ENTERING THE ANTHROPOCENE: HOW OCEAN ACIDIFICATION AND WARMER TEMPERATURES AFFECT THE SYMBIOTIC SEA ANEOMONE *EXAIPTASIA PALLIDA*

by

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ABSTRACT

Here I report the effects of long-term elevated CO_2 combined with two subsequent elevated temperature intervals on the model symbiotic anemone *Exaiptasia pallida.* A central goal of this thesis was to investigate how altered CO_2 and temperature affect the symbiotic relationship while this anemone hosted three different strains of endosymbiotic dinoflagellates (*Symbiodinium minutum, Symbiodinium* A4a, and *Symbiodinium* A4b). Exposure to elevated CO_2 (930µatm) alone for 42 days led to no significant changes in either the anemone or the algae physiological response, with the exception of some separation between the photosynthesis to respiration ratio of *S.* A4a and *S.* A4b control and treatment animals. Exposure to both elevated CO_2 (930µatm) and a moderate elevation in temperature (29°C) for 49 days led to a significant increase in the net maximal photosynthesis (normalized to algal cell density) between the treatment and controls of all three holobionts. Exposure to both elevated CO_2 (930µatm) and an even higher temperature (33°C) for up to 20 days led to a significant decrease in photobiology and algal cell density, along with visible bleaching in the *S. minutum* holobiont. All three holobionts displayed a significant decrease in the photosynthesis to respiration ratio, thereby providing evidence for temperature having a greater impact on the phototrophic response of these anemones. However, anemones harboring the two A4 *Symbiodinium* did not show as large of a negative response in photosystem II photochemistry when compared to anemones with *S. minutum*. The high temperature treatment also resulted in juvenile mortality in all three holobionts, with the greatest mortality seen in the *S. minutum* holobiont. The differential response to both elevated CO_2 and elevated temperature between the three holobionts highlights the thermal sensitivity of the *S. minutum* symbiosis, and the

thermal tolerance of the *S.* A4 holobionts. Thermal tolerance may enable these anemones to survive and thrive in future climate change conditions, while the effects of higher CO_2 appear to be more neutral.

Chapter 1

HOW OCEAN ACIDIFICATION AND WARMER TEMPERATURES AFFECT THE SYMBIOTIC SEA ANEMONE *EXAIPTASIA PALLIDA*

1.1 Introduction

Studying the biological responses of coral reef organisms is becoming increasingly important in light of future global projections of carbon dioxide (CO_2) release and subsequent temperature rise. Anthropogenic inputs of CO_2 are expected to continually rise in the next 100 years, causing the acidification of the ocean (Krief et al. 2010) by as much as 0.5 pH units (NOAA, Caladeira and Wickett 2003, IPCC 2014). Increased atmospheric temperatures by over 4°C are also projected to occur by 2100 (Newell 1979, IPCC 2014). These environmental changes have direct effects on reef ecosystems, which are generally restricted to areas where temperatures vary between 23-30°C and pH is relatively stable at 8.2 (NOAA). Additionally, reefs support vast amounts of biodiversity, buffer shorelines, protect wetlands, and provide about $9.9 trillion every year in human services (Costanza et al 2014), making them a highly valuable asset. Because of their environmental and human importance, coral reef builders are vital to the success of future ecosystems.

Climate change due to anthropogenic release of carbon dioxide (CO_2) has affected the global oceans since industrial times. CO_2 in the atmosphere has increased from ~280 ppm (pre-industrial) to over 400 ppm today (Raven 2005, NOAA). Increased CO_2 in the atmosphere leads to decreased surface ocean pH through CO_2 hydrolysis in the seawater (Krief et al. 2010). The decline in surface ocean pH is

called “ocean acidification”, with the surface oceans pH already decreased by 0.1 unit since pre-industrial times (Raven 2005), bringing the average surface ocean pH to about 8.1 today (IPCC 2014). Not only does increased CO_2 decrease the surface ocean pH, it also decreases the calcium carbonate saturation state by reducing carbonate ion concentrations (Orr et al. 2005). It is predicted that by 2100 the carbonate ion concentration could decrease by 56% if CO_2 emissions remain high (IPCC 2014). This has major implications for calcifying organisms, such as stony corals (order scleractinian), which rely on carbonate to build their calcium carbonate skeletons.

Scleractinian corals’ success in the low nutrient environments they inhabit is due to their relationship with endosymbiotic dinoflagellates in the genus *Symbiodinium* (Muscatine and Porter 1977). *Symbiodinium* provide the coral with photosynthetically fixed organic carbon that is translocated to the host coral. In return, the coral provides the algae with concentrated CO_2 and other nutrients, such as nitrogen and phosphorus, which the algae need for photosynthesis (Muscatine 1981, Muscatine et al. 1989, Rahav et al. 1989). The genus *Symbiodinium* is highly diverse and includes nine described clades (designated A-I) (Pochon and Gates 2010). These clades are identified by several nuclear genetic markers, including the ribosomal internal transcribed spacer 2 (ITS2) non-coding region of the rDNA (LaJeunesse 2002). Clades A through D are most commonly associated with metazoan hosts like corals and sea anemones (Pochon and Powlowski 2006), while clades E and F are better known as free-living or residing within foraminiferans (Jeong et al. 2013, Baker 2003). These algae not only have high inter-cladal (between clade) diversity, but they also have high intra-cladal (within clade) diversity. LaJeunesse et al. (2003) suggests there could be over 100 *Symbiodinium* types (within the eight described clades at the

time). Rowan et al. (1997) noted the diversity in *Symbiodinium* may act as a source of variation in the stress response of corals. This was also noted by Tchernov et al. (2004) when they noted a range of thermal tolerance to elevated temperatures among closely related sister *Symbiodinium*. In particular, thermal sensitivity and tolerance was noted among cultured algal isolates within many of these clades (Tchernov et al. 2004). Sampayo et al. (2008) also observed that thermal tolerance within several corals is variable down to the sub-clade level, and is most likely variable for individual genotypes of the same species. Differences in thermotolerance between individuals of the same species were also seen between two ITS2 type A4 *Symbiodinium* recently isolated from a sea anemone in Florida (Hawkins et al. 2016). Similar to the variability in thermal response, closely related *Symbiodinium* in culture prefer different forms of inorganic carbon. For example, individuals within clade A preferentially acquired different forms of dissolved organic carbon (DIC) as HCO_3^- and CO_2 (Brading et al. 2013), further demonstrating the high physiological diversity within this genus. Notably, the intra-cladal diversity is largely unexplored, especially regarding the consequences of hosting these divergent algae, and how the symbionts influence their hosts, through carbon translocation and their contribution to the nutritional balance of the animal-algal unit (Leal et al. 2015).

High genetic diversity in both stony corals and *Symbiodinium* has led to different ways of coping with decreased pH and carbonate ion concentration. Corals have displayed delayed and variable responses to ocean acidification (OA) (Anthony et al. 2008), largely due to their ability to regulate pH in the subcalicoblastic medium (SCM) where aragonite crystals of their skeleton are formed (Reis et al. 2009, Venn et al. 2013). OA causes a decrease of the internal pH in the SCM, but some corals may

be able to mitigate the effects through this regulation. Coral's external epidermal layer that covers their calcium carbonate skeleton also provides a barrier to the reduction of calcium carbonate saturation (particularly aragonite concentration) via increase of CO_2 in the ocean. Some *Symbiodinium* may also "fertilize" their coral hosts with more organic carbon (closely linked to calcification) because of their increased photosynthesis due to the saturation of CO_2 (Reis et al. 2009). These mixed responses to OA greatly affect the calcification of each coral-symbiont pairing. Rodolfo-Metalpa et al. (2011) found that the temperate corals *Cladocora caespirosa* and *Balanophyllia europaea* grew faster and calcified faster near high CO_2 vents (pH 7.3, 3 months), whereas Gómez et al. (2014) found a significant negative relationship between calcification and CO_2 concentration (pH 7.1, 4 weeks) in *Eunicea fusca.* Different outcomes in these studies may also arise due to the length of the experiments. The study by Gómez et al. (2014) may have potentially missed a different outcome due to its limited time frame. Many ocean acidification studies are short term and may run for a few days to less than 2 months (Reis et al. 2009, Gómez et al. 2014, Gibbin and Davy 2014, Rodolfo-Metalpa et al. 2011, Sinutok et al. 2012, Anthony et al. 2008, Comeau at al. 2014), and may not assess longer term effects or potential recovery. The treatment of decreasing pH (8.12 to 7.08) was only conducted for 30 days, finding a linear decay in skeleton growth. A similar experiment conducted by Krief et al. (2010) had a treatment period of 13 months with three pH levels (8.09. 7.49, 7.19). This study also showed a decrease in skeleton growth from 8.09 to 7.49, but no difference between pH 7.49 to 7.19.

Symbiodinium, like their cnidarian hosts, display variability in their responses to ocean acidification. Anthony et al. (2008) determined that OA decreased the

productivity of *Symbiodinium* in the coral *Acropora intermedia* once the pH dropped below 7.7-7.6, and below 7.95-7.85 in the coral *Porites lobata*. Decreased photosynthetic efficiency leads to a decrease in coral calcification and reduced productivity of the coral-symbiont association (Sinutok et al. 2012, Anthony et al. 2008). High variability in the response to OA by both *Symbiodinium* and their coral hosts is not surprising due to their high diversity and unique host-symbiont pairings.

Increasing sea surface temperature also has physiological effects on *Symbiodinium* and their host cnidarians. The response of *Symbiodinium* to increased temperature is less variable than to the response of ocean acidification. Response to OA has likely been less explored than response to temperature effects due to the assumption that the alga is protected from OA by its host. Increased temperature can lead to a breakdown of the photosynthetic pathway, particularly through photosystem II (PSII) reaction center damage, prompting a decrease in photosynthetic electron transport efficiency (Warner et al. 1996, Iglesias-Prieto et al. 1992, Robison and Warner 2006, Fujise 2014). Coral response to elevated temperature is usually an effect of *Symbiodinium* dysfunction, which triggers the coral to remove the damaged algae via apoptosis or autophagy (Paxton et al. 2013). Increased temperature can also cause an elevated release of reactive oxygen species (ROS), though not in all *Symbiodinium* (McGinty et al. 2012). In many cases, the release of ROS is considered the first step towards symbiont expulsion, referred to as bleaching (Paxton et al. 2013). Bleaching may also occur when the *Symbiodinium* lose a large portion of their chlorophyll *a* pigment (Venn et al. 2006). As mentioned above, some *Symbiodinium* types (even at the scale of different genotypes within the same species) are thermally tolerant, while other genotypes are susceptible to thermal stress. Certain coral–*Symbiodinium*

combinations are more tolerant to thermal stress than others, and thus are less susceptible to bleaching. For example, *Pocillopora damicornis*, which hosted C1 and C3 *Symbiodinium*, was thermally sensitive and bleached readily with increased temperature (Gibbin et al. 2015). This is likely due to the thermally sensitive *Symbiodinium* it hosted, as *Pocillopora damicornis* was more thermally tolerant (having survived in temperatures up to 35°C) when it hosted a clade D symbiont in addition to C1 (Oliver and Palumbi 2011). In contrast, *Montipora capitata*, which hosted C3, C17, C21, C31, and D1 *Symbiodinium* simultaneously, was thermally resistant and resisted bleaching at higher temperatures (Gibbin et al. 2015). This is likely due, in part, to its thermally tolerant D1 symbiont as well. Symbiont based bleaching was also seen between *Symbiodinium* type C3 and C15 hosted in four different coral species. At 34°C for 4 days, corals hosting C3 symbionts showed signs of thermal stress, noted as a drop the maximum quantum efficiency of photosystem II (F_v/F_m), where corals with C15 symbiont did not, regardless of the coral species (Fisher et al. 2012). These differences in thermal stress are also seen in other symbiotic cnidarians, such as anemone–*Symbiodinium* combinations. For example, clade A symbionts in sea anemone *Exapitasia pallida* showed higher thermal tolerance than clade B symbionts in the same host (Goulet et al. 2005).

While the effects of ocean acidification and temperature on corals and their symbionts are highly variable, combined OA and elevated temperature is the most detrimental. Elevated temperatures are found to compound the effects of OA in multiple studies (Sinutok et al. 2012, Rodolfo-Metalpa et al. 2011, Martin and Gattuso 2009), making climate change a real threat to coral reefs in the future. While these results are not always the case, as corals display a high variability in their responses to

OA and temperature (Schoepf et al. 2013, Edmunds et al. 2012), it is likely some level of acclimation and/or adaptation will be key to their survival.

Climate change is not only affecting stony corals on reefs, it is also affecting soft corals and other non-calcifiying anthozoans like sea anemones. Octocorals are the second most important cnidarian fauna on many coral reefs and can occupy up to ~17% of the reef substrate (Gabay et al. 2013, Benayahu and Loya 1977, Fabricius 1997). When three species of octocorals were exposed to pH as low as 7.3 there was no significant change in symbiont density, chlorophyll *a* concentration, sclerite weight, or pulsation rate. No change in these parameters was inferred to mean no change in *Symbiodinium* health or coral health (Gabay et al. 2013). The resilience to low pH was attributed to the octocoral tissues acting as a protective barrier, against pH keeping all internal functions normal. Conversely, Gomez et al. (2015) noted a decrease in sclerite calcification in *Eunicea fusca* at pH 7.1 for four weeks. This is likely due to the extreme low pH exposure, and it also points to species specific responses in octocorals to OA.

Similar to octocorals, many sea anemones appear to be quite resistant to OA. One species of sea anemone (*Anemonia viridis*), occurring along a natural CO_2 gradient near volcanic seeps in the Mediterranean Sea, displays increased growth, abundance, and size when compared to sea anemones occurring in higher pH and lower pCO_2 (pH 7.6 vs. 8.2) outside of the seep zone. Additionally, photosynthetic electron transport and gross photosynthesis were also higher in the low pH gradient (Suggett et al. 2012). Jarrold et al. (2013) noted a similar outcome when exposing the sea anemone *Anemonia viridis* to pH 7.4 and observed high plasticity in the symbiont, as it was able to acclimate to the low pH environment. Towanda and Thuesen (2012)

also observed high resistance to OA for the temperate sea anemone *Anthopleura elegantissima*. When the pH was lowered from 8.1 to 7.3 (6 weeks) the *Symbiodinium* in this anemone also increased photosynthesis and increased their carbon translocation to the host. There were significant decreases in *Symbiodinium* photosynthetic efficiency and cell density, but also a significant increase in chlorophyll *a* content. Although there was a loss of *Symbiodinium*, the authors suggested the increase in chlorophyll *a* content per algal cell, preservation of the metabolic relationships (productivity and respiration), and photosynthetic capacity were evidence of acclimatory abilities in this symbiosis. Because of the sea anemone's advantageous plasticity and resilience to ocean acidification, Weis et al. (2008) suggested that sea anemones could be used as model organisms to further investigate the underlying cellular processes in cnidarian-algae symbioses. While many coral species may survive future ocean conditions, we may nevertheless see a phase shift from stony corals to soft corals, sponges, and even sea urchins (Gómez et al. 2014, Norström et al. 2009). Thus, it has become even more important to study these soft-bodied anthozoans as well as scleractinians.

The sea anemone *Exaiptasia pallida* (*E. pallida*) is recognized as a good model species to study the cnidarian–*Symbiodinium* relationship, not only in the context of soft-bodied anthozoans but for stony corals as well (Weis 2008). *Exaiptasia,* (formerly placed in the genus *Aiptasia,* Grajales and Rodriguez 2014) is a good model system as one can readily render these animals aposymbiotic (lacking symbiotic algae) and then 're-infect' them with many different types of *Symbiodinium* (Kinzie et al. 2001, Belda-Baillie et al. 2002, Hunter 1984). *Exaiptasia* are also able to host different types of *Symbiodinium* (Thornhill et al. 2013). When compared to a scleractinian coral,

Exaiptasia is also easier to maintain in a laboratory setting, grows quickly and tends to reproduce well by asexual pedal laceration (Grajales and Rodriguez 2014), making it convenient to develop into a large experimental population. The majority of recent studies to date that have used this anemone system typically use one of two common clonal lines (named "CC7" and "H2"), with the CC7 genome being recently published (Baumgarten et al. 2015). While these clonal lines have provided important information toward our understanding of how this symbiosis functions, much less is known with regard to the physiological response of natural populations of this anemone or its resident symbionts.

