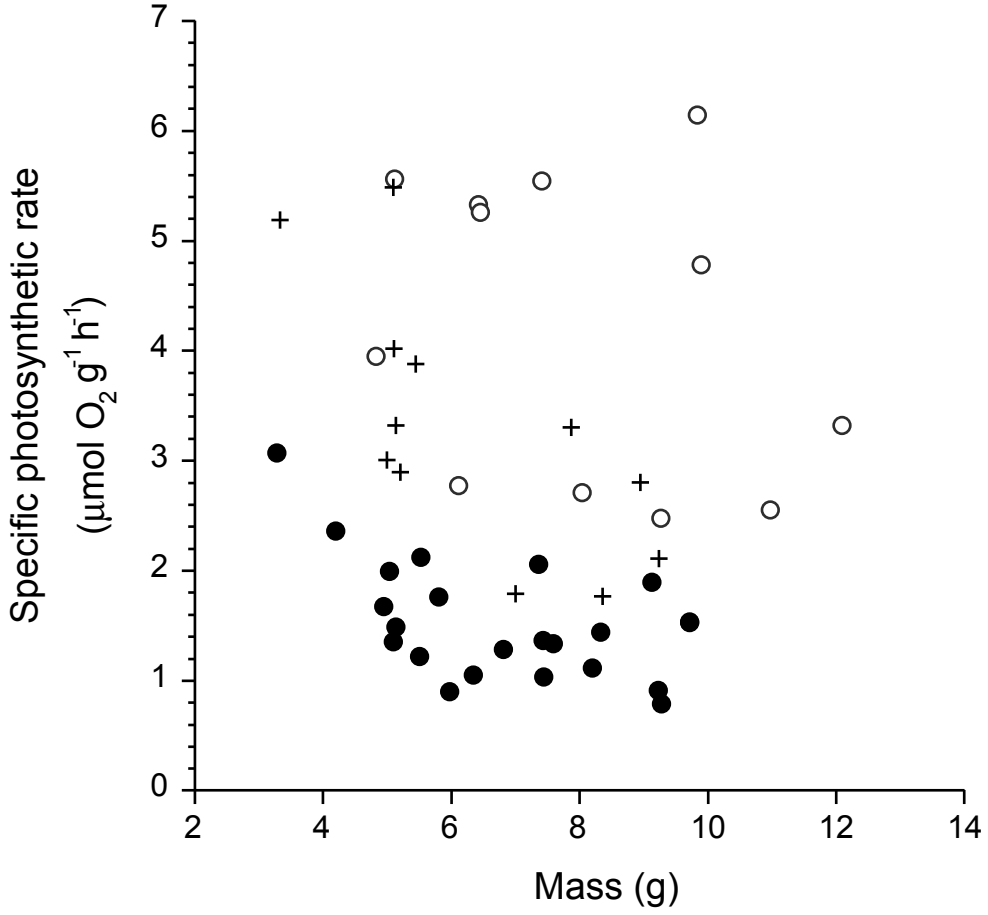

Figure 5. Mass-specific rate of photosynthesis of *Anthopleura elegantissima* at PCO_2 of 368 ppmv (●, n = 22), 450 ppmv (○, n = 12) and 2340 ppmv (+, n = 12) in relation to body wet weight. There was no significant effect of body weight on photosynthetic rates (linear regression analysis, $p > 0.05$).

