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Partner preference in the intertidal: possible benefits of ocean acidification to sea anemone-algal symbiosis

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By

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Natalie Coleman

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A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Natalie Coleman
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Abstract

Ocean acidification (OA) threatens many marine species and is projected to become more severe over the next 50 years. Areas of the Salish Sea and Puget Sound that experience seasonal upwelling of low pH water are particularly susceptible to even lower pH conditions. While ocean acidification literature often describes negative impacts to calcifying organisms, including economically important shellfish, and zooplankton, not all marine species appear to be threatened by OA. Photosynthesizing organisms, in particular, may benefit from increased levels of CO_2.

The aggregating anemone (*Anthopleura elegantissima*), a common intertidal organism throughout the northeast Pacific, hosts two photosynthetic symbionts: *Symbiodinium muscatinei* (a dinoflagellate) and *Elliptochloris marina* (a chlorophyte). The holobiont, therefore, consists of both a cnidarian host and a photosymbiont that could be affected differently by the changing levels of environmental CO_2. To determine the effects of OA on this important marine organism, *A. elegantissima* in each of four symbiotic conditions (hosting *S. muscatinei*, hosting *E. marina*, hosting mixed symbiont assemblages, or symbiont free) were subjected to one of three pCO_2 levels (800 ppm, 1200 ppm, or 1800 ppm) of OA for 10 weeks. At regular intervals, gross photosynthesis and density of the symbionts, respiration rate of the hosts, levels of reactive oxygen species (ROS) in the host, and percent of organic carbon received by the host from the symbiont (CZAR) were measured. Over the 10-week period of the experiment, the densities of symbionts responded differently to an increase in pCO_2, increasing in anemones hosting *S. muscatinei* but decreasing for those hosting *E. marina.* Similarly, anemones of mixed symbiont complement that started with approximately 50% of each symbiont type shifted toward a higher percentage of *S. muscatinei* with higher pCO_2. Both gross photosynthesis and dark respiration were significantly affected by pCO_2 and symbiont state, though we cannot say that the symbionts

responded differently to increased OA. Symbiont state was a significant predictor for ROS concentration, with greatest levels seen in anemones hosting *E. marina* and for CZAR score, with greatest levels in anemones hosting *S. muscatinei*, our linear models did not reveal pCO_2 as a significant factor in these responses. Together, these results suggest that *S. muscatinei* may benefit from elevated pCO_2 levels and that *A. elegantissima* hosting that symbiont may have a competitive advantage under some future scenarios of ocean acidification.

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Introduction

Since the industrial revolution, anthropogenic forces have increasingly altered Earth's ecosystems. One such force is the ever-increasing release of CO_2 into the atmosphere. Oceans cover over 70% of Earth's surface, providing a large surface area that absorbs much of that atmospheric CO_2 (Doney et al. 2009), which decreases the pH and increases the partial pressure of carbon dioxide (pCO_2) in seawater (Keeling et al. 1976). The diffusion of CO_2 into the ocean shifts the chemical equilibria of the carbonate system (pH, pCO_2, CO_3^{-2}, HCO_3^-), decreasing the pH, the saturation state of $CaCO_3$, and the available CO_3^{-2} ions in a process known as ocean acidification (Orr et al. 2005, Feely et al. 2010). Ocean acidification is a widespread problem affecting global marine and coastal habitats with differing degrees of severity.

Calcifying marine organisms build their shells and other structural elements from calcium carbonate ($CaCO_3$) and rely on high saturation states of carbonate ions (CO_3^{-2}) to do so (Orr et al. 2005). Ocean acidification not only dissolves the hard structures of calcifying organisms, but also, due to the lower availability of CO_3^{-2}, makes it more difficult for the organisms to build calcium carbonate structures without a major energy expense. Thus, OA poses a significant threat to calcifying organisms like corals (Langdon and Atkinson 2005), mollusks (Doney et al. 2009), and some algae and plankton (Orr et al. 2005; Wooten et al. 2008; Byrne and Przeslawski 2013; Kroeker et al. 2013). However, ocean acidification may not threaten all marine species.

Photosynthetic organisms may benefit from the increased levels of pCO_2 that lead to ocean acidification. Several studies have shown that marine micro- and macroalgae and seagrasses have greater photosynthetic activity when exposed to levels of pCO_2 higher than the average ambient levels in their environments (Zimmerman 1997, Palacios and Zimmerman 2007, Alexandre et al. 2012, Tan et al. 2019). And, seagrasses and other marine plants can utilize the increased levels of HCO_3^- provided by OA as an inorganic carbon source for photosynthesis

(Alexandre et al. 2012). The current levels of dissolved inorganic carbon (DIC) in the oceans are generally not high enough to saturate photosynthesis for most species, resulting in higher photosynthetic rates in areas of low pH and high DIC (Invers et al. 2001, Koch et. al. 2013). Marine plants, however, are not the only organisms that may be able to utilize the higher HCO_3^- concentration in low pH water for a photosynthetic benefit.

Cnidarians, including many corals and anemones, can form symbiotic relationships with photosynthetic algae. The symbionts provide the host with oxygen and fixed organic carbon and benefit from host-derived nutrients (Engebretson and Muller-Parker 1999, LaJeunesse and Trench 2000, Verde and McCloskey 1996) and are likely to benefit from higher pCO_2. For example, Brading et al. (2011) showed that two species of *Symbiodinium,* when cultured outside their coral host, had increased photosynthetic rates and growth rates under higher pCO_2 (1100 ppm) conditions. Another study of *Symbiodinium* in corals (Crawley et al. 2010) found that photosynthetic capacity increased under increased pCO_2 conditions (700 ppm) but decreased under more pessimistic pCO_2 projections (1500 ppm).

While it is clear that some photosymbionts may benefit from some OA conditions, it is less clear whether those benefits will translate to the animal host. Langdon and Atkinson (2005) found that an increase in pCO_2 increased the translocation of carbon from zooxanthellae symbionts to their host coral, but the lower pH led to breakdown of the coral's $CaCO_3$ skeleton. Suggett et al. (2012), studying symbiotic sea anemones within a natural pCO_2 gradient near an undersea volcano in Italy, found that the anemone *Anemonia viridis* increased in abundance and size in higher pCO_2 waters due to increased photosynthesis and translocation from their symbiotic S*ymbiodinium*. A recent laboratory study on the temperate sea anemone *Anthopleura elegantissima* found that their photosymbiont *Symbiodinium muscatinei* had a higher growth rate

and higher rate of photosynthesis in waters with a lower pH and higher pCO_2 (2200 ppm), suggesting these intertidal anemones might benefit from increased pCO_2 (Towanda and Thuesen 2012).

A. elegantissima is the most abundant intertidal anemone along the west coast of North America and can inhabit mid-intertidal to subtidal waters from Baja, California to British Columbia (Kozloff 1983). Populations exist in aggregations of genetically identical clones that can reproduce asexually by fission or sexually by spawning sperm or eggs (Sebens 1982). This anemone is unusual in that it can host two very different photosymbionts: *Symbiodinium muscatinei* (LaJeunesse and Trench 2000), which are dinoflagellates also called zooxanthellae and *Elliptochloris marina* (Letsch et al. 2009), which are chlorophyte algae also called zoochlorellae.

The abundance of these two symbionts within *A. elegantissima* varies with abiotic factors including latitude, intertidal height, temperature, and light (LaJeunesse and Trench 2000, Secord and Augustine 2000). Anemones hosting *S. muscatinei* dominate in high light, higher intertidal, and warmer environments. Anemones hosting *E. marina* tend to occur in low light, low intertidal, high latitude, and colder habitats (Secord and Augustine 2000). *A. elegantissima* can host either symbiont, both symbionts at once (creating "mixed" individuals) or can exist symbiont-free. Such aposymbiotic individuals ("apos") are generally found in very shaded environments (Fitt and Pardy 1981, Baker 2003, Dimond et al. 2011, Hiebert and Bingham 2012). Symbiotic *A. elegantissima* comprise a significant percent of the biomass in some intertidal communities, contributing to high rate of primary production there (Fitt et al. 1982).

Environmental conditions influence the complement of symbionts *A. elegantissima* host and the symbiont complement influences how the anemones reproduce. Bingham et al. (2014)

showed that *A. elegantissima* hosting *S. muscatinei* are more likely to reproduce asexually through fission while those hosting *E. marina* primarily reproduce sexually. A shift to a symbiont complement dominated by *S. muscatinei* could, therefore, lower genetic diversity, with asexually reproducing populations creating monocultures. Populations with less genetic diversity are likely to be less resilient to environmental catastrophes or pathogens, indicating that the symbiont complement could influence survivorship of *A. elegantissima* populations (Hughes and Stachowicz 2004).

Given that symbiont identity can impact reproductive strategy of these anemones, it is interesting to note that the relative abundances of these two symbionts individual anemones can shift in individual *A. elegantissima* in response to environmental changes (Baker 2003, Dimond et al. 2013). Increased temperature and irradiance benefit *S. muscatinei* over the less thermotolerant *E. marina*, but the relative effect of pH and pCO_2 on the two symbionts is not well understood (Dimond et al. 2013). While *A. elegantissima* can host both symbiont species at the same time, laboratory studies suggest that a mixed symbiont complement is an unstable equilibrium with symbiotic condition over time tending toward dominance by one or the other symbiont (Saunders and Muller-Parker 1997).

One metric that measures the competitive advantage of *A. elegantissima* hosting one symbiont type or another is the CZAR score (contribution of zooxanthellae or zoochlorellae to animal respiration). CZAR is the amount of the total carbon fixed by the symbiont that is used by the host for respiration. A higher CZAR score signifies that the anemones rely less on heterotrophic feeding due to input of carbon from the symbiont (Shick and Dykens 1984). Temperate corals show CZAR scores of up to 95%, with the score increasing with irradiance until photoinhibition occurs at extremely high light levels (Muscatine et al. 1984, Ferrier-Pages

et al. 2014, Hawkins et al. 2016). CZAR score can also be influenced by temperature (Gibbons 2008) and can differ among *Symbiodinium* species (Starzark et al. 2014).

Anthopleura species that host *Symbiodinium* show CZAR scores of 98% and above under some conditions (Muscatine et al. 1981, 1983; Gibbons 2008), and Verde and McCloskey (2007) found that *A. elegantissima* hosting *S. muscatinei* had a consistently higher CZAR score than those hosting *E. marina*, suggesting that, energetically, *E. marina* may be a poorer symbiotic partner. Studying potential effects of changing ocean pCO_2, Towanda and Theusen (2012) found that photosynthetic rate, CZAR, and respiration all increased when *A. elegantissima* hosting *S. muscatinei* were exposed to increased pCO_2 (up to 2340 ppm). To date, no such tests have been done with *A. elegantissima* hosting *E. marina* or living with mixed symbiont complements.

While it appears that anemones hosting *S. muscatinei* reap certain benefits from higher pCO_2, there are potential negative implications of living in these conditions. For example, high photosynthetic rates of the symbionts can create a hyperoxic environment within the host, producing singlet oxygen and hydrogen peroxide (reactive oxygen species or ROS) as byproducts of photosynthesis (Harland and Davies 1995, Lesser 2006). The hyperoxic environment and ROS can reduce photosynthetic efficiency and increase respiration rates of the host, effects that can be exacerbated by other forms of stress (Asada and Takahashi 1987, Harland and Davies 1995, Laloi and Havaux 2015). Accessory pigments in chloroplasts, including carotenoids, normally dissipate excess light energy as heat by converting triplet chlorophyll through a series of conformational changes in the xanthophyll cycle, a form of nonphotochemical quenching (NPQ) (Miki et al. 1994). When these and other enzyme-based degradation systems become overwhelmed, ROS levels rise (Harland and Davies 1995; Dimond et al. 2017). Intracellular pH, which is affected by pCO_2 levels, affects the state of the ROS

species (i.e., protonated or unprotonated), changing their concentrations and biological impacts (Dykens et al. 1992). Combined stresses on the symbiont and their host can compromise this mutualistic relationship, leading the host to expel the symbionts in a phenomenon known as bleaching (Dykens and Shick 1982; Dykens et al. 1992; McCloskey et al. 1996; Hainey 2008; Suggett et al. 2008; Fransolet 2013, Wooldridge 2014).

Dimond et al. (2017) demonstrated that, under high light conditions, the more productive *S. muscatinei* create a significantly greater burden of reactive oxygen species (ROS) for the *A. elegantissima* host than do the less productive *E. marina*. If individuals hosting *S. muscatinei* face ROS burdens that outweigh any benefit received from higher photosynthetic rates, their symbiont complements could shift toward *E. marina*, with potential impacts on the reproductive strategy of the *A. elegantissima*. This differential sensitivity of the symbionts and the flexibility of this temperate symbiotic relationship thus provides an outstanding opportunity to study the potential ecological impact of OA.

My research objective was to expose *A. elegantissima* in each of four symbiotic conditions (hosting *S. muscatinei*, hosting *E. marina*, hosting mixed symbiont assemblages, or symbiont free) to elevated pCO_2 treatments to determine:

1. How OA affects photosynthetic processes of the symbionts and respiration of the host.
2. Whether symbiont complement affects CZAR or levels of ROS.
3. Whether competitive exclusion or symbiont shifting occurs between *S. muscatinei* and *E. marina* under OA conditions.

While several studies have focused on the effects of increased temperature and light on *A. elegantissima* symbiosis, this study is the first to compare effects of pCO_2 on multiple

symbiotic states with the goal of understanding how this important symbiotic system might respond to changing ocean conditions.

