ENERGY IN SYMBIOSIS: CARBON FLUX IN ALGAL MUTUALISMS INVOLVING VERTEBRATE AND INVERTEBRATE HOSTS

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ABSTRACT

Symbiosis has been an important factor in evolution, and continues to drive speciation and allows organisms to fill new ecological niches. Symbiotic relationships in which both partners benefit from the association, or mutualisms, are ubiquitous in both terrestrial and aquatic ecosystems. Many of the symbionts in these associations are photosynthetic algae or cyanobacteria that fix carbon through photosynthesis and translocate a portion of this energy to their hosts. Host organisms utilize this fixed carbon for a variety of physiological processes, including growth and development, thus, photosynthetically-fixed carbon is vital for many hosts. The following chapters will describe carbon fixation and translocation in two algal symbioses: the freshwater association between the alga *Oophila* and the eggs of *Ambystoma maculatum* salmanders, and the relationship between the dinoflagellate *Symbiodinium* and marine zoanthids. These chapters will discuss carbon flux in symbiosis, and reveal some of the ways in which environmental factors alter photosynthesis in algal mutualisms.

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CHAPTER 1

INTRODUCTION

Symbiosis, or "the living together of unlike organisms" (DeBary, 1879) is responsible for the early success of eukaryotic organisms (Douglas 2012; Margulis 1991). Indeed, all eukaryotic organisms either contain a mitochondrion, or have a eukaryotic ancestor bearing a mitochondrion, which evolved from a symbiotic relationship with an α-proteobacterium. Today, symbiotic relationships are ubiquitous among eukaryotic organisms and continue to be a driving force in evolution and speciation (Brucker and Bordenstein 2012; Douglas 2012). The term symbiosis was originally coined to refer to a variety of interactions among organisms including what we now refer to as mutualism, parasitism, and commensalism, with interactions occurring on both short and long time scales (see Douglas 1994). Much symbiosis research has since focused on relationships that occur over long time scales (i.e. the life of the host partner) and involve benefits to both partners (i.e. mutualism). For the purpose of this paper, symbiosis will be defined by these terms.

Symbiotic relationships can be found throughout most of the major eukaryotic groups. However, there are several well-studied mutualisms that are commonly used as model systems. These include the relationship between corals and the dinoflagellate *Symbiodinium*, lichens, or the association of fungus with a photosynthetic symbiont, mycorrhizal fungi symbiotic with the roots of plants, and *Wolbachia* bacterial symbionts in a variety of insects. Despite the importance of symbiosis in the evolution of eukaryotic organisms, symbiosis research has tended to focus largely on these few well-studied

systems, therefore, the true quantity of symbiotic associations is likely orders of magnitude higher than reported.

There are many benefits that can be conferred by a symbiont. Some marine organisms including a variety of telost fish, as well as some cephalopods, urochordates, and nematodes acquiring luminescence by bacterial symbionts (see Douglas 1994). Mutualism can also confer protection to both the host and symbiont. For example, the bacteria *Hamiltonella defensa* protects its pea aphid hosts from parasitic wasps by disrupting the life cycle of the parasitoid, and the antibiotic-producing symbiont *Streptomyces philanthi* inhibits fungal attacks on its host, the European beewolf. Indeed, a variety of microbial symbionts protect their hosts from fungal, viral, and other pathogens, and even predation (see Oliver et al. 2013). Symbionts can even increase their host's physiological thermal tolerance and raise host reproductive rates (see Oliver et al. 2013). In many organisms, including humans, symbionts perform or assist in vital physiological processes such as digestion. Gastrointestinal bacteria digest cellulose in herbivorous mammals, and in a few birds and reptiles, allowing plant materials to be utilized for nutrition more efficiently.

Perhaps the most common benefit from symbiosis is the acquisition of nutritional compounds or metabolites. Symbionts, such as bacteria and yeast in a number of insect species, synthesize amino acids, sterols and vitamin B. Numerous plants, animals, fungi and protists have nitrogen-fixing symbionts that provide their hosts with fixed nitrogen as ammonia. Finally, carbon fixed by chemosynthetic or photosynthetic symbionts is translocated to many host organisms, especially in the marine environment.

Mutualisms between eukaryotes and photosynthetic symbionts are widespread. Hosts may be terrestrial, marine or freshwater organisms, and symbionts include a several algal and cyanobacterial species. Some common algal symbionts are the green alga *Chlorella* sp., which is symbiotic with a number of freshwater organisms—amoeba, ciliates, sponges, hydras, tubellarian worms, and mollusks—and the dinoflagellate *Symbiodinium* sp., which is prevalent in marine cnidarians and appears in some mollusks, protists, sponges, and a ciliate. There are also other, lesser-studied algal mutualisms including *Tetraselmis convolutae* in marine acoelomorph worms, and, as will be discussed in this paper, *Oophila amblystomatis* in salamander eggs. The common thread in all of these algal mutualisms is that inorganic carbon is fixed during photosynthesis and translocated to the host as a variety of compounds. Thus, the symbiont provides its host with energy that, in some cases, is enough to completely fulfill the metabolic demands of the host (Muscatine et al. 1984b).

Photosynthetic symbionts can release over 90% of their photosynthate to their hosts (Muscatine et al. 1981). Photosynthetically-fixed carbon is delivered to host tissues as a variety of "mobile" compounds, which vary depending on the symbiosis. The majority of these compounds are glucose, glycerol, amino acids, and lipids, but may also include maltose, fructose, or xylose (see Ahmadjian 2000; Venn et al. 2008). In photosynthetic associations, substrates and materials required for photosynthesis, such as inorganic carbon, nitrogen and phosphorus, are provided entirely by the host, and nutrient exchange is tightly regulated by the host.

The question of whether fixed carbon from photosynthesis is necessary for the survival of hosts remains debated, especially since most hosts feed heterotrophically.

Although many algal symbioses are described as facultative, the absence of symbiont-free hosts suggests that there is a clear benefit to the mutualism, perhaps even beyond fixed carbon. For example, in the salamander *Ambystoma maculatum*, embryos lacking algal symbionts in their eggs, or embryos in eggs maintained in darkness, have significant delays in growth and development, as well as increased (Gilbert 1942; Gilbert 1944; Goff and Stein 1978). Likewise, there are numerous accounts of inhibited growth in algal symbioses placed in the dark (Venn et al. 2008). Fixed carbon certainly cannot sustain the host entirely, as the host still has to acquire nutrients such as nitrogen and phosphorus, however, fixed carbon does provide the energy necessary for many cellular processes, and therefore, may be critical in some associations.

The following three chapters will discuss carbon flux in algal mutualisms and address the roles that fixed carbon plays in these symbioses. Chapter two describes the discovery of fixed carbon translocation in the lesser-known symbiosis between the algae *Oophila amblystomata*, and embryos of the North American Spotted Salamander, *Ambystoma maculatum*. Chapter three shifts to carbon flux in the well-studied cnidarian-*Symbiodinium* symbiosis, and discusses the effects of climate change on carbon flux in this mutualism. Finally, chapter four expands on chapter three by revealing climate-related changes in dissolved inorganic carbon acquisition that directly influence carbon fixation and translocation in the cnidarian-*Symbiodinium* symbiosis. This body of work supports the hypothesis that fixed carbon from photosynthetic symbionts significantly contributes to the health of the host organism.

CHAPTER 2

INTRACAPSULAR ALGAE PROVIDE FIXED CARBON TO DEVELOPING EMBRYOS OF THE SALAMANDER *AMBYSTOMA MACULATUM*

2.1 Abstract

Each spring, North American spotted salamander (*Ambystoma maculatum*) females each lay hundreds of eggs in shallow pools of water. Eggs are surrounded by jelly layers and deposited as large gelatinous masses. Following deposition, masses are penetrated by a mutualistic green alga, *Oophila amblystomatis*, which enters individual egg capsules, proliferates, and aggregates near the salamander embryo, providing oxygen that enhances development. I examined the effects of population density of intracapsular *Oophila* on *A. maculatum* embryos, and show that larger algal populations promote faster embryonic growth and development. Also, I show that carbon fixed by *Oophila* is transferred to the embryos, providing the first evidence of direct translocation of photosynthate from a symbiont to a vertebrate host.

2.2 Introduction

Over 120 years ago it was discovered that a mutualistic, flagellated green alga, *Oophila amblystomatis* (Lambert) inhabits the eggs of a few species of ambystomatid salamanders and ranid frogs (Orr 1888). The spotted salamander, *Ambystoma maculatum*, is common throughout North America, and has served as a model organism for studying this unique algal-vertebrate symbiosis. *A. maculatum* females deposit hundreds of eggs in shallow vernal pools each spring. The eggs are surrounded by multiple jelly layers and

deposited as masses enclosed in a thick jelly matrix that protects the embryos from pathogens, predators, and contaminants (Altig and Mcdiarmid 2007). Egg masses remain below the surface of the water, and are often attached to vegetation. Within hours of deposition, the masses are penetrated by benthic *Oophila* that swim to the egg masses (Gilbert 1944), however, the mechanism by which *Oophila* locate *Ambystoma* eggs remains unknown. Once inside, *Oophila* multiplies and traverses the middle membranes of the eggs, ultimately aggregating as nonmotile spherical or ovoid cells on the inner membrane of individual eggs, adjacent to the developing salamander embryo (Gilbert 1942). A recent discovery revealed that *Oophila* is also endosymbiotic; a portion of algal cells penetrate the embryo near the blastopore and settle within tissues and cells of the embryonic salamander (Kerney et al. 2011).

Oophila benefit *A. maculatum* embryos, presumably by increasing oxygen within the egg capsules (Pinder and Friet 1994; Valls and Mills 2007), which contributes to faster embryonic growth and development, increased survivorship, and more synchronous hatching (Gilbert 1942; Gilbert 1944; Tattersall and Spiegelaar 2008). In turn, *Oophila* receive embryonic nitrogenous waste (Goff and Stein 1978) and possibly CO_2 (Hammen, 1962). Another possible benefit for the salamander embryo is contribution of photosynthetically-fixed carbon from *Oophila* (reviewed in Kerney 2011), which could provide additional nutrition during development. Translocation of sugars from symbiont to host is common among algal-invertebrate symbioses, such as in tropical cnidarian-zooxanthellae symbioses, but remains unverified in an algal-vertebrate mutualism.

The recent work of Kerney et al. (2011) has increased our understanding of the *Oophila- A. maculatum* mutualism, yet several questions regarding this relationship remain unanswered. First, is there a direct relationship between *Oophila* abundance and *A. maculatum* growth and development? Second, aside from increasing oxygen in egg capsules, does *Oophila* provide any additional benefits to salamander embryos, such as photosynthetic products? To address these questions, I tested the effects of *Oophila* abundance on growth and development of *A. maculatum* embryos by comparing egg masses maintained in light, darkness, and with a photosynthesis inhibitor. I hypothesized that algal population density would be positively correlated with embryonic development and growth, irrespective of light. Although *Oophila* increase intracapsular oxygen during photosynthesis, eggs inhabited by algae have been shown to experience periods of anoxia during the night when the combination of algal and salamander respiration are not countered by oxygen production from photosynthesis (Pinder and Friet 1994). Therefore, I hypothesized that oxygen may not be the primary benefit for the embryos, and that photosynthetically-fixed carbon may accelerate growth and development. I used [^{14}C]bicarbonate to test the hypothesis that *Oophila* provides *A. maculatum* embryos with fixed carbon from photosynthesis. My results reveal that fixed carbon is translocated from *Oophila* to salamander embryos, and that there is a direct relationship between *Oophila* abundance and *A. maculatum* embryonic growth and development. These results also suggest that algae protect embryos from harmful microorganisms, possibly by inhibiting bacterial growth. This study provides the first evidence of direct translocation of carbon from a photosynthetic symbiont to a vertebrate host, and demonstrates that *Oophila* contributes multiple benefits to the salamander in this mutualism.