E. pallida, similar to other symbiotic cnidarians, has species specific variability to ocean acidification and increased temperature. Gibbin and Davy (2014) exposed *Exaiptasia* sp. (*Symbiodinium* B1) to a pH of 7.54 (from 8.14) for two months and noted a significant increase in *Symbiodinium* cells to host protein, F_v/F_m, P_{gross} to host protein, P_{gross} per algal cell, and respiration to host protein. All of these parameters point to an anemone that was thriving in its environment. Hoadley et al. (2015) also noted an increase in photosynthesis in three *E. pallida – S. minutum* combinations when they were exposed to pH 7.95 for 28 days, but these anemones had no change in cell density and also no change in host protein. Although ocean acidification had an overall positive photosynthetic effect on *E. pallida*, the anemones did not respond in the same manner when exposed to high temperature. Hanes and Kempf (2013) exposed *E. pallida* (A4 *Symbiodinium)* to 32.5°C for 12 hours and 24°C for 12 hours two times (for a total of 48 hours) and quantified the amount of host driven (and damaging) autophagy. They found short term thermal stress induced a cellular stress response in the host resulting in symbiont cell expulsion. Thermal stress leading to

bleaching was also noted by Hillyer et al. (2016). Hillyer exposed *Exaiptasia* sp. (*Symbiodinium* B1) to 32°C for 6 days, this resulted in a reduction of fatty acids in the host (energy reserves) and bleaching from the thermal stress. While we have an understanding about the separate effects of OA and elevated temperature, the combined effects on *E. pallida* is relatively unknown. This experiment was designed to begin to understand this interaction.

This study sought to describe the response of two ITS2-type A4 *Symbiodinium* isolated from a natural *E. pallida* population from Florida, as well as an isolate of *Symbiodinium minutum* (ITS2-type B1) that was originally isolated from *E. pallida* as well. We examined the physiological response of a natural population of *E. pallida* (originally collected from Florida) to the combined stressors of elevated CO_2 and temperature. Three sets of anemones were used, with each hosting one of the three naturally occurring *Symbiodinium* isolates. Anemone treatments consisted of an initial treatment to high CO_2 alone, followed by a slight thermal rise and then an acute thermal shock. Based on previous short-term experiments with other *E. pallida* / *Symbiodinium* combinations as well as cultured *Symbiodinium*, we anticipated a positive response to OA and a moderate temperature increase. However, we also expected that such positive physiological responses would be diminished by further application of higher temperature, which would be expected to lead to *Symbiodinium* photosystem damage and possible patterns of whole animal bleaching. Different thermal tolerances between each algal type were also expected, such that the two A4 isolates would display similar physiological response and a greater tolerance to the combined effects of high CO_2 and temperature when compared to the more thermally sensitive *S. minutum*.

1.2 Materials and Methods

1.2.1 Anemone Collection, Husbandry, and Characterization

Individual *E. pallida* anemones were collected from Key Largo, FL, USA in August 2014. They were generously donated by Dr. William K. Fitt, University of Georgia, and collected under FWCC permit DD-J2T15642566. The anemones were held at 26°C in 30L tanks with continuously flowing (1L min^{-1}) artificial seawater (Instant Ocean, 1-micron filtered and UV sterilized, salinity 33ppt, pH 8.1). Light was provided at 100µmol photons m^{-2} s^{-1} (12h:12h light:dark cycle, lights on at 06:00) by a bank of LEDs (Cree XP-G2, LED Supply, Randolf, VT, USA). Temperature and recirculating pumps were set and monitored with a digital control system (Neptune Systems Apex Jr., Morganhill, CA, USA) and titanium heaters (Finnex 500+, Chicago, IL, USA). Anemones were fed once per week with freshly hatched *Artemia* napulii.

In order to characterize the *Symbiodinium* within the anemones, tentacles (n = 3) were removed from approximately 10 randomly selected anemones using small stainless steel scissors. DNA extraction was performed using a commercial kit (Promega Wizard Prep) as previously described in Warner et al. (2006). The internal transcribed spacer 2 (ITS2) region of the symbiont rDNA was amplified using "ITS2intfor2" and "ITS2clamp" polymerase chain reaction (PCR) primers as previously described (LaJeunesse and Trench 2000, LaJeunesse 2002). The ITS2 region is highly variable between *Symbiodinium* types and has a high molecular resolution, making it a good indicator of algal type at the intracladal level. The ITS2 fingerprint was visualized using denaturing gradient gel electrophoresis (DGGE) as

described in LaJeunesse (2001), and algal identification confirmed the presence of type-A4 symbionts in all sampled anemones.

After initial characterization and identification of the A4 *Symbiodinium,* two randomly selected anemones were used for *Symbiodinium* isolation and culturing. The anemones were homogenized in 0.22µm-filtered sea water using a glass tissue grinder, and single cells were picked and placed into uni-algal cultures as described in Hawkins et al. (2016). Visual examination of the isolated cultures revealed two potentially phenotypically different A4 type *Symbiodinium* (different growth patterns and visual culture colors). As noted in Hawkins et al. (2016), the cultures were further characterized by sequencing the ITS2, cp23S, and the non-coding region of the plastid encoded *psbA* gene minicircle ($psbA^{ncr}$). This analysis revealed that the two isolates were different genotypes of the same A4 ITS2 type and were designated as KLAp1 and KLAp 2 (Hawkins et al 2016). Herein these two A4 isolates are referred to as *Symbiodinium* A4a and *Symbiodinium* A4b respectively.

1.2.2 *Symbiodinium* Removal and Re-infection

In order to obtain aposymbiotic anemones (anemones lacking symbiotic algae), *Symbiodinium* were removed from a batch of anemones (n > 200) using the menthol exposure method (Matthews et al. 2016) combined with brief periods of cold shock at 4°C (Muscatine et al. 1991). Complete aposymbiosis was confirmed using a fluorescence microscope (EVOS system, ThermoFisher, Life Technologies, Waltham, MA, USA) in order to observe any algal cells by their chlorophyll *a* auto fluorescence. Once complete removal of all *Symbiodinium* was confirmed, aposymbiotic anemones were held in a 30L tank containing filtered and UV sterilized Instant Ocean at room temperature (~25°C) until re-infection.

Aposymbiotic anemones were split into 3 groups (n > 20 each) for algal reinfection. Group 1 was infected with the *S.* A4a culture, group 2 was infected with the *S.* A4b culture, and group 3 was infected with an isolate of *Symbiodinium minutum* (ITS2 type B1) that was originally isolated from a Caribbean soft coral and placed into culture ((Pk702 *S.minutum*), donated by Dr. Mary-Alice Coffroth, University of New York, Buffalo, NY, USA). Prior to infection, all culture designations were confirmed by ITS2 DGGE analysis (described above) and were re-infected in accordance with infection methods described in Hoadley et al. (2015) (~1000 cells mL^{-1} in 500 mL seawater, with two rounds of algal exposure in tandem with feeding). Re-infected anemones were periodically checked using PCR and DGGE to verify that they harbored only their assigned *Symbiodinium* introduced by the infection series.

1.2.3 Experimental Setup

The experimental design consisted of three seawater systems. Each system consisted of one sump (~360L) which circulated water to three 30L tanks (water flow and light conditions were as described above). The systems were randomly assigned one of three groups: Treatment 1, Treatment 2, or Control. The tank assignments allowed for staggered (1-day difference) treatment and sampling. The Treatment 1 system had two tanks, one containing *S.* A4a anemones and the other containing *S.* A4b anemones. The Treatment 2 system had one tank containing *S. minutum* anemones. The Control system had three tanks, one tank containing *S.* A4a anemones, one containing *S.* A4b anemones, and the last containing *S. minutum* anemones. The anemone types were randomly assigned to a tank within each respective system. Each tank contained four glass bowls to provide separation for ease of cleaning, counting,

and sampling. Anemones were randomly split into these four bowls (n = 7 or 8 $bowl^{-1}$). A schematic of the experimental system is shown in Figure 1.

Random assignments of systems and tanks were conducted using R's sample function, which randomly orders a given amount of numbers (e.g. Sample(1:3) ← 2,1,3). The parameters (in the order they appear above) were paired with the numbers assigned to each system and subsequently each tank after they had been randomly ordered using the sample function.

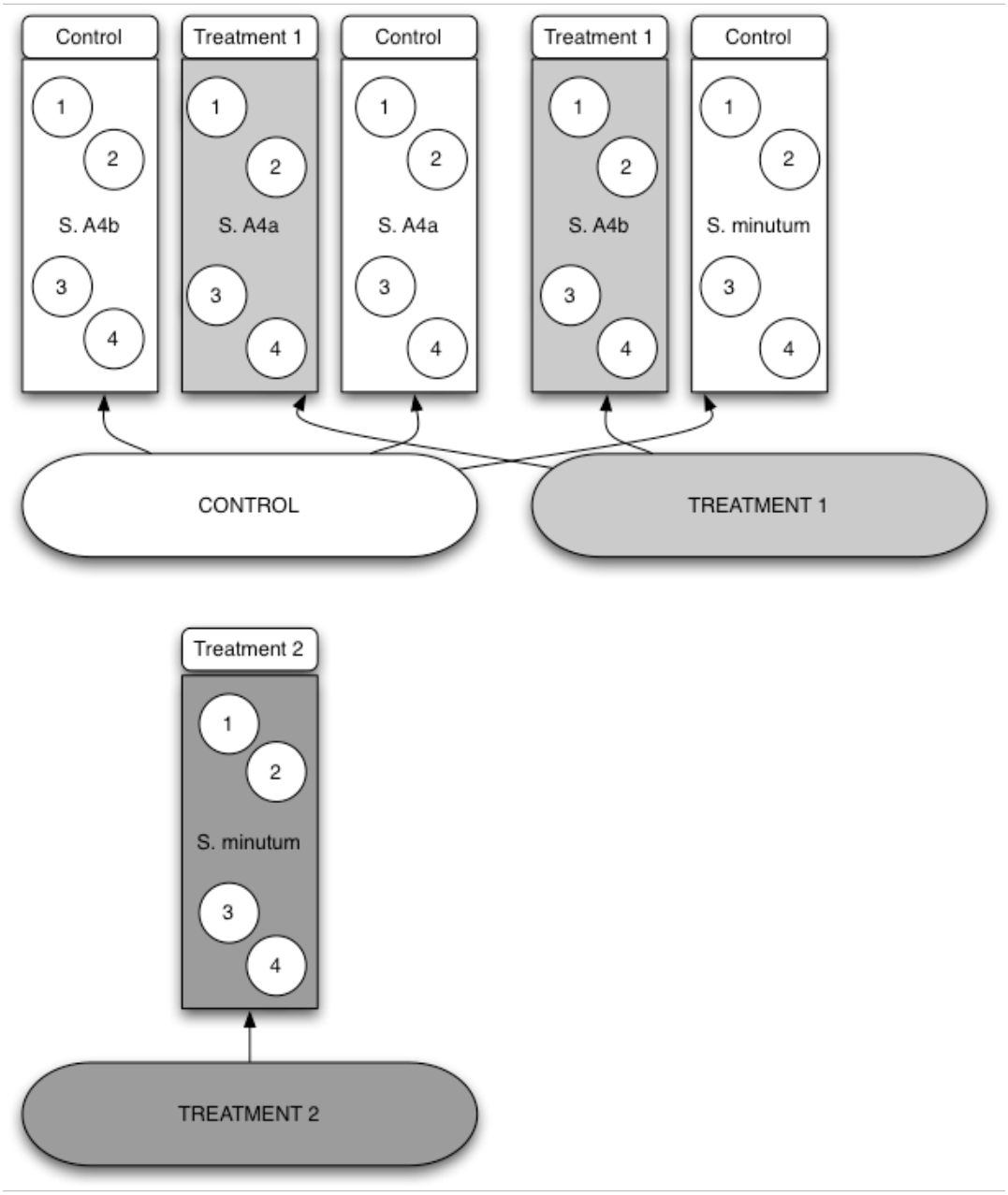

Figure 1. Layout of the experimental system. Numbered circles represent bowls containing anemones. An additional bowl was added 2 weeks into the experiment to hold the previously counted juvenile anemones (not shown).

In order to more closely mimic the pH and temperature conditions seen in a natural tropical environment, these parameters (Table 1) were programmed to change on a diel cycle (Table 2). The pH of each system was controlled using a custom designed pH stat system composed of a series of solenoid valves connected to a supply of pure CO_2 or CO_2-free air. Seawater pH was monitored throughout the experiment by a single pH electrode in each sump connected to a digital monitoring system (Hach sc200, Hach, Loveland, CO, USA). Measurements from the pH probes were recorded using a custom-built software program (P. Grecay). Diel pH shifts were designed from data presented in Hofmann et al. (2011) and pre-programed into the pH stat software. Initial tests of the pH electrodes were cross-checked with an additional laboratory pH system and it was determined that pH electrode re-calibration was necessary at approximately two-week intervals. All calibrations were performed with NBS pH buffer standards. The temperature was controlled by titanium heaters as described above. All three systems were held at the control conditions (pH 8.1 ± 0.1, 26°C) for two weeks prior to the start of the experiment to acclimate the anemones to the experimental system. The control system remained at these conditions for the duration of the experiment.

The anemones in the treatment systems were initially exposed to long term high CO_2 alone, followed by two subsequent periods of elevated temperature. From Day 0 to Day 7, the pH was ramped from 8.1 to 7.7 (0.05 pH units day^{-1}), with the temperature remaining at 26°C. The final pH was 7.7 (pCO_2 ~920µatm, Table 1) for the remainder of the experiment. From Day 42 to Day 48 the temperature was ramped from 26°C to 29°C (at 0.5°C day^{-1}), where it was held until day 91. From Day 91 to

Day 97 the temperature was ramped from 29°C to 33°C (at 0.5°C day^{-1}), where it was held until the end of the experiment on Day 111.

Anemones were sampled from the control and treatment systems (n = 4, with 1 anemone removed from each bowl) on Days 0, 14, 42, 91, and 111. At each of these time points, anemones were sampled for photochemistry, photosynthesis, respiration, ash-free dry weight, animal protein, animal lipids, animal carbohydrates, animal citrate synthase activity, chlorophyll *a*, and *Symbiodinium* cell number (described in further detail below). Due to some mortality during the highest temperature treatment, some sample sizes were reduced to a n of 3. Anemones were also collected (n = 3) on day 42 and 111 for RNA extraction and subsequent carbonic anhydrase (CA) analysis (not shown here). Day 0 encapsulated the baseline responses, Day 14 the acute response to high CO_2, Day 42 the long-term response to high CO_2, Day 91 the combined effects of long-term high CO_2 and moderate temperature increase, and Day 111 tested the combined effects of long-term high CO_2 and acute high temperature exposure. The timeline of the experimental treatments and sampling days is shown in Figure 2.

In order to quantify reproductive output, all bowls of anemones were examined at the end of each week for asexually produced juveniles with visible tentacles at the base of the pedal disk (i.e. the foot) of each adult. Juveniles were counted and then transferred to a separate bowl and held within the same respective system as their parents.

Table 1. Seawater chemistry. pCO_2 and pH seawater scale ($_{SWS}$) were calculated using the carb() function in the "seacarb" statistical package for R.

	Treatment 1	Treatment 2	Control
pH_{SWS}	7.73 ± .05	7.73 ± .04	8.07 ± .08
pCO_2 (µatm)	911 ± 97	931 ± 75	391 ± 79
TA (µmol kg^{-1})	2239 ± 114	2364 ± 136	2360 ± 192
DIC (µmol kg^{-1})	2178 ± 186	2227 ± 212	2149 ± 280
Salinity (ppt)	33 ± 2	33 ± 2	33 ± 2
Temperature (°C)	26 ± 1, 29 ± 1, 33 ± 1	26 ± 1, 29 ± 1, 33 ± 1	26 ± 1

Table 2. Averaged carbonate parameters recorded over a 12 hour cycle (5:00 – 17:00 with one recording $hour^{-1}$ in 2015. pCO_2 and pH_{SWS} were calculated using the carb() function in the "seacarb" package in R.

	Treatment 1	Treatment 2	Control
pH_{SWS}	7.74 ± .05	7.75 ± .04	8.11 ± .04
pCO_2 (µatm)	900 ± 92	935 ± 101	343 ± 35
DIC (µmol kg^{-1})	2116 ± 40	2016 ± 34	2227 ± 37

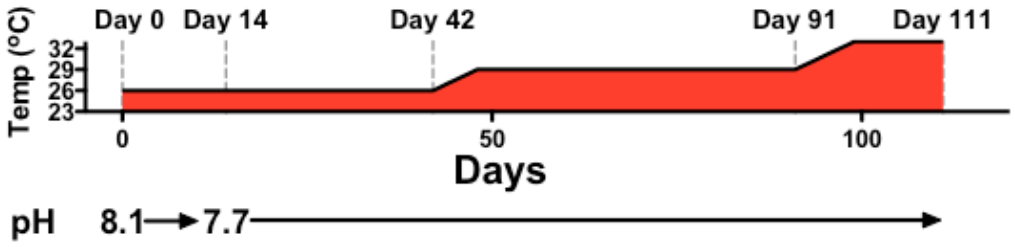

Figure 2. Experimental timeline for the change in temperature, pH and anemone destructive sampling. "Day" values along the top of the figure demarcated by the dashed grey vertical lines are the anemone sampling days. Temperature ramping intervals were on days 42 - 48 and 91 - 97. The pH ramping period is not explicitly labeled, but occurred during the first 7 days of the experiment.