Materials and Methods

Anemone Collection and Experimental Setup

Eighteen individual *A. elegantissima* of each symbiont state, hereafter referred to as "brown" (hosting *S. muscatinei*), "green" (hosting *E. marina*), "mixed" (hosting a near 50:50 mix of *S. muscatinei* and *E. marina*), and "apo" (with extremely low or no symbionts), were collected from various microhabitats around Point Lawrence in the San Juan Islands (48°37'58.5"N, 122°47'13.3"W) and transported to the Shannon Point Science Center in Anacortes, WA. In the laboratory, a single tentacle was clipped from each anemone and examined via light microscopy to confirm the symbiotic state. Only anemones with approximately 50% of each symbiont type (+/- 5%) were used for the mixed group and the apo group included only anemones with <100 cells/mg of host protein. All anemones thus identified to symbiotic state were transferred into individual labeled glass watch glasses and acclimated for two weeks in an indoor, flow-through sea-table exposed to natural sunlight through large windows. Seawater temperature during the acclimation period was 13-14°C and ambient pCO_2 levels were 690-800 ppm.

An experimental system was set up in a modification of the design described by Jokiel et al. (2014). To create the experimental pCO_2 conditions, 18 50-L flow-through glass tanks were set up on an outdoor platform exposed to full sunlight. These tanks, which received running seawater input directly from the marine center seawater system, served as insulating jackets to maintain seawater temperature at ambient levels for the duration of the experiment. A 40 L

acrylic aquarium, in which a pCO_2 treatment was created, was placed inside each of the insulating jackets. Independent seawater lines fed ambient seawater directly through each of these aquaria.

Three pCO_2 treatments, with 6 replicate aquaria per treatment, were created by using peristaltic pumps to control the amount of CO_2 reaching the aquaria from a food-grade CO_2 tank. A length of silicone tubing from a CO_2 gas tank was split and directed to two peristaltic pumps set at different speeds. CO_2 at a pressure of 10 psi was bled from the source tank, moving through the peristaltic pumps at one of the two speeds, and bubbling into the aquaria. A submerged Aquanet powerhead pump in each tank ensured that the water and CO_2 were well mixed. The different rates of CO_2 delivery from the peristaltic pumps created six aquaria with a pCO_2 level of 1800 ppm and six with a level of 1200 ppm. The remaining six tanks received no bubbling, creating an ambient control with a pCO_2 of approximately 800 ppm (Fig. 1). The arrangement of the pCO_2 treatment aquaria on the platform was randomized.

To start the experiment, one arbitrarily chosen anemone in each of the four symbiotic conditions was placed in each of the aquaria (i.e., each aquarium held four *A. elegantissima,* one of each symbiotic states). The entire platform was then covered with a black nylon shade cloth to reduce PAR by approximately 30%. To control growth of fouling organisms in the experiment, the aquaria were carefully cleaned 1-2 times per week throughout the experiment. At each of four time points (initial, 3 weeks later, 6 weeks later, and after 10 weeks), two tentacles were taken from each anemone and homogenized with 1.0 mL filtered seawater using a tissue tearer to break animal cells but leave photosymbiont cells intact. This sample was then split in half, with one half immediately going to ROS processing and the other held for later protein and symbiont density analysis.

Monitoring of pCO_2 levels in the experiment

To ensure the experimental pCO_2 levels remained near their desired levels, measurements of DIC were taken at 3 to 13-day intervals from each of the 18 aquaria throughout the 10-week experiment. Sterile syringes with attached 45μm filters were used to collect 20 mL samples from the center of the aquaria. The sample was used to fill 20-ml scintillation vials from the bottom up with no head space, overflowing until the volume of the vile was replaced one full time, being careful to minimize exposure of the sample to air. The samples were either run immediately on an Agilent DIC analyzer or were poisoned with 20 μL $HgCl_2$ to stop all respiration and photosynthesis. The poisoned samples were refrigerated at 5°C and analyzed for DIC content within a week of collection. pCO_2 was then calculated from DIC using CO2SYS (Pierrot et al. 2006) with K1 and K2 equilibrium constants from Mehrbach et al. (1973) and refit from Dickson and Millero (1987). Using the m-cresol method modified by Dickson et al. (2007), we measured the pH of our samples on the same day DIC was measured by filling a 5 cm triple-rinsed cuvette with a seawater sample using a syringe. After a baseline spectrum was taken, 30 μL of m-cresol dye was added, and a second spectrum collected, both using an Ocean Optics FlameS-UV-VIS spectrophotometer.

To document day-to-night fluctuation in pH of the system, we continuously monitored pH for 11 hours during the fifth week of the experiment using an Orion Star A121 portable electronic glass electrode tip pH meter with a sensor placed in two randomly chosen aquaria in each of the three pCO_2 treatments. This same portable pH probe was used to spot check pH in all the aquaria twice a day, three times a week to ensure system conditions were not changing. To ensure accuracy of the measurements, the pH probe was recalibrated between the pCO_2

treatments with NHS buffer solutions of pH 4, 7, and 10. Photosynthetically active radiation (PAR) was measured four times a week throughout the day with a Biospherical Instruments QSL-100 4π quantum sensor. This was done to ensure irradiance was similar in all treatment aquaria throughout the day. Average ambient PAR levels over the course of the experiment were estimated from data collected by the Padilla Bay National Estuarine Research Reserve at a sensor located 29 kilometers away from the experimental site (http://nerr.noaa.gov).

Reactive oxygen species measurements

To measure ROS production of the anemones and their symbionts under each of the treatment conditions, the homogenized tentacle samples were dosed with Amplex Red fluorescent probe (Molecular Probes, Eugene, Oregon) to obtain a final concentration of 100μM. The samples were then incubated in a temperature-controlled photosynthetron (OHPT Inc., Lewes, Delaware) at 100 μmol quanta m^{-2} s^{-1} for thirty minutes using a rose-colored light filter to exclude wavelengths that cause Amplex Red photobleaching. The supernatant from each sample was then placed in a 96-well microplate with 0 to 10μ H_2O_2 standards and absorbance was read on a microplate reader according to methods from Dimond et al. (2017). The hydrogen peroxide (an ROS) concentration in each sample was calculated using a standard curve for each run calculated from the H_2O_2 standards. All tentacle samples were collected at a similar time of day (12 PM-2 PM) to control for differences in ROS that can occur throughout a 24-hour cycle due to changes in photosynthetic activity and irradiance.

Measurements of respiration and gross photosynthesis

To obtain a measure of the gross photosynthesis of the symbionts and respiration of the *A. elegantissima* hosts in different symbiotic states and under different experimental pCO_2 treatments, Presens Oxygen spot P3 sensors were fixed to the inside of 500 mL quartz jars using aquarium-grade silicone. A magnetic stir bar was placed in the bottom of each jar beneath a round, perforated Plexiglas stand upon which an anemone in its watch glass was placed (Fig. 2A). The jars were filled with UV-sterilized, filtered seawater then placed in a shallow flow-through tank, which maintained ambient seawater temperature during incubations. The flow-through tank sat on a large outdoor stir table that accommodated eight anemones in their jars and a UV-sterilized seawater "blank" jar for correction of background microbial respiration. (Fig. 2B). The runs were conducted during the same time period on each sampling day (10am - 2pm).

To start the analysis, we measured the oxygen concentration in each jar via the sensor spots then covered the entire setup with a blackout tarp that eliminated all light. After one hour, the oxygen concentration was measured in each darkened jar to permit later calculation of dark respiration. We then removed the tarp, exposing the anemones in their sealed jars to one hour of natural sunlight then took a final oxygen measurement. This allowed us to calculate respiration and photosynthesis in the light. To measure respiration of all 64 anemones, multiple runs had to be spread over three days at each sampling interval with the anemones from the experiment randomly assigned to each run. To permit comparison of those samples, it was critical that light exposure remained as consistent as possible not only within a set of trials, but also across multiple trials during the 10-week experiment. We achieved this by adding or removing layers of window screen as necessary to ensure the PAR stayed near 1500 $\mu mol/m^{-2}/S^{-1}$ during the light incubation, enough to saturate photosynthesis but minimize or eliminate photorespiration in the

symbiotic individuals. All PAR measurements were made with a Biospherical Instruments QSL-100 4π quantum wand sensor.

Data from the oxygen sensors in each sealed jar were collected from outside the jars with a Presens fiberoptic sensor reader. This was done before the dark incubation, after the dark incubation, and after the light incubation, allowing us to calculate total oxygen flux from which we could determine respiration and photosynthesis rates. To correct for differences in biomass of the anemones, we measured the oral disk diameter of each anemone when they were submerged and fully open the day before each of the respiration trials and converted the value to biomass using the equation of Dimond et al. (2011). These data were then used to calculate biomass-specific respiration rates as in Verde and McCloskey (2007). Dark-exposed and light-exposed oxygen fluxes were used to calculate gross photosynthetic rate as measured by oxygen production per algae biomass over time for each anemone. To do this we used the following equation, where 0.095 is the estimated mean algal biomass ratio calculated for *A. elegantissima* (McKinney 1978). Because there was no measurable respiration in the blank jars, no correction was necessary.

$$P_G=((O_{AL}-O_{BL})+R_{AD})/(AB*0.095)$$

P_G =gross photosynthesis as oxygen production per algal biomass per hour
O_{AL} =oxygen production in the anemone jar in the light incubation
O_{BL} = oxygen production in the blank jar in the light incubation
R_{AD} =oxygen consumption of the anemone jar in the dark incubation
AB=Anemone biomass estimated from oral disk diameter

Symbiont Density, Growth, and Anemone Protein Biomass

The half of the homogenate sample from the tentacle clips not used for ROS was frozen at -40°C within 30 minutes of collection and later processed for symbiont cell density, protein, and mitotic index. Symbiont identity and number were determined by placing a sample of the thawed and

vortex-mixed homogenate on a hemocytometer and counting the number of symbionts in four replicate grids. The symbiont mitotic index (later used for CZAR calculation) was determined by examining 1000 cells under light microscopy and determining the percentage in the process of dividing as described by Verde and McCloskey (1996). For anemones with mixed symbiont complements, the number of both *S. muscatinei* and *E. marina* cells, which can be readily distinguished by size and color, were calculated and the proportion of each was determined.

To normalize measurements and permit comparison of data collected from tentacles of different sizes, we determined the total protein concentration of each clipped tentacle sample using a Pierce BCA protein micro-assay (Pierce Biotechnology, Rockford, Illinois) with a standard of bovine serum albumin (BSA). The absorbance of the processed homogenates at 562 nm was read on a microplate reader using a spectrophotometer, and protein content of the samples was then determined from standard curves according to protocols from Noble and Bailey (2009). This allowed us to compare cell densities and ROS content on a per µg anemone protein basis.

CZAR Score

The calculation of carbon from symbiont photosynthesis that went toward animal respiration (CZAR) was estimated with the formula provided by Muscatine et al. (1981) and modified by Verde and McCloskey (1996, 2007), assuming the mean algal biomass ratio of 0.095 (McKinney 1978). Due to our inability to distinguish algal and anemone respiration in the light, the daytime algal respiratory rate was estimated from the total dark respiration rate of the holobiont as a ratio of biomass. This calculation included a daily gross photosynthetic rate equal to the sum of the oxygen production rate in the light and the oxygen consumption rate in the dark and a conversion

ratio of carbon to oxygen equivalents of 12:32 (Verde and McCloskey 2007) The photosynthetic quotient, animal respiratory quotient, and algal respiratory quotient were assumed to be 1.1, 0.9 and 1.0, respectively (Verde and McCloskey 1996, 2007). The algal-specific growth rate was calculated assuming a fixed value for the duration of cytokinesis and incorporating our measurement of symbiont mitotic indices (Verde and McCloskey, 1996). The algal carbon-specific growth rates were also determined using standing stock estimated from algal cell density and the same algal-specific growth rate. Because of the difficulty in calculating separate algal growth rates for *S. muscatinei* and *E. marina* in mixed anemones, we were unable to calculate CZAR scores for those individuals. Aposymbiotic anemones also did not receive CZAR scores as the symbiont cell densities in those individuals were too low to provide any meaningful carbon to the host.

Statistical Analyses

All statistical analysis was done in R version 3.3.4 using linear mixed effects models, which tested the fixed effects of symbiotic state, pCO_2 treatment, time, all possible two-way interactions, and the interaction of all three fixed variables while accounting for repeated measures of the same individuals over time and repeated sampling of the same aquaria (i.e., multiple anemones were in the same aquaria). The LME function implemented from the "nlme" package built under R 3.3.1 (R Core Team 2016, Pinheiro et al. 2017) was used for all analyses.

Separate analyses were run to test for treatment effects on symbiont cell density, ROS concentration per symbiont cell, ROS concentration per anemone protein, CZAR, respiration rate, gross photosynthesis, and proportion of *S. muscatinei* in the *A. elegantissima* with mixed symbionts complements. We built each model by first including all fixed and random effects,

then using log likelihood ratio tests with REML estimation to determine which random effects improved the model. Random intercepts and slopes were tested for each anemone across time and for anemones nested in random tanks. We then sequentially removed fixed variables and compared AIC values to arrive at the most parsimonious model (i.e., the one with the lowest AIC). We used residual plots to test assumptions of the models and adjusted covariance matrices, where necessary, to improve the fit. Marginal R^2, which describes the proportion of variance explained by the fixed factors alone, and conditional R^2, which describes the proportion of variance explained by both the fixed and random factors, were calculated for all final models.