2.3 Materials and Methods

A. maculatum egg masses were collected from a vernal pool in Bucks County, PA, on March 28, 2011 and March 15, 2012. Clutches were attached to tree branches approximately 10 cm below the water surface. On the dates of collection, the water temperature was 4˚C and 10˚C, respectively. Clutches were transferred to the laboratory where they were rinsed in dechlorinated tap water and staged using an axolotl embryo staging series (Ambystoma Genetic Stock Center, University of Kentucky. Viewed at www.ambystoma.org/education/embryo-staging-series). To test the effects of algal population density on embryonic growth and development, ten egg masses were separated into three portions each and placed into 1L of dechlorinated tap water assigned to one of three treatments: 1) control 2) photosynthesis inhibitor (80µl of 100mM 3-3,4-dichlorophenyl-1,1-dimethylurea [DCMU], created by dissolving 200mg DCMU in 8.6 mL 95% ethanol) or 3) dark. 80µl of 95% ethanol was also added to all control and dark containers to account for the ethanol solvent used in the DCMU solution. To approximate natural conditions, all eggs were maintained at approximately 1.5 x 10^{15} quanta sec^{-1} cm^{-2} at 8˚C on a 12:12 light:dark cycle for 30 days. Dark treatment eggs were housed in a light-tight box in the same incubator with other treatments. Water was changed every 5 days. In addition, five uncut egg clutches were maintained in pond water in the incubator under the same light and temperature conditions. Eggs from these clutches were used to determine algal density in the inner and outer egg membranes as well as in embryonic tissue, and carbon translocation (described below).

2.3.1 Algal and embryo measurements

Eggs were collected from treatment and control clutch portions on days 10, 20, and 30. Each egg and embryo were only used for one measurement and then discarded. Algal population abundance was determined by rinsing individual eggs with phosphate-buffered saline (PBS), rupturing the egg and removing the embryo, which were rinsed with PBS and placed in 10% formalin in PBS in individual microcentrifuge tubes. The egg fluid and membranes were transferred to a separate microcentrifuge tube and 1150 µL deionized (DI) water and 50µL 70% ethanol (ETOH, to emulsify large globules) were added. The egg fluid mixture was vortexed, then centrifuged at 2,000 g for ten minutes. After centrifugation, the supernatant was removed and the algal pellet resuspended in 1200 µL DI water. Samples were vortexed, loaded into a Phycotech settling chamber, and algal cells were counted with a Zeiss Axiovert inverted microscope at 1000 X. Cell population density was determined by averaging cell counts of 50 grids. Embryos were staged and measured using a Wild M8 dissecting microscope at 12 X. To compare the outer and inner egg membranes, the outer membrane only was ruptured first and collected, and then the vitelline membrane was ruptured and collected. To examine algae contained inside embryo tissues, fixed embryos were rinsed with PBS, and homogenized in 500 µL PBS using a mortar and Teflon pestle. The homogenate was centrifuged at 2,000 g for eight minutes, then the supernatant was discarded and the pellet resuspended in dechlorinated water, vortexed, and loaded into a settling chamber. Algal cells were counted as described above.

2.3.2 DNA extraction and amplification

I extracted DNA from algae using a cetyltrimethylammonium bromide (CTAB) / hot detergent extraction protocol (Gast et al. 2004; Kuske et al. 1998). Algal DNA was PCR amplified using eukaryotic 18S rDNA gene primers EukA (5'AACCTGGTTGA TCCTGCCAGT-3') and EukB (5'-TGATCCTTCTGCAGGTTCACCTAC-3') (Medlin et al. 1988). Amplified DNA was directly sequenced (Genewiz, NJ) and the resulting 1,689 bp consensus sequence was deposited into GenBank (JQ779881).

2.3.3 Carbon translocation

Carbon translocation from *Oophila* to *A. maculatum* embryos was measured using methods modified from Engebretson and Muller-Parker (1999) for sea anemones. Salamander eggs (stages 37 to 41) were placed in replicate scintillation vials (2 eggs per vial) containing 5 ml dechlorinated water with 3 µl of sodium [^{14}C]bicarbonate for a final specific activity of 3µ Ci per sample (1.5 µCi per egg). Eggs were incubated in the light (1.5 x 10^{15} quanta sec^{-1} cm^{-2}) at 10˚C for 1.75 hours. Control samples were incubated in darkness for the same duration. Following incubation, eggs were transferred to non-labeled dechlorinated water and placed in the dark for a dark chase period (2 hours). Embryos were removed from their egg capsules, rinsed twice in dechlorinated water, and homogenized in 3 ml clean dechlorinated water. Subsamples of homogenized embryos (0.5 ml) were transferred to clean vials. The remaining embryo homogenate was combined with its respective egg capsule material and mixed. Subsamples (0.5 ml) of the total egg homogenate (embryo, algae, and capsule) were taken. All samples were acidified with 6M HCl in a fume hood overnight to remove unincorporated ^{14}C, then

neutralized with 6M NaOH before adding scintillation fluid. Radioactivity was measured using a scintillation counter (Bekman LS-3801); average counts per minute were converted to disintegrations per minute (DPM) using a quench correction curve. Average DPM of dark controls for embryo alone and total egg were subtracted from the light incubations of embryo alone and total egg, respectively, to determine carbon fixation. After correction with dark controls, the percent translocation was calculated from the ratios of radioactivity of embryo only to that of the total egg for each sample. Actual rates of carbon fixation and translocation were calculated using the equation of Engebretson and Muller-Parker (1999): carbon fixation (or translocation) per hour = DIC in sample x (DPM total egg (or embryo fraction) / DPM of ^{14}C added / hours incubation). DIC was estimated according to Wetzel and Likens (Wetzel 2000).

To test for ^{14}C assimilation by endosymbiotic algae, I incubated newly hatched embryos in sodium [^{14}C]bicarbonate under the conditions described above, with one salamander larvae per vial. Seven salamanders were incubated in the light and six in darkness. After incubation and dark chase period, we homogenized salamanders and collected 0.5 ml samples for analysis. I counted algal cells in the remaining homogenate using a settling chamber at 400X as previously described.

2.3.4 Statistical analysis

To examine the effects of algal population density and treatment on embryonic growth and development, random effects regression models ($y_{it} = \alpha + x_{it} + \nu_i + \varepsilon_{it}$; Stata 12) were estimated with robust standard errors to allow for potential heteroskedasticity. These models were compared to corresponding fixed-effects models using Hausman

specification tests, and no evidence of unobserved heterogeneity was detected. Algal densities were transformed by natural logarithms (ln (x + 1)) prior to analysis. Due to the death of all embryos in the DCMU treatment, two sets of analyses were performed: one set consisted of all treatments (control, DCMU, and darkness) at Day 10 only, and the second set compared control and darkness treatments only from days 0-30. For the first analysis, models were fitted independently to determine the effects of treatment and clutch on algal population density, and to determine the effects of treatment, clutch, and algal population density on embryonic growth and development. In the second set of analyses, I examined algal population density using time and treatment as main effects as well as the time × treatment interaction, and clutch membership. Models predicting length and development over 30 days in control and darkness were estimated using time, treatment (and the time-treatment interaction), clutch membership, and log-transformed algal density as variables. Algal densities in the inner and outer egg envelopes and carbon translocation data were analyzed using SPSS 17.0.

2.4 Results

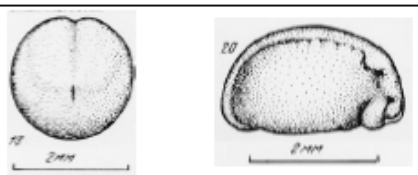
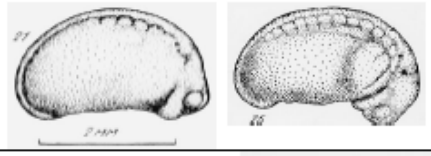
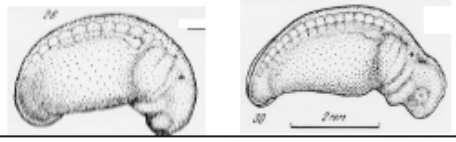
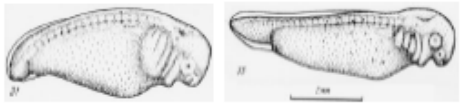
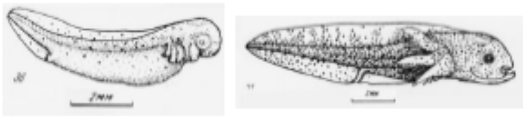
2.4.1 Location and morphology of *Oophila*

Algal DNA sequences from *A. maculatum* eggs were 99.8% similar, 1685/1689 base pairs, to GenBank HM590634, confirming affinity to *Oophila amblystomatis* (Kerney et al. 2011). During the tailbud period of embryonic development (Table 2.1), *Oophila* were abundant throughout the outer and middle egg envelopes. As embryonic development progressed, *Oophila* were significantly more abundant in the inner envelope and vitelline membrane surrounding the embryo (Figure 2.1A). I discovered *Oophila*

cysts in eggs of prehatched embryos (Figure 2.1B). In addition, *Oophila* transferred to DYIV culture media (Sanders et al. 2001) became cysts which have remained in the cyst form in culture for over one year.

Oophila were observed within intact salamander embryos using dissecting and compound fluorescence microscopy; these masses were located near the developing heart. Internal examination of pond-water embryos in developmental stages ranging from early tailbud (25) to hatchling (44) revealed that twenty-five of twenty-eight (89%) embryos contained endosymbiotic *Oophila*. The population density of internal *Oophila* cells ranged from 17,560 to 526,822 cells per ml of homogenate, however, there was no correlation between developmental stage and algal cell abundance (data not shown). The three embryos that did not contain endosymbiotic algae were in different developmental stages (41, 28, 35), and originated from different egg clutches.

Table 2.1. Major time points during *A. maculatum* development.

Period of Development	Period Beginning and End Images	Stages	Time * (hours)
Fertilization through gastrulation		1- 12.5	49-51
Neurula		13- 20	70.5
Early Tailbud		21- 25	83
Middle Tailbud		26 - 30	102
Late Tailbud		31 - 35	122
Pre-hatched through Hatched		36 - 44	342

Embryonic and larval illustrations, as well as stage and time data, are from Bordzilovskaya et al. (Bordzilovskaya et al., 1989), reproduced with permission from Oxford University Press. *Time indicates hours elapsed post-fertilization for the latest stage shown.

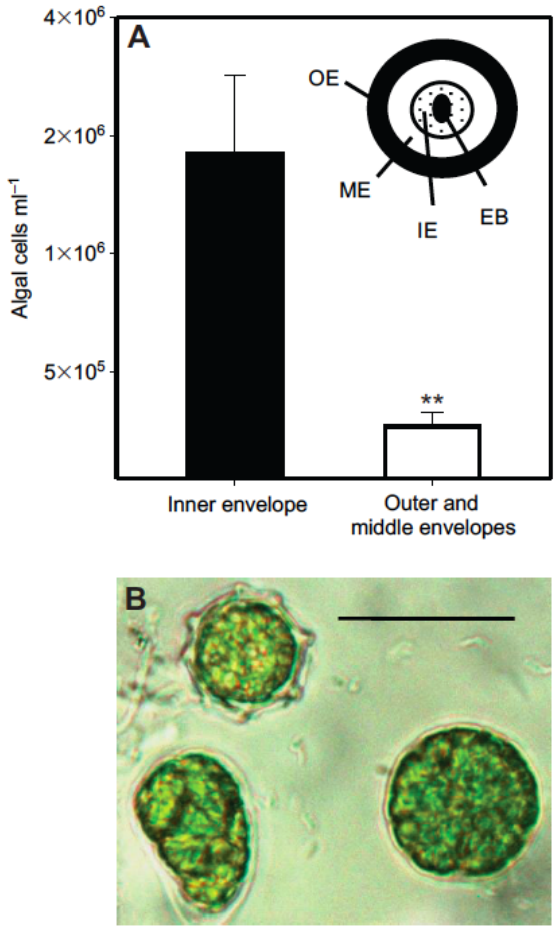


Figure 2.1. Characteristics of *O. amblystomatis* in *A. maculatum* eggs of embryos in hatchling stages (36-44). (a) Algal cells are more abundant in the inner envelope and vitelline membrane (black bar), although some remain in the middle and outer egg envelopes (open bar). Mean and standard error of the mean are shown (n=17). Inset shows eggs layers OE= outer envelope, ME=middle envelope, IE= inner envelope, EB= embryo surrounded by vitelline membrane. Statistical significance was evaluated by Mann-Whitney (***p = 0.001, **p =0.01, *p =0.05). (b) Algal cells observed in the inner envelope were predominantly spherical (lower right), but included a few cyst-like (upper left) and ovoid cells (lower left). Scale bar = 10µm.