1.2.4 Seawater Chemistry

Seawater carbonate chemistry was confirmed by measuring the dissolved inorganic carbon (DIC) and total alkalinity (TA) of each sump. DIC measurements were performed using a 20mL water sample taken at approximately 09:00 once per week and fixed with 200µl mercuric chloride (5% solution). Fixed water samples were kept in air tight glass vials at 4°C if they were not measured immediately. DIC was measured using a LI-COR LI-6252 CO_2 analyzer, Lincoln, NE, USA. Seawater standards of a known DIC and TA (Carbon Dioxide Information Analysis Center, Scripps, UCSD, USA) were used for the standardization of these measurements. TA measurements were performed using the pH bromocresol purple based method of Yao and Byrne (1998) with a Metrohm 876 Dosimat Plus Titrator (Herisau, Switzerland) connected to a spectrometer (Ocean Optics USB2000). TA measurements were also standardized with the same seawater standards (Carbon Dioxide Information Analysis Center, Scripps, UCSD, USA). Actual pCO_2 and pH (sea water scale) were calculated using the "seacarb" R package with the DIC, TA, salinity, and temperature measurements. These values are described in Table 1.

1.2.5 Photochemistry, Photosynthesis, and Respiration

In addition to the destructive sampling described below, anemone symbiont photosystem II (PSII) photochemistry was monitored twice per week, and daily from Days 91 to 111, by measuring active chlorophyll *a* fluorescence of the symbiotic algae with a pulse amplitude modulation (PAM) fluorometer (Diving PAM, Walz, Effeltrich, Germany). Anemones were dark acclimated for approximately 30 minutes before maximum potential quantum efficiency of photosystem II (F_v/F_m^{MT}) was recorded in triplicate (n = 2 anemones $bowl^{-1}$) for all control and treatment anemone-

algal combinations (Figure 3). Maximal fluorescence (F_m^{MT}) was recorded by a single saturating pulse of light provided by the halogen light source of the fluorometer.

At each destructive sampling point, anemones (n = 3 or 4, depending on treatment and timepoint) were removed from their systems and sampled for active chlorophyll *a* fluorescence as well as oxygen production and respirometry. Active chlorophyll *a* fluorescence measurements, in the dark and light acclimated state, were conducted with a Fluorescence Induction and Relaxation (FIRe) fluorometer (Satlantic, Halifax, Canada). This provided measurements of the maximum quantum efficiency of photosystem II (F_v/F_m^{ST}) and the quantum efficiency of photosystem II photochemistry in the light activated state ($F_q'/F_m'^{ST}$). FIRe measurements were taken using anemones that were held in a glass bowl containing water from their respective system and light acclimated to 100 µmol photons m^{-2} s^{-1} white LED light for 15 minutes. The anemones were then exposed to a series of five single turnover flashes of blue light (each 120µs long) to obtain the minimum (F_s') and maximum (F_m') fluorescence in the light. The anemones were then dark acclimated for 15 minutes, and subsequently exposed to another series of five single turnover flashes of blue light to obtain the minimum (F_o) and maximum (F_m) fluorescence in the dark. Fluorescence parameter estimates were taken from non-linear curve fitting, and calculations of F_v/F_m^{ST} and $F_q'/F_m'^{ST}$ were performed in the FIRePRO software using these equations: $F_v/F_m = ((F_m - F_o)/F_m)$, and $F_q'/F_m' = ((F_m' - F_s')/F_m')$.

The F_v/F_m^{MT} values recorded from the PAM fluorometer are slightly higher than those given from the FIRe fluorometer due to the fundamental differences in the light pulses provided by the two instruments. The FIRe fluorometer provides a very short (100 - 400µs) and intense (> 20,000µmol photons m^{-2} s^{-1}) pulse of light, whereas

the PAM provides multiple saturating pulses (20kHz) of a much lower intensity (~3000 – 10,000µmol photons m^{-2} s^{-1}) light. As the multiple turnover saturating light pulse of the PAM fluorometer completely reduces the plastoquinone pool (PQ) as well as PSII, the F_v/F_m^{MT} values are typically higher than those recorded by the FIRe fluorometer that only provides a single turnover of the PSII reaction center (Sugget et al. 2003).

After recording the chlorophyll *a* fluorescence, the anemones were placed in individual water-filled 20mL glass vials fitted with a mesh bottom and magnetic stir bar and held at the pCO_2 and temperature of the respective system. Anemones were allowed to relax and open their tentacles before respirometry measurements. The respirometry system consisted of a 4-vial rack with a stir plate and was placed in a temperature controlled water bath set to the temperature of the control or treatment system. Once the anemones were relaxed, the vials were sealed with a rubber stopper that was fitted with a fiber optic oxygen optode attached to a digital recorder (Fibox, Presens Gmbh, Regensburg, Germany). Anemones were placed in the dark for 15 minutes prior to measurement. Dark respiration was then measured in the dark for 15 minutes. Oxygen production was then recorded by switching on a set of white LEDs (200µmol photons m^{-2} s^{-1}) for 30 minutes. After the light period, dark respiration was recorded for an additional 30 minutes. Anemones were then removed and the volume of water in each vial was recorded. Anemones were then blot-dried on paper towels, placed in 2mL screw cap vials, snap-frozen in liquid nitrogen, and immediately stored at -80°C until further processing.

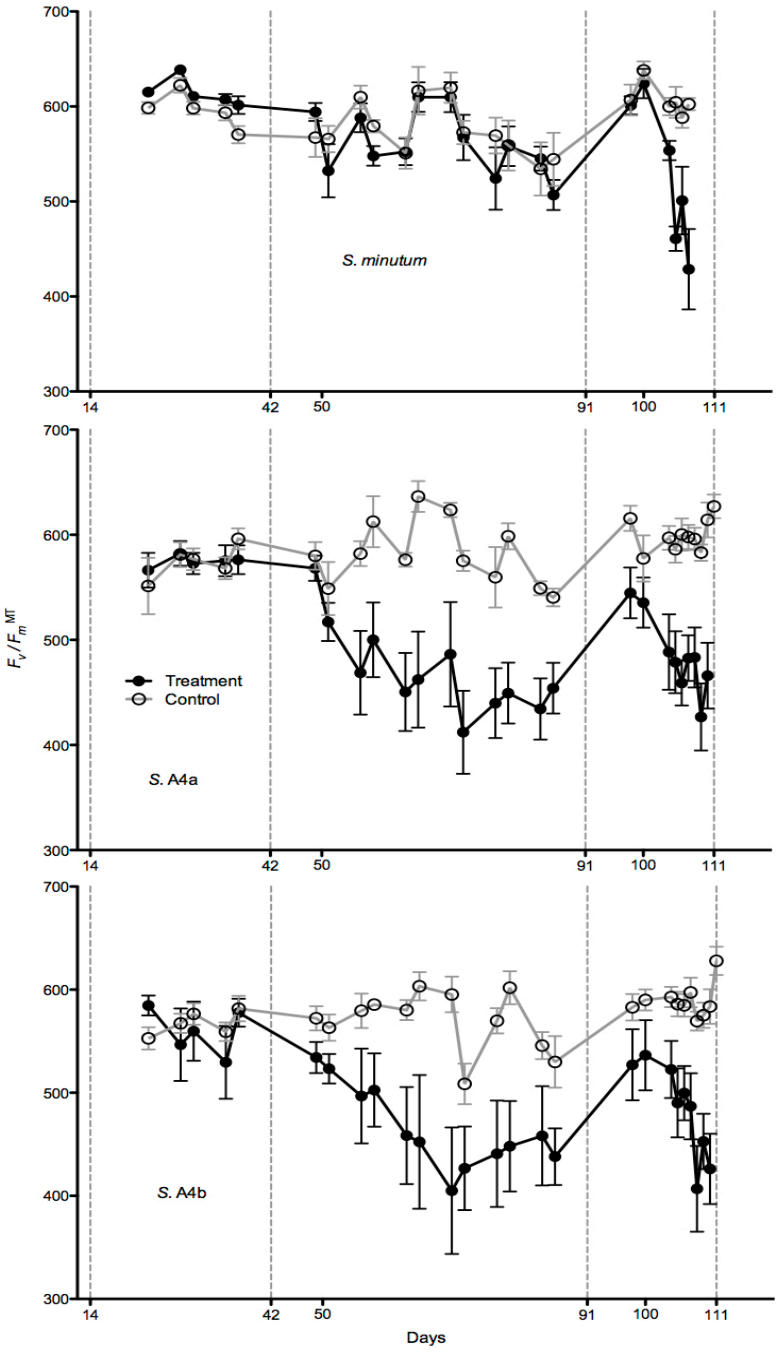

Figure 3. Maximum quantum yield of PSII recorded by PAM fluorometry (F_v/F_m^{MT}) (n = 3 or 4 ± SE). Gaps in the data are during sampling points and the first two weeks of the experiment when measurements were taken with a FIRe fluorometer.

1.2.6 Anemone Processing

All anemone processing was carried out on ice or at 4°C to minimize enzyme degradation. The sample processing protocol is shown in Figure 4. Anemones were thawed on ice and 0.8mL ice cold lysis buffer (25mM Tris, 1mM EDTA, 10% glycerol, pH 7.8) was added to each screwcap vial along with a stainless steel ball bearing (5mm diameter). The samples were homogenized with a chilled bead beater (Fast Prep-24, MP Bio, Santa Ana, CA, USA) for 60s at 6m s^{-1}. 150µL of the homogenate was removed for ash-free dry weight (AFDW) quantification. The remaining homogenate was centrifuged at 3000 x g for 30s to quickly pellet the majority of the *Symbiodinium* cells without removing anemone material. The supernatant was transferred to a new 1.5mL plastic tube and centrifuged at 16,000 x g for 20 minutes to pellet any remaining *Symbiodinium* cells (only very small algal pellets indicated low algal cell carryover into the supernatant at this step). Further algal pellet processing is described below. The supernatant was then divided into separate fractions for protein, lipid and carbohydrate extraction (40µL, 150µL, and 150µL respectively) and stored at -20°C. 70µL of the supernatant was then snap frozen in liquid nitrogen, and stored at -80°C for subsequent citrate synthase (CS) activity measurement. Any remaining supernatant was also snap frozen and stored at -80°C. The *Symbiodinium* pellet was re-suspended in 500µL filtered sea water (FSW) and 100µL was removed for cell counts and fixed with 5uL glutaraldehyde (0.4% final concentration), and stored at 4°C. 100µL was removed for chlorophyll *a* extraction, and stored at -20°C. The remaining algal cell samples were centrifuged at 5000 x g for 5 minutes, snap frozen in liquid nitrogen, and stored at -80°C.

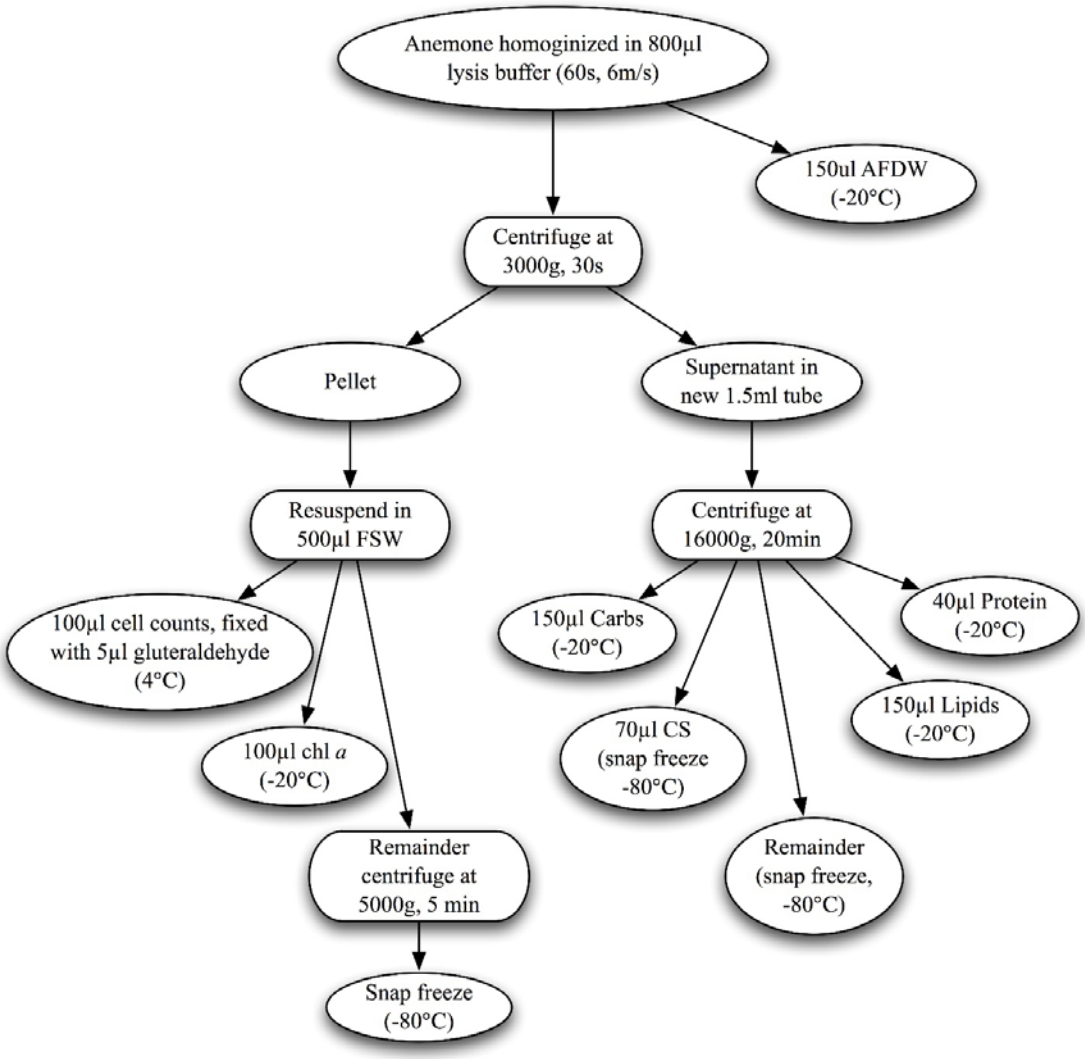

Figure 4. Flow schematic of the anemone processing. Supernatant and pellet processing occurred simultaneously to minimize enzyme and pigment degradation.

1.2.7 Symbiodinium Analysis

Algal cell number was recorded using an Improved Neubauer hemocytometer (n = 6 replicate counts sample^{-1}) and an epifluorescence microscope (EVOS system). Algal cells were identified (4x objective) by chlorophyll *a* fluorescence using a CY5

filter (ex. 628 ± 40 nm, em. 692 ± 40 nm). Each field of view was photographed and the cells were counted using the "analyze particles" function in ImageJ (NIH, Bethesda, MD, USA). Chlorophyll *a* content was quantified by exacting the pigments with methanol, then reading the pigment absorbances at 632nm, 665nm and 750nm wavelengths using a plate reader, adapted from Ritchie (2006) and Warren (2008). Absorbance at 632nm and 665nm were corrected for turbidity by subtracting the absorbance recorded at 750nm.

1.2.8 Anemone Analysis

Anemone analysis consisted of both biomass and enzymatic measurements. Ash-free dry weight (AFDW, a combination of both anemone and *Symbiodinium* material) samples were first placed in pre-weighed aluminum boats and dried in an oven for 3 weeks at 60°C. Dried material was then weighed again on a microbalance to obtain the dry weight. The boats were combusted at 500°C for four hours and allowed to cool overnight, then re-weighed to record the ash weight. The ash weight was subtracted from the dry weight to calculate the ash-free dry weight of each sample. Biomass measurements were all conducted in microplates via color-metric assays adjusted for the small sample volume. Protein content was quantified using a modified version of the Bradford method (Bradford 1976) which measured absorbance at 595nm and corrected for 465nm (595nm $465nm^{-1}$). Lipid content was quantified using a bulk lipid extraction adapted from Cheng et al. (2011). Background absorbance was measured at 540nm, and final absorbance was measured at the same wavelengths after a five minute incubation with 0.2mg mL^{-1} vanillin. Carbohydrate content was quantified using an assay adapted from Dubois et al. (1956), with absorbance measured at 485nm. All absorbance measurements were corrected for

turbidity at 750nm. Protein, lipids, and carbohydrates were all normalized to AFDW. Citrate synthase (CS) enzyme activity was also measured using a color-metric assay adapted from Srere (1969). The baseline absorbance (412nm, 3 min) was measured prior to CS catalyzation with oxaloacetate and acetyl coenzyme A which was measured again at 412nm for 3 minutes. CS enzyme activity was normalized to the protein content. Detailed methods and calculations are described in Hawkins et al. (2016b). All absorbance based assays were conducted on a FLUOstar Omega microplate reader (BMG LABTECH, Ortenburg, Germany).