Results

Despite our best efforts to keep the experimental aquaria clean over the 10 weeks of the experiment, it was not possible to prevent some fouling by diatoms. The photosynthetic activity of those diatoms, photosynthetic activity of organisms in the incoming seawater, or photosynthesis and respiration of the *A. elegantissima* themselves may have produced the diurnal pattern of pH shown in Figure 3. Despite the apparent diurnal rise and fall in pH within the tanks, the separation of the treatments remained very consistent, suggesting that the anemones in different treatments were always experiencing different treatment levels of acidification. Furthermore, pCO_2 levels (calculated regularly from DIC values measured in the experimental aquaria) revealed that the mean levels in the treatments fell within ± 5% of the target values over the entire 10-week experiment (Fig. 4). Spectrophotometric pH measurements from the same discrete samples used for the DIC and pCO_2 calculations above always fell within 0.07 pH units of the intended level for all treatments (Fig. 5).

H_2O_2 production

Our approach to measuring ROS focused on measuring the concentration of H_2O_2 in tentacle homogenate containing burst anemone animal cells with intact, suspended symbiont cells rather than in isolated symbiont cells alone. When calculated on a per-symbiont-cell basis (and thus adjusting for different symbiont densities), *A. elegantissima* hosting *E. marina* consistently showed greater quantities of H_2O_2 than did individuals hosting *S. muscatinei*. Under ambient conditions, the production of H_2O_2 by *E. marina* was nearly twice that of *S. muscatinei* (Fig. 6). With pCO_2 levels raised to 1800 ppm, *E. marina* appeared to respond over time by increasing H_2O_2 production. In contrast, the *S. muscatinei* appeared to acclimate to the conditions, showing slight decreases in H_2O_2 levels in the 1200 ppm treatment and to a lesser degree in the 1800 ppm treatment (Fig. 6).

Statistical analysis (LMM) with random intercepts for aquaria and random slopes and intercepts for individuals nested in aquaria showed an optimal model consisting only of symbiont and pCO_2 as factors (Table 1). The absence of any interaction effects in the final model indicates that any differences in the apparent slopes of brown and green anemones in Figure 6 were not meaningful against the background of anemone-to-anemone variability. Marginal and conditional R^2 values for this model were 0.76 and 0.94 respectively (Table 8).

In an effort to clarify the role of symbiont versus host tissue in producing the H_2O_2, production was also expressed per μg of *A. elegantissima* protein. Doing this permitted the inclusion of aposymbiotic and mixed symbiont complement anemones in the analysis. The results (Fig. 7) show that, under ambient conditions, all symbiont-hosting anemones produced similar levels of H_2O_2 that were higher than those of the aposymbiotic anemones. At higher pCO_2 levels, the concentration of H_2O_2 increased and accumulated over time in the aposymbiotic

individuals, but still stayed well below the levels measured in the anemones hosting photosymbionts.

The slope of the regression line over time for green anemones mirrored that seen for aposymbiotic anemones, suggesting that the *E. marina* were doing little to increase production of H_2O_2 over what was happening in the homogenate itself. The slope for brown anemones in the 1200 and 1800 ppm treatments appeared slightly steeper than that of the other symbiotic states suggesting that, while something in the homogenate itself was producing H_2O_2, the *S. muscatinei* may have been exacerbating the problem (Fig. 7). The pattern for mixed anemones was nearly identical to the green anemones in all treatments.

Statistical analysis produced a model for H_2O_2 production per anemone protein biomass that included day, symbiotic state, pCO_2 treatment, the interaction of symbiont state*day, and the interaction of day*pCO_2 (Table 2). The interactions that appear in this model indicate that, over time, ROS production of green and brown symbionts was different and that ROS production differed according to pCO_2 treatment, but the absence of a symbiont state*pCO_2 interaction indicates that the apparent difference in the responses of the anemones in different symbiotic states described above were not sufficient to be meaningful in building the explanatory model. The optimal model, with random intercepts for each anemone, produced marginal and conditional R^2 values of 0.84 and 0.94 respectively (Table 8).

Symbiont productivity

Symbiont productivity, as measured by gross photosynthesis, was only determined for anemones that were either brown or green, excluding mixed and aposymbiotic individuals. Figure 8 suggests that the photosynthetic activity of *S. muscatinei* and *E. marina* responded differently to

pCO_2. Under ambient pCO_2 (800 ppm), *E. marina* were less productive than *S. muscatinei* and performance changed little in either group over the 10-week experiment. Exposing the anemones to a pCO_2 to 1200 ppm, however, more than doubled the gross photosynthesis of *S. muscatinei* over time while that of *E. marina* showed only a minor upward trend. The effect of an even higher pCO_2 (1800 ppm) on *E. marina* appeared to be negative as the productivity showed a gradual decline over time (Fig. 8). In contrast, the performance of *S. muscatinei* at both 1200 ppm and 1800 ppm was apparently enhanced over time, albeit with a smaller increase in the 1800 ppm treatment.

The LMM analysis, with random slopes and intercepts for anemones nested within aquaria, produced a best fit model with fixed effects of day, symbiont, pCO_2, and symbiont*day interactions (Table 3). The primary trend in the data, indicated by the inclusion of the symbiont*day interaction, was for a change in the relative gross productivity of both brown and green anemones over time, independent of pCO_2 treatment. So, the apparent differences in responses of the different symbiotic states was again not statistically meaningful. The marginal and conditional R^2 values for this model were 0.72 and 0.91 respectively (Table 8).

Respiration

To examine potential stress responses of *A. elegantissima* exposed to increasing pCO_2, we periodically measured respiration rates of the anemones over the duration of the experiment. The dark respiration rate was nearly unchanged over time for all symbiont states in the ambient treatment and only increased slightly in the 1200 ppm treatment. However, respiration rate appeared to increase more among anemones in all symbiotic states held under 1800 ppm pCO_2 (Fig. 9). Aposymbiotic individuals showed the greatest apparent change, followed by anemones

hosting *E. marina*. Mixed anemones and those hosting *S. muscatinei* showed a more moderate increase.

Statistical analysis with random intercepts for individuals and for aquaria produced an optimal model with day, symbiont state, pCO_2, and the interaction of day*pCO_2 (Table 4). The inclusion of symbiont state in the final model indicates that anemones in different symbiont states respired at different rates with aposymbiotic individuals showing the highest rates, followed by green, brown, then mixed individuals in that order. The results also show that an increase in pCO_2 levels increased respiration rates and that the effects increases over time. However, there was again no symbiont state*pCO_2 interaction indicating that the respiration rates of the different symbiont states were affected similarly by the pCO_2 treatments. The final model produced a marginal R^2 of 0.41 and a conditional R^2 of 0.78 (Tables 4, 8).

Symbiont density

Under ambient pCO_2 conditions of 800 ppm, density of *S. muscatinei* in brown anemones was approximately twice that of *E. marina* in green anemones and those densities changed little during the 10-week experiment (Fig. 10). Increasing pCO_2 affected the two symbionts very differently. While *S. muscatinei* cell density increased with higher pCO_2 levels, *E. marina* densities stayed nearly constant or slightly decreased over time. The difference in the response was particularly evident in the 1200 ppm pCO_2 treatment.

The best fit LMM model, based on AIC values, included day, symbiont, pCO_2 level, and the interactions of symbiont*day and symbiont*pCO_2 (Table 5). The inclusion of the symbiont*pCO_2 interaction indicates that the symbionts were responding differently to the pCO_2 treatments with green anemones showing a significantly lower slope than brown anemones,

highlighting their opposite responses. Random effects in the final model include individual slopes and intercepts for anemones nested within aquaria and the marginal and conditional R^2 values were 0.62 and 0.97 respectively (Table 8). During the analysis, the covariance structure was adjusted to allow unique variances for each level of pCO_2 treatment.

CZAR score

To examine how changes in pCO2 might affect the relationship between *A. elegantissima* and its symbionts, we also examined how CZAR (the percent of carbon for animal respiration coming from symbiont carbon fixation) differed with OA conditions for *S. muscatinei* and *E. marina*. Under all conditions, *S. muscatinei* provided more carbon to the anemone host (Fig. 11). Under ambient pCO_2, the CZAR for *S. muscatinei* increased slightly over time while that of *E. marina* decreased slightly. Under 1200 pCO_2, *S. muscatinei* reaching its highest CZAR score (65%) and nearly doubled the CZAR score of the *E. marina* by the end of the experiment. Interestingly this increase in performance of *S. muscatinei* did not occur in the 1800 ppm treatment where the CZAR score was nearly unchanged over time while that of *E. marina* dropped at a rate very similar to that seen in the 1200 ppm treatment (Fig 11).

LMM analysis including random slopes and intercepts for individual anemones nested within aquaria produced an optimal model that included day, symbiont state, pCO_2, the interaction between symbiont state*day, and the interaction of pCO_2*day (Table 6).The symbiont state*day interaction shows that the slope of the CZAR for green anemones was significantly lower than that of brown individuals, but the pattern of the response was similar across pCO_2 levels. The marginal and conditional R^2 values for this model were 0.46 and 0.75 respectively (Table 8).

Symbiont shifts

To better understand the potential relative effects of ocean acidification on the two photosymbionts, we measured the densities of both *E. marina* and *S. muscatinei* in the mixed anemones over the course of the 10-week experiment. The proportion of *S. muscatinei* cells in the mixed anemones increased with increasing pCO_2 over time (Fig. 12). The mixed anemones started with a near 50:50 proportion of the symbionts (± 5%) and stayed near that ratio in the ambient 800 ppm treatment. However, in the enriched treatments (1200ppm and 1800 ppm) a shift to a higher proportion of *S. muscatinei* started to take place, particularly after day 42, approaching a proportion of 70% *S. muscatinei* cells in the 1800 ppm treatment (Fig. 12). The statistical analysis for these data included significant treatment effects of day and pCO_2 as fixed effects with the pCO_2 term indicating that the treatment affected symbiont complement in the anemones (Table 7). The final model, which also included random intercepts for individual anemones nested within aquaria, produced a marginal R^2 of 0.45 and a conditional R^2 of 0.55 (Table 8).

To determine whether the shifting of the symbiont complement towards *S. muscatinei* was due to an increase in *S. muscatinei* density, a decrease in *E. marina* density or some combination of both, we also looked at the absolute cell densities of both over the period of the experiment. Figure 13 shows stable populations of both symbionts in the ambient pCO_2 treatment. However, as pCO_2 level increased, there appeared to be an increase in *S. muscatinei* and a corresponding decrease in *E. marina* density. However, the LMM analysis, produced an optimal model that included no fixed effects, suggesting the absence of any pattern or, we believe, the presence of non-linear behavior in the system, a possibility that is supported by

evidence of a shift in the patterns of the 1200 ppm and 1800 ppm treatments after day 42 (Fig. 13).

Discussion

We studied the impact of OA on the sea anemone *A. elegantissima* with its two algal photosymbionts and found that OA conditions affect ROS concentration, respiration rate, gross photosynthesis, symbiont cell density, CZAR score, and the proportion of *S. muscatinei* in mixed anemones. While our linear model approach did not reveal a significant pCO_2*symbiont state interaction in any of our measured response variables except symbiont cell density, there were a number of patterns that suggest impacts may be more evident with longer experiments, greater replication, or statistical analyses that include non-linear responses.

Our results suggest that *S. muscatinei* may benefit from increasing pCO_2 at levels of 1200 ppm and 1800 ppm. The symbiont cell density, gross photosynthesis, and CZAR of *S. muscatinei* all appeared to increase with increasing pCO_2 (Figs. 8, 10, 11), which is consistent with previous studies showing increased individual and population performance in marine diatoms, dinoflagellates, and coccolithophores in response to increased pCO_2 (Feng et al. 2008; Towanda and Theusen 2012). Weis (1993) suggested that *S. muscatinei* is carbon limited under ambient DIC/pCO_2 conditions and showed increasing photosynthetic rates and carbonic anhydrase activity with increasing DIC. Koch et al. (2020) subsequently demonstrated higher carbonic anhydrase activity in anemones hosting *S. muscatinei* over those hosting *E. marina*. The higher HCO_3^- in the water as a result of OA may be converted by the rubisco enzyme system in the symbionts, providing more CO_2 for carbon fixation. This process was likely responsible for the significant effect we saw of pCO_2 on gross photosynthesis (Fig. 8).

Like other dinoflagellates, *S. muscatinei* has the less efficient form II rubisco while *E. marina* has the more efficient form I rubisco that has a higher affinity for CO_2 over O_2; *S. muscatinei* may overcome this through carbon concentrating mechanisms such as carbonic anhydrase (Morse et al., 1995; Tabita et al., 2008; Koch et al. 2020). The pattern we observed that *S. muscatinei* gross photosynthesis and density both increased with elevated pCO_2 while that of *E. marina* appeared to remain constant or even decreased is consistent with the hypothesis that *S.muscatinei* is carbon-limited (Figs. 8, 10).

Wu et al. (2010) found that marine diatoms reared in waters with a pH of 7.73 show higher gross and net photosynthesis than those raised at ambient levels. However, carbon concentrating mechanisms (CCMs), including carbon anhydrase, were downregulated in higher pCO_2 and NPQ dropped due to the decreased CCMs. This contrast shows how diverse carbon fixation responses can be in photosynthetic organisms of different groups, particularly when there is an animal host involved. The Wu et al. (2010) study also showed an increase in dark respiration of the diatoms, showing that higher environmental pCO_2 can lead to an increase in photosynthesis while having a dual negative affect of increased respiration (see also Gao et al. 2012). This matches the trend we observed in anemones hosting *S. muscatinei* and provides support for the dual positive (photosynthesis/cell density/CZAR) and negative (respiration/ROS) effects for anemones hosting symbionts in our study.