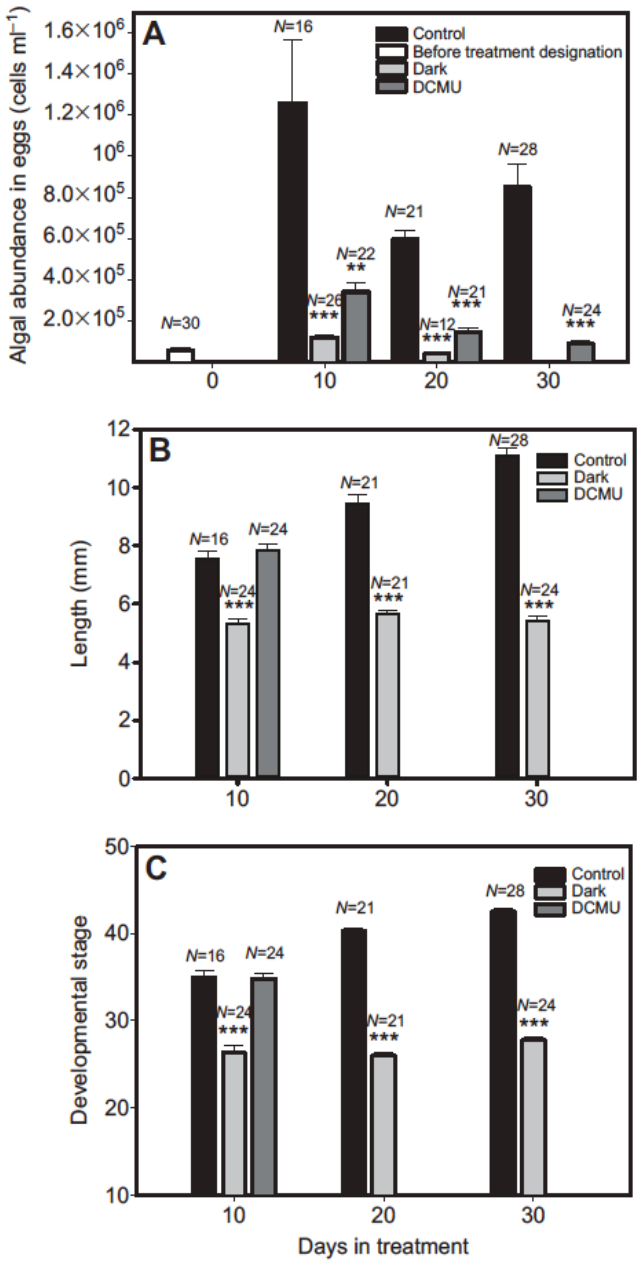


Figure 2.2. The effects of darkness and algaecide on (a) *Oophila* abundance in *A. maculatum* eggs (b) growth (length) and (c) development of *A. maculatum* embryos within eggs inhabited by *Oophila.* Bars and represent means with standard errors (n= 10 for all bars). Statistical significance was evaluated by ANOVA (***p = 0.001, **p =0.01, *p =0.05).

Table 2.2. Summary of z and p values for the random effects regression models testing the effects of darkness and algaecide on *Oophila* abundance in eggs and *A. maculatum* growth and development.

Day 10	Algaecide		Darkness		Algal abundance	
	z	*p*	z	*p*	z	*p*
Algal abundance (*n*=64)	-5.64	0.000	-2.28	0.023	–	–
Length (*n*=62)	0.9	0.371	-5.45	0.000	0.71	0.479
Developmental Stage (*n*=62)	1.59	0.112	-7.18	0.000	2.66	0.008

Days 20-30	Darkness		Time		Darkness x Time		Algal abundance	
	z	*p*	z	*p*	z	*p*	z	*p*
Algal abundance (*n*=134)								
overall	-2.24	0.025	–	–	–	–	–	–
Day 20	–	–	-0.81	0.419	-1.08	0.279	–	–
Day 30	–	–	-0.4	0.687	-2.2	0.028	–	–
Length (*n*=131)								
overall	-5.52	0.000	–	–	–	–	2.35	0.019
Day 20	–	–	5.8	0.000	-5.57	0.000	–	–
Day 30	–	–	10.18	0.000	-7.41	0.000	–	–
Developmental Stage (*n*=131)								
overall	-7.7	0.000	–	–	–	–	3.27	0.001
Day 20	–	–	6.03	0.000	-6.64	0.000	–	–
Day 30	–	–	6.19	0.000	-4.38	0.000	–	–

2.4.2 *Oophila* population density and embryonic growth and development

Darkness and photosynthesis inhibitor (DCMU) significantly reduced *Oophila* abundance in *A. maculatum* eggs. After ten days, eggs reared in darkness and DCMU contained less than one-third and one-tenth, respectively, of the amount of algal cells in control eggs reared in light without DCMU. This result was primarily due to treatment conditions (r^2= 0.54, p <0.001; Figure 2.2A), though egg clutch accounted for approximately 16% of the variance (data not shown). Algal density was significantly lower in the DCMU (121,650 +/- 13,293 cells ml^{-1} ; p<0.001) and darkness (341, 715 +/-

46,709 cells ml^{-1}; p=0.023) at day 10 compared to eggs reared in the light (1,261,344+/-304,413 cells ml^{-1}) (Figure 2.2A, Table 2.2).

In addition to differences in algal population densities, there were significant differences in both the length and developmental stage of embryos reared in DCMU and darkness compared to control eggs. Throughout the experiment, embryo length was strongly correlated with developmental stage (r=0.89, p <0.001) in control eggs. Fifty-eight percent of the variance in salamander length was due to treatment condition (p<0.001), and 27% of the variance was associated with clutch membership. There was no significant difference between length of embryos reared in control versus DCMU conditions, however, embryos reared in darkness were an average 2.08 mm shorter at ten days of development (p<0.001; Figure 2.2B, Table 2.2). Apart from treatment condition, algal density alone did not add significantly to predicted length (Table 2.2). Similar to the model predicting embryo length, a parallel model predicting embryo developmental stage assigned 68% of the variance to differential treatment conditions (p<0.001). Embryos reared in darkness were an average of 7.6 developmental stages behind those reared under control (light) conditions (Figure 2.2C, Table 2.2). Again, clutch membership accounted for 25% of the variability in embryonic development. Unlike the length model, however, there was a significant effect of algal population density on embryo development, above and beyond treatment condition, such that higher algal populations in the eggs corresponded to more developed embryos (p=0.008; Table 2.2).

At day twenty, no live embryos remained in the DCMU treatment. For this reason, analysis at days 20 and 30 includes control and darkness treatments only. From Day 10 to 30, algal population density was affected primarily by treatment condition

(R^2=0.69, p<0.001), and to a lesser extent by clutch membership (accounted for 10% variance). Over the 30 days, darkness was a significant predictor of embryo length (r^2= 0.89, p<0.001) and development (r^2= 0.90, p<0.001) (Figure 2.2B-C, Table 2.2). Embryos reared in the dark averaged 1.94 mm shorter and were an average 8.02 developmental stages behind those in control conditions (p<0.001) (Fig. 2.2B, Table 2.2). Above and beyond treatment condition (darkness), algal population density had a significant positive effect on embryo length (p=0.019) and development (p=0.001) from Day 10 to 30. Clutch membership still contributed 13% of the variance in both the models predicting length and development. There were also significant effects of time on both length and development, as well as time x treatment interactions in both models (Table 2.2).

2.4.3 Bacterial colonization of DCMU eggs

On day 20, all of embryos in each of the ten masses in the DCMU treatment were completely disintegrated, appearing as dark streaks inside the egg capsules. Microscopical analysis of egg capsules (n=12) revealed the presence of bacteria throughout all of the capsules. *Oophila* population density was extremely low (average 41, 770 cells mL^{-1}), and algal cells remaining in the eggs were degraded and/or lysed. All samples in DCMU were discarded before the 30-day analysis due to bacterial colonization and deterioration of the egg masses.

2.4.4 Carbon translocation

Salamander eggs incubated in the light (n=10) had higher radioactivity than those maintained in the dark (n=5; Mann-Whitney, $p < 0.001$). The corresponding embryos extracted from eggs incubated in the light (n=10) also had significantly higher activity than embryos from eggs maintained in the dark (n=5; Mann-Whitney, $p = 0.018$). In other words, all embryos in the light received carbon from the surrounding egg capsule or from endosymbionts. Translocation percentages (embryo DPM / total egg DPM) ranged from 0.5% to 13.2% (n=10), with an average of 6.3% (+/- 1.3 S.E). The estimated average hourly rate of carbon fixation was 1.3 (+/- 0.8) ng carbon per sample, or 0.6 ng carbon per egg. An estimated average 0.03 ng fixed carbon was translocated to each egg per hour.

Endosymbiotic algae were observed in all salamander larvae examined (n=13). The average population density of algae inside embryonic tissue was 14,314 +/- 2500 cells ml^{-1} (approximately 715 cells per larvae) and ranged from 2954 cells ml^{-1} to 25,021 cells ml^{-1}. Despite the presence of endosymbiotic *Oophila* in all of the salamander larvae, there was no significant difference in radioactivity between salamander larvae incubated in the dark versus light (Mann-Whitney, $p = 0.554$).

2.5 Discussion

2.5.1 *Oophila* morphologies and locations

Algae in this study were genetically identified as *Oophila amblystomatis*, the same species reported by Kerney et al. (2011) from *A. maculatum* egg masses collected in Nova Scotia, Canada. This supports that mutualism is exclusive between *A. maculatum*

and *O. amblystomatis*. Similar to others (Gilbert 1942; Kerney et al. 2011), I observed several protist species within *A. maculatum* eggs, however, they did not appear to affect salamander embryos or *Oophila*.

Three morphologies of *Oophila* have been described previously: spherical and ovoid cells 6-30 μm in diameter and an elongated biflagellate form 15-25 μm long (Gilbert 1942). I observed these forms in addition to a cyst form that was present in eggs in the final stages of development. The cysts observed in this study are morphologically distinct from encysted *Oophila* observed on the inner egg membranes of early stage embryos and intracellular algae with cyst-like envelopes observed by Kerney et al. (2011). It is possible that the thicker cyst form observed in this study may represent the morphology of free-living *Oophila* that inhabit soils until vernal pools form in the spring and new egg masses are deposited.

During neurula and early tailbud (stages 13-25), eggs in all treatments contained primarily tear-drop (ovoid) shaped, motile algae, which were replaced by spherical non-motile algae as development progressed. Some eggs maintained in the dark contained elongated algae. Algal population densities were significantly higher in the inner envelope and vitelline membrane compared to the outer egg membranes. This supports the hypothesis that *Oophila* enters the egg, travels through the outer and middle egg envelopes, and attaches to the inner envelope as embryonic development progresses (Gilbert 1942).

Similar to Kerney et al. (2011), I observed endosymbiotic algae in embryos throughout their development. I initially observed clumps of algal cells near the blastopore of embryos in late neurula and early tailbud (stages 17-25). In stage 25-44

embryos, algal population density was approximately twenty-six fold lower in embryonic tissue compared to the egg capsule, however, 89% of the embryos examined contained algae, suggesting that intraembryonic invasion is common. This invasion does not appear to affect growth, development, or hatching. I observed no correlation between abundance of intraembryonic algal cells and developmental stage of the embryo. Endosymbiotic algae were observed in thirteen hatched salamander larvae examined in this study. Kerney et al. (2011) also observed intracellular algae in liver and cartilage of salamander larvae, although intracellular algae were much less abundant in larvae compared to stage 35-44 embryos.