1.2.9 Statistical Analysis

1.2.9.1 Linear Mixed Effects Modeling

The majority of the experimental data collected, $F_vF_m^{ST}$, $F_q'/F_m'^{ST}$, P:R, Protein AFDW^{-1}, Lipid AFDW^{-1}, Carb AFDW^{-1}, CS Protein^{-1}, Cells AFDW^{-1}, Respiration Cell^{-1}, P_{net} Cell^{-1}, Chl *a* Cell^{-1}, were examined with a linear mixed effects model (R package "nlme") to determine statistical significance. All data were tested with a Shapiro-Wilk test of normality, visually assessed for normality (due to the smaller sample size n = 4), and tested with a Levene Test for homogeneity of variance prior to being tested in the linear model. A model was constructed for each subject of experimental data, using the between-subjects of "Treatment" (treatment or control), and "Zoox" (algae type), and a within-subject factor of "Time" (day). A random effect of "Rep" (replicate measurement) was also included. F-statistics were obtained using the anova() function, and only constructions with $p < 0.05$ were used in a subsequent pairwise *post hoc* analysis, using the glht function (R package "multcomp") and

Tukey's correction for multiple comparisons. Statistical outputs are located in Appendix A.

1.2.9.2 Polynomial Modeling

The asexual reproduction data were not normally distributed or homogeneous but showed a parabolic distribution pattern. Therefore, a polynomial mixed model (R package lme4) was used to match the similarity of the data to a polynomial function, versus a linear function. This allowed statistical comparison between the polynomial pattern of the control data and the polynomial pattern of the treatment data in order to test for a difference in the overall trend of the juvenile:adult animal values. Hence, this model examined the overall difference in the polynomial trends between control and treatments, but it did not compare the individual time points throughout the experiment.

1.3 Results

Seawater chemistry parameters of pH_{SWS}, pCO_2, total alkalinity (TA), dissolved inorganic carbon (DIC), salinity, and temperature remained within expected ranges over the course of the experiment and are presented in Table 1. The summary statistical outputs from the linear mixed-effects model *post hoc* analysis, and the polynomial model are presented in Tables 3 and 4 respectively. All p values listed below are from the *post hoc* analysis with the exception of the reproduction results.

Table 3. Statistical output of the linear mixed model *post hoc* analysis of significant ANOVA results. Significant p-values are in bold. TR = Treatment, CT = Control. Numbers 1 – 5 represent the five sampling days: 0, 14, 42, 91, and 111.

	F_v/F_m ST	F_q'/F_m' ST	Cells AFDW^{-1}	P_{net} Cell^{-1}	P:R	R Cell^{-1}
Degrees of Freedom	24	24	24	39	34	43
	TR A4a 1 TR A4a 4	TR A4a 1 TR A4b 1	CT A4a 5 TR A4a 5	TR 1 TR 4	TR 2 TR 5	TR 1 TR 5
p-value	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.001**	**< 0.01**	**< 0.001**
	TR A4a 1 TR A4a 5	TR A4a 1 TR B1 1	TR A4b 2 TR A4b 4	TR 2 TR 4	TR 3 TR 5	TR 2 TR 4
p-value	**< 0.01**	**< 0.01**	**0.0138**	**< 0.001**	**< 0.01**	**0.02866**
	TR A4a 3 TR A4a 4	TR A4a 3 TR A4a 4	TR A4b 2 TR A4b 5	TR 3 TR 4	TR 4 TR 5	TR 2 TR 5
p-value	**< 0.01**	**0.0289**	**0.0266**	**< 0.001**	**< 0.01**	**< 0.001**
	TR A4b 3 TR A4b 4	CT A4b 4 TR A4b 4	TR B1 1 TR B1 3	TR 4 TR 5	CT 5 TR 5	TR 3 TR 5
p-value	**< 0.01**	**0.0137**	**0.0150**	**< 0.001**	**< 0.01**	**< 0.001**
	TR A4b 3 TR A4b 4	TR B1 1 TR B1 5	TR B1 1 TR B1 5	CT 4 TR 4		CT 5 TR 5
p-value	**< 0.01**	**< 0.01**	**0.0130**	**< 0.001**		**< 0.001**
	TR B1 4 TR B1 5	TR B1 2 TR B1 5	CT B1 5 TR B1 5			
p-value	**< 0.01**	**< 0.01**	**0.0128**			
	CT B1 5 TR B1 5	TR B1 3 TR B1 5				
p-value	**< 0.01**	**< 0.01**				
		TR B1 4 TR B1 5				
p-value		**< 0.01**				
		CT B1 5 TR B1 5				
p-value		**< 0.01**				

Table 4. Statistical output of the polynomial mixed model comparing the patterns of juvenile:adult anemones over time between control and treatment trends within each holobiont combination. Significant p-values in bold.

Variable	Degrees of Freedom	t-value	p-value
S. A4a	108	0.953	0.3427
S. A4b	104	-3.629	**0.0004**
S. minutum	108	-1.783	0.0774

1.3.1 Static Variables

Over the course of this experiment there were multiple variables that did not display any significant responses to the high CO_2 or high temperature conditions in any of the three anemone-alga (holobiont) combinations. Namely, the anemone biomass parameters of protein, bulk lipids, and carbohydrates, all normalized to ash-free dry weight (Protein $AFDW^{-1}$, Lipid $AFDW^{-1}$, Carb $AFDW^{-1}$), remained static. The citrate synthase enzyme activity normalized to the anemone protein (CS $Protein^{-1}$) also showed no significant changes. The symbiont chlorophyll *a* normalized to the total *Symbiodinium* cells (Chl *a* $Cell^{-1}$) was the only symbiont related variable that did not change significantly over the course of this experiment (Figure 5).

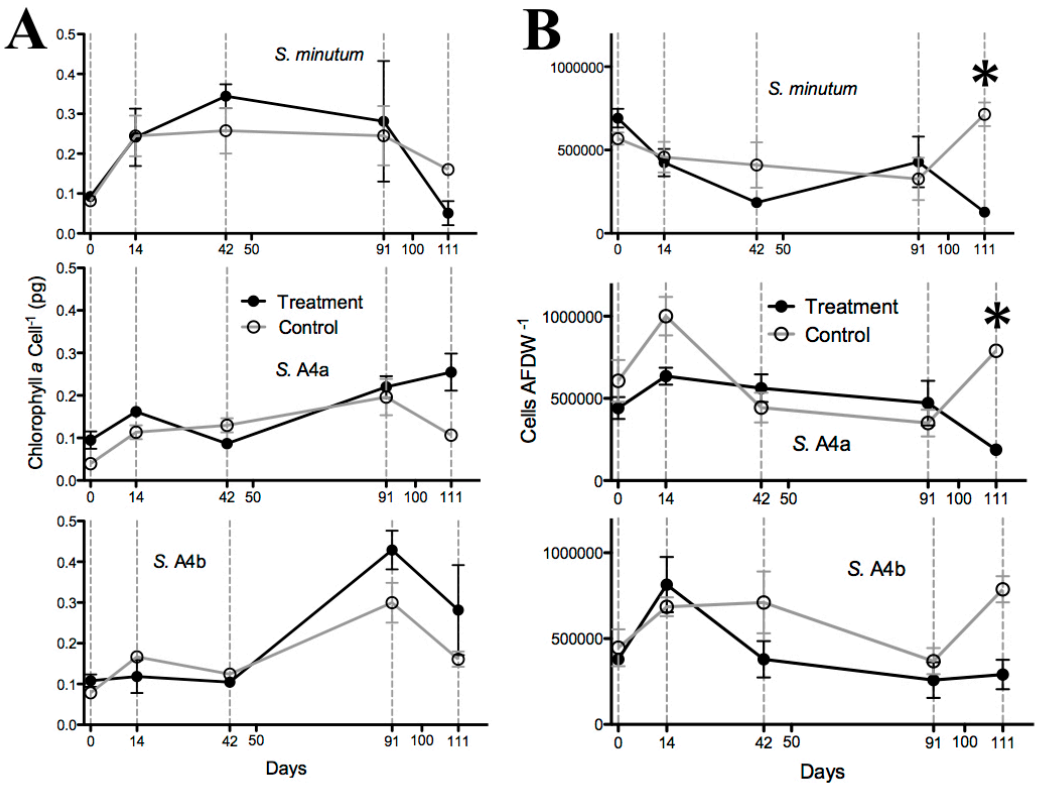

Figure 5. Picograms chlorophyll *a* algal cell^{-1} (A) and algal cells to milligrams ash-free dry weight^{-1} (B). The asterisk signifies a significant difference ($p < 0.05$) between the treatment and control for the particular holobiont type at that time point (n = 3 or 4 ± SE).

1.3.2 Effects of Long Term CO_2 Exposure

During the first 42 days of the experiment, when treatment anemones were exposed to high CO_2 alone, there were minimal changes in the physiological variables measured. Symbiont photochemistry remained static during this period, showing no deviation from the control measurements. Gross photosynthesis to respiration (P:R) of *S.* A4a and *S.* A4b holobionts during this period appeared higher than their respective controls (albeit not statistically based on the linear mixed model used for the analysis), but there was no difference between *S. minutum* holobiont control and treatment P:R

(Figure 10). There was however a significant decrease ($p = 0.0150$) of treatment *S. minutum* cells normalized to ash-free dry weight (Cells $AFDW^{-1}$) between day 0 and day 42, with no significant difference in the symbiont cell density $AFDW^{-1}$ in either of the A4 anemones (Figure 5).

1.3.3 Effects of Combined High CO_2 and a Moderate Temperature Increase

After exposure to CO_2 alone for 42 days, the treatment anemones were exposed to a moderate temperature increase, from 26°C to 29°C, for 49 more days (day 42 to day 91). The maximum quantum efficiency of photosystem II (PSII), as measured by FIRe fluorometry (F_v/F_m^{ST}), changed significantly at this temperature (Figure 6). Specifically, F_v/F_m^{ST} was significantly lower in the treatment anemones harboring the *Symbiodinium* A4b than the control *S.* A4b anemones on day 91 ($p = 0.0137$). Interestingly, F_v/F_m^{ST} increased significantly between day 42 and day 91 in the treatment *S.* A4a anemones ($p = 0.0289$). Meanwhile, there was no significant change detected in maximal PSII efficiency in treatment *S. minutum* holobionts over the same time period. By day 91, the quantum efficiency of PSII photochemistry in the light activated state ($F_q'/F_m'^{ST}$) was significantly lower in the *S.* A4b treatment as compared to its control ($p = 0.0137$). However, no other changes in $F_q'/F_m'^{ST}$ were noted over this time period in the other two anemone-algal combinations (Figure 7).

Photosynthesis and respiration were also affected in the treatment holobionts at this temperature. Treatment anemones harboring *S. minutum* had lower a P:R than the control, though this difference was not significant. The treatment A4 anemones both had P:R ratios higher than their respective controls, with *S.* A4a anemones showing a slight increase between day 42 and day 91, and *S.* A4b anemones showing a slight decrease over that time period (Figure 10). Net photosynthesis normalized to total

Symbiodinium cells (P_{net} $Cell^{-1}$) was significantly higher in all three treatment holobionts compared to their controls ($p < 0.001$), while respiration normalized to total *Symbiodinium* cells (R $Cell^{-1}$) showed a decrease in the treatment *S.* A4b anemones compared to its control (albeit not significant) at this temperature (Figure 8, Figure 9). The other two holobionts displayed no change in R $Cell^{-1}$ on day 91.

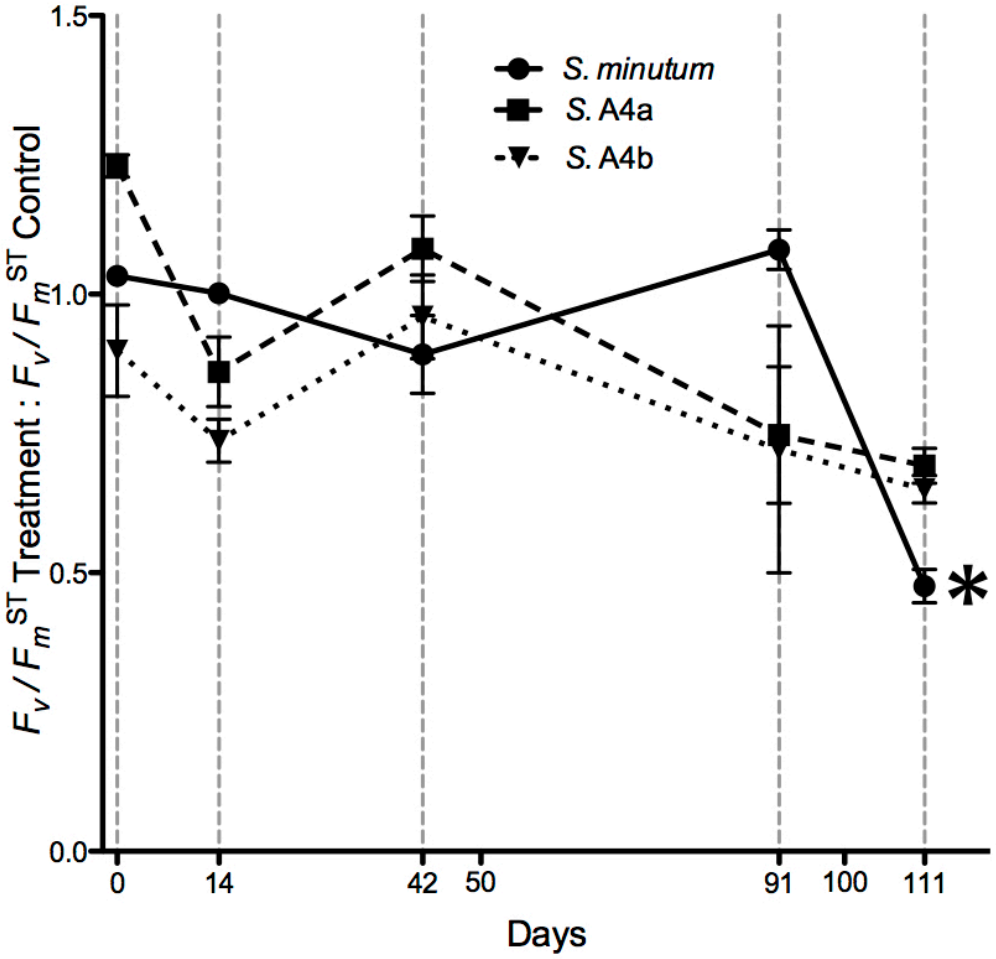

Figure 6. Normalized maximum quantum yield of PSII (F_v/F_m^{ST}, treatment:conrol) recorded by FIRe fluorometry. The controls had no significant change over time. The asterisk signifies a significant difference ($p < 0.05$) between the treatment and control for the particular holobiont type at that time point (n = 3 or 4 ± SE).

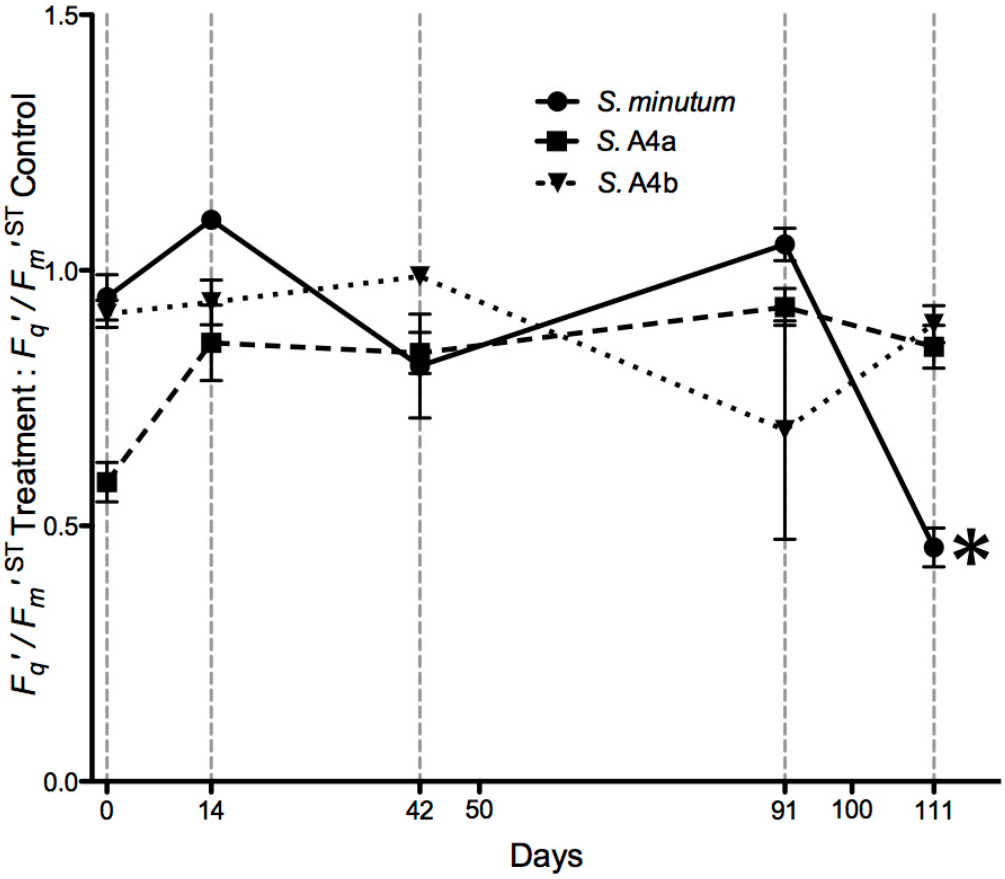

Figure 7. Normalized efficiency of PSII in the light activated state ($F_q'/F_m'^{ST}$ treatment:control) recorded by FIRe fluorometry. The controls had no significant change over time. The asterisk signifies a significant difference ($p < 0.05$) between the control and the treatment for the *S. minutum* anemones (n = 3 or 4 ± SE).