Previous studies on *A. elegantissima* have shown that symbiotic *E. marina* populations are more sensitive to increasing temperature, irradiance, and ROS levels than are those hosting *S. muscatinei.* (Engebretson and Muller-Parker 1999; Lesser 1996, 2006; Verde and McCloskey 2007; Dimond et al. 2017). Our study, which measured concentration of ROS in the host tissues,

adds pCO_2 as another stressor that affects *S. muscatinei* and *E. marina,* with the latter potentially being the more sensitive symbiont.

While a pattern of tolerance of *S. muscatinei* for elevated pCO_2 appears in several variables of our study, that is not true for all cnidarian/algal symbioses. In experiments with scleractinian corals, researchers found decreasing densities of *Symbiodinium* in response to increasing levels of OA. This underscores the potential differences between temperate and tropical symbioses. The synergistic effects of multiple environmental factors (e.g., pCO_2 and temperature), and the differing environmental tolerances of differing *Symbiondiaceae* species can differentially impact their hosts, leading to the competitive advantage of certain symbionts (Grottoli et al. 2006, 2014; Mason 2018)

Tropical corals can "shift" or "shuffle" their symbiont partners depending on, for example, the temperature and light levels they experience. Some species (or clades) of photosymbionts are more stress tolerant than others (LaJeunesse et al. 2009, Ladner et al. 2012) and there is evidence that the host can discharge "sensitive" algae in exchange for more tolerant partners (Brading et al. 2011; Yamashita et al. 2011; Grottoli et al. 2014). Similar results have been found in *A. elegantissima*. Individuals with mixed complements of *S. muscatinei* and *E. marina* shift toward *S. muscatinei* (the anemones "brown") when they are exposed to increased irradiance and temperature. This process occurs relatively slowly, requiring a 3-month lag between temperature maxima and symbiont shifts (Dimond et al. 2013).

In our study, an increase in pCO_2 led to a significant increase in *S. muscatinei* density and likely contributed to an increase in the proportion of *S. muscatinei* in mixed anemones, but the process occurred in just 10 weeks. The majority of change in symbiont complements of the mixed anemones appeared to happen after just 42 days (Fig. 12). This shift in the symbiont

complement suggests a competitive advantage of *S. muscatinei* over *E. marina* under conditions of increased pCO_2.

The increased respiration rate of both *S. muscatinei* and *E. marina* in response to increased pCO_2 levels may be connected to the production of reactive oxygen species. Increased photosynthetic activity can create a hyperoxic environment near PSII in the chloroplast of the symbionts, leading to increased production of ROS in surrounding cells (Suggett et al. 2008). This increased photosynthetic activity appeared to be present in *S. muscatinei* in the enriched treatments and might account for this symbiont state having the highest ROS production on a per-protein basis (Fig. 7). We expected *A. elegantissima* hosting photosymbionts to have higher baseline levels of H_2O_2 (an ROS species) than aposymbiotic anemones, and this pattern is suggested in our results (Fig. 7) though it was not significant in our statistical analysis (Table 2).

Wu et al. (2010) found that nonphotochemical quenching capacity (a protective energy sink for excess excited electrons) was reduced in diatoms raised in waters with elevated pCO_2. This reduced capacity to handle excess electron energy could lead to the increased ROS production and reduced gross photosynthesis patterns we observed in all CO_2-enriched treatments for *E. marina*. Based on a per-protein-biomass measurement, *A. elegantissima* hosting *S. muscatinei* appeared to have higher H_2O_2 production than those hosting *E. marina* (Fig. 7). However, while *S. muscatinei* cell density significantly increased with pCO_2, *E. marina* density decreased. As a result, on a per cell basis, *E. marina* showed the highest concentrations of H_2O_2, indicating either a lower capacity for scavenging ROS species or that *S. mucatinei* may have a greater ability to keep ROS from forming in the first place through mechanisms such as NPQ (Fig. 6).

In evaluating the results of our ROS measurements, we cannot overlook the fact that we measured only H_2O_2 and none of the other ROS species that may have been present in the anemones. Nor can we ignore photochemical production of H_2O_2, which occurs when light interacts with the surface of the ocean. This background process can confound measurements of ROS in samples of tissues or even free-living microalgae in seawater (Zinser 2018). The amount of filtered seawater we added to homogenize our anemone tentacles was relatively small and we believe the impacts of photochemically-produced H_2O_2 were small, but this should be examined further.

Nii and Muscatine (1997) studied effects of sublethal acute temperature stress in a tropical anemone/zooxanthellae symbiosis that included aposymbiotic individuals and determined that ROS production was primarily an animal response. Exposure to light did not increase the ROS production and ROS production tracked with an increase in dark respiration, leading to the conclusion that the root cause was increased mitochondrial activity in response to the temperature increase. It is clear, however, that more study of photochemical production of ROS in laboratory experiments is necessary to ensure that background abiotic processes do not confound measurements.

Analyzing the aposymbiotic anemones allowed us to look at H_2O_2 production by the host without the complication of symbiont presence. While the aposymbiotic individuals had the lowest overall H_2O_2 production values, they still showed an increase in H_2O_2 production with increasing pCO_2. This increase suggests a possible baseline host stress response to OA independent of the photosymbionts, though, again, there is no way with our data to eliminate abiotic photochemistry as a possible contributing factor. Many external stressors (hypoxia, temperature, irradiance, heavy metals, and herbicides) increase ROS concentrations in non-

photosymbiotic marine organisms by inhibiting the action of ROS scavenging enzymes like superoxide dismutase and catalase (Mallick and Mohn 2000; Lesser 2006). These effects are often coupled with increased host respiration, which also increases ROS production. We saw this combination in our aposymbiotic *A. elegantissima* (Figs. 7, 9). The presence of either *S. muscatinei* or *E. marina* was associated with overall lower respiration rates and the patterns were different when pCO_2 was increased.

The ability of symbionts to reduce respiration may be related to light-protective pigments, another source of ROS scavenging compounds, and additional NPQ processes including the xanthophyll cycle that dissipates excess light energy as heat in many algae species (Brown et al. 1999; Ferrier-Pages et al. 2014; Laloi and Havaux 2015; Shick and Dykens 1984). Another possibility is that the higher intracellular pH created by the photosynthesizing cells in the anemone tissue creating a buffering effect that compensated for the lower pH the animals were experiencing (Koch et al. 2020). However, it should also be recognized that, while the anemones in our respiration-measuring jars never experienced oxygen concentrations below the EPA ambient water quality guidelines of 5.5 mg/L, aposymbiotic anemones may have experienced some form of hyperventilation and were working harder to acquire oxygen than were symbiotic anemones, potentially with residual oxygen from their symbionts persisting in their tissues (Shick 1990).

Studies investigating *Symbiodinium* symbioses in corals have shown that, when ROS concentrations increase, several photosynthetic parameters of the symbionts decrease. The corals in one study responded with an increase in ROS-scavenging species on the time scale of minutes to hours (Levy et al. 2006 and Nishiyama et al. 2006). In general, different species of *Symbiodinium* show different tolerances for temperature, light, and ROS, with tolerance tied to

more efficient ROS scavenging, maintenance of adequate NPQ, and a slower electron transport rate of PSII (Suggett et al. 2008; Jones et al. 2012, and Levy et al. 2006).

ROS, as measured by H_2O_2 concentration, rose over time in all states and all treatments in our study, and pCO_2 had a significant effect on gross photosynthesis (Figs. 7, 8). Previous studies using a variety of methods have demonstrated that baseline photosynthetic activity is higher in *S. muscatinei* than *E. marina* and that *S. muscatinei* responds to higher irradiance and temperature by increasing photosynthetic rates whereas *E. marina* respond with stagnant or decreasing photosynthetic rates depending on the length of exposure (Verde and McCloskey 1996, 2007; Secord and Augustine 2000; Bergschneider and Muller-Parker 2008; Dimond et al. 2013). Our experiment probably exaggerates potential effects of OA: the design exposed the anemones to chronic stress without the regular emersion that would occur with daily tidal exchanges. Indeed, Dimond et al. (2017) found that, under shorter exposures to high light and temperature, anemones hosting *S. muscatinei* produced more H_2O_2 than those hosting *E. marina* when measured on a per symbiont cell basis, which is the opposite of what we found. The authors of that study also used rapid light curves to demonstrate that NPQ is higher in *E. marina*, which also tend to have higher concentrations of carotenoids (another avenue for NPQ). The longer-duration stress our experiment exposed the *A. elegantissima* to may have overcome these systems, leading to the decline of photosynthesis and rise of H_2O_2 in *E. marina.* It is also possible that the higher H_2O_2 on a per-protein basis in *S. muscatinei* is the result of the hyperoxic environment created by increased photosynthetic rates in this group. The O_2 generated by photosynthesis is a strong oxidant and can lead to the production of ROS species.

We expected that OA-related effects would be evident in the respiration rates of *A. elegantissima* exposed to high pCO_2 levels and would differ among symbiotic groups. All

anemones showed an increase in respiration rate in higher pCO_2 treatments and those increased over time, suggesting a stress response. Respiration was greatest in aposymbiotic individuals and individuals hosting *E. marina* and lowest in anemones hosting *S. muscatinei*, suggesting an animal-level stress response (Fig. 9). This is supported by previous studies pointing to increased mitochondrial activity both in animal cells and free-living microalgae in response to increasing temperature and pCO_2 and lower pH (Nii and Muscatine 1997, Geider and Osborne 1989). While we did not experiment with temperature, the exposure to low pH caused by the high pCO_2 environments may have led to a similar increase in energy demand to counteract the low external pH, spurring mitochondrial activity and therefore increasing respiration in our aposymbiotic anemones. The low pH experienced by the animal cells could have been mitigated by the presence of carbonic anhydrase, an enzyme that is more abundant in anemones hosting *S. muscatinei* and helps maintain acid/base balance and pH homeostasis. Weis and Reynolds (1999) showed that *A. elegantissima* expression of CA increased in the presence of symbionts, indicating that presence of the symbiont can cause changes in expression of host genes.

Studies of temperate coral-dinoflagellate symbioses show a positive connection between holobiont respiration and symbiont cell density (Hoogenboom et al. 2010, Starzark et al. 2014). Increased host respiration can lead to an increase in CO_2 concentrations in the host tissues, fueling symbiont population growth (Harland and Davies 1995). We observed an increase in symbiont density with elevated respiration in anemones hosting *S. muscatinei*, but not in those hosting *E. marina*, likely because *E. marina* is not carbon-limited like *S. muscatinei* (Weis 1993). This again suggests that *S. muscatinei* is a more beneficial symbiont under OA conditions in that these conditions relieve the carbon limitation for that symbiont and the responding

increase in cell density for that symbiont may offer 1) a buffering effect through enhanced CA activity and 2) higher potential for translocation of fixed carbon to the host.

The combined impacts on symbionts and host in the present study show that the consequences of increasing pCO_2 may be different for *A. elegantissima* in different symbiotic states. Particularly interesting is the shifting of symbiont complements toward *S. muscatinei* under elevated pCO_2 conditions. Anemones hosting *S. muscatinei* at intermediate (1200 ppm) pCO_2 levels showed a trend toward higher gross photosynthesis, higher cell densities, and lower respiration rates. These were all coupled with the highest CZAR scores in our treatments (Fig. 11), suggesting that anemones hosting *S. muscatinei* could, at least initially, benefit from OA with more carbon being translocated to the host where it can be used for growth and reproduction. Previous work suggests that *E. marina* is a poorer symbiotic partner under normal conditions (Engbretson and Muller-Parker 1999; Dimond et al. 2017) and our results indicate that increasing pCO_2 may only exacerbate this difference.

If increasing pCO_2 does favor symbiosis with *S. muscatinei* over *E. marina*, we are likely to see a shift in *A. elegantissima* symbiosis with an increasing proportion of the population hosting *S. muscatinei* (i.e., a "browning" of the *A. elegantissima*). This effect will be in addition to the browning that may occur in response to a concurrent increasing global temperature since *S. muscatinei* is the more thermotolerant of the symbionts (Secord and Augustine 2000; Verde and McCloskey 2007). *A. elegantissima* are important members of intertidal communities and function as both primary producers and heterotrophic predators. A shift from "green" to "brown" with the resulting changes in symbiont productivity and CZAR could produce changes in heterotrophic feeding patterns and primary productivity in the intertidal environments in which these anemones are abundant (Fitt et al. 1982; Hiebert and Bingham 2012). More importantly,

such a shift could have profound consequences for the anemones themselves. Bingham et al. (2014) showed that reproduction of *A. elegantissima* is related to symbiotic state. Individuals that host *E. marina* primarily reproduce sexually through spawning of sperm and eggs. Individuals hosting *S. muscatinei*, in contrast, primarily reproduce asexually through fission. A loss of *E. marina* from the landscape of *A. elegantissima* symbiotic states could, therefore, have far-reaching implications for the genetic diversity and habitat extent of this important species.

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Tables

Table 1. LMM output for best fit model explaining H_2O_2 production on a per symbiont cell basis, based on pCO_2 level, symbiotic state, and days in the experiment. Model building was based on removal of terms with p values >0.1 and examination of resulting AIC values.

	Value	Std.Err	DF	t-value	p-value
(Intercept)	1.4756	0.1279	108	11.5301	<0.001
symbiont_green	1.2062	0.0528	17	22.8315	<0.001
pCO_2	-0.0001	0.0001	16	-1.3520	0.095

Table 1. LMM output for best fit model explaining H_2O_2 production on a per anemone biomass basis, based on pCO_2 level, symbiotic state, and days in the experiment. Model building was based on removal of terms with p values >0.1 and examination of resulting AIC values.