2.5.2 *Oophila* – salamander interactions

Several studies have concluded that *Oophila* promote faster embryonic growth and development and more synchronous hatching (Gilbert 1942; Gilbert 1944; Tattersall and Spiegelaar 2008). In addition, Marco and Blaustein (2000) reported that algal population density was positively correlated with reduced mortality and larger, more developed embryos in *A. gracile* (Marco and Blaustein 2000). My study also found a significant positive relationship between algal population density and embryonic growth and development. Although treatment condition was the primary predictor of embryo length and development throughout the experiment, increased *Oophila* abundance alone also had a significant positive effect on both length and development over thirty days. These positive effects are likely due to increased oxygen from photosynthesis (Pinder and Friet 1994; Valls and Mills 2007) in addition to receiving fixed carbon (discussed below).

Eggs maintained in the dark had lower algal populations and embryos were smaller and less developed. Several studies have reported that *A. maculatum* embryos reared in darkness averaged 1-4 developmental stages behind those reared in the light (Gilbert 1944; Goff and Stein 1978) and 1.3 mm shorter in length (Gilbert 1944). Embryos in this study averaged 7 stages behind and 1.94 mm shorter than eggs reared in light; this increased effect may be due to the lower incubation temperatures, typical of local early spring, that we used. Similar to Gilbert (1944) this study shows that delays in growth and development in embryos maintained in the dark are not simply due to the absence of light, but are effects caused by *Oophila* being maintained in darkness. In the absence of photosynthesis, *Oophila* respiration contributes to anoxia in egg capsules (Pinder and Friet 1994), therefore, eggs with *Oophila* maintained in the dark not only miss out on photosynthetic oxygen, but become more susceptible to anoxia when *Oophila* respire. Similar results were reported by Gilbert (1944), who created algae-free egg masses by breeding adult salamanders in the lab and preventing their exposure to *Oophila*. In his study, embryos from algae-free egg masses maintained in both light and darkness had similar growth and development to control (*Oophila*-inhabited) eggs in the light; however, embryos from *Oophila*-inhabited eggs in the dark were significantly smaller and less developed than both control eggs in the light and algae-free eggs, indicating that *Oophila* benefit salamander embryos as long as photosynthesis is occurring.

During my experiments, embryos maintained in DCMU suffered mass mortality within 20 days. It is unlikely that DCMU directly killed the salamander embryos. The effects of DCMU has been studied in several species of amphibian tadpoles and eggs, and

the lowest reported LC_{50} (dose resulting in 50% mortality) was 5.4 mg/L in *Xenopus laevis* tadpoles maintained in DCMU for 14 days; this is nearly three-fold more than my dose of 1.9 mg/L (Schuytema and Nebeker 1998). In this same study, lowest observed adverse effect level (LOAEL) values were above 14.5 mg/L for all amphibians maintained at the dose concentration of DCMU for 10-21 days (embryos of *X. laevis* and *Pseudocriss regilla* and tadpoles of *X. laevis*, *P. regilla*, *Rana catesbeiana* and *R. aurora*). In this study, *A. maculatum* embryos in the DCMU treatment appeared to have died following bacterial colonization in the eggs. Brodman (1995) suggested that *Oophila* protects *A. maculatum* eggs from pathogenic microorganisms, and this study strongly supports the hypothesis that *Oophila* prevent bacterial colonization (Brodman 1995). Embryos placed in DCMU continued to grow and develop at the same rate as control embryos until there were no observable healthy *Oophila* cells remaining. At this time (approaching day 10) bacteria began colonizing the egg capsules, and by day 20, all of the embryos were dead and bacteria were abundant throughout the egg capsules. Bacterial colonization following the death of *Oophila* could have caused embryonic mortality by depleting oxygen or by excreting toxins. In either case, the absence of healthy algal symbionts appears to have facilitated the colonization. This hypothesis is further supported by the fact that bacteria did not appear in eggs maintained in the dark, despite low numbers of *Oophila* cells, because the remaining algal cells were not lysed and appeared healthy apart from some being reduced in size. I propose that the presence of a small population of healthy *Oophila* inhibited bacterial colonization in the dark treatment, compared to the DCMU treatment, where all *Oophila* were deteriorated. I did not test for the mechanism by which *Oophila* inhibits bacteria, however, some eukaryotic

algae, including the closely related genus *Chlamydomonas*, can exude antibiotics (Cembella 2003; Proctor 1957). Additional studies are needed to confirm if *Oophila* also releases antibiotics in egg capsules.

2.5.3 Translocation of carbon

Fixed carbon was translocated from *O. amblystomatis* to salamander embryos in all of the samples radiolabeled with ^{14}C. Our translocation values likely underestimate the amount of fixed carbon translocated from *O. amblystomatis* to *A. maculatum* embryos in nature because I was not able to create higher light intensities representative of those in the field in vitro without affecting the incubator temperature. However, light intensity was sufficient enough to encourage algal growth when eggs were placed in the incubator (Figure 2.2A). *Oophila-A. maculatum* translocation (maximum 13%, average 6.3%) was similar to percentages reported for the freshwater sponge *Ephydatia fluviatilis*, which receives 9-17% of photosynthetically-fixed carbon from its facultative algal symbiont (Wilkinson 1980), and the freshwater hydra, *Chlorohydra viridissima*, which acquires 10-20% of its symbiont's photosynthate (Muscatine and Lenhoff 1965). My results were, however, lower than amounts reported for marine algal symbionts using ^{14}C methods (see Table 2.3). For example, photosynthetic dinoflagellates (zooxanthellae) in temperate sea anemones translocate as much as 50% of their fixed carbon (Muller−Parker and Davy 2001) and tropical zooxanthellae give over 50% and as much as 97% of their photosynthate to invertebrate hosts (Fitt and Cook 2001; Muscatine et al. 1984a; Steen and Muscatine 1984). However, these latter symbioses are often obligate and intracellular, suggesting a long evolutionary relationship, and may involve a "host factor"

that stimulates release of algal photosynthate (Gates et al. 1995). The amount of carbon translocated from *Oophila* to salamander embryos is expected to vary because translocation can be affected by several factors including temperature and irradiance. Moreover, length of the dark-chase period can alter translocation estimates (Engebretson and Muller-Parker 1999), and using ^{14}C to quantify translocation can alone lead to underestimation (reviewed byMuller−Parker and Davy 2001). Hatched salamander larvae did not incorporate ^{14}C on their own, nor did their endosymbiotic algae. If any ^{14}C was assimilated by algae within the salamanders, the quantity was too small to identify using my standard method.

Fixed carbon can be translocated to hosts as several compounds. The most common products translocated in algal-invertebrate symbioses include glycerol and glucose, although C4 acids, succinate/fumerate, maltose, lipids, and amino acids can also be transferred (Whitehead and Douglas 2003; Yellowlees et al. 2008). Algal release products can also change in response to environmental conditions, therefore, *Oophila* may translocate a number of photosynthetic compounds depending on the environmental conditions surrounding the egg mass. In addition to acquiring nutrition from algal symbionts, studies on algae in eggs of other organisms revealed that embryos actually consumed algae in the egg capsule. Embryos of the marine polychaete *Axiothella mucosa* have a fully-developed digestive system within their first five days, which allows them to graze on diatoms in their eggs during the rest of development (Peyton et al. 2004). Although *Oophila* likely provide nutrition to hatched salamanders during their initial foraging, it is unlikely that algae are consumed by embryos. The digestive system of *A.*

maculatum is not functional until stage 44-45 and doesn't complete development until after the salamander hatches (Harris 1967).

Amphibian eggs deposited in aquatic environments usually have a lower amount of yolk than those deposited terrestrially, and the yolk is incorporated into the gut at an early stage. The additional nutrition provided by photosynthate would ensure that adequate nutrition was available to embryos until hatching. Yet, is carbon translocation necessary for the development and survival of *A. maculatum* embryos? Although rare, algal-free egg masses can be found in nature (Gilbert, 1942), indicating that embryos do survive without photosynthetically-derived carbon. But the positive relationship between *Oophila* abundance and *A. maculatum* embryo survival and development argues for the overall importance of the relationship for *A. maculatum*. A recent study suggests that associations between egg masses and photoautotrophs may be more prevalent than previously thought, and more heterotrophic organisms may rely on products from photoautotrophs during development. Woods and Podolsky (2007) examined aquatic egg masses spanning a variety of invertebrate species and found that eggs often were either deposited on substrates covered by macrophytes, or if not, the masses contained microalgae. No egg mass in the Woods and Podolsky (2007) study was associated with both macrophytes and microalgae, however, in either case, oxygen in the egg masses was increased from association with a phototroph(Woods and Podolsky 2007). The apparent facultative nature of the *Oophila – A. maculatum* mutualism may be influenced by the presence of macroalgae or macrophytes in the water surrounding the egg masses. It is possible that masses lacking *Oophila* (e.g., Gilbert 1942) were deposited on macrophyte-covered surfaces, and despite the absence of an intracapsular algae, embryos would still

benefit from the associated photoautotroph. Overall, however, the importance of *Oophila* to development in *A. maculatum* is emphasized by the rarity of algal-free clutches in nature and the high mortality rate of those clutches (Gilbert 1942; Gilbert 1944).

Table 2.3. Percentages of fixed carbon translocated from photosynthetic symbionts to hosts based on ^{14}C measurements.

Species	Photosynthetic Symbiont	Translocation %	Reference
Sea anemones			
Aulactinia stelloides	zooxanthellae	21-29%	Smith, 1986
Aiptasia pulchella	*Symbiodinium*	12-22%	Muller-Parker & Davy, 2001
Anemonia viridis		50%	
Coral			
Plesiastrea versipra	zooxanthellae	10%	Hinde 1987
Stylophora pistillata	*Symbiodinium*	>95%	Muscatine et al., 1984
Heteroxenia fuscescens (octocoral)		17%	Schlichter et al., 1983
Zoanthids			
Zoanthus robustus	zooxanthellae	12-42%	Hinde 1987
Palythoa variabilis	*Symbiodinium*	89%	Steen and Muscatine, 1984
Zoanthus sociatus		95%	
Molluscs	chemoautotrophic bacteria	>45%	Fisher & Childress, 1986
Solemya reidi	zooxanthellae	39-45%	Trench et al., 1981
Tridacna maxima			
Other marine invertebrates			
Pteraeolidia ianthina	zooxanthellae	25-50%	Hinde 1987
Cassiopea andromeda	*Symbiodinium*	5-10%	Hofmann & Kremer, 1981
Myrionema amboinense		52%	Fitt and Cook, 2001
Freshwater invertebrates			
Ephydatia fluviatilis	green algae	9-17%	Wilkinson, 1980
Chlorohydra viridissima	green algae	10-20%	Muscatine & Lenhoff, 1965
Vertebrates			
Ambystoma maculatum	*Oophila*	0.5-13.2%	this study

2.5.4 Conclusions

This study shows a direct positive relationship between *Oophila* abundance and the growth and development of salamander embryos, and suggests that *Oophila* prevent bacteria from invading *A. maculatum* eggs. In addition, I have identified a cyst form of *Oophila* that can be maintained in laboratory culture, the likely form in which *Oophila* survive outside of eggs in vernal pools. *Oophila* benefit *A. maculatum* embryos by increasing oxygen content and probably by removing nitrogenous waste in the egg capsule. Furthermore, as shown in this study, *Oophila* provide supplemental nutrition from photosynthetic products. Although additional experiments are required to identify what compounds are exchanged between *Oophila* and *A. maculatum*, it is clear that salamander embryos do incorporate fixed carbon from algal photosynthesis. To my knowledge, this is the first study to identify carbon translocation in an algal-vertebrate symbiosis. Other salamander and frog species have similar algal-egg symbioses, and it is likely that translocation occurs in those associations as well. Indeed, there may be algal-egg relationships that have not yet been identified, and phenomena similar to those known for *Oophila* and *A. maculatum* may be more common than suspected from current reports.