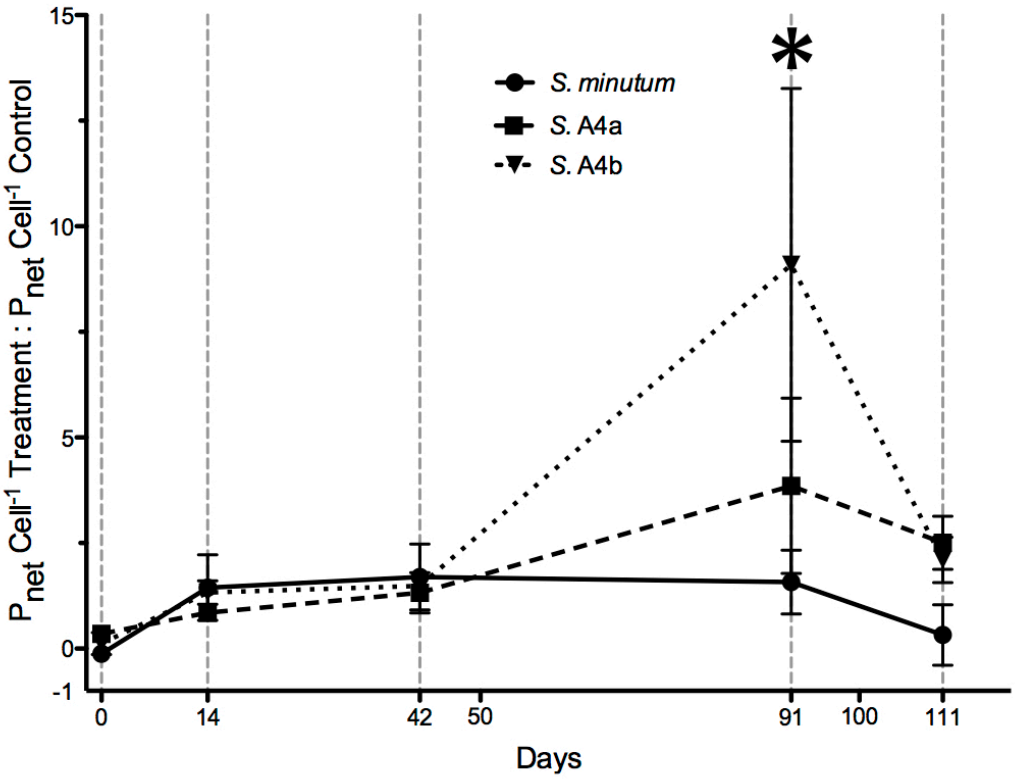

Figure 8. Normalized net photosynthesis algal cell^{-1} (treatment:control). The controls had no significant change over time. The asterisk signifies a significant difference ($p < 0.05$) in all treatments relative to their respective control values (n = 3 or 4 ± SE).

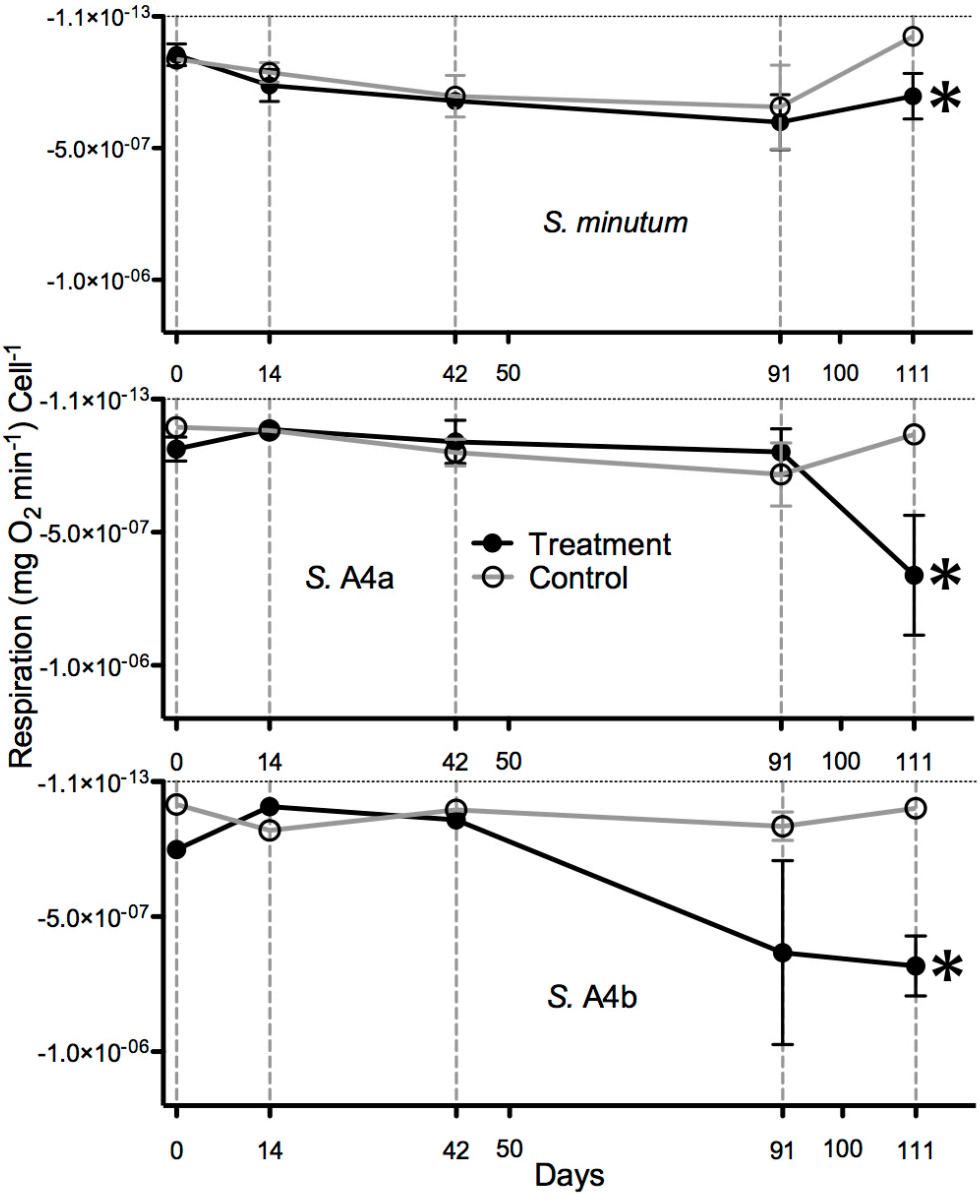

Figure 9. Whole animal respiration algal cells^{-1}. Asterisks signify a significant difference ($p < 0.05$) between the treatment and the control at Day 111 (n = 3 or 4 ± SE).

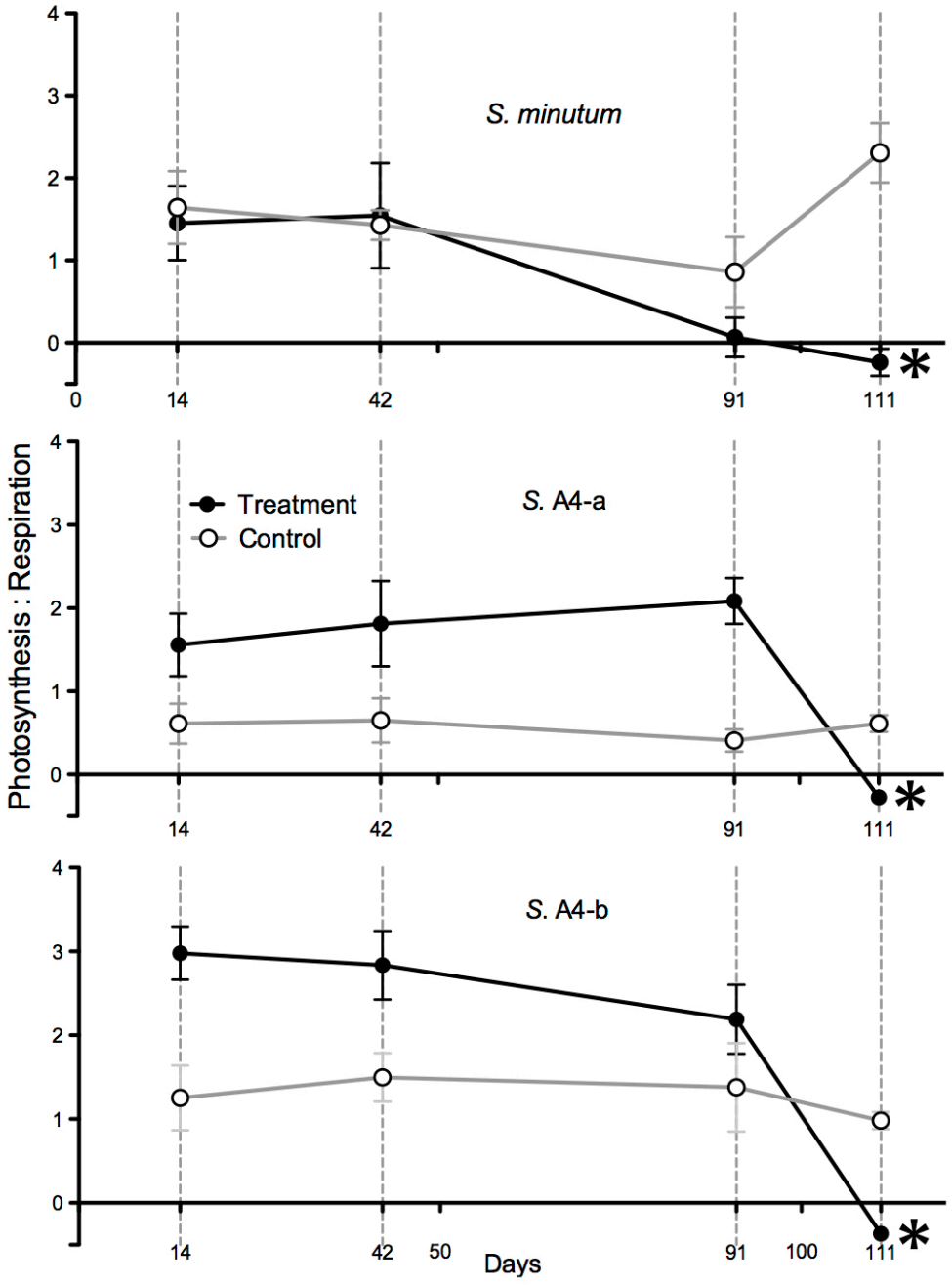

Figure 10. Gross photosynthesis to respiration for *E. pallida* hosting three different *Symbiodinium*. Asterisks signify significant differences ($p < 0.05$) between the treatments and the controls at Day 111 (n = 3 or 4 ± SE).

1.3.4 Effects of Combined High CO_2 and a High Temperature Anomaly

While there was no significant change in the photochemical activity in any of the control anemone-algal combinations throughout the course of the experiment, several changes were noted when treatment anemones were exposed to high CO_2 and a temperature increase from 29°C 33°C (day 91 to day 111). By day 111, the maximum PSII efficiency (F_v/F_m^{ST}) of the treatment anemones harboring *S. minutum* was significantly lower than that of its control ($p < 0.01$), while the treatment *S.* A4a anemones F_v/F_m^{ST} was significantly lower when compared to day 0 ($p < 0.01$). Likewise, the $F_q'/F_m'^{ST}$ of treatment anemones harboring *S. minutum* was also significantly lower than the *S. minutum* control ($p < 0.01$), and all other treatment time points ($p < 0.01$). However, no $F_q'/F_m'^{ST}$ changes were noted in the other two holobionts at this temperature. Symbiodinium density normalized to ash-free dry weight (Cells $AFDW^{-1}$) changed significantly within the high temperature time period as well. Cells $AFDW^{-1}$ in both the treatment anemones harboring *S. minutum* and *S.* A4a were significantly lower than their respective controls ($p = 0.0128$, $p < 0.01$), though only the *S. minutum* anemones showed visible signs of bleaching.

Along with changes in photochemistry and symbiont density, photosynthesis and respiration were also affected by the highest temperature treatment. The P:R ratios of all three holobiont treatments were significantly lower than the controls ($p < 0.01$) and significantly lower than all the other treatment measurements ($p < 0.01$) by day 111. Although treatment P:R was lower than the controls at this temperature, P_{net} $Cell^{-1}$ for the treatment anemones harboring *S.* A4a and *S.* A4b was higher than their controls, while the treatment *S. minutum* anemones had a P_{net} $Cell^{-1}$ lower than its control. While these changes were not significant, the respiration per cell (R $Cell^{-1}$) of

all three treatment holobions was significantly lower than their controls ($p < 0.001$) at 33°C.

The highest temperature treatment also led to juvenile mortality in the treatment anemones. Juvenile anemone mortality was calculated at the end of the experiment by subtracting the number of juvenile survivors on Day 111 from the total counted (the recorded counts from each week added together) number of juveniles. Juvenile mortality was highest in the *S. minutum* anemones at 67%, and lower in *S.* A4a and *S.* A4b at 32% and 50% respectively. Juvenile mortality only occurred in the treatment anemones.

1.3.5 Effects on Reproduction

The ratio of anemone juveniles per adult over the course of the experiment revealed a cyclic pattern of reproduction (Figure 11). This pattern was seen in all three anemone *Symbiodinium* combinations in both the control and the treatment conditions. The *S.* A4b anemones were the only holobionts which displayed a significant difference between the reproduction pattern of the control and treatment, as the treatment ratio was lower than the control for the entire experiment ($p = 0.0004$).

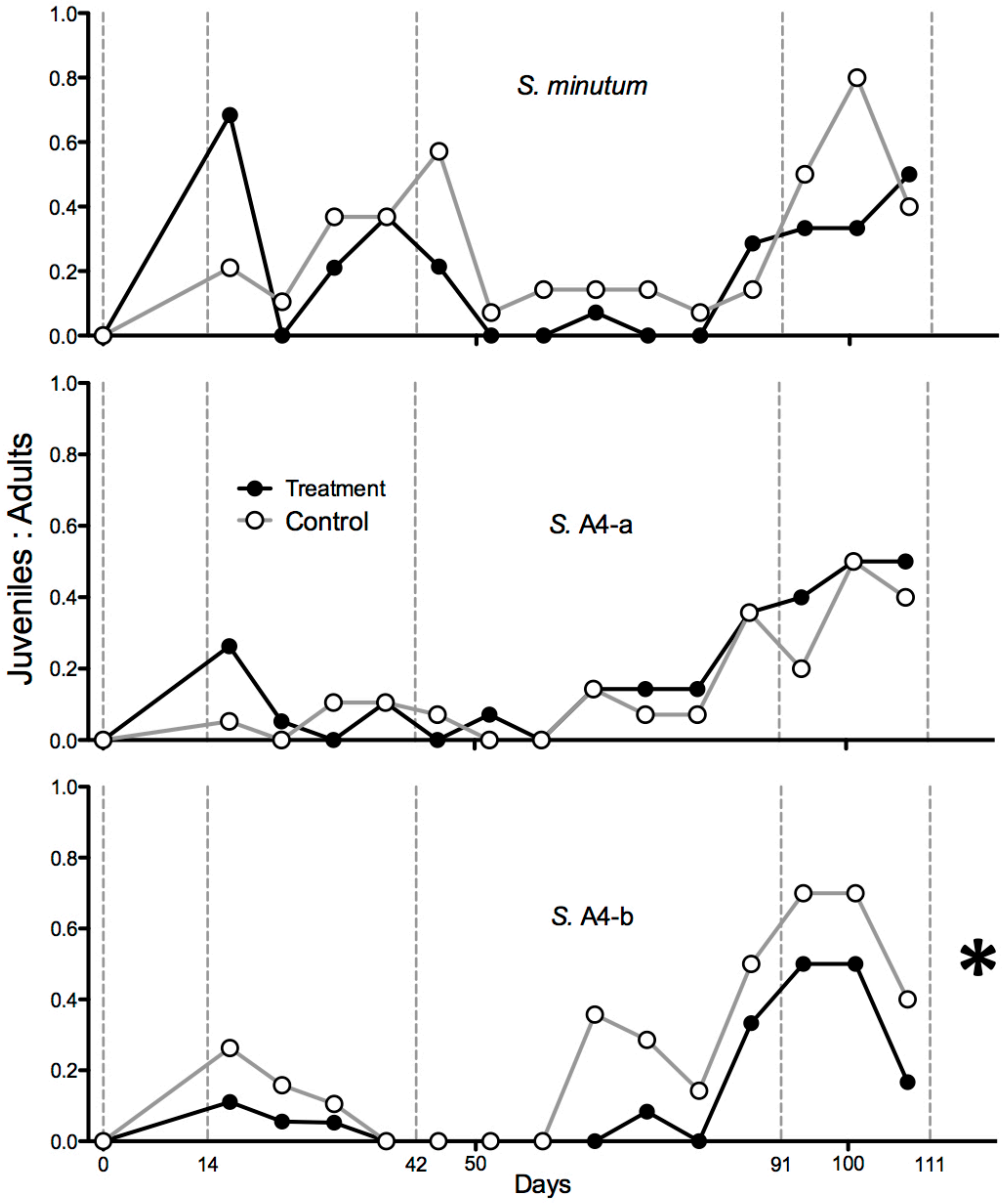

Figure 11. The Juvenile:Adult ratio designating the reproductive output of each holobiont combination during the course of the experiment. The asterisk indicates significant difference ($p < 0.001$) between the control and treatment for the overall time series (n = 19 for *S. minutum*, n = 19 for *S.* A4a, and n = 19 for *S.* A4b).