	Value	Std.Err	DF	t-value	p-value
(Intercept)	5.4394	0.0212	211	257.0668	<0.001
day	-0.0022	0.0004	211	-5.9244	<0.001
symbiont_brown	0.4258	0.0189	67	22.4969	<0.001
symbiont_green	0.3570	0.0189	67	18.8637	<0.001
symbiont_mixed	0.3522	0.0189	67	18.6073	<0.001
pCO_2	$-1.0 \cdot 10^{-5}$	0.0001	67	-0.4772	0.635
day:symbiont_brown	0.0022	0.0003	211	6.8088	<0.001
day:symbiont_green	0.0002	0.0003	211	0.6321	0.528
day:symbiont_mixed	0.0005	0.0003	211	1.4738	0.142
day: pCO_2	$3.0 \cdot 10^{-6}$	$2.36 \cdot 10^{-7}$	211	14.0500	<0.001

Table 3. LMM output for best fit model explaining gross photosynthesis by oxygen production and based on pCO_2 level, symbiotic state, and days in the experiment. Model building was based on removal of terms with p values >0.1 and examination of resulting AIC values.

	Value	Std.Error	DF	t-value	p-value
(Intercept)	19.0499	1.2799	106	14.8832	<0.001
day	0.2139	0.0335	106	6.3743	<0.001
symbiont_green	-11.8775	0.9249	33	-12.8418	<0.001
pCO_2	0.0001	0.0009	33	0.1875	0.009
day:symbiont_green	-0.2057	0.0474	106	-4.3345	<0.001

Table 4. LMM output for best fit model explaining biomass-specific dark respiration rate, based on pCO_2 level, symbiotic state, and days in the experiment. Model building was based on removal of terms with p values >0.1 and examination of resulting AIC values.

	Value	Std.Error	DF	t-value	p-value
(Intercept)	9.7196	0.3393	214	28.6458	<0.001
day	-0.0152	0.00711	214	-2.1498	0.033
symbiont_brown	-1.1262	0.1848	51	-6.0921	<0.001
symbiont_green	-0.5522	0.1848	51	-2.9869	0.004
symbiont_mixed	-1.1782	0.1848	51	-6.3734	<0.001
pCO_2	0.0005	0.0002	16	1.8332	0.045
day: pCO_2	0.0001	0.0001	214	4.6253	<0.001

Table 5. LMM output for best fit model explaining symbiont cell density based on pCO_2 level, symbiotic state, and days in the experiment. Model building was based on removal of terms with p values >0.1 and examination of resulting AIC values.

	Value	Std.Err	DF	t-value	p-value
(Intercept)	3.9374	0.1960	106	20.0861	<0.001
day	0.0161	0.0025	106	6.3590	<0.001
symbiont_green	-1.7166	0.1828	16	-9.3912	<0.001
pCO_2	0.0005	0.0001	16	3.1524	0.006
symbiont_green:pCO_2	-0.0004	0.0001	16	-2.7628	0.014
day:symbiont_green	-0.0171	0.0032	106	-5.2909	<0.001

Table 6. LMM output for best fit model explaining CZAR scores, based on pCO_2 level, symbiotic state, and days in the experiment. Model building was based on removal of terms with p values >0.1 and examination of resulting AIC values.

	Value	Std.Error	DF	t-value	p-value
(Intercept)	54.5163	0.8267	105	65.9398	<0.001
day	0.1714	0.0299	105	5.7306	<0.001
symbiont_green	-10.4748	0.5846	33	-17.9178	<0.001
pCO_2	0.0001	0.0005	33	0.3163	0.754
day:symbiont_green	-0.2012	0.0211	105	-9.5163	<0.001
day: pCO_2	-0.0001	0.00002	105	-2.8997	0.005

Table 7. LMM output for best fit model explaining the proportion of brown symbionts in mixed anemones based on pCO_2 level and days in the experiment. Model building was based on removal of terms with p values >0.1 and examination of resulting AIC values.

	Value	Std.Error	DF	t-value	p-value
(Intercept)	0.4831	0.0211	53	22.906970	<0.001
day	0.0019	0.0002	53	7.631377	<0.001
pCO_2	0.0001	0.0001	16	0.836961	0.015

Table 8: Summary of best fit LMM models with marginal and conditional R^2 for each variable tested. Best fit model was based on lowest AIC score.

Measured outcome	Final Model	Marginal R^2	Conditional R^2
H_2O_2 per symbiont cell	Symb + pCO_2	0.76	0.94
H_2O_2 per anemone biomass	Day + Symb + pCO_2 + Symb*Day + Day*pCO_2	0.84	0.94
Gross Photosynthesis	Day + Symb + pCO_2 + Symb*Day	0.65	0.82
Respiration	Day + Symb + pCO_2 + Day*pCO_2	0.41	0.78
Symbiont density	Day + Symb + pCO_2 + Symb*pCO_2 + Symb*Day	0.62	0.97
CZAR	Day + Symb + pCO_2+ Symb*Day+ Day*pCO_2	0.46	0.75
Prop. brown symbionts	Day+pCO_2	0.45	0.55

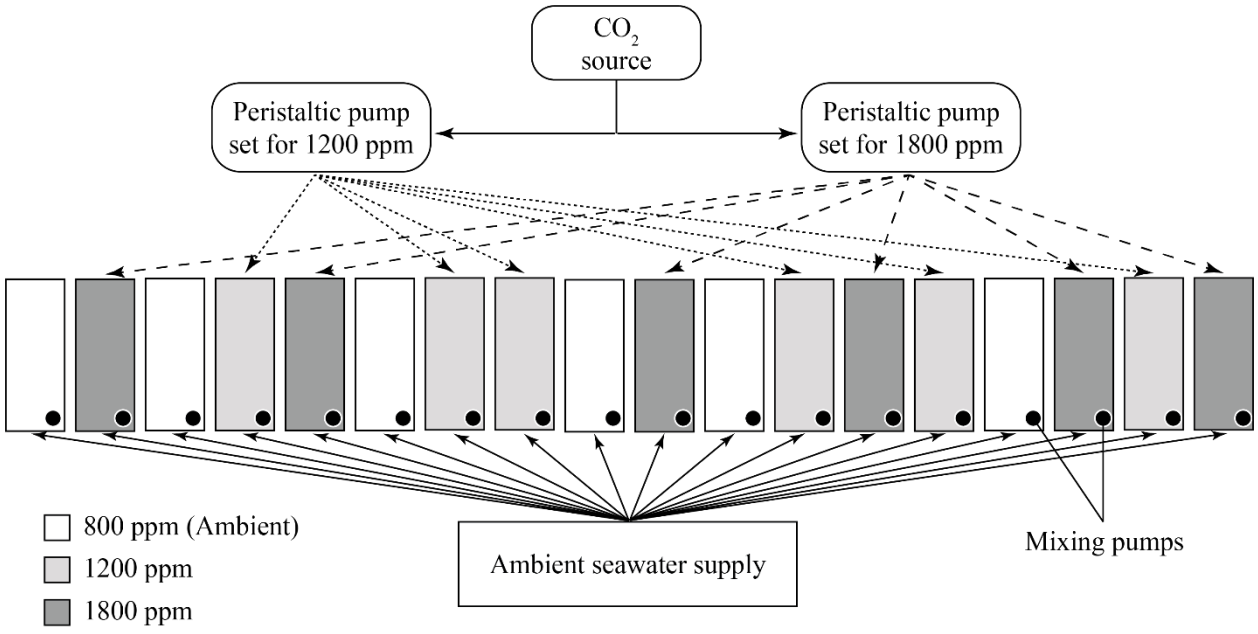

Figure 1. Experimental setup of 18 aquaria used to create pCO_2 levels of 800 ppm (ambient), 1200 ppm, and 1800 ppm. Four *A. elegantissima*, one in each symbiont state were placed in each aquarium. The flow-through cooling jackets around each aquarium are not shown.

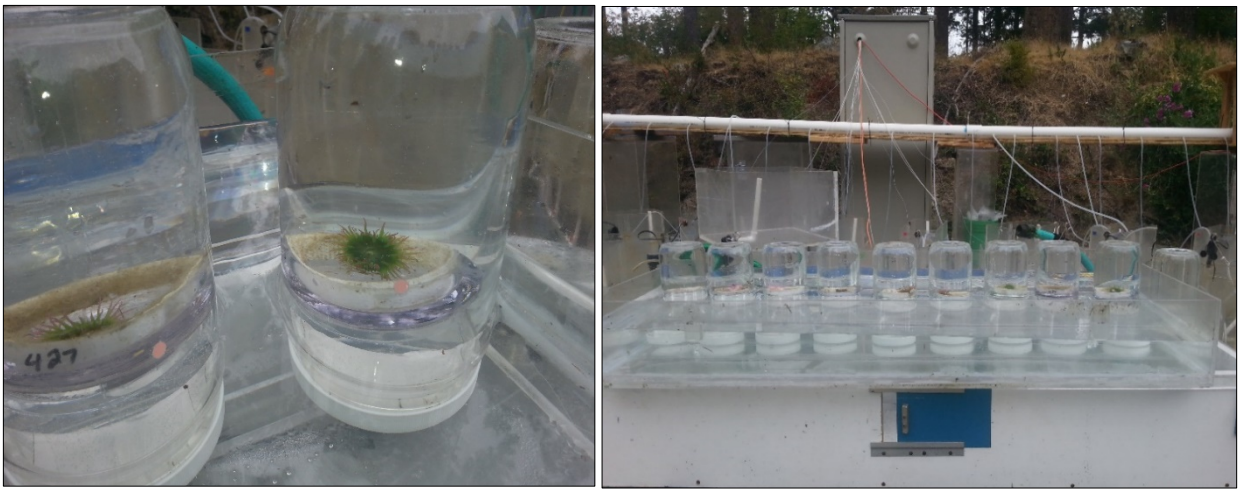

Figure 2. A) Respirometry jar with perforated anemone stand and pink oxygen sensor dot, B) magnetic stir table with one run of anemones in their water bath to control temperature (photo taken during the light incubation).

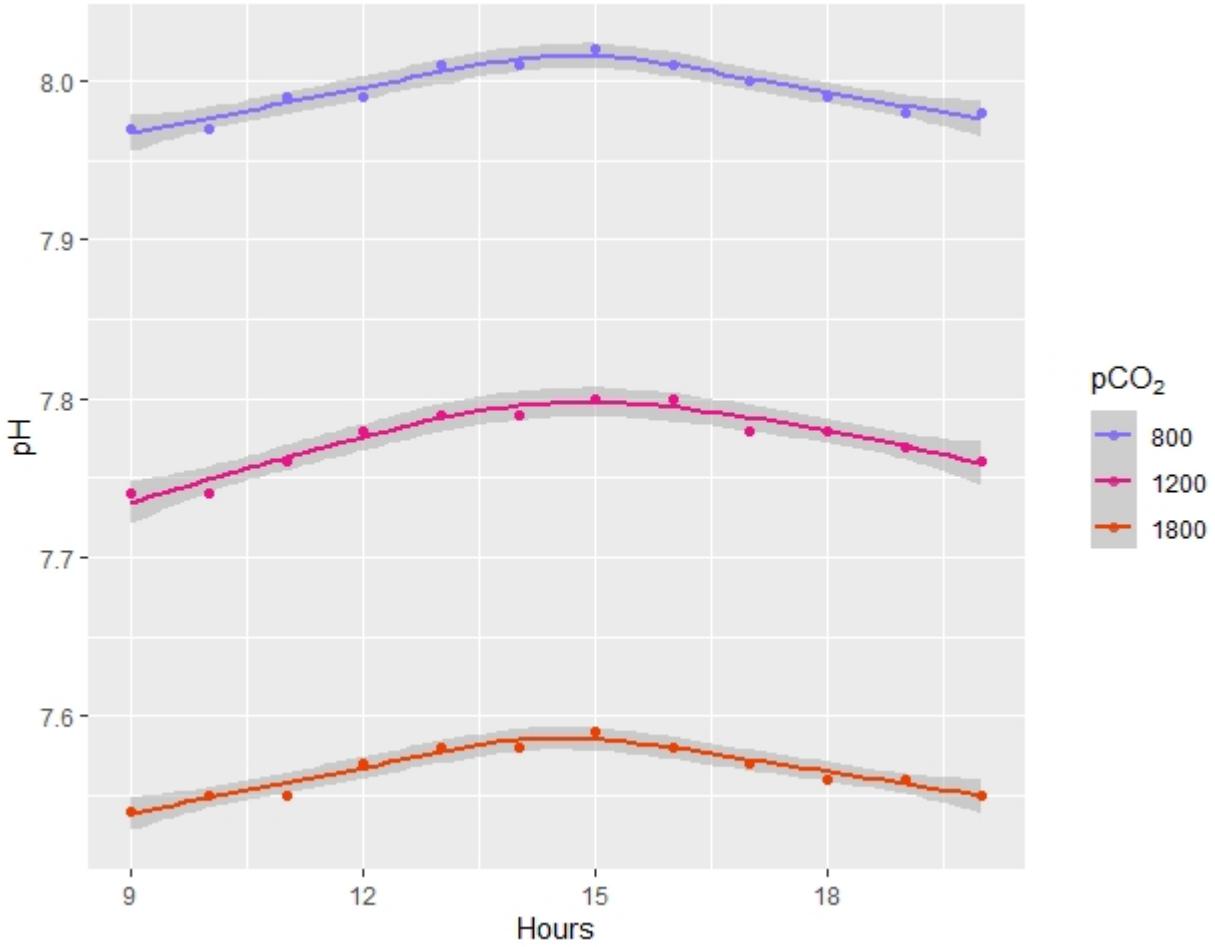

Figure 3. pH levels measured with *in-situ* electrode sensors over an 11-hour period from 9am to 8pm to show diurnal pH variability in the pCO_2 treatments. Best-fit loess lines with 95% confidence intervals are shown. Each point represents the mean of two randomly chosen aquaria in each pCO_2 treatment.