CHAPTER 3

SPECIES-SPECIFIC PHOTOSYNTHETIC RESPONSES OF SYMBIOTIC ZOANTHIDS TO CLIMATE CHANGE AND OCEAN ACIDIFICATION

3.1 Abstract

Increasing sea surface temperatures and ocean acidification (OA) are impacting physiological processes in a variety of marine organisms. Many sea anemones, corals, and jellies in the phylum Cnidaria, form endosymbiotic relationships with the dinoflagellate *Symbiodinium* spp., which supplies the hosts with fixed carbon from photosynthesis. Much work has focused on the effects of climate change and OA on calcification in *Symbiodinium*-coral symbioses, but has not related this work to symbiont photosynthesis or nutrient exchange in the symbiotic animal (the holobiont). *Symbiodinium* species or types vary in their environmental tolerance and photosynthetic capability, therefore, primary production in the holobiont is directly related to symbiont type. However, symbiont type has not been identified in a large portion of *Symbiodinium*-cnidarian studies. Future climate conditions may favor non-calcifying, soft-bodied cnidarians, including zoanthids, over coral species. Here I show that two zoanthid species, *Palythoa* sp. and *Zoanthus* sp., harboring different symbiont types (C1 and A4), had very different responses to increased temperature and increased pCO_2/low pH. Thermal stress did not affect photosynthesis or fixed carbon translocation in the *Zoanthus* sp./A4 association, and high pCO_2/low pH increased carbon fixation and translocation. In contrast, both thermal stress and high pCO_2/low pH greatly inhibited the *Palythoa* sp./C1 association. However, synchronous increases in temperature and pCO_2 stabilized carbon

fixation and translocation in this host-symbiont pair. My observations support the growing body of evidence that demonstrates that the response of symbiotic cnidarians to climate change and OA must be considered on a host-specific and symbiont-specific basis. Moreover, the combined effects of OA and thermal stress are not necessarily consistent with the responses of the individual treatments. Understanding how photosynthesis varies with both cnidarian host and *Symbiodinium* type is critical to predicting which host-symbiont combinations will persist in warm, acidified oceans.

3.2 Introduction

Climate change is reshaping aquatic and terrestrial ecosystems worldwide. Sea surface temperatures have increased as much as 2˚C over the last four decades and are predicted to rise an additional 2-6˚C by the end of the century (Meehl 2007). In addition, dissolution of increased atmospheric CO_2 in the oceans has led to ocean acidification (OA), or a decline in ocean pH. Surface water pH is predicted to further decline by 0.3-0.4 units by the end of the century (Guinotte and Fabry 2008; Meehl 2007). Rising sea water temperature negatively affects marine ecosystems by reducing vertical mixing, promoting stratification, and extending the spatial range of nutrient-poor waters (Behrenfeld et al. 2006; Bopp et al. 2003). OA typically reduces photosynthesis and species diversity, increases mortality in marine organisms, and inhibits calcification in mollusks, foraminifera, echinoderms, polychaetes, and coral (Doney et al. 2009; Fabricius et al. 2011; Fabry et al. 2008; Hall-Spencer et al. 2008; Kroeker et al. 2010; Ries et al. 2009). The combined effects of temperature and OA on marine organisms are predicted to be largely negative (Hoegh-Guldberg et al. 2007; Rodolfo-Metalpa et al.

2011). Indeed, it is evident that increased temperature and OA will continue to transform

Cnidarians form the foundation of diverse benthic ecosystems in tropical and subtropical waters. Their success is largely due to symbiotic relationships with photosynthetic dinoflagellates (zooxanthellae) of the genus *Symbiodinium*. Corals, sea anemones, zoanthids, and other shallow-water cnidarians contain zooxanthellae within endodermal cells. These symbionts fix CO_2, using C3 photosynthesis (Streamer et al. 1993), translocating as much as 95% of their fixed carbon in the form of glycerol, glucose, amino acids, and other organic acids (Gates et al. 1995; Muscatine 1967; Trench 1971; Whitehead and Douglas 2003), and providing up to 100% of the host‘s daily carbon requirement (Muscatine et al. 1984b).

Over the past three decades, research has revealed the detrimental effects of climate change on zooxanthellae-cnidarian symbiosis. Hyperthermal stress damages the CO_2-fixation mechanism in zooxanthellae, and affects gross photosynthesis, photosynthetic efficiency, and host and symbiont respiration rates (Al-Horani 2005; Buxton et al. 2009; Fitt et al. 2009; Goulet et al. 2005; Iglesias-prieto et al. 1992; Jones et al. 1998; Lesser 1997; Warner et al. 1999). Increased temperature is also a primary cause of bleaching, or expulsion of zooxanthellae by hosts, which can devastate coral reef communities (Brown 1997; Brown et al. 1999; Gates 1990; Gates et al. 1992). OA has multiple effects on cnidarian symbioses. High pCO_2 reduces calcification in scleractinian corals, inhibits coral growth, and reduces species diversity (Anthony et al.; Fabricius et al. 2011; Hall-Spencer et al. 2008; Kroeker et al. ; Langdon et al. 2000; Leclercq et al. 2000; Leclercq et al. 2002; Renegar and Riegl 2005).

However, despite numerous investigations reporting negative impacts of climate

change and OA on coral, some cnidarian symbioses occur near shallow volcanic seeps where pH conditions can fall well below predicted OA values for the upcoming century (Fabricius et al. 2011; Meron et al. 2013; Meron et al. 2012; Suggett et al. 2012). Moreover, increased primary production, symbiont population density, and growth were observed in anemones exposed to natural and simulated increases in pCO_2 (Suggett et al. 2012; Towanda and Thuesen 2012). Even some corals have survived incubations in low pH for over a year, actually increasing their soft tissue biomass and living as soft-bodied polyps after dissolution of their calcium carbonate skeleton (Fine and Tchernov 2007; Krief et al. 2010). The ability of some cnidarians (even calcifying species) to persist in high pCO^2, combined with the fact that some cnidarians live where maximum temperatures often exceed 32° C, the threshold maximum for bleaching (Baker et al. 2013a; Baker et al. 2004; Oliver and Palumbi 2011), suggest that certain cnidarian symbioses may survive the warmer acidified waters predicted for the next century.

Symbiodinium are a genetically diverse genera with nine clades (A-I) and numerous phylotypes (e.g. A3, B1). Multiple studies have established that *Symbiodinium* clades vary in their environmental tolerance and adaptive physiology (Pochon et al. 2006; Stat et al. 2006) as well as their photobiology (Hennige et al. 2008). Clade D is recognized as a thermally tolerant clade, prevalent in high temperature regions (Baker et al. 2013a; Fabricius et al. 2004; Garren et al. 2006; LaJeunesse et al. 2008; LaJeunesse et al. 2010; Oliver and Palumbi 2011), while clade C, the most common clade worldwide (Baker et al. 2013a; LaJeunesse et al. 2010) is more thermally sensitive (Baird and Marshall 2002; Dove et al. 2006). Recent studies showed that environmental tolerance varies not only among, but within clades. For example, types of clade C (i.e. C1 and C15)

have higher thermal tolerance than other C types (Abrego et al. 2008; LaJeunesse et al. 2003) and clade A types in culture have discrete responses to both temperature and pCO_2 manipulations (Brading et al. 2011; Robison and Warner 2006). In addition, placing the same symbiont type in different host species results in dissimilar photophysiological responses (Goulet et al. 2005). This effect also occurs when different symbiont types are placed in the same host species (Loram et al. 2007). Indeed, there is growing support for the importance of the host-symbiont combination to environmental tolerance and health of the symbiosis (Bellantuono et al. 2012; D'croz and Maté 2004; Fitt et al. 2009; Middlebrook et al. 2008).

Identifying the responses of various host-symbiont combinations is a critical step in predicting shifts in community composition and ecosystem functioning, and managing marine protected areas. Despite the evidence that different host taxa and symbiont types have varying degrees of tolerance to climate change and OA, very few studies have examined these combined effects on photosynthesis and carbon translocation in zooxanthellae-cnidarian symbioses *in hospite*, or using multiple host-symbiont combinations. Moreover, there is no information about these effects in zoanthids, (subclass Hexacorallia, Cnidaria, Anthozoa), despite their ubiquity as hosts of *Symbiodinium* and wide geographic distribution. In this study, I determined carbon fixation and translocation rates *in hospite* for two *Symbiodinium*-zoanthid symbioses exposed to thermal stress and OA, both as singular and combined treatments. The results of our study show several differences in physiological responses to environmental stress between the two *Symbiodinium*-zoanthid associations, and provide further evidence that certain host-symbiont combinations are more likely to prevail in warm acidified seas.

3.3 Materials and Methods

3.3.1 Organism collection and maintenance

The Florida Keys National Marine Sanctuary (FKNMS) hosts a wide variety of cnidarian-zooxanthellae symbioses, and is home to the third longest barrier reef in the world, and the only barrier reef in the United States (http://floridakeys.noaa.gov). The Sanctuary also contains mangrove and seagrass habitats that support numerous fish and invertebrate species. Current summer seawater temperatures within the Sanctuary often reach 32˚C (NOAA National Data Buoy Center data), and summer maximum temperatures are sure to exceed the threshold for bleaching in upcoming years. For this study, I selected two zoanthid species common to the FKNMS, harboring different *Symbiodinium* types.

Polyps of the green zoanthid *Zoanthus* sp., and the cinnamon zoanthid *Palythoa* sp. were collected from Duck Key and Tavernier, Florida, respectively in October and November 2012, and January 2013. Organisms were maintained in glass aquaria under cool white fluorescent lights (80 μmol photons m^{-2} s^{-1}) on a 12:12 light dark cycle. Aquaria were filled with artificial seawater (Instant Ocean®, United Pet Group, Blacksburg, VA) at 27˚C and specific gravity 1.026 (35 practical salinity units) and equipped with individual filtration units. Organisms were fed twice weekly with freshly hatched *Artemia sp*. nauplii.

During acclimation to laboratory conditions, zoanthid polyps were divided into clusters consisting of 1-3 polyps and assigned identification numbers to insure that polyps within the same cluster were not used more than once for an experiment. Experimental aquaria were maintained at 27˚C (control), 31˚C or 33˚C using aquarium heaters. Three

target pH values selected were: 8.1 (control), 7.85, and 7.65. Due to space and tank availability, some experimental treatments were conducted in two tanks simultaneously, while others were carried out using a single tank only (Table 3.1). I adjusted total alkalinity (TA) to 2300 μmol kg^{-1} in all pCO_2 treatment tanks using 12M hydrochloric acid, and equipped tanks with automatic CO_2 injection systems (Drs. Foster and Smith Rhinelander, WI). Temperature, salinity and pH were monitored daily. Water samples were collected weekly during the twelve-week experiments to measure pH and TA. Carbonate chemistry was determined using CO2calc (Robbins 2010) using CO_2 constants K1, K2 from Hansson (1973), refit by Dickson and Millero (1987), $KHSO_4$ from Dickson (1990) and Total pH scale (Table 3.1) (Dickson and Millero 1987; Dickson 1990; Hansson 1973).

3.3.2 Organism and symbiont identification

Symbiont cells were separated from host tissue by homogenization and centrifugation. DNA was extracted from *Symbiodinium* using a modified *Promega Wizard* protocol (see LaJeunesse et al. 2003). I used denaturing gradient gel electrophoresis (DGGE) to determine the dominant symbiont clade in both zoanthid species. The internal transcribed spacer 2 region (ITS 2) was amplified for DGGE fingerprinting using 'ITS 2 clamp' and 'ITSintfor 2' (LaJeunesse and Trench 2000) with the thermal cycle described in LaJeunesse (2002). PCR products were electrophoresed for 16 h at 115 V on denaturing gradient gels (45–80%) using a CBScientific system (Del Mar, CA). The dominant band of the DGGE was excised, reamplified, and sequenced directly (Penn State Genomics Core Facility, University Park, PA). Subsequent

Symbiodinium samples were amplified using *Symbiodinium* LSU rDNA gene primers (Forward 5'-CCCGCTGAATTTAAGCATATAAGTAAGCGG-3', Reverse 5'-GTTAGACTCCTTGGTCCGTGTTTCAAGA-3') (Zardoya et al. 1995) with PCR conditions as follows: 2 minutes at 90°C, then 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by 72°C for 5 minutes. To further resolve clade C symbionts, I also amplified the non-coding region of the plastid psbA minicircle ($psbA^{ncr}$) using primers (7.4-Forw, 5'-GCATGAAAGAAATGCACACAACTTCCC-3', and 7.8-Rev, 5'-GGTTCTCTTATTCCATCAATATCTACTG-3' (Moore et al. 2003). The PCR conditions for $psbA^{ncr}$ amplifications were: 94°C for 2 min; 40 cycles of 94°C 10 s, 55°C for 30 s and 72°C for 2 min; followed by a final extension at 72°C for 10 min (LaJeunesse and Thornhill 2011). The internal transcribed spacer region of ribosomal DNA (ITS-rDNA) was amplified for zoanthid hosts using anthozoan-specific primer pairs for the complete ITS region: ITSf (5′-CTAGTAAGCGCGAGTCATCAGC-3′**)** and ITSr (5**'**-GGTAGCCTTGCCTGATCTGA-3′) (Swain 2009). PCR conditions consisted of 94°C for 3 min, 32 cycles of 94 °C for 30 s, 50 °C for 60 s, 72 °C for 90 s, with a final extension step of 72 °C for 10 min. All PCR products were purified by enzymatic digestion (ExoSAP-IT®; USB Corporation, Santa Clara, CA). Amplified DNA was sequenced directly (Genewiz, South Plainfield, NJ) and representatives of the resulting consensus sequences were deposited into GenBank.