1.4 Discussion

1.4.1 Effects of Long Term High CO_2 Exposure

While previous studies have investigated the impact of high CO_2 on symbiotic sea anemones and corals, this study is the first of its kind to investigate how anemones respond to chronic ocean acidification (OA) under an extended period of time and under the combined stressors of high CO_2 and high temperature. It is not surprising that all three combinations of *E. pallida* and their three respective symbionts had a positive photosynthetic response (via oxygen production) to increased CO_2. Other studies have noted an increase in *Symbiodinium* photosynthesis under similar conditions, particularly for *S. minutum* (Hoadley et al. 2015, Gibbin and Davy 2012) and also in an A type *Symbiodinium* within a temperate anemone living near a natural volcanic CO_2 seep (Suggett et al. 2012). While we did not see the four-fold increase in *S. minutum* photosynthesis observed by Gibbin and Davy (2012), we did note a two-fold increase in P_{net} $cell^{-1}$ under high CO_2 in all holobionts tested. Gibbin and Davy normalized photosynthesis to host protein, not *Symbiodinium* cells, which may explain the differences observed. Additionally, their P:R did not change, pointing to a decrease in the respiration when the photosynthesis increased. Towanda and Thuesen (2012) also noted that high CO_2 (2280µatm, pH 7.3) promoted the photosynthesis in the symbiotic anemone *Anthopleaura elegantissima*. In this current study, P:R increased in *S.* A4a (one-fold) and *S.* A4b (two-fold), which further agrees with the positive response to increased CO_2 described in the other studies.

Although many studies point to an increase in photosynthesis with increased CO_2, several studies describe no change in photosynthesis under elevated CO_2. One example is Brading et al. (2011), where cultured *Symbiodinium* types A1 and B1

showed no change in photosynthesis when CO_2 exposure was doubled (pCO_2 ~390 to 800 ppm). P_{net} $cell^{-1}$ for the B1 *Symbiodinium* in that study was the lowest of all algae tested, including algae from clade A. The treatment B1 alga in this study (*S. minutum*) also had a lower average P_{net} $cell^{-1}$ as compared to the two A4 isolates, along with a lower P:R, pointing to a species-specific response to high CO_2. Increased photosynthesis in this experiment did not lead to any significant changes in anemone biomass (protein, lipids or carbohydrates). It is possible the increased photosynthetic carbon produced by the symbionts was not transferred to the anemone, or anemone growth increased while the relative ratio of these parameters did not change when normalized to the total organic mass of the animal. Hoadley et al. (2015) noted an increase in the rate of carbon translocation with increased photosynthesis, but no change in the total percent of carbon translocated to the host. It is likely that the increased amount of photosynthate was sequestered by the algae. Unfortunately, we were unable to track carbon translocation (via C^{14}) in this experiment due to a mass anemone die off prior to the experiment, limiting the amount of sample animals available. This limited our ability to track carbon movement within the holobiont, outside of measuring the biomass parameters. Additionally, normalization of the biomass parameters to AFDW may have skewed any significant changes we could have seen had the data been normalized differently. As noted above, comparisons between studies can be difficult due to the differences in the normalization unit (e.g. ash-free dry weight, wet weight, protein content, symbiont cells, etc.) used for each study, which may weaken comparisons. The large variety in size of the anemones used in this experiment may also further compound the difficulty of normalization, as the

relative proportion of protein, lipids, and carbohydrates may change across anemone size.

1.4.2 Moderate Temperature Increase with Elevated CO_2

Moderately increasing the temperature, while maintaining a high level of CO_2, revealed some interesting similarities in the response between the different holobionts. When the temperature was increased to 29°C there were no significant changes in either productivity or photobiology. The lack of significant effects corresponds to the thermal tolerance of the *Symbiodinium* and is also influenced by the fact that these anemones and symbionts used here came from environments that typically experience this temperature each summer. Krueger et al. (2014) exposed cultured *S. minutum* to a temperature increase from 25°C to 29°C for 14 days and also observed no changes in photochemistry (F_v/F_m), concluding the algae were not stressed at this temperature. Likewise, Hawkins et al. (2016) noted that the *S.* A4 isolates also responded similarly with no change in photochemistry when the cultures were exposed to 30.5°C (1.5°C higher than this study).

1.4.2.1 Differential Responses in the A4 *Symbiodinium*

While there were no significant differences in control to treatment F_v/F_m in the anemones harboring either of the *S.* A4 isolates, there were some differential responses in the ratio of photosynthesis to respiration (P:R). *S.* A4a displayed a slight increase in P:R at 29°C, where *S.* A4b displayed a slight decrease. While this difference was not significant, it is suggestive of possible intraspecific differences in productivity while under moderately high temperature scenarios. Intraspecies differences were expected to reveal themselves, as they have been noted in other

studies. Hoadley et al. (2015) recently reported differences in productivity and carbon translocation between three different isolates of *S. minutum* within *E.* pallida while exposed to low pH (7.95) at 26.5°C for 28 days. Hawkins et al. (2016) also noted thermal differences between the two *S.* A4 isolates used here while in culture, and in particular, once a 32°C threshold was passed, F_v/F_m declined significantly within the *S.* A4b isolate, while *S.* A4a photochemistry was unaffected.

Differential thermal performance among closely related *Symbiodinium* has also been described in *Symbiodinium* within reef-building corals. For example, Howells et al. (2012) noted the same algae (type C1) in colonies of *Acropora tenuis* in a nearshore (warm) reef and an offshore (cool) reef in Australia, with the offshore algae showing a greater stress response to higher temperature. Intraspecific differences in response to climate change are just beginning to be fully appreciated on a fine scale level, with intraspecific physiological differences in thermal tolerance noted between different clones of the host animal as well. For example, Parkinson et al. (2015) noted thermal differences across different colonies of the coral *Acropora palmata* (n=6, genetically different from each other) while these corals harbored a single clonal line of *Symbiodinium* '*fitti*'. When these corals were exposed to a cold shock (shifting from 27°C to 20°C) algal photochemistry differed by up to 3.6 fold between each colony (Parkinson et al. 2015). Further study of the differences among different genotypes of the same host and algal species will continue to tease apart the specificity in response to climate change seen in symbiotic cnidarians.

1.4.2.2 Animal-Algal Interactions

Due to the symbiotic nature of *E. pallida*, photosynthesis to respiration (P:R) measurements takes into account the respiration of not only the algae but also the

animal. When the respiration in either the anemone or the alga (or both) increases, possibly due to stress, the overall P:R is lowered if photosynthesis remains constant. If photosynthesis increases as the respiration rises, then no change in P:R would occur. Such a pattern may explain the data noted at 29°C. The P_{net} $cell^{-1}$ dramatically increased in all the algal types, but particularly in *S.* A4b, while the P:R ratio remained rather constant. This significant increase in P_{net} $cell^{-1}$ could be masked in the P:R ratio by an increase in respiration. An inherent difficulty in using bulk P:R in symbiotic organisms is the inability to separate the metabolic demand of each partner. One way to begin to tease apart the respiratory demand of the animal host and symbiont is by using specific mitochondrial enzyme assays such as citrate synthase (CS). The CS activity correlates with respiration, providing a way to relate the two (Hawkins et al. 2016b). While the small size of the available algal pellets precluded CS activity quantification of the algae alone, CS activity of the anemone did not change significantly over the duration of the experiment, thereby suggesting that the that animal respiration may not have changed as much as that of the algae. Hence, there is a possibility that greater respiratory changes were driven by the algae and these affected the P:R values. When whole animal respiration was normalized to algal cell at 29°C, there was an increasing trend in the data, especially for the A4b alga and the holobiont with the largest increase in P_{net} $cell^{-1}$ (Figure 9). It is possible that the A4 algae are at a thermal optimum at 29°C, which would explain the drastic rise in P_{net} $cell^{-1}$ during this time period.

While it appears that the net photosynthesis increases in *E. pallida* at a moderate temperature, not all coral species seem to have the same positive response. Towle et al. (2016) examined the response of the coral *Porites porites* (a Caribbean

branching coral, *Symbiodinium* type unspecified) to a temperature increase from 26°C to 31°C (2 months) after being previously exposed to high CO_2 (900ppm, 3 months). These corals showed a decrease in photochemical efficiency, even after already starting at a lower F_v/F_m than the control at the beginning of the heating. While this study does not describe the productivity of the coral, or describe the type of symbiotic algae, decreased F_v/F_m points to an algal stress response, which we did not see in this study at a moderate temperature.

1.4.3 Acute Shifts in Temperature While Under High CO_2

While the three *Symbiodinium* isolates used here did not have any significant responses to moderate temperature, exposure to a simulated high temperature anomaly (33°C) brought about multiple negative effects. *S. minutum* had the most severe response to the high temperature, displaying a significant decrease in F_v/F_m, F_q'/F_m', P:R, and Cells AFDW^{-1}. The anemones hosting *S. minutum* also showed signs of visual bleaching, and some adult and juvenile mortality was noted, which led to the early termination of the experiment. The P_{net} cell^{-1} also declined, but this was not significant. The decrease in the P:R of the holobiont was most likely driven by the loss of *Symbiodinium* cells. The *S.* A4 algae proved to be slightly more thermally tolerant than *S. minutum*, yet they still displayed significant decreases in P:R, P_{net} cell^{-1} and F_v/F_m at high temperature. Unlike the *S. minutum* anemones, there was no visible paling/bleaching, though *S.* A4a had a significant decrease in *Symbiodinium* cells compared to the control (it is possible that pigmentation of the remaining cells remained highly concentrated, keeping the anemone from looking bleached). Only one other study has examined the effects of high temperature stress on these particular type A4 *Symbiodiniu*m. Hawkins et al. (2016) documented the survival of both *S.* A4a and

S. A4b at 33.5°C, but with a ~50% decrease in the (F_v/F_m) for *S.* A4b. Graham and Sanders (2015) showed that type A4 *Symbiodinium* were not affected by increased temperature, up to 33°C, and variable responses to combined high temperature and high CO_2 were noted. While there is variability in the thermal tolerance for different isolates of A4 *Symbiodinium*, their overall ability to survive and even thrive at these high temperatures could mean anemones with these algae have a competitive advantage in a high CO_2 and high temperature world. Goulet et al. (2005) also observed that a type A *Symbiodinium* isolated from *E. pallida* from Florida displayed a higher tolerance (quantified as maximal net photosynthesis Chl a^{-1}) to 34°C for one hour as compared to a B type alga (most likely *S. minutum* as well) that originated from anemones collected from Bermuda. The Bermuda anemones containing the Florida type A symbiont were also tested and displayed greater tolerance than the Bermuda anemones with the B symbiont. This provides further evidence of the general thermal tolerance of the A type *Symbiodinium* since it performs similarly regardless of the anemone host, and the sensitivity of the B type alga.

Similar bleaching and stress responses in *S. minutum* were seen in several other studies with reef corals. Grottoli et al. (2014) observed *S. minutum* bleaching in corals with increased temperature over multiple years and even the replacement of some algae by other algal types due to its inability to recover from these stress events. Wang et al. (2011) used the activation energy needed to inhibit photosystem II activity of different algal types to determine their thermal tolerance and found *S. minutum* to be one of the least tolerant. Krueger et al. (2014) also observed a rapid decline in *S. minutum* F_v/F_m, in culture, at 33°C, eventually causing mass mortality by day 14 of the experiment (we began seeing mortality after 5 days in this experiment).

The inability of *S. minutum* to survive in long term high temperature environments could have severe ecological implications for the cnidarians harboring this symbiont. These animals will need to adapt in ways of movement, acclimation or plasticity, or "winning" the genetic lottery, making them less sensitive to the changes. Stony corals are sessile organisms, limiting their ability to migrate to the span of their juvenile spawning. Juvenile spreading is limited as they tend to only settle in areas with crustose coralline algae, which are also depleted in OA conditions (Doropoulos et al. 2012). Most corals also only broadcast spawn once per year, further decreasing the likelihood of migrating out of unsuitable conditions fast enough (Harrison et al. 1984). Coral plasticity and acclimation has been noted in many studies and could be a defense against future environment conditions. Palumbi et al. (2014) studied the effect of short-term (3 hours) and long-term (two years) temperature stress (34°C) on the environmentally sensitive, dominant reef-building coral *Acropora hyacinthus,* found in locations with highly variable thermal conditions (30°C - 35°C) and moderately variable conditions (static 32°C). They observed greater plasticity in the corals from the highly variable location, with a loss of only 20% of their chlorophyll *a* (minimal bleaching). Acclimatization was also observed in the long-term temperature stress experiments, noting changes in gene expression (e.g. heat shock protein upregulation) in corals from both conditions. Acclimation may also occur in the form of symbiont "shuffling", where certain thermal tolerant symbionts become dominant in the coral, or infect corals after bleaching events. The shift to or acquisition of these symbionts would reduce their sensitivity to thermal stress (Baker 2001). Coral success in future ocean conditions could also be determined by their innate genetics and the genetics of their symbionts, making "winning" or "losing" combinations. "Winning" genetic

pairings that have resistance to environmental stressors may result in the natural selection of those tolerant individuals. Certain coral-*Symbiodinium* combinations have been shown to survive ocean acidification and temperature stress better than others. Baird et al. (2008) noted that differential bleaching depended on both the coral and symbiont species, and also the combinations of each. Parkinson et al. (2015) also observed differential responses of photochemical stress to temperature change (27°C to 20°C) in six genetically different coral colonies with clonal symbionts. The anemones used for this experiment are a natural population that came from a large standing population in Florida that grow on mangrove roots. The genetic diversity in this population is potentially larger than expected given the clonal growth this animal utilizes when held in the laboratory (preliminary data based on 7 microsatellite loci). Therefore, further work with this 'model' system will need to account for the possible population variability of both the host and the symbiont in order to draw a more complete picture of the range in thermal response.

1.4.3.1 Juvenile Mortality

Juvenile anemone mortality between each holobiont was not statistically different, but corresponded well with the stress responses seen in the adult anemones. The anemones hosting *S. minutum* had the highest juvenile mortality at 67%. This alga also showed the most severe decreases in F_v/F_m, F_q'/F_m', P_{net} $cell^{-1}$ and P:R. These anemones most likely did not survive due to the low thermal tolerance of *S. minutum*. Anemones hosting *S.* A4b had a 50% juvenile mortality, and anemones hosting *S.* A4a had a 32% juvenile mortality. The A4 type *Symbiodinium* are more thermally tolerant than *S. minutum*, which most likely decreased the likelihood of mortality from the high temperature stress the juveniles experienced. Corals have also been shown to have

reduced juvenile recruitment, likely due to mortality, in low pH environments (Fabricius et al. 2011). In contrast, Hoadley et al. (2015) noted a marked increase in *E. pallida* juveniles produced per month under high CO_2 (pH 7.95) along with an increase in P_{net} $Cell^{-1}$ at those conditions. While high CO_2 alone appears to enhance photosynthesis, it does not seem to provide any sort of buffer against acute thermal shock.