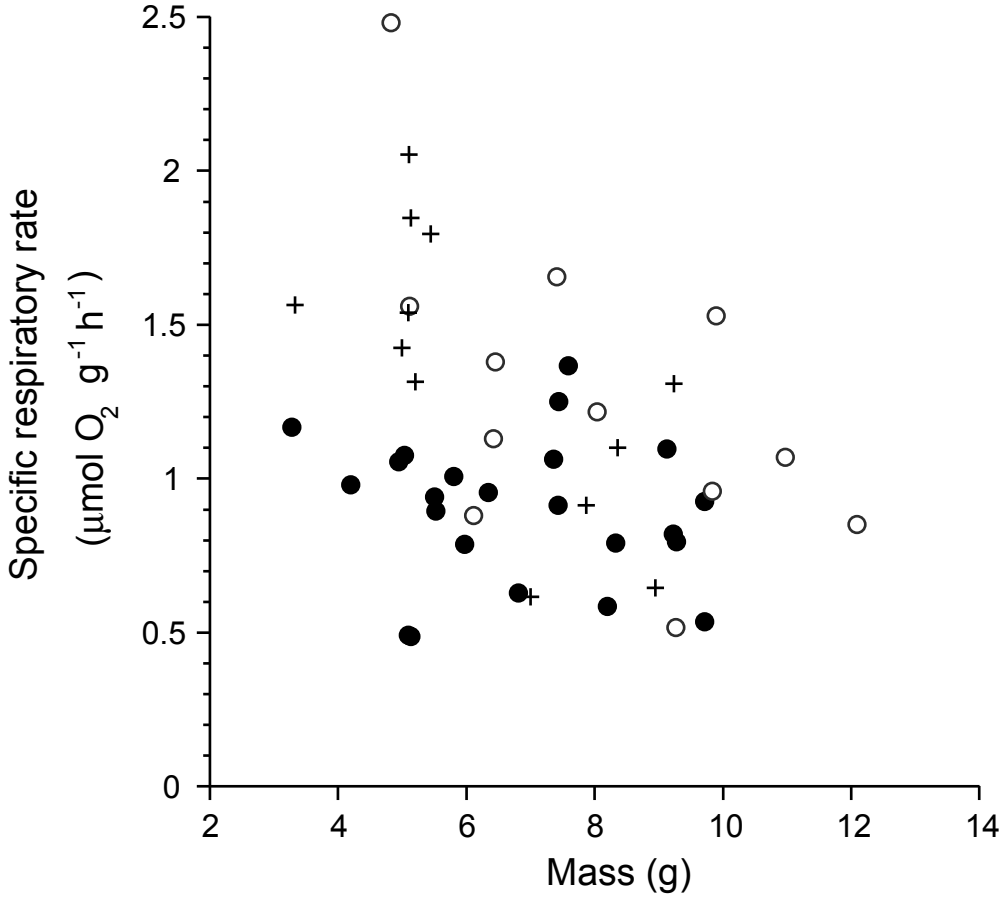

Figure 6. Mass-specific rate of respiration of *Anthopleura elegantissima* at PCO_2 of 368 ppmv (●, n = 22), 450 ppmv (○, n = 12) and 2340 ppmv (+, n = 12) in relation to body wet weight. There was no significant effect of body weight on photosynthetic rates (linear regression analysis; $p > 0.05$).