3.3.3 Carbon fixation and translocation

Carbon fixation by *Symbiodinium* and translocation to the host was measured using methods of Engebretson and Muller-Parker (1999). Zoanthid polyps were placed in 5 ml ASW with 6 µl of sodium [^{14}C] bicarbonate for a final specific activity of 6 µCi per sample. Polyps were incubated in the light (80 µmol photons m^{-2} s^{-1}) under their corresponding treatment conditions for 1.5 hours. Control samples were incubated in darkness for the same duration. Following incubation, polyps were transferred to non-labeled ASW for a dark chase period (1.75 hours). Polyps were then rinsed and homogenized in 3 ml clean ASW. Subsamples (0.5 ml) of the total homogenate and the host fraction (supernatant after centrifugation) were collected and acidified with 6M HCl in a fume hood overnight to remove unincorporated ^{14}C, then neutralized with 6M NaOH before adding scintillation fluid. Average disintegrations per minute (DPM) of dark controls were subtracted from the light incubations, and the percent translocation was calculated from the ratios of radioactivity of host supernatant only to that of the total homogenate for each sample. Actual rates of carbon fixation and translocation were calculated using the equation: carbon fixation (or translocation) per hour = DIC in sample x (DPM total homogenate [or supernatant] / DPM of ^{14}C added / hours incubation) (Engebretson and Muller-Parker 1999). DIC for each treatment was calculated using CO2calc (see above).

3.3.4 Chlorophyll

Isolated zooxanthellae were placed in an acetone/ethanol chlorophyll extraction medium and stored at -20˚C for 12 hours. Following extraction, samples were centrifuged

and chlorophyll a was measured in the supernatant using a TD-700 laboratory fluorometer (Turner Designs, Sunnyvale, CA).

3.3.5 Host protein and symbiont population density

Zoanthid polyps were homogenized in fresh ASW, and the homogenate was centrifuged for 10 minutes at 1600g to separate host material from zooxanthellae. A portion of the host supernatant was reserved for protein analysis determined by a biconchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). The algal pellet was rinsed and centrifuged twice, then resuspended in fresh ASW for enumeration in a Phycotech settling chamber using a Zeiss Axiovert inverted microscope. Cell population density was determined by averaging cell counts of 50 grids, and normalizing to host protein.

3.3.6 Statistical analysis

Carbonate chemistry in the experimental tanks was compared using a two-way ANOVA with Restricted Maximum Likelihood (REML) Method. In cases where there were duplicate tanks for a single treatment, treatment was a fixed effect, and tank was a random effect nested within treatment. Tank effect was minimal for all role variables, therefore tank effect was eliminated from subsequent anayses of photosynthetic parameters. The effects of pH, temperature, and species on carbon fixation and translocation were analyzed using MANOVA with an identity matrix and post hoc contrasts. MANOVA data reported are based on Wilks' lambda distributions. Data were transformed to meet the assumptions of normality and equal variance using log

transformations. Carbon translocation per cell was found to be highly correlated with carbon fixation per cell (r = 0.8212), therefore it was eliminated from the MANOVA analysis. Additional analyses of each independent variable were conducted using one-way ANOVA with Tukey post-hoc analysis. Host protein levels were compared using Dunnett's Method with a control. Finally, the effects of chlorophyll, *Symbiodinium* population density, and host protein on carbon fixation and translocation were analyzed using pairwise correlations estimated by the REML method. All statistical tests were performed using JMP 10.0.0 statistical software (SAS, Cary, NC).

3.4 RESULTS

3.4.1 Tank conditions in pH/ pCO_2 treatments

Temperature was checked daily and was maintained within 0.5°C of the target temperature. pH and pCO_2 differed among treatments ($F = 313.17$, $p < 0.0001$; $F = 482.37$, $p < 0.0001$, respectively) but not between tanks within treatments (REML variance ratio= -0.04 for pCO_2 and 0.01 for pH) (Table 3.1). DIC differed among treatments ($F = 9.29$, $p = 0.0301$), but this difference was only significant between the control tanks (27 °C, pH 8.1) and the 31°C, pH 7.65 tank ($p = 0.0216$), and was near significant between the control tanks and the pH 7.65 tanks ($p = 0.0517$). Again, there was no effect of tanks within treatments on DIC (variance ratio= -0.02). TA did not vary among pCO_2 treatments ($F=3.8$, $p =0.1105$) or between tanks within treatments (-0.025 variance ratio).

Table 3.1. Temperature and carbonate chemistry for high *p*CO2/ low pH experimental tanks. Values for pH, *p*CO2, dissolved inorganic carbon (DIC), and total alkalinity (TA) represent means with S.E.M. in parenthesis. Treatment names are as follows: high temperature/control pH (HT-CPH), control temperature/low pH (CT-LPH), and high temperature/low pH (HT-LPH). *n*= number of water samples characterized for the corresponding tank.

Treatment	Tank	Temp. (°C)	pH	pCO_2	DIC	TA	*n*
Control	1	27	8.05 (0.01)	361.4 (8.09)	1848.1 (26.9)	2157.7 (32.0)	18
	2	27	8.05 (0.01)	354.4 (18.0)	1796.5 (71.1)	2118.3 (76.5)	11
CT-LPH	3	27	7.86 (0.01)	604.3 (18.2)	1913.9 (25.7)	2120.3 (27.6)	17
	4	27	7.89 (0.01)	584.0 (21.2)	1965.8 (39.5)	2188.2 (41.6)	14
CT-LPH	5	27	7.69 (0.01)	1003.1 (32.0)	2056.4 (20.9)	2188.9 (20.3)	12
	6	27	7.69 (0.01)	1048.9 (27.1)	2164.5 (15.0)	2302.1 (12.1)	12
HT-CPH	7	31	8.10 (0.01)	321.8 (14.5)	1841.7 (100.3)	2223.5 (118.9)	13
HT-LPH	8	31	7.89 (0.01)	604.3 (26.6)	1997.7 (31.6)	2249.9 (31.0)	12
HT-LPH	9	31	7.69 (0.01)	1037.6 (22.4)	2160.3 (24.3)	2326.3 (24.9)	12

3.4.2 Host and symbiont identification

Palythoa sp. ITS rDNA sequences were > 99% similar to specimens 1562 and 1563 from Reimer et al. (2012) identified as *Palythoa* aff. *clavata* (Duchassaing 1850) from Pompano Beach and Miami Beach, Florida (GenBank JX119126 and JX119125, respectively) (Reimer et al. 2012) and from Ascension Island (unpublished, JD Reimer personal communication). The *Zoanthus* species is an unidentified species closely related (704/709 bp) to both *Z. sociatus* (GenBank JX119131.1 through JX119133.1) and *Z. pulchellus* (GenBank EU418342.1 through EU418346.1) *Zoanthus* sp. contained a single *Symbiodinium* phylotype that was similar (>99%) to clade A4 (formerly *Gymnodinium*

linucheae; GenBank AF060893) and with additional A4 unpublished sequences (LaJeunesse, personal communication). *Palythoa* sp. also contained a single symbiont >99% similar to C1 sequences isolated from *Acropora cervicornis* in the Florida Keys (GenBank KF572154 and KF572153).

3.4.3 Carbon fixation and translocation.

Temperature, pH, and species had significant effects on carbon fixation per symbiont cell and percent fixed carbon translocated (MANOVA, pH $F=3.93$, $p = 0.004$; temperature $F=4.76$, $p = 0.0011$, species $F=34.66$, $p <0.0001$). Separate tests for each dependent variable revealed that temperature, pH, and species all had significant effects on carbon fixation per cell ($F=5.22$, $p = 0.007$; $F=6.61$, $p = 0.002$; $F=27.1$, $p <0.001$, respectively). Although temperature and species had significant effects on percent carbon translocated (temperature $F=5.92$, $p = 0.0037$; species $F=50.62$, $p <0.0001$), pH did not. Carbon fixation and carbon translocation per cell were highly correlated ($r = 0.8118$), therefore, it is highly likely that pH, temperature, and species would have significant effects on fixed carbon translocation per cell as well, although a parallel MANOVA was not conducted. Carbon fixation per cell and fixed carbon translocated per cell were highly correlated (0.8212, $p <0.0001$), and the percentage of fixed carbon translocated was negatively correlated with fixation per cell (-0.5545, $p < 0.0001$), but not with translocation per cell.

3.4.4 *Symbiodinium* in *Palythoa* sp.

Both increased temperature and pCO_2/low pH had significant negative effects on carbon fixation and translocation to *Palythoa* sp. from C1 symbionts. At 27°C, C1 symbionts fixed 0.59 pg carbon $cell^{-1}$ $hour^{-1}$, and translocated approximately 0.24 pg carbon $cell^{-1}$ $hour^{-1}$ of fixed carbon to their hosts. At 31°C, fixation and translocation decreased to 0.1 and 0.03 pg carbon $cell^{-1}$ $hour^{-1}$, respectively, and further decreased at 33°C (Figure 3.1A-B). Interestingly, despite a 92% decrease in carbon fixation, the percent of fixed carbon translocated per cell increased significantly for *Palythoa* sp. at 33°C compared to lower temperatures (Figure 3.1C). Carbon fixation normalized to host protein also decreased significantly as temperature increased; however, temperature did not significantly affect fixed carbon translocation per µg protein (Figure 3.2A-B).

At the control temperature (27°C) and high pCO_2/low pH, carbon fixation and translocation decreased over 90% both on a per cell and per unit host protein basis for *Palythoa* sp. (Figure 3.3A-B; Figure 3.4A-B). Unlike in the temperature treatments, the percent of fixed carbon translocation from symbiont to host did not change concomitantly with decreased fixation in low pH, and remained around 25-30% (Fig. 3.3C).