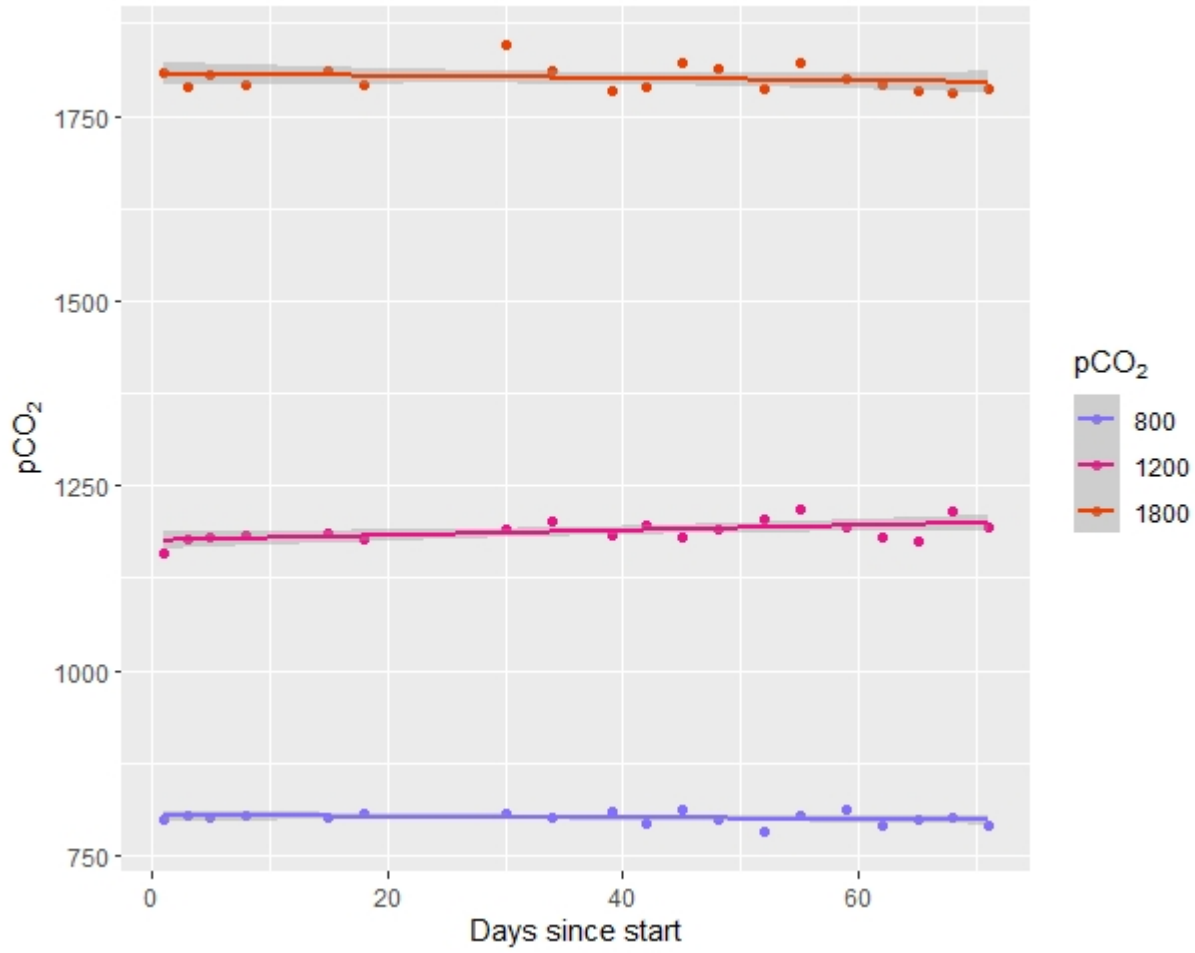

Figure 4. pCO_2, calculated from DIC and spectrophotometric pH measurements made during daylight hours in each of the treatments. Results indicate that the levels were stable and stayed close to the intended levels of 800, 1200, 1800 ppm. Best-fit regression lines with 95% confidence intervals are shown. Each point represents the mean of 6 aquaria in each pCO_2 treatment.

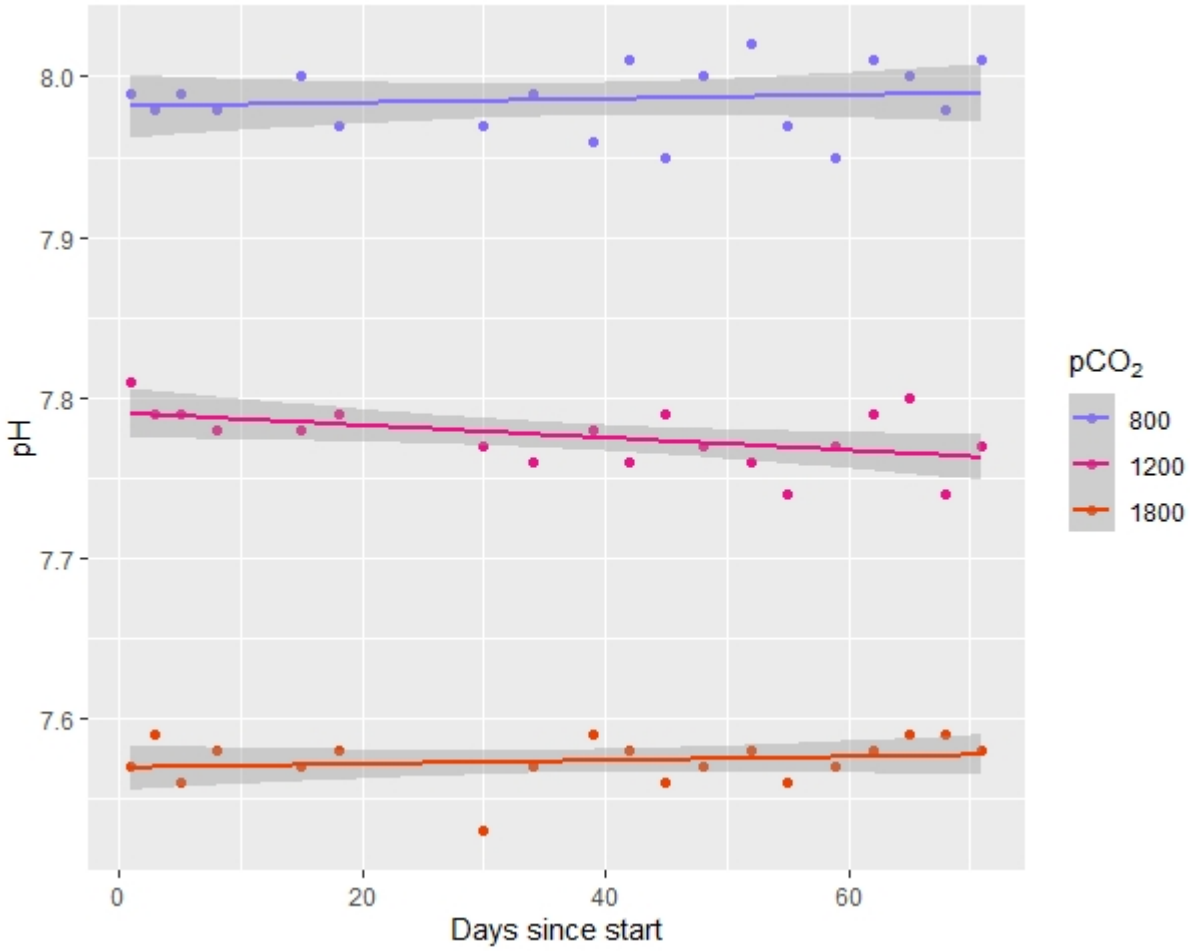

Figure 5. pH as measured spectrophotometrically in each of the pCO_2 treatments over the 10-week experimental period. Best-fit regression lines with 95% confidence intervals are shown. Each point represents the mean of 6 aquaria in each pCO_2 treatment.

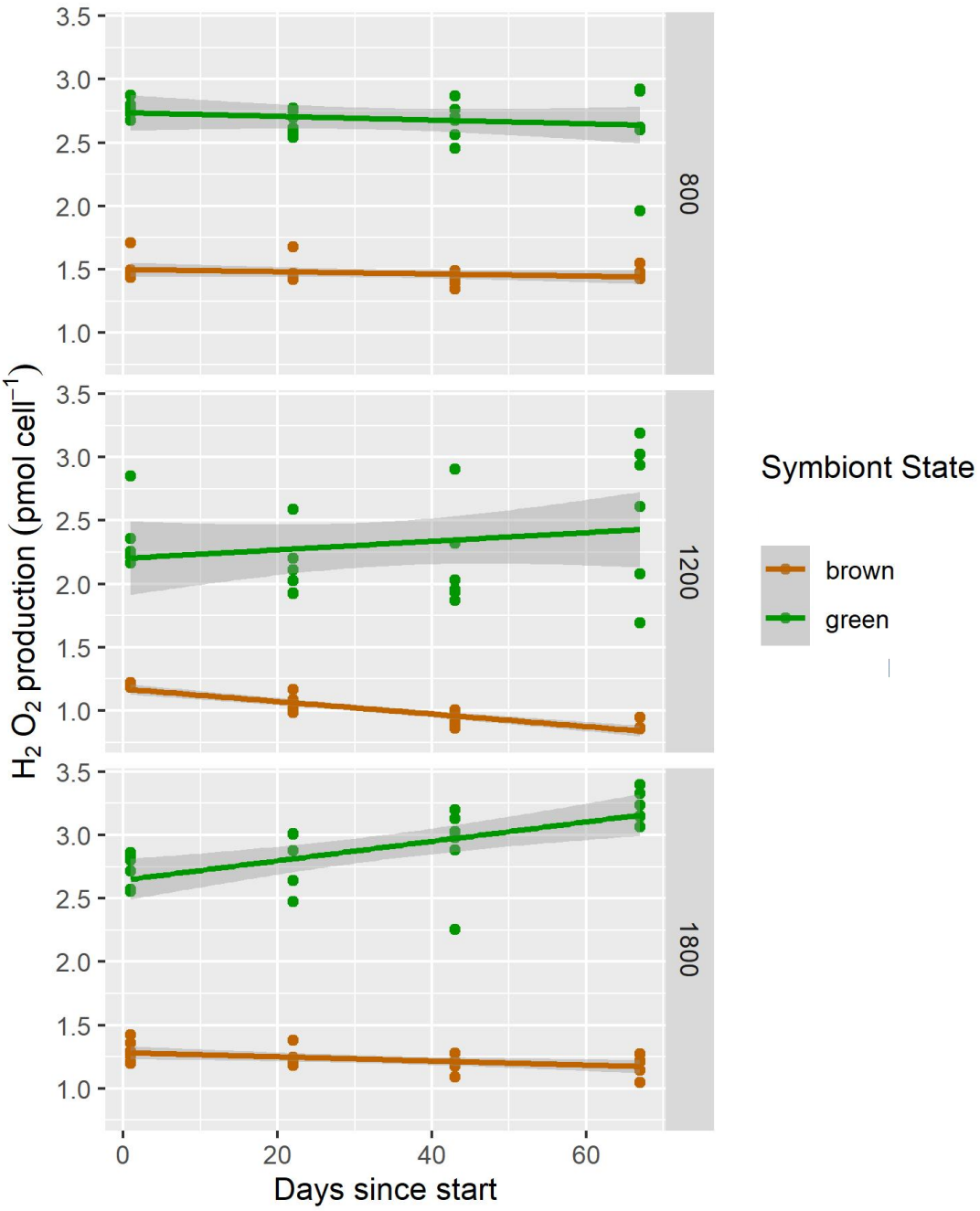

Figure 6. H_2O_2 concentrations (measured on a per-symbiont-cell basis) for *A. elegantissima* hosting *S. muscatinei* (brown) or *E. marina* (green) in pCO_2 levels of 800, 1200, or 1800 ppm across the sampling period. Regression lines with 95% confidence intervals are shown.