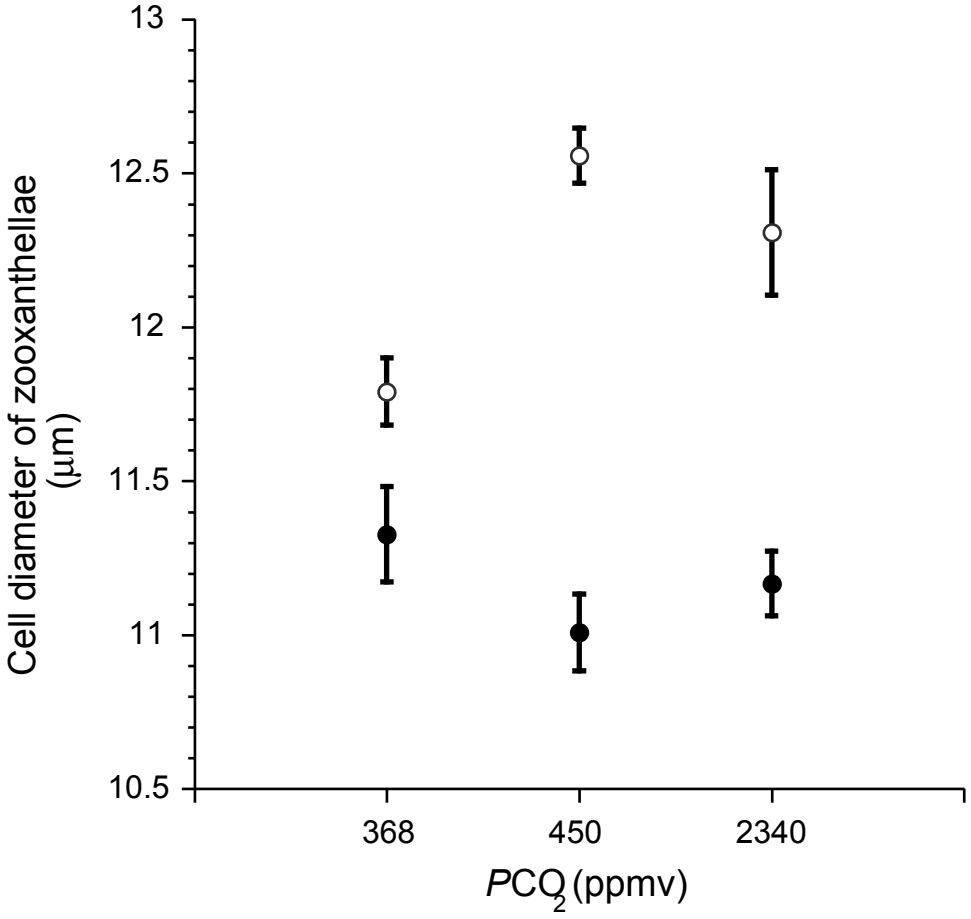

Fig. 7. Zooxanthellal (ZX) cell diameter of *Anthopleura elegantissima* at PCO_2 of 368 ppmv, 450 ppmv, and 2192 ppmv in tentacles (○, n = 11) and oral disk with intact tentacles (●, n = 11). Error bars represent ± one standard error. Highly significant differences between cell diameter of tentacles at PCO_2 368 ppmv and the other two conditions (paired t-test; ***: $p < 0.001$). Cell diameter of tentacle samples are significantly different from oral disk and tentacle samples in all cases (paired t-test, $p<0.01$).

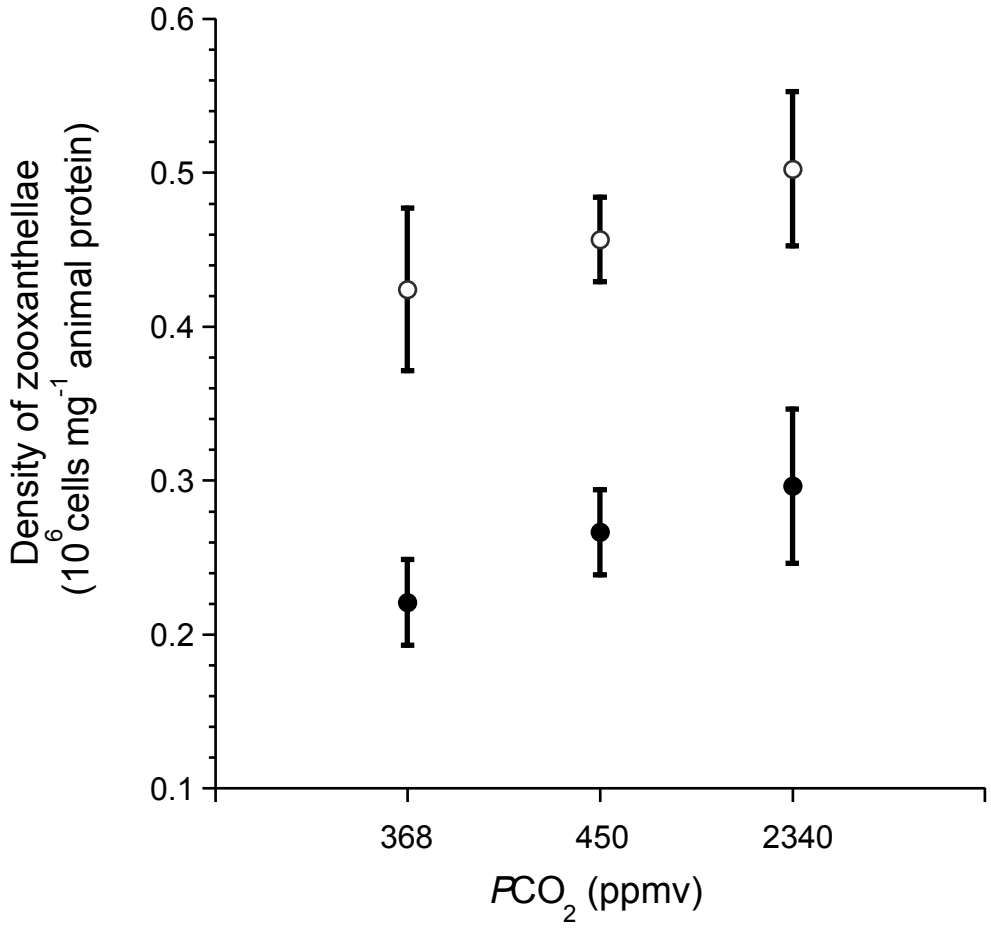

Fig. 8. Zooxanthellal (ZX) density of *Anthopleura elegantissima* at PCO_2 of 368 ppmv, 450 ppmv, and 2340 ppmv in tentacles (●, n = 11) and oral disk with intact tentacles (○, n = 11). No significant difference between any conditions (ANOVA ≥ 0.27). Error bars represent ± one standard error; ZX density in tentacle samples are significantly different from oral disk and tentacle samples in all cases (paired t-test, $p \leq 0.01$).