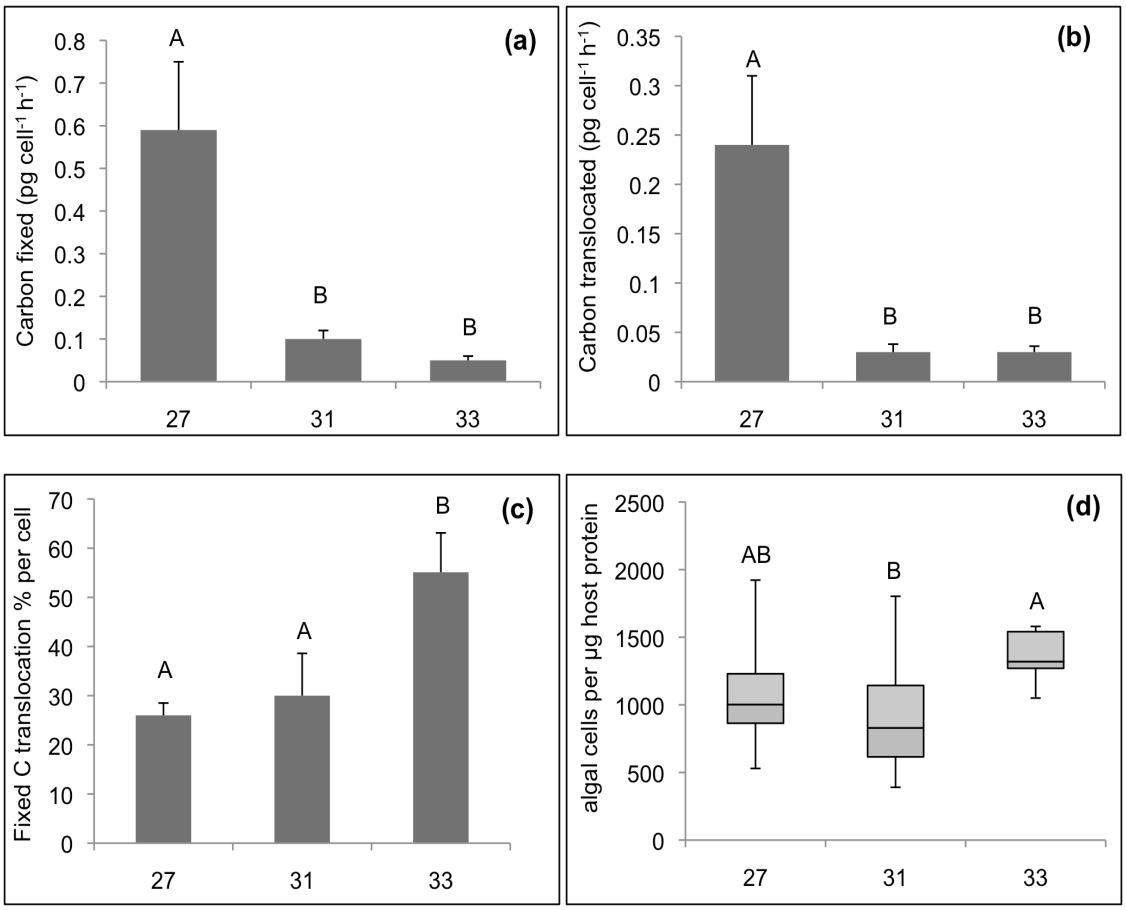


Figure 3.1. The effects of temperature (°C) on C1 *Symbiodinium* in *Palythoa* sp. maintained in control pH (8.1). Panels show carbon fixation per symbiont (a), fixed carbon translocated per symbiont (b), percent of total fixed carbon translocated from symbiont to host (c), and symbiont population density (d) at the control temperature (27°) and two treatment temperatures (31° and 33°). Bars in a, b, and c represent means with S.E.M. Boxes in panel d represent the first and third quartiles and the whiskers represent the range of algal cells per µg host protein. For each panel, matching letters indicate means that are not significantly different, while non-matching letters indicate significant differences (ANOVA with Tukey post-hoc, $p < 0.05$). Sample sizes for panels a,b, and c are n=7 (27°), *n*=9 (31°) and *n*= 5 (33°). Samples sizes for panel d are *n*=14, *n*=17, and *n*=7, respectively.

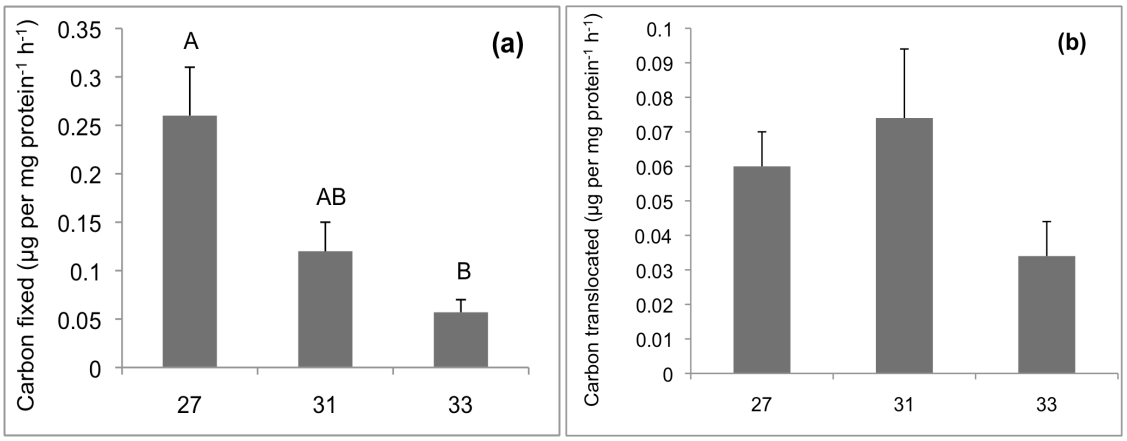


Figure 3.2. The effects of temperature (°C) on C1 carbon fixation and translocation maintained in control pH (8.1), normalized to host protein. Panels show carbon fixation per host protein (a) and fixed carbon translocated per host protein (b) at the control temperature (27°) and two treatment temperatures (31° and 33°). Symbols and statistical indicators are the same as in Figure 3.1. Sample sizes are n=7 (27°), n=9 (31°) and n= 5 (33°).

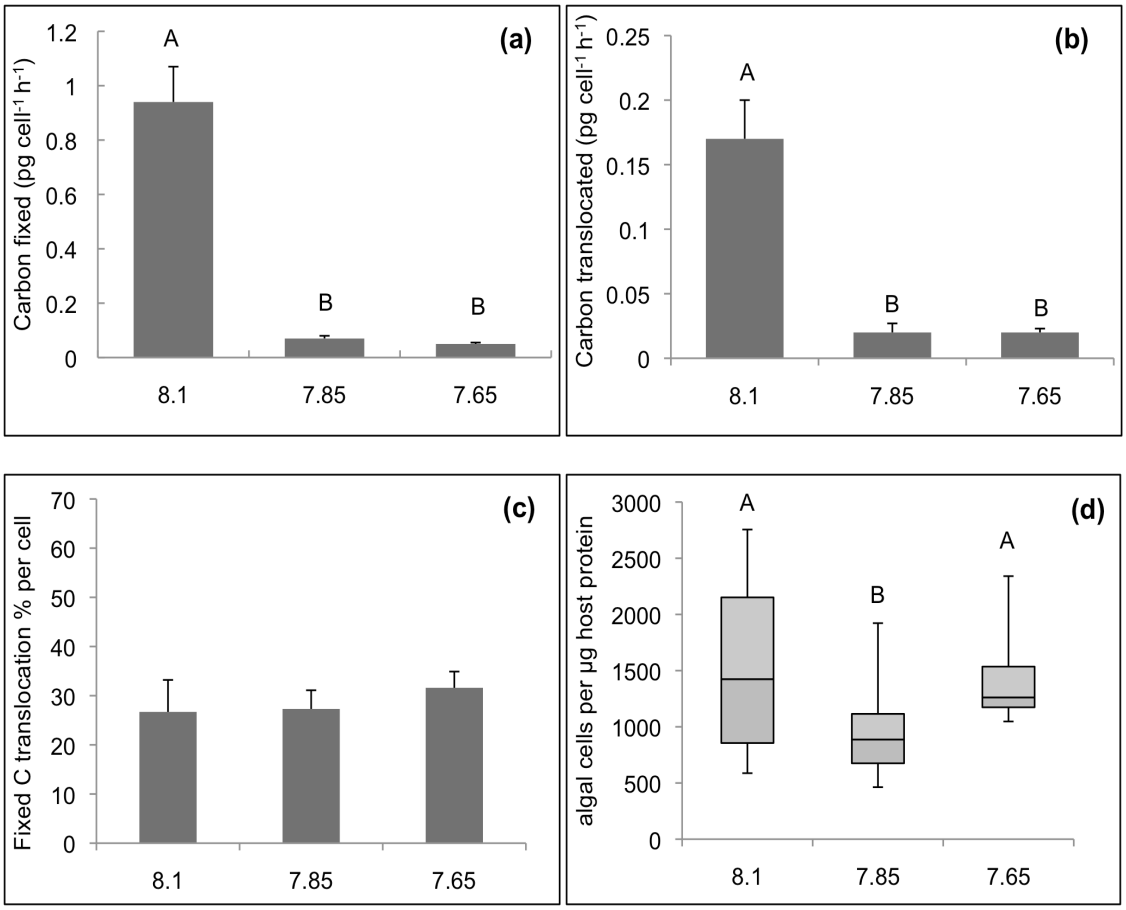


Figure 3.3. The effects of pH on C1 *Symbiodinium* in *Palythoa* sp. maintained in control temperature (27°). Panels show carbon fixation per symbiont (a), fixed carbon translocated per symbiont (b), percent of total fixed carbon translocated from symbiont to host (c), and symbiont population density (d) at the control pH (8.1) and two treatment pH values (7.85 and 7.65). Symbols and statistical indicators are the same as in Fig. 3.1. Sample sizes for panels a, b, and c are n=11 (8.1), n=10 (7.85) and n= 13 (7.65). Samples sizes for panel d are n=18, n=16, and n=20, respectively.

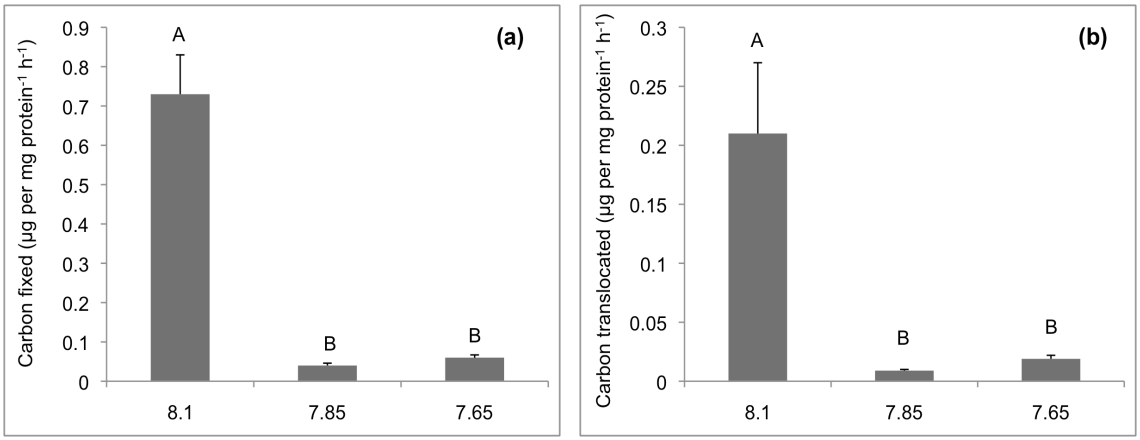


Figure 3.4. The effects of pH on C1 carbon fixation and translocation maintained in control temperature (27°), normalized to host protein. Panels show carbon fixation per host protein (a) and fixed carbon translocated per host protein (b) at the control pH (8.1) and two treatment pH values (7.85 and 7.65). Symbols and statistical indicators are the same as in Fig. 3.1. Sample sizes are n=11 (8.1), n=10 (7.85) and n=12 (7.65).

3.4.5 *Symbiodinium* in *Zoanthus* sp.

Unlike C1 symbionts in *Palythoa* sp., there were no significant differences in carbon fixation or translocation rates per cell for clade A4 symbionts in *Zoanthus* sp. among the temperature treatments (Figure 3.5A-B). Translocation percentages averaged between 50% and 60% (Figure 3.5C). These trends did not change when carbon fixation and translocation were normalized to host protein content (Figure 3.6A-B). In contrast to the temperature treatments, high pCO_2 had significant positive effects on photosynthesis. A4 symbionts fixed 65% more and translocated 58% more carbon per cell (Figure 3.7A-B), or 53% and 49% more fixation and translocation per µg protein (Figure 3.8A-B)

when in pH 7.65 compared to pH 8.1. The percent of fixed carbon translocated remained at approximately 55% for all pCO_2 treatments (Figure 3.7C).