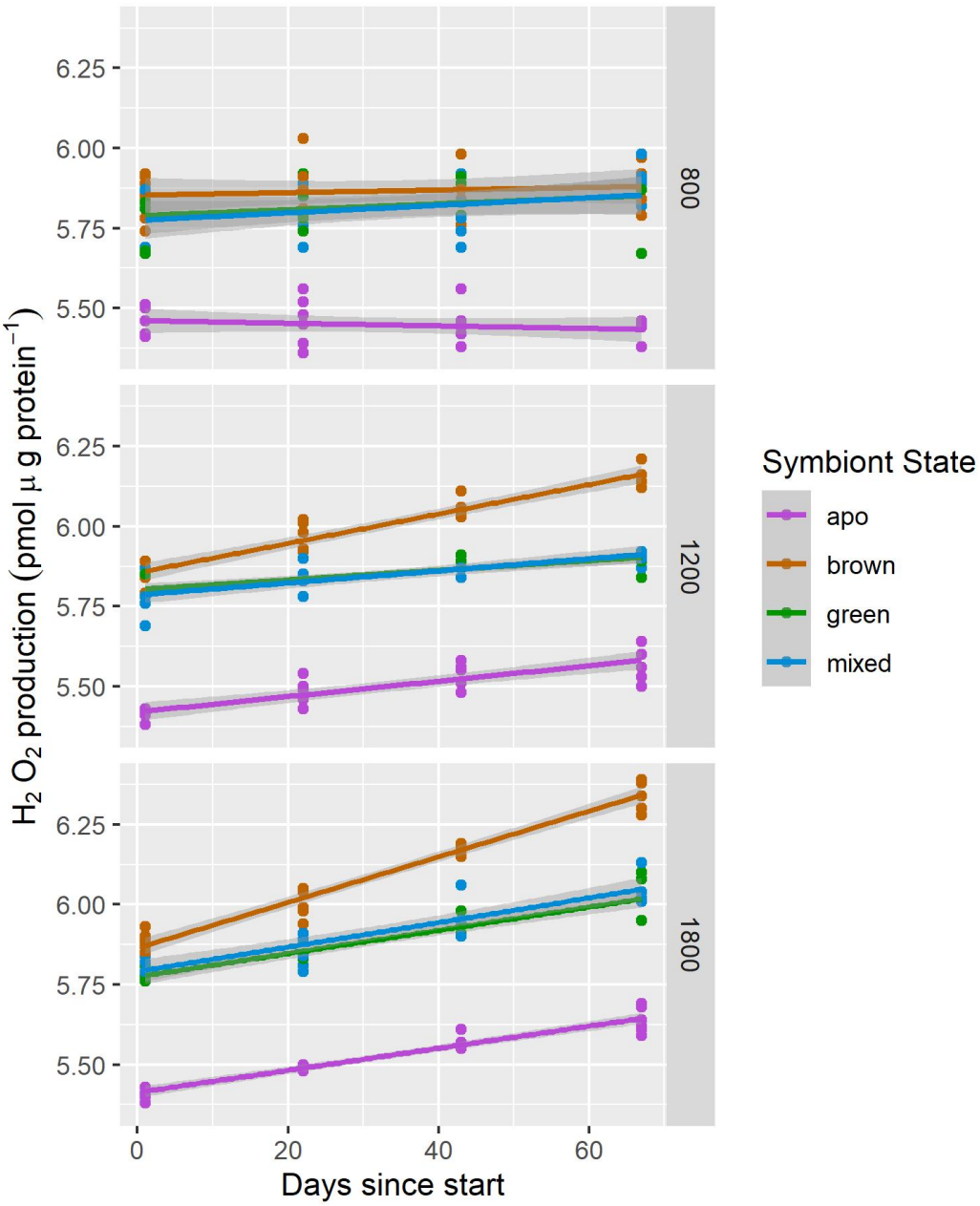

Figure 7. H_2O_2 concentrations (measured on the basis of host protein biomass) for *A. elegantissima* hosting *S. muscatinei* (brown), *E. marina* (green), with near 50/50 populations of *S. muscatinei* and *E. marina* (mixed) and lacking symbionts ("apo"). Experimental conditions were pCO_2 levels of 800, 1200, or 1800 ppm across a 10-week sampling period. Regression lines and 95% confidence intervals are shown.

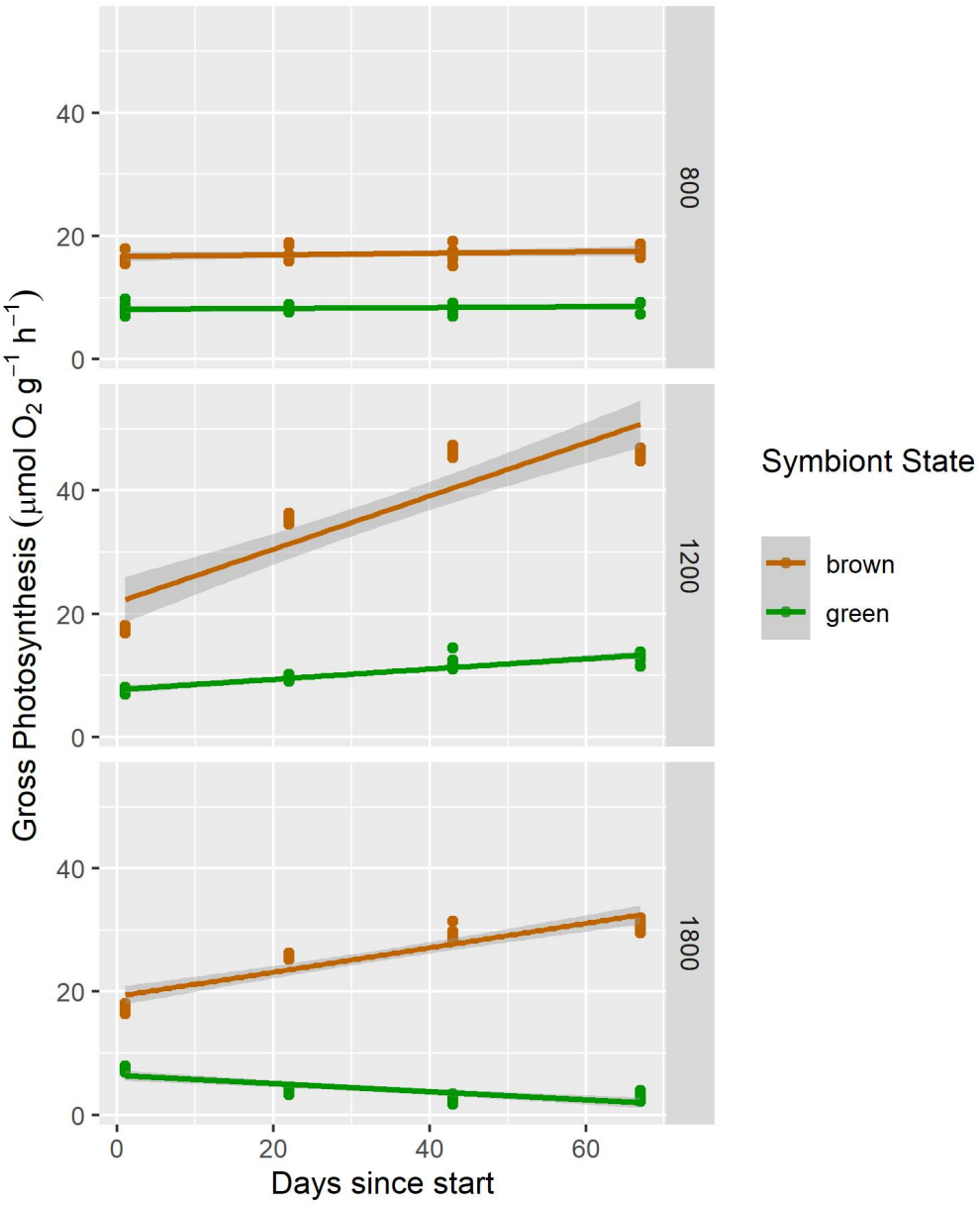

Figure 8. Gross photosynthesis as measured by oxygen production for *A. elegantissima* hosting *S. muscatinei* (brown) or *E. marina* (green) in pCO_2 levels of 800, 1200, or 1800 ppm across the sampling period. Regression lines and 95% confidence intervals are shown.

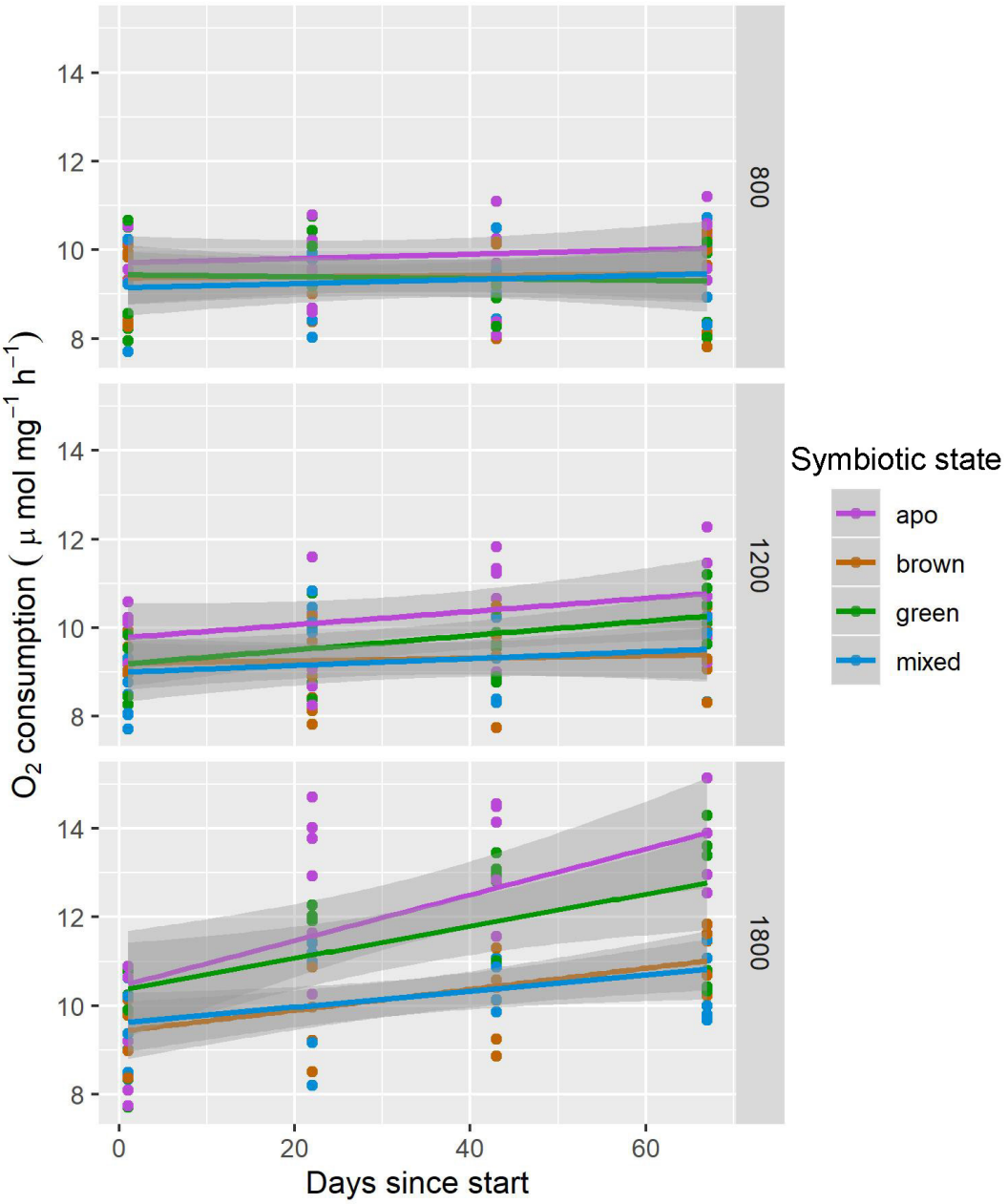

Figure 9. Dark respiration rate of *A. elegantissima* in pCO_2 treatments of 800, 1200, and 1800 ppm. Best-fit regression lines with 95% confidence intervals are shown.

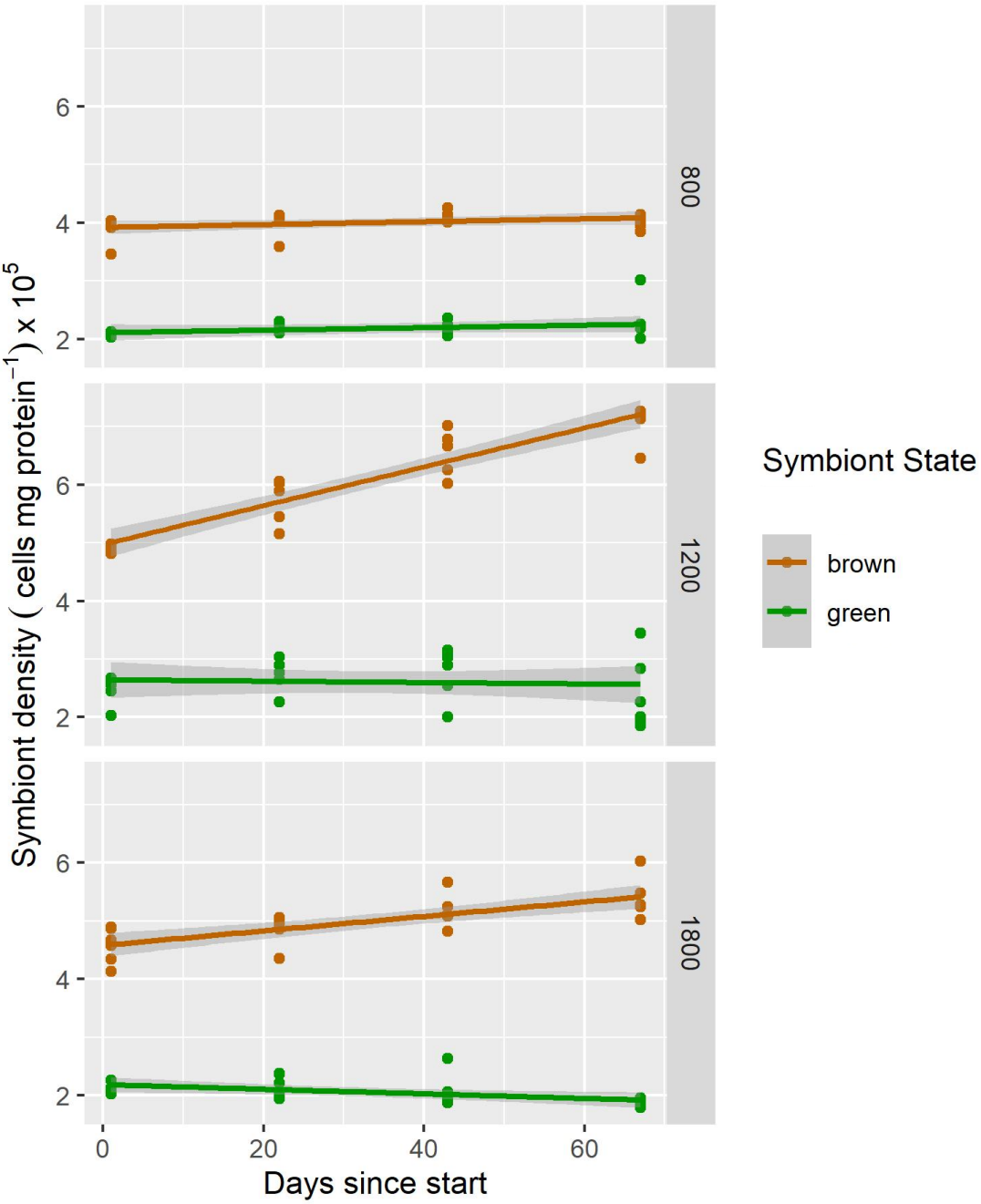

Figure 10. Densities of *S. muscatinei* and *E. marina* in green and brown anemones in each pCO_2 treatment (800 ppm, 1200 ppm, 1800 ppm) over time. Regression lines with 95% confidence intervals are shown.