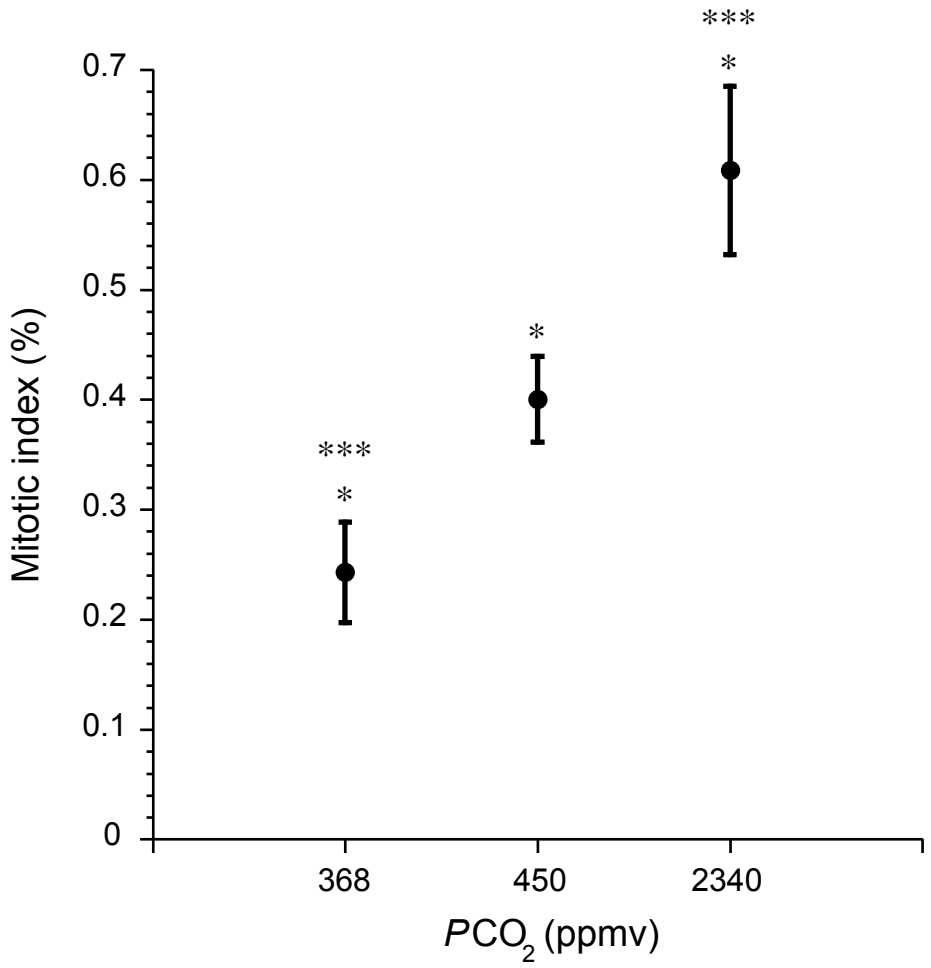

Figure 9. Mitotic index of zooxanthellae in *Anthopleura elegantissima* oral disk with tentacles at PCO_2 concentrations of 368 ppmv (n=11), 450 ppmv (n=11) and 2340 ppmv (n=12). Error bars represent ± one standard error. Significant differences between MI at PCO_2 450 ppmv and the other two conditions (ANOVA; *: $p<0.05$) and highly significant differences between PCO_2 368 and 2340 (ANOVA ; ***: $p<0.001$).