1.4.4 Overall Experimental Effects

1.4.4.1 Anemone Biomass

While there was a wide range in some of the algal physiological parameters measured, animal biomass (protein, carbohydrates, and lipids) was highly variable and did not change over time. Also, the absence of any change in the CS activity (and one proxy for mitochondrial function) is also suggestive of minimal host stress. While this result may suggest that the animal was less affected by the increased CO_2 and/or increased temperature this may also be a result of the duration of the experiment and the longer time interval needed to see significant changes in some parameters. However, this does not align with what other studies have found, particularly with lipids and proteins. Krief et al. (2010) found that corals exposed to OA have an increase in protein concentration yet that study did not manipulate temperature. Revel et al. (2015) noted that total lipids (symbiont and host) decreased in the sea anemone *Anemonia viridis* with a thermal stress as low as 29°C. They also found evidence for lipid translocation from the symbiont to the host, which could remediate lipid loss in the host in temperature exposure. Differences in study findings are most likely attributed to the interspecies differences in both the anemones and their symbionts

between each study, and also the normalization methods used in each study Lack of photosynthetic carbon incorporation from the holobiont may also explain the static anemone biomass. Hoadley et al. (2016) observed decrease in carbon translocation, with no change in the percent of the translocated carbon incorporated into the host, when the corals *Montipora hirsute* (C15 symbiont) and *Pocillopora damicornis* (D1 symbiont) were exposed to high CO_2 (~830µatm, pH 7.9) and high temperature (31.5°C), even though there was increased photosynthesis. The difficulty in normalizing data to size when the size of the anemones changed over the course of the experiment may also be a reason for the lack of significance in the biomass data, as other studies have noted animal stress responses to OA and temperature. Likewise, other stress responses may have been occurring in the animal at the cellular level that were not quantified here. For example, Paxton et al. (2013) observed host (*E. pallida*) apoptosis (and symbiont cell ejection) at 33°C for 8 hours and anemone bleaching. Dunn et al. (2002) also observed *E. pallida* cell necrosis and programmed cell death (also resulting in symbiont cell death in those anemone cells) when exposed to a temperature increase from 26°C–27°C to 33–34°C for 7 days.

The anemones in this current study were also fed throughout the entire experiment, which could account for the lack of change in biomass. Heterotrophy is quite important to *E. pallida* as they only receive roughly 20% of photosynthetically fixed carbon through symbiont translocation (Hoadley et al. 2015, Hoadley et al. 2016). On the other hand, reef-building corals and other symbiotic cnidarians rely heavily on the autotrophic contribution of their resident *Symbiodinium*. For example, the corals *Montipora hirsute* and *Pocillopora damicornis,* and the corallimorph *Discosoma nummiforme* receive 45%, 46%, and 78% of photosynthetically fixed

carbon respectively, leading to the assumption that these cnidarians are not as reliant on heterotrophy as *E. pallida* (Hoadley et al. 2016). This reliance on feeding may cause biological components such as proteins, lipids, and carbohydrates to remain more static than they would in a symbiosis with a greater reliance on algal carbon translocation.

A fundamental difference between anemone and scleractinian coral stress response is also the lack of a calcium carbonate skeleton and the additional energetic demand of maintaining calcification and accretion under OA conditions (Reis et al. 2009, Hoegh-Guldberg et al. 2007, Albright et al. 2010, Anthony et al. 2008). A recent meta-analysis on 228 studies examining biological responses (survival, calcification, growth, photosynthesis, development, abundance, and metabolism) to ocean acidification, performed by Kroeker et al. (2013), found that ocean acidification significantly reduced calcification and abundance in corals. Conversely, not all corals experience depressed skeletal growth rates when exposed to low pH. Brown and Edmunds (2016) noted a decrease in calcification in only one (*Pocillopora meandrina*) of three corals (*Porites* spp., *Acropora pulchra*) exposed to a temperature increase from 28°C to 30.1°C and a pH decrease from 8.1 to 7.8. Importantly, none of the corals experienced depressed calcification in low pH and 28°C. Likewise, Grottoli et al. (2014) noted bleaching and decreased calcification in *Porites divaricata, Porites asteroides,* and *Orbicella faveolata* after exposure to 31.5°C (from 30.5°C) for 17 days, but also recovery in skeletal growth and partial recovery in symbiont density after a year back in 30.5°C. Comeau et al. (2014) also saw no decrease in calcification, which is closely coupled with photosynthesis and carbon translocation (Camp et al. 2016), in either *Pocillopora damicornis* or massive *Porites* at 1000µatm pCO_2 (low

pH) and 27°C. While the experimental *E. pallida* align with the "low host stress response" observations, the conflicting responses to increased temperature and ocean acidification are likely due to the large variation in coral-*Symbiodinium* combinations, resulting is species specific responses to these conditions.

1.4.4.2 Reproduction and Productivity Interactions

Asexual reproduction in anemones occurs through budding by pedal laceration and was rather variable over time in both the control and treatment. While this variability may be due to a cyclic nature of anemone reproduction, the overall average reproduction ratio (juveniles:adults) of the control anemones harboring *S.* A4b and *S. minutum* was higher than each of their respective CO_2 / temperature treatments, with control *S.* A4b being significantly higher. The *S.* A4a and *S. minutum* anemones had no significant differences between the control and treatment. This points to a species-specific response in reproduction to combined high CO_2 and temperature.

Variability in asexual reproduction was also noted within the control holobionts. Importantly, there was a positive correlation between the average reproduction rate and the respective average P:R of each control holobiont (Figure 12). While this is a small data set that represents the long-term average trend over time with a limited number of individuals, it shows that a higher amount of symbiont productivity (possibly correlating with excess energy for reproduction) correlates with a higher rate of asexual reproduction and hence fitness. This correlation of possible resource partitioning toward asexual reproduction is strengthened by the fact that the anemone biomass did not change over time nor did algal cell density increase.

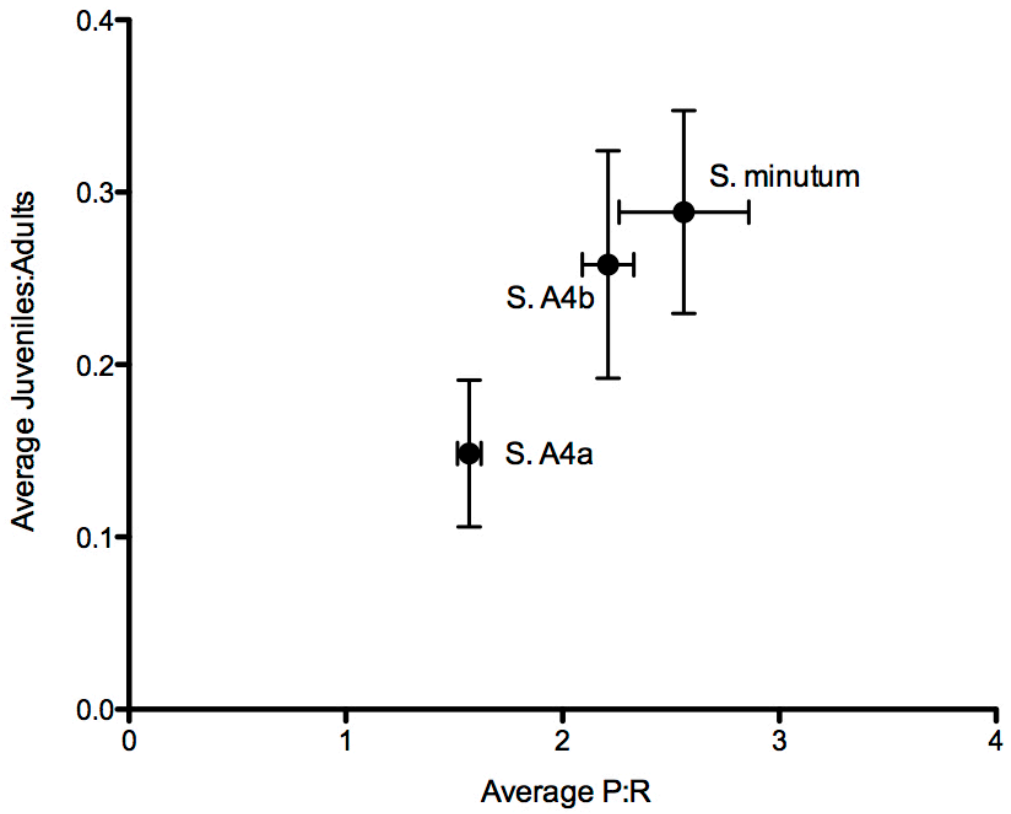

Figure 12. Average juvenile per adult of each control *Symbiodinium* type compared to the average P:R of each control *Symbiodinium* type (n = 3 or 4 ± SE for P:R, n = 19 ± SE for Juvenile:Adult).

1.5 Summary and Conclusions

This study highlights the differences in the response of three holobionts of a common symbiotic sea anemone to changing ocean conditions. Although all three displayed some level of thermal stress at high temperature, the inter-species differences in thermotolerance between the *S. minutum* and the two A4 algae is clear given the significant decline in F_v/F_m and F_q'/F_m' and visual bleaching in *S. minutum* at high temperatures that were not seen in the A4 algae. This difference was also seen

in the P:R of the holobionts, where *S. minutum* displayed no difference when exposed to high CO_2 and decreased when exposed to moderate temperatures, while the A4 algae responded differently and displayed an increased P:R with high CO_2 and minimal cell loss until they were exposed to the highest temperature. Changes in P:R also revealed intraspecific differences between *S.* A4a and *S.* A4b.

Similar to reef corals, anemones will ultimately face the projected increased CO_2, and temperature expected to occur within the next 100 years. Several lines of evidence suggest that anemones may fare better in a high CO_2 ocean when temperatures do not pass a 29–30°C threshold. However, once high temperature anomalies (32–33°C +) are introduced, a divergence in successful anemone-algal combinations may appear which is driven, in part, by the thermal tolerance of the resident symbionts. Anemones hosting *S. minutum*, and other less thermally tolerant *Symbiodinium* species, will likely face higher levels of bleaching stress and greater mortality, while anemones hosting more thermally tolerant symbionts, such as *S.* A4a and *S.* A4b, will likely show some stress responses, but may have a better chance for long-term survival.

REFERENCES

Albright, R., et al. (2016). "Reversal of ocean acidification enhances net coral reef calcification." Nature 531(7594): 362-365.

Anthony, K. R. N., et al. (2008). "Ocean acidification causes bleaching and productivity loss in coral reef builders." PNAS 105: 17442–17446.

Baird, A. H., et al. (2009). "Coral bleaching: the role of the host." Trends Ecol Evol 24(1): 16-20.

Baker, A. (2001). "Reef corals bleach to survive." Nature 411: 765-766.

Baker, A. C. (2003). "Flexibility and Specificity in Coral-Algal Symbiosis: Diversity, Ecology, and Biogeography of Symbiodinium." Annual Review of Ecology, Evolution, and Systematics 34: 661-689.

Baumgarten, S., et al. (2015). "The genome of Aiptasia, a sea anemone model for coral symbiosis." Proc Natl Acad Sci U S A 112(38): 11893-11898.

Belda-Baillie, C. A., et al. (2002). "Specificity of a model cnidarian-dinoflagellate symbiosis." Biol Bull 202(1): 74-85.

Benayahu, Y. and Y. Loya (1977). "Space partitioning by stony corals soft corals and benthic algae on the coral reefs of the northern Gulf of Eilat (Red Sea)." Helgol~inder wiss. Meeresunters 30: 362-382.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Analytical Biochemistry 72: 248-254.

Brading, P., et al. (2011). "Differential effects of ocean acidification on growth and photosynthesis among phylotypes ofSymbiodinium(Dinophyceae)." Limnology and Oceanography 56(3): 927-938.

Brading, P., et al. (2013). "Contrasting modes of inorganic carbon acquisition amongst Symbiodinium (Dinophyceae) phylotypes." New Phytol 200(2): 432-442.

Brown, D. and P. J. Edmunds (2016). "Differences in the responses of three scleractinians and the hydrocoral Millepora platyphylla to ocean acidification." Marine Biology 163(3).

Caldeira, K. and M. E.Wickett (2003). "Anthropogenic carbon and ocean pH The coming centuries may see more ocean acidification than the past 300 million years." Nature 425: 365.

Camp, E. F., et al. (2016). "Acclimatization to high-variance habitats does not enhance physiological tolerance of two key Caribbean corals to future temperature and pH." Proc Biol Sci 283(1831).

Cheng, Y. S., et al. (2011). "Rapid quantitative analysis of lipids using a colorimetric method in a microplate format." Lipids 46(1): 95-103.

Comeau, S., et al. (2014). "Pacific-wide contrast highlights resistance of reef calcifiers to ocean acidification." Proc Biol Sci 281(1790).

Costanza, R., et al. (2014). "Changes in the global value of ecosystem services." Global Environmental Change 26: 152-158.

Doropoulos, C., et al. (2012). "Ocean acidification reduces coral recruitment by disrupting intimate larval-algal settlement interactions." Ecol Lett 15(4): 338-346.

Dubois, M., et al. (1956). "Colorimetric Method for Determination of Sugars and Related Substances." ANALYTICAL CHEMISTRY 28.

Dunn, S. R., et al. (2002). "Programmed cell death and cell necrosis activity during hyperthermic stress-induced bleaching of the symbiotic sea anemone Aiptasia sp." Experimental Marine Biology and Ecology 272: 29-53.

Edmunds, P. J., et al. (2012). "Interactive effects of ocean acidification and temperature on two scleractinian corals from Moorea, French Polynesia." Global Change Biology 18(7): 2173-2183.

Fabricius, K. E. (1997). "Soft coral abundance on the central Great Barrier Reef: e§ects of Acanthasterplanci, space availability, and aspects of the physical environment." Coral Reefs 16: 159—167.

Fabricius, K. E., et al. (2011). "Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations." Nature Climate Change 1(3): 165-169.

Fisher, P. L., et al. (2011). "The effect of temperature stress on coral–Symbiodinium associations containing distinct symbiont types." Coral Reefs 31(2): 473-485.

Fujise, L., et al. (2014). "Moderate Thermal Stress Causes Active and Immediate Expulsion of Photosynthetically Damaged Zooxanthellae (Symbiodinium) from Corals." PLoS One 9(12): e114321.

Gabay, Y., et al. (2013). "Does elevated pCO2 affect reef octocorals?" Ecol Evol 3(3): 465-473.

Gibbin, E. M. and S. K. Davy (2014). "The photo-physiological response of a model cnidarian–dinoflagellate symbiosis to CO2-induced acidification at the cellular level." Journal of Experimental Marine Biology and Ecology 457: 1-7.

Gibbin, E. M., et al. (2015). "Species-specific differences in thermal tolerance may define susceptibility to intracellular acidosis in reef corals." Marine Biology 162: 717–723.

Gómez, C. E., et al. (2014). "Responses of the tropical gorgonian coral Eunicea fusca to ocean acidification conditions." Coral Reefs 34(2): 451-460.

Goulet, T. L., et al. (2005). "Effect of short-term exposure to elevated temperatures and light levels on photosynthesis of different host–symbiont combinations in the Aiptasia pallida/ Symbiodinium symbiosis." Limnology and Oceanography 50: 1490–1498.

Graham, E. R. and R. W. Sanders (2016). "Species-specific photosynthetic responses of symbiotic zoanthids to thermal stress and ocean acidification." Marine Ecology 37(2): 442-458.

Grajales, A. and E. Rodriguez (2014). "Morphological revision of the genus Aiptasia and the family Aiptasiidae (Cnidaria, Actiniaria, Metridioidea)." Zootaxa 3826(1): 55-100.

Grottoli, A. G., et al. (2014). "The cumulative impact of annual coral bleaching can turn some coral species winners into losers." Glob Chang Biol 20(12): 3823-3833.

Hanes, S. D. and S. C. Kempf (2013). "Host autophagic degradation and associated symbiont loss in response to heat stress in the symbiotic anemone,Aiptasia pallida." Invertebrate Biology 132(2): 95-107.

Harrison, P. L., et al. (1984). “Mass spawning in tropical reef corals”. Science: 1186-1189.

Hawkins, T. D., et al. (2016). "Partitioning of Respiration in an Animal-Algal Symbiosis: Implications for Different Aerobic Capacity between Symbiodinium spp." Front Physiol 7: 128.

Hawkins, T. D., et al. (2016). "Temperature moderates the infectiousness of two conspecific Symbiodinium strains isolated from the same host population." Environ Microbiol.

Hillyer, K. E., et al. (2016). "Metabolite profiling of symbiont and host during thermal stress and bleaching in the coral Acropora aspera." Coral Reefs.

Hoadley, K. D., et al. (2016). "Contrasting physiological plasticity in response to environmental stress within different cnidarians and their respective symbionts." Coral Reefs 35(2): 529-542.

Hoadley, K. D., et al. (2015). "Differential carbon utilization and asexual reproduction under elevatedpCO2conditions in the model anemone,Exaiptasia pallida, hosting different symbionts." Limnology and Oceanography 60(6): 2108-2120.