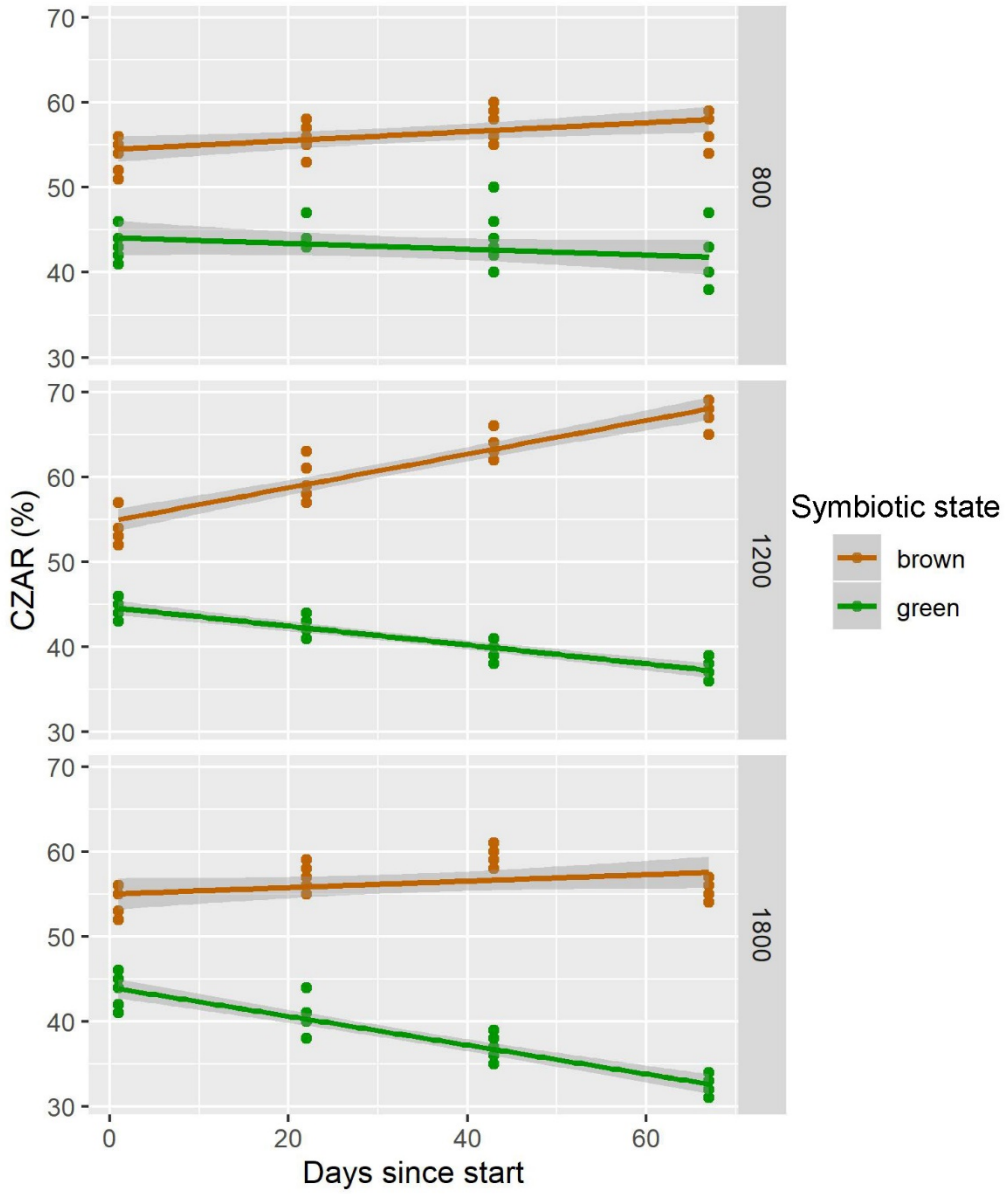

Figure 11. CZAR scores of *A. elegantissima* in pCO_2 treatments of 800, 1200, and 1800 ppm. Best-fit regression lines with 95% confidence intervals are shown.

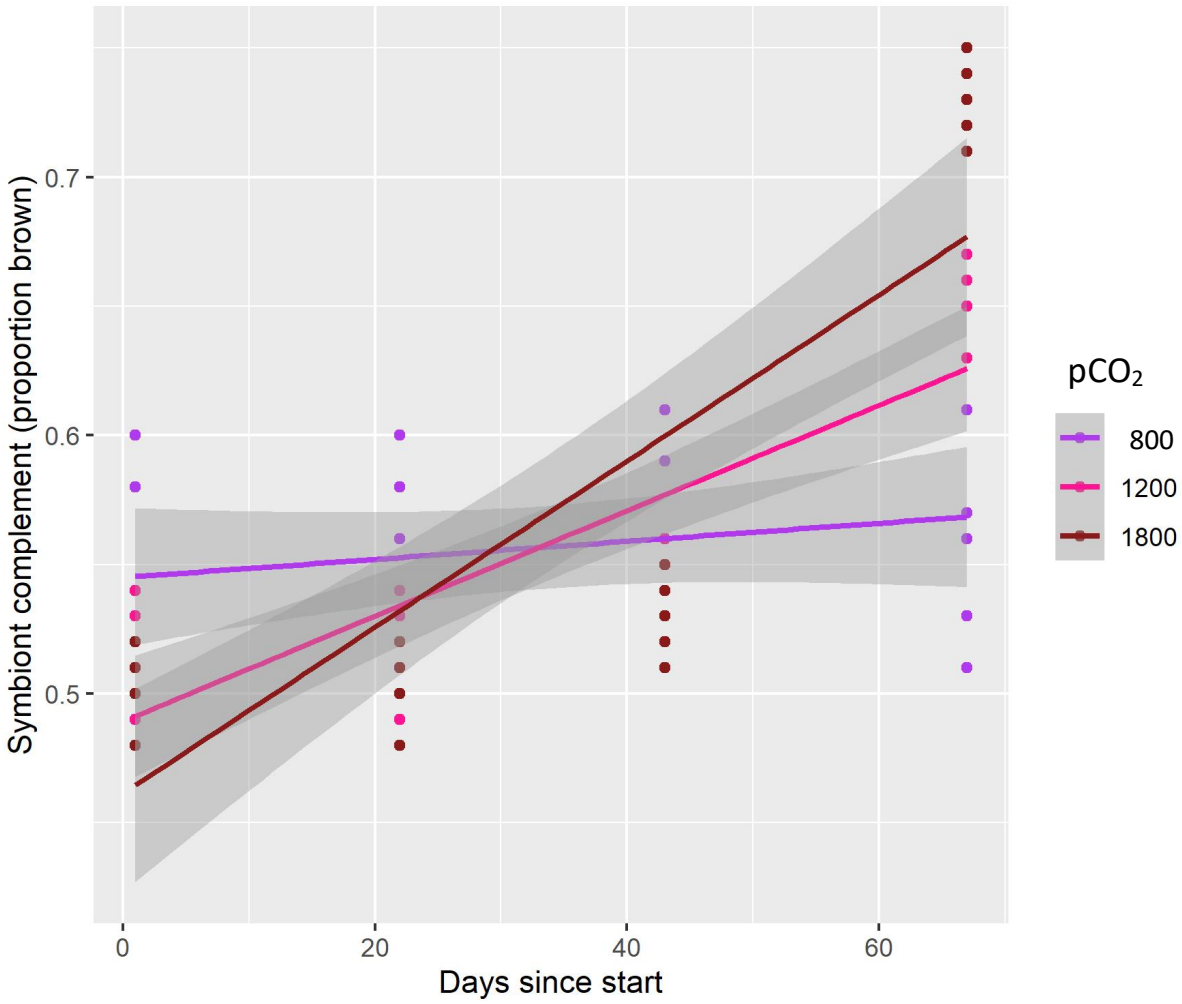

Figure 12. Proportion of the symbiont complement comprised of *S. muscatinei* (brown) in *A. elegantissima* with mixed symbiont complements. Best-fit regression lines with 95% confidence intervals are shown.

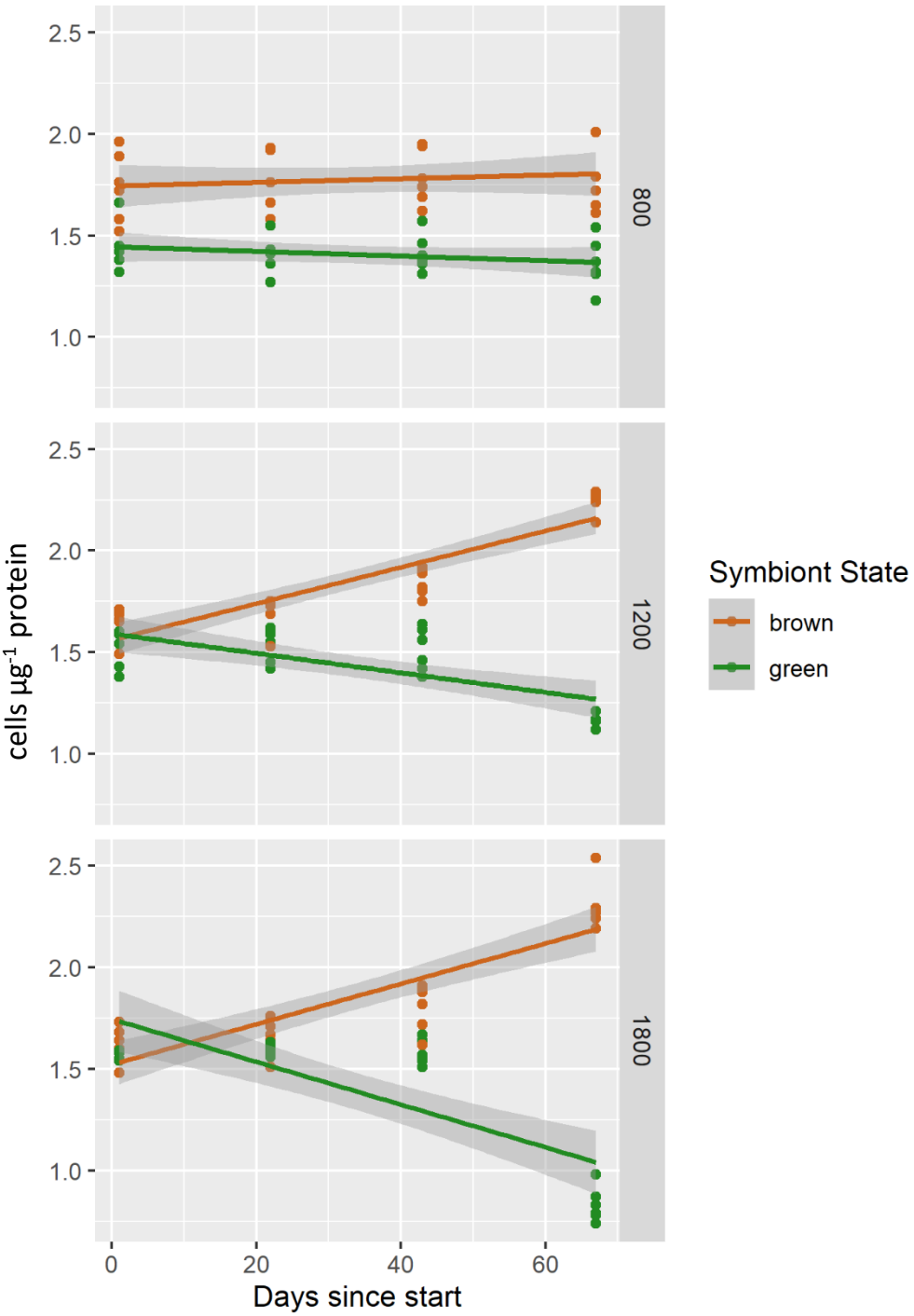

Figure 13. Densities of *S. muscatinei* and *E. marina* in *A. elegantissima* in mixed symbiont complements when exposed to pCO_2 treatment of 800, 1200, and 1800 ppm). Best-fit regression lines with 95% confidence intervals are shown.