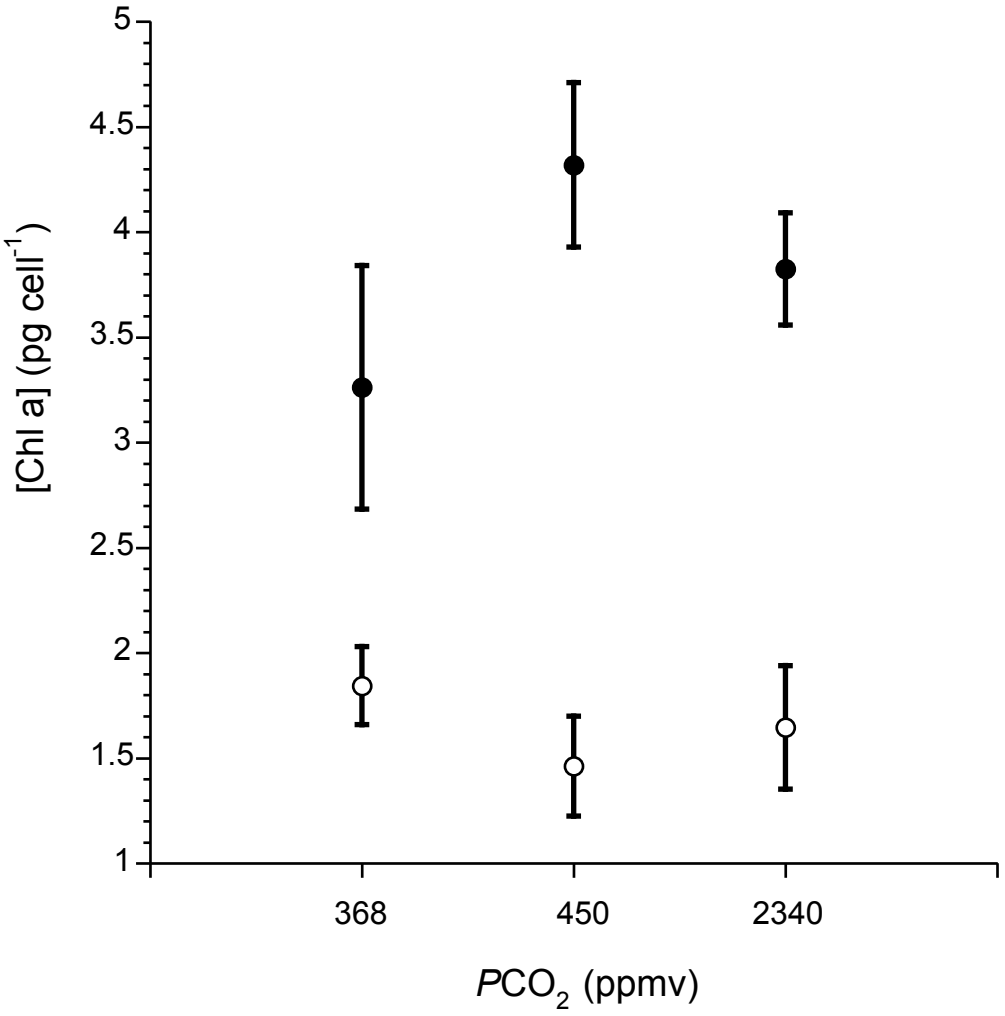

Figure 10. Chlorophyll *a* concentrations of zooxanthellae in *Anthopleura elegantissima* at PCO_2 concentrations of 368 ppmv (n=12), 450 ppmv (n=11) and 2340 ppmv (n=11) in tentacles (●, n = 11) and oral disk with intact tentacles (○, n = 11). Error bars represent ± one standard error. No significant difference between any conditions (ANOVA, $p \geq 0.10$). Chl *a* in tentacle samples are significantly different from oral disk and tentacle samples in all cases (ANOVA, $p<0.01$).

Table 1. Carbonate parameters of aquaria to investigate effects of hypercapnic acidification on *Anthopleura elegantissima*. NBS: National Bureau of Standards, ANC: acid neutralizing capacity, PCO_2: partial pressure of carbon dioxide. Values are mean ± SD.

	pH_{NBS} ± SE	ANC ± SE (μmol/kg SW)	PCO_2 ± SE (ppmv)
Current PCO_2 (n=2)	8.10 ± 0.02	2403	368
Moderate PCO_2 (n=14)	8.08 ± 0.04	2566 ± 120	448 ± 48
High PCO_2 (n=14)	7.35 ± 0.02	2119 ± 43	2342 ± 151

Table 2. Oxygen and carbon flux in *Anthopleura elegantissima* at PCO_2 of 368, 450 and 2340 ppmv. CZAR: percent of carbon provided by zooxanthellae to animal respiration.