Hoegh-Guldberg, O., et al. (2007). "Coral Reefs Under Rapid Climate Change and Ocean Acidification." Science 318.

Hofmann, G. E., et al. (2011). "High-frequency dynamics of ocean pH: a multi-ecosystem comparison." PLoS One 6(12): e28983.

Howells, E. J., et al. (2011). "Coral thermal tolerance shaped by local adaptation of photosymbionts." Nature Climate Change 2(2): 116-120.

Hunter, T. (1984). "THE ENERGETICS OF ASEXUAL REPRODUCI'ION: PEDAL LACERATION IN THE SYMBIOTIC SEA ANEMONE AZPTASZA PULCHELLA." J Exp Mar Biol Ecol 83: 127-147.

Iglesias-Prieto, R., et al. (1992). "Photosynthetic response to elevated temperature in the symbiotic dinoflagellate Symbiodinium microadriaticum in culture." Proc. Natl. Acad. Sci. USA 89: 10302-10305.

IPCC (2014). "Climate Change 2014 Mitigation of Climate Change Working Group III Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change." Cambridge University Press.

Jarrold, M. D., et al. (2013). "Physiological plasticity preserves the metabolic relationship of the intertidal non-calcifying anthozoan-Symbiodinium symbiosis under ocean acidification." Journal of Experimental Marine Biology and Ecology 449: 200-206.

Jeong, H. J., et al. (2014). "Genetics and morphology characterize the dinoflagellate Symbiodinium voratum, n. sp., (Dinophyceae) as the sole representative of Symbiodinium Clade E." J Eukaryot Microbiol 61(1): 75-94.

Kinzie, R. A., 3rd, et al. (2001). "The adaptive bleaching hypothesis: experimental tests of critical assumptions." Biol Bull 200(1): 51-58.

Krief, S., et al. (2010). "Physiological and isotopic responses of scleractinian corals to ocean acidification." Geochimica et Cosmochimica Acta 74(17): 4988-5001.

Kroeker, K. J., et al. (2013). "Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming." Glob Chang Biol 19(6): 1884-1896.

Krueger, T., et al. (2014). "Antioxidant plasticity and thermal sensitivity in four types of Symbiodinium sp." J Phycol 50(6): 1035-1047.

LaJeunesse, T. C. (2001). "INVESTIGATING THE BIODIVERSITY, ECOLOGY, AND PHYLOGENY OF ENDOSYMBIOTIC DINOFLAGELLATES IN THE GENUS SYMBIODINIUM USING THE ITS REGION: IN SEARCH OF A "SPECIES" LEVEL MARKER " J. Phycol. 37: 866–880.

LaJeunesse, T. C., et al. (2003). "Low symbiont diversity in southern Great Barrier Reef corals, relative to those of the Caribbean." Limnology and Oceanography 48: 2046–2054.

LaJeunesse, T. C. and R. K. Trench (2000). "Biogeography of Two Species of Symbiodinium (Freudenthal) Inhabiting the Intertidal Sea Anemone Anthopleura elegantissima (Brandt)." Biologial Bullitin 199: 126–134.

Leal, M. C., et al. (2014). "Molecular assessment of heterotrophy and prey digestion in zooxanthellate cnidarians." Mol Ecol 23(15): 3838-3848.

Martin, S. and J.-P. Gattuso (2009). "Response of Mediterranean coralline algae to ocean acidification and elevated temperature." Global Change Biology 15(8): 2089-2100.

Matthews, J. L., et al. (2016). "Menthol-induced bleaching rapidly and effectively provides experimental aposymbiotic sea anemones (Aiptasia sp.) for symbiosis investigations." J Exp Biol 219(Pt 3): 306-310.

McGinty, E. S., et al. (2012). "Variations in reactive oxygen release and antioxidant activity in multiple Symbiodinium types in response to elevated temperature." Microb Ecol 64(4): 1000-1007.

Muscatine, L. (1980). Productivity of Zooxanthellae. Primary Productivity in the Sea. P. G. Falkowski. Boston, MA, Springer US: 381-402.

Muscatine, L., et al. (1991). "Release of symbiotic algae by tropical sea anemones and corals after cold shock." Marine Ecology Progress Series 77: 333-243.

Muscatine, L. and J. W. Porter (1977). "Mutualistic Symbioses Adapted to Nutrient-Poor Environments." BioScience 27: 454-460.

Muscatine, L., et al. (1989). "Resource partitioning by reef corals as from stable isotope composition*." Marine Biology 100: I85-193.

Newell, R. E. and T. G. Dopplick (1979). "Questions concerning the possible influence of anthropogenic CO2 on atmospheric tempearture." Journal of Applied Meteorology 18: 822-825.

Norström, A. V., et al. (2009). "Alternative states on coral reefs: beyond coral–macroalgal phase shifts." Marine Ecology Progress Series 376: 295-306.

Oliver, T. A. and S. R. Palumbi (2010). "Many corals host thermally resistant symbionts in high-temperature habitat." Coral Reefs 30(1): 241-250.

Orr, J. C., et al. (2005). "Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms." Nature 437(7059): 681-686.

Palumbi, S. R., et al. (2014). "Mechanisms of reef coral resistance to future climate change." Science 344(6186): 895-898.

Parkinson, J. E. and I. B. Baums (2014). "The extended phenotypes of marine symbioses: ecological and evolutionary consequences of intraspecific genetic diversity in coral-algal associations." Front Microbiol 5: 445.

Paxton, C. W., et al. (2013). "Stress and death of cnidarian host cells play a role in cnidarian bleaching." J Exp Biol 216(Pt 15): 2813-2820.

Pochon, X. and R. D. Gates (2010). "A new Symbiodinium clade (Dinophyceae) from soritid foraminifera in Hawai'i." Molecular Phylogenetics and Evolution 56: 492–497.

Pochon, X. and J. Pawlowski (2006). "Evolution of the soritids-Symbiodinium symbiosis." Symbiosis 42: 77–88.

R., R., et al. (1997). "Landscape ecology of algal symbionts creates variation in episodes of coral bleaching." Nature 388: 265-269.

Rahav, O., et al. (1988). "Ammonium metbolism in the zooxanthellate coral, Stylophora pistillata." Proc. R Soc. Lond. 236: 325-337.

Raven, J., et al. (2005). "Ocean acidification due to increasing atmospheric carbon dioxide." The Royal Society.

Revel, J., et al. (2016). "Differential distribution of lipids in epidermis, gastrodermis and hosted Symbiodinium in the sea anemone Anemonia viridis." Comp Biochem Physiol A Mol Integr Physiol 191: 140-151.

Ries, J. B., et al. (2009). "Marine calcifiers exhibit mixed responses to CO2-induced ocean acidification." Geology 37(12): 1131-1134.

Ritchie, R. J. (2006). "Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents." Photosynth Res 89(1): 27-41.

Robison, J. D. and M. E. Warner (2006). "Differential Impacts of Photoacclimation and Thermal Stress on the Photobiology of Four Different Phylotypes of Symbiodinium (Pyrrhophyta)1." Journal of Phycology 42(3): 568-579.

Rodolfo-Metalpa, R., et al. (2011). "Coral and mollusc resistance to ocean acidification adversely affected by warming." Nature Climate Change 1(6): 308-312.

Sampayo, E. M., et al. (2008). "Bleaching susceptibility and mortality of corals are determined by fine-scale differences in symbiont type." Proc Natl Acad Sci U S A 105(30): 10444-10449.

Schoepf, V., et al. (2013). "Coral energy reserves and calcification in a high-CO2 world at two temperatures." PLoS One 8(10): e75049.

Sinutok, S., et al. (2012). "Microenvironmental changes support evidence of photosynthesis and calcification inhibition in Halimeda under ocean acidification and warming." Coral Reefs 31(4): 1201-1213.

Srere, P. A. (1969). "CITRATE SYNTHASE."

Suggett, D. J., et al. (2012). "Sea anemones may thrive in a high CO2world." Global Change Biology 18(10): 3015-3025.

Suggett, D. J., et al. (2003). "Fast repetition rate and pulse amplitude modulation chlorophyllafluorescence measurements for assessment of photosynthetic electron transport in marine phytoplankton." European Journal of Phycology 38(4): 371-384.

T, L. (2002). "Diversity and community structure of symbiotic dinoflagellates from Caribbean coral reefs." Marine Biology 141(2): 387-400.

Tchernov, D., et al. (2004). "Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals." PNAS 101: 13531–13535.

Thornhill, D. J., et al. (2013). "Population genetic data of a model symbiotic cnidarian system reveal remarkable symbiotic specificity and vectored introductions across ocean basins." Mol Ecol 22(17): 4499-4515.

Towanda, T. and E. V. Thuesen (2012). "Prolonged exposure to elevated CO(2) promotes growth of the algal symbiont Symbiodinium muscatinei in the intertidal sea anemone Anthopleura elegantissima." Biol Open 1(7): 615-621.

Towle, E. K., et al. (2016). "Preconditioning to high CO2 exacerbates the response of the Caribbean branching coral Porites porites to high temperature stress." Marine Ecology Progress Series 546: 75-84.

Venn, A. A., et al. (2013). "Impact of seawater acidification on pH at the tissue–skeleton interface and calcification in reef corals." PNAS 110: 1634–1639

Venn, A. A., et al. (2006). "The impact of coral bleaching on the pigment profile of the symbiotic alga, Symbiodinium." Plant, Cell and Environment 29: 2133–2142.

Wang, J. T., et al. (2012). "Physiological and biochemical performances of menthol-induced aposymbiotic corals." PLoS One 7(9): e46406.

Warner, M. E., et al. (1996). "The effects of elevated temperature on the photosynthetic efficiency of zooxantheliae in hospiteUom four different species of reef coral: a novel approach." Plant, Cell and Environment 19: 291-299.

Warner, M. E., et al. (2006). "The ecological distribution and comparative photobiology of symbiotic dinoflagellates from reef corals in Belize: Potential implications for coral bleaching." Limnology and Oceanography 51: 1887–1897.

Warren, C. R. (2008). "Rapid Measurement of Chlorophylls with a Microplate Reader." Journal of Plant Nutrition 31(7): 1321-1332.

Weis, V. M. (2008). "Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis." J Exp Biol 211(Pt 19): 3059-3066.

Weis, V. M., et al. (2008). "Cell biology in model systems as the key to understanding corals." Trends Ecol Evol 23(7): 369-376.

Appendix A

STATISTICAL OUTPUT

Statistical analysis (Linear Mixed Effects Model) of the $F_vF_m^{ST}$, $F_q'/F_m'^{ST}$, P:R, Protein AFDW^{-1}, Lipid AFDW^{-1}, Carb AFDW^{-1}, CS Protein^{-1}, Cells AFDW^{-1}, Respiration Cell^{-1}, P_{net} Cell^{-1}, and Chl *a* Cell^{-1}. Significant p-values are in bold. Asterisk signifies ANOVA used for the *post hoc* analysis.

Variable	Effect	Error Degrees of Freedom	Effect Degrees of Freedom	F-value	p-value
$F_vF_m^{ST}$	Time	4	66	18.686	< **0.0001**
	Zoox	2	18	57.938	< **0.0001**
	Treatment	1	18	32.245	< **0.0001**
	Time:Zoox	8	66	5.209	< **0.0001**
	Time:Treatment	4	66	11.436	< **0.0001**
	Zoox:Treatment	2	18	3.207	0.0644
	Time:Zoox:Treatment	8	66	3.543	**0.0018***
$F_q'/F_m'^{ST}$	Time	4	66	10.868	< **0.0001**
	Zoox	2	18	7.343	0.0047
	Treatment	1	18	36.471	< **0.0001**
	Time:Zoox	8	66	6.780	< **0.0001**
	Time:Treatment	4	66	8.646	< **0.0001**
	Zoox:Treatment	2	18	0.910	0.4204
	Time:Zoox:Treatment	8	66	7.135	< **0.0001***
P:R	Time	3	53	11.0765	< **0.0001**
	Zoox	2	20	2.3854	0.1177
	Treatment	1	20	0.1734	1.6816
	Time:Zoox	6	53	2.1791	0.0595
	Time:Treatment	3	53	17.6617	< **0.0001***
Protein AFDW^{-1}	Time	4	63	0.4177	0.7953
	Zoox	2	18	1.0237	0.3792
	Treatment	1	18	3.5971	0.0740
	Time:Zoox	8	63	1.1883	0.3204
	Time:Treatment	4	63	1.5612	0.1957
	Zoox:Treatment	2	18	0.2534	0.7788
	Time:Zoox:Treatment	8	63	1.1323	0.3545*

Lipid AFDW^{-1}	Time	4	64	6.9209	**0.0001**
	Zoox	2	18	0.8394	0.4482
	Treatment	1	18	1.5227	0.2331
	Time:Zoox	8	64	1.0128	0.4356
	Time:Treatment	4	64	0.7955	0.5325
	Zoox:Treatment	2	18	5.0927	**0.0177**
	Time:Zoox:Treatment	8	64	1.8417	0.0854*
Carb AFDW^{-1}	Time	4	64	2.6058	0.0439
	Zoox	2	18	1.3388	0.2870
	Time	1	18	1.2082	0.2862
	Time:Zoox	8	64	1.1939	0.3169
	Time:Treatment	4	64	0.3747	0.8259
	Zoox:Treatment	2	18	1.2150	0.3199
	Time:Zoox:Treatment	8	64	1.0663	0.3978*
CS Protein^{-1}		4	66	3.1618	**0.0192**
	Zoox	2	18	0.0513	0.9501
	Treatment	1	18	0.1122	0.7415
	Time:Zoox	8	66	1.0548	0.4053
	Time:Treatment	4	66	1.2408	0.3023
	Zoox:Treatment	2	18	1.0101	0.3839
	Time:Zoox:Treatment	8	66	0.4523	0.8848*
Cells AFDW^{-1}	Time	4	66	9.4931	**< 0.0001**
	Zoox	2	18	2.7568	0.0903
	Treatment	1	18	14.6042	**0.0012**
	Time:Zoox	8	66	3.6961	**0.0013**
	Time:Treatment	4	66	9.9803	**< 0.0001**
	Zoox:Treatment	2	18	0.1927	0.8264
	Time:Zoox:Treatment	8	66	2.4271	**0.0230***
Respiration Cell^{-1}	Time	4	73	4.6389	**0.0022**
	Zoox	2	20	0.3127	0.7350
	Treatment	1	20	13.2476	**0.0016**
	Time:Zoox	8	73	1.8538	0.0807
	Time:Treatment	4	73	6.3469	**0.0002***
P_{net} Cell^{-1}	Time	4	65	10.3601	**< 0.0001**
	Zoox	2	18	1.3504	0.2842
	Treatment	1	18	6.2943	**0.0219**
	Time:Zoox	8	65	2.0508	0.0539
	Time:Treatment	4	65	3.0953	**0.0215***
	Zoox:Treatment	2	18	2.7273	0.0923
	Time:Zoox:Treatment	8	65	2.0245	0.0571

Chl *a* Cell^{-1}	Time	4	64	14.1928	< **0.0001**
	Zoox	2	18	6.4994	**0.0075**
	Treatment	1	18	5.0310	**0.0377**
	Time:Zoox	8	64	4.5958	**0.0002**
	Time:Treatment	4	64	0.7633	0.5530
	Zoox:Treatment	2	18	0.3734	0.6936
	Time:Zoox:Treatment	8	64	1.7278	0.1090*

Appendix B

CHALLENGES AND FUTURE DIRECTIONS

Multiple challenges and set-backs were faced over the course of this experiment. The initial experimental plan was much more in depth than the experiment displayed here. The initial experiment consisted of a larger anemone sample size (n = 6), an additional holobiont type (n = 4), and an additional sampling point (n = 6) after the high temperature exposure, that would have encapsulated 'recovery' responses. Carbon translocation was also going to be measured using C^{14} tagging. This initial experiment was cut short on its first week after a power failure which resulted in the release of an entire CO_2 cylinder into the system, bringing the pH of the water to ~3. This occurred overnight and resulted in complete mortality. Due to the loss of all the experimental anemones, a new experiment (this thesis project) was drawn up, much reduced in scope, in consideration of concrete time constraints. The stock anemone population was matured and developed until there were enough anemones for the new experiment. Due to time constraints, the first anemones large enough to sample were used in the experiment, resulting in a range of anemone sizes, which later presented major difficulties in normalization. The population growth time also constrained the amount of time available for running the experiment, so the total study was shortened to ~3 months, versus the original 6-month plan. While these set-backs were difficult to overcome, a complete and novel experiment was still conducted.

Moving forward, if I had the chance to revisit this research study I would increase the sample size to > 6, include a recovery step, and include a juvenile transplant portion as well. I would make sure all anemones at each sampling point

were the same size to minimize normalization error, and include carbon translocation measurements, as well as carbonic anhydrase transcription and activity. I would also track juvenile mortality throughout the entire experiment, not just at the last time point.