Parameter: Mean ± SE	PCO_2 (ppmv)		
	Current - 386 n=23	Moderate - 450 n=11	High - 2340 n=12
Photosynthetic rate- Gross μmol O_2 g wet weight^{-1} h^{-1}	1.53 ± 0.11	3.30 ± 0.35	4.20 ± 0.40
Photosynthetic rate- Net μmol O_2 g wet weight^{-1} h^{-1}	0.69 ± 0.08	2.86 ± 0.31	2.03 ± 0.23
Respiratory rate μmol O_2 g wet weight^{-1} h^{-1}	0.94 ± 0.05	1.34 ± 0.13	1.27 ± 0.15
Gross Photosynthesis: Respiration	1.02 ± 0.08	1.92 ± 0.16	1.53 ± 0.11
Czar (%)	66.6 ± 6.6	137.3 ± 13.0	78.2 ± 8.2

Table 3. Biomass and growth parameters of zooxanthellae *Symbiodinium muscatenei* (Zx) exposured to PCO_2 368, 450 and 2340 ppmv. OD (oral disk with intact tentacles) values were after 6 weeks of exposure to experimental conditions. Tentacles were measured after 3 weeks of exposure. Values are pooled where there are no significant differences in mean.

	PCO_2 (ppmv)			
Parameter: Mean ± SE (n)	Current- 386	Moderate- 450	High- 2340	Pooled
Mitotic Index- % Zx cells dividing				
OD	0.24 ± 0.05 (11)	0.40 ± 0.04 (11)	0.61 ± 0.08 (12)	
Tentacles	no data	0.48 ± 0.04 (11)	1.20 ± 0.27 (12)	
Zx cell diameter- μm				
OD	11.33 ± 0.16 (11)	11.17 ± 0.11 (12)	11.01 ± 0.43 (12)	11.16 ± 0.08 (35)
Tentacles	11.79 ± 0.11 (22)	12.31 ± 0.20 (12)	12.56 ± 0.09 (12)	12.43 ± 0.11 (46)
Zx cell density-10^6 cells mg protein^{-1}				
OD	0.22 ± 0.03 (12)	0.29 ± 0.05 (12)	0.27 ± 0.03 (11)	0.26 ± 0.02 (35)
Tentacles	0.48 ± 0.06 (22)	0.50 ± 0.04 (12)	0.46 ± 0.05 (12)	0.48 ± 0.03 (46)
Chlorophyll *a*- pg ZX cell^{-1}				
OD	3.26 ± 0.58 (11)	4.32 ± 0.39 (11)	3.83 ± 0.27 (12)	3.80 ± 0.25 (34)
Tentacles	1.85 ± 0.25 (23)	1.46 ± 0.24 (12)	1.65 ± 0.29 (12)	1.70 ± 0.13 (47)

Appendix I. Abbreviations

C	carbon
$CaCO_2$	calcium carbonate
CO^{-2}_3	carbonate
CO_2	carbon dioxide
CZAR	contribution of zooxanthellae to animal respiration as % carbon
C_μ	carbon-specific growth rate
DI	de-ionized water
f	fraction of cells in the division phase
h	hour
H_2SO_4	sulfuric acid
HA	hypercapnic acidification
HCl	hydrochloric acid
HCO^-_3	bicarbonate
IPCC	Intergovernmental Panel on climate Change
L	liter
mg	milligram
$MgCO_3$	magnesium carbonate
MI	mitotic index
mL	milliliter
$\dot{M}O_2$	hourly mass-specific respiration rate
N	normal (one gram equivalent of a solute per liter of solution)
NaOH	sodium hydroxide
O_2	oxygen
OD	oral disk and tentacles
PCO_2	partial pressure of carbon dioxide
pg	picogram
$\dot{P}_g$	hourly mass-specific photosynthetic rate (O_2)
P_g^O	hourly gross PS rate (O_2)
ppmv	parts per million by volume
PQ_Z	photosynthetic quotient of zooxanthellae
$\dot{P}_g$: MO_2	ratio of daily gross photosynthetic rate to respiration rate
PS	photosynthesis, photosynthetic
psu	practical salinity units
R	respiration, respiratory
R_{ae}^O	daily respiratory rate of the anemone (combined animal and zooxanthellae)
RQ_{ae}	respiratory quotient of the anemone (combined animal and zooxanthellae)
RQ_{al}	respiratory quotient of the animal
RQ_z	respiratory quotient of zooxanthellae
SD	standard deviation
SE	standard error
SS	standing stock
ß and 1- ß	animal and algal proteins expressed as fractions of total protein, respectively
t_d	duration of cytokinesis
USGS	United States Geological Service
ZX	zooxanthellae, zooxanthellal
μ_z	specific growth rate of zooxanthellae
Ω $CaCO_3$	saturation state of calcium carbonate