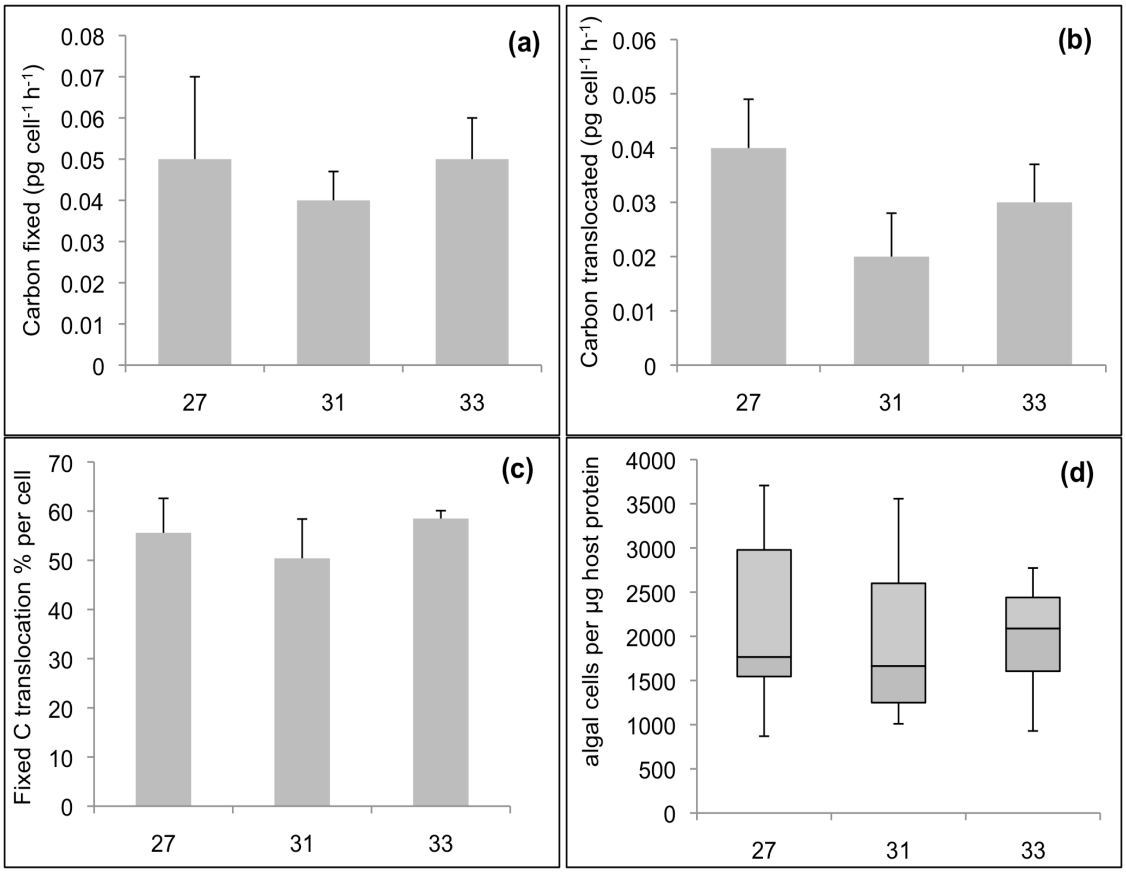


Figure 3.5. The effects of temperature (°C) on A4 *Symbiodinium* in *Zoanthus* sp. maintained in control pH (8.1). Panels show carbon fixation per symbiont (a), fixed carbon translocated per symbiont (b), percent of total fixed carbon translocated from symbiont to host (c), and symbiont population density (d) at the control temperature (27°) and two treatment temperatures (31° and 33°). Symbols and statistical indicators are the same as in Figure 3.1. Sample sizes for panels a,b, and c are n=10 (27°), n=8 (31°) and n= 6 (33°). Samples sizes for panel d are n=17, n=14, and n=7, respectively.

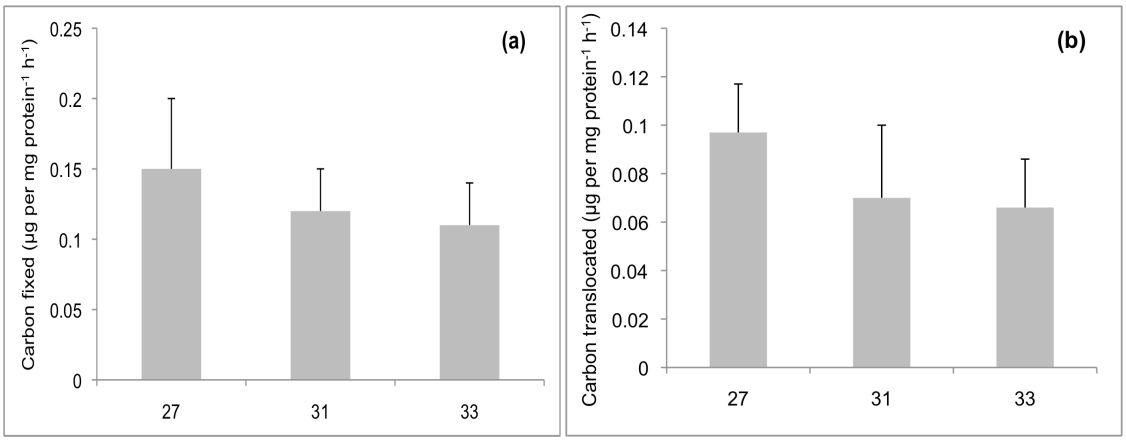


Figure 3.6. The effects of temperature (°C) on A4 carbon fixation and translocation maintained in control pH (8.1), normalized to host protein. Panels show carbon fixation per host protein (a) and fixed carbon translocated per host protein (b) at the control temperature (27°) and two treatment temperatures (31° and 33°). Symbols and statistical indicators are the same as in Figure 3.1. Sample sizes are *n*=10 (27°), *n*=8 (31°) and *n*= 6 (33°).

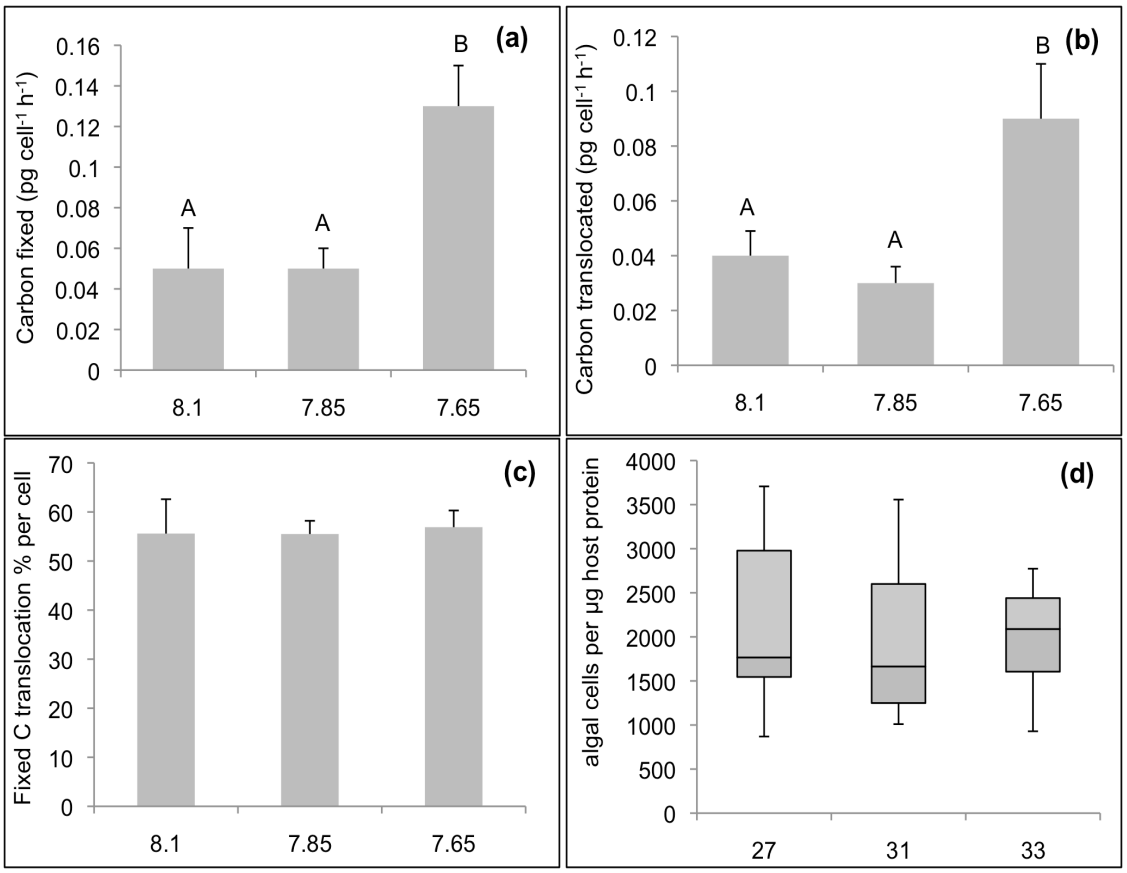


Figure 3.7. The effects of pH on C1 *Symbiodinium* in *Zoanthus* sp. maintained in control temperature (27°). Panels show carbon fixation per symbiont (a), fixed carbon translocated per symbiont (b), percent of total fixed carbon translocated from symbiont to host (c), and symbiont population density (d) at the control pH (8.1) and two treatment pH values (7.85 and 7.65). Symbols and statistical indicators are the same as in Fig. 3.1. Sample sizes for panels a, b, and c are n=10 (8.1), *n*=12 (7.85) and *n*= 7 (7.65). Samples sizes for panel d are *n*=17, *n*=15, and *n*=12, respectively.

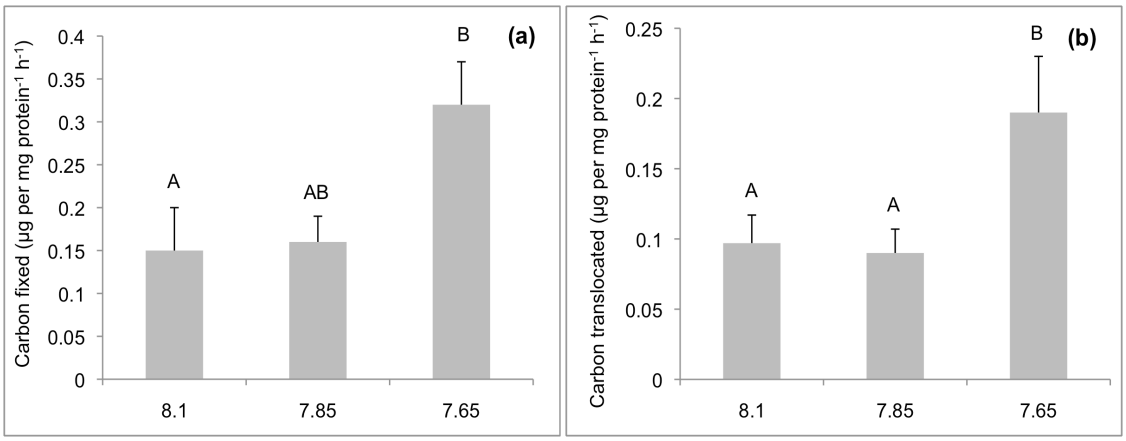


Figure 3.8. The effects of pH on A4 carbon fixation and translocation maintained in control temperature (27°), normalized to host protein. Panels show carbon fixation per host protein (a), and fixed carbon translocated per host protein (b) at the control pH (8.1) and two treatment pH values (7.85 and 7.65). Symbols and statistical indicators are the same as in Fig. 3.1. Sample sizes are n=10 (8.1), *n*=12 (7.85) and *n*= 7 (7.65).

3.4.6 Interactive effects of temperature and pCO_2 on *Palythoa* sp.

Only *Palythoa* sp. with C1 symbionts was followed experimentally in combination high temperature and high pCO_2/low pH treatments. At 31°C and pH 8.1, C1 symbionts fixed 0.1 pg carbon cell^{-1} hour^{-1} and translocated 0.02 pg carbon cell^{-1} hour^{-1} to their hosts. In contrast to the negative effects of high pCO_2 at 27°C, carbon fixation and translocation per cell were significantly higher at 31°C and pH 7.65 compared to 31°C and pH 8.1 (Figure 3.9A-B). However, when normalized to host protein, carbon fixation rates were not different among pH treatments, and fixed carbon translocated was significantly lower in 31°C and pH 7.85 compared to 31°C and pH 8.1 (Figure 3.10A-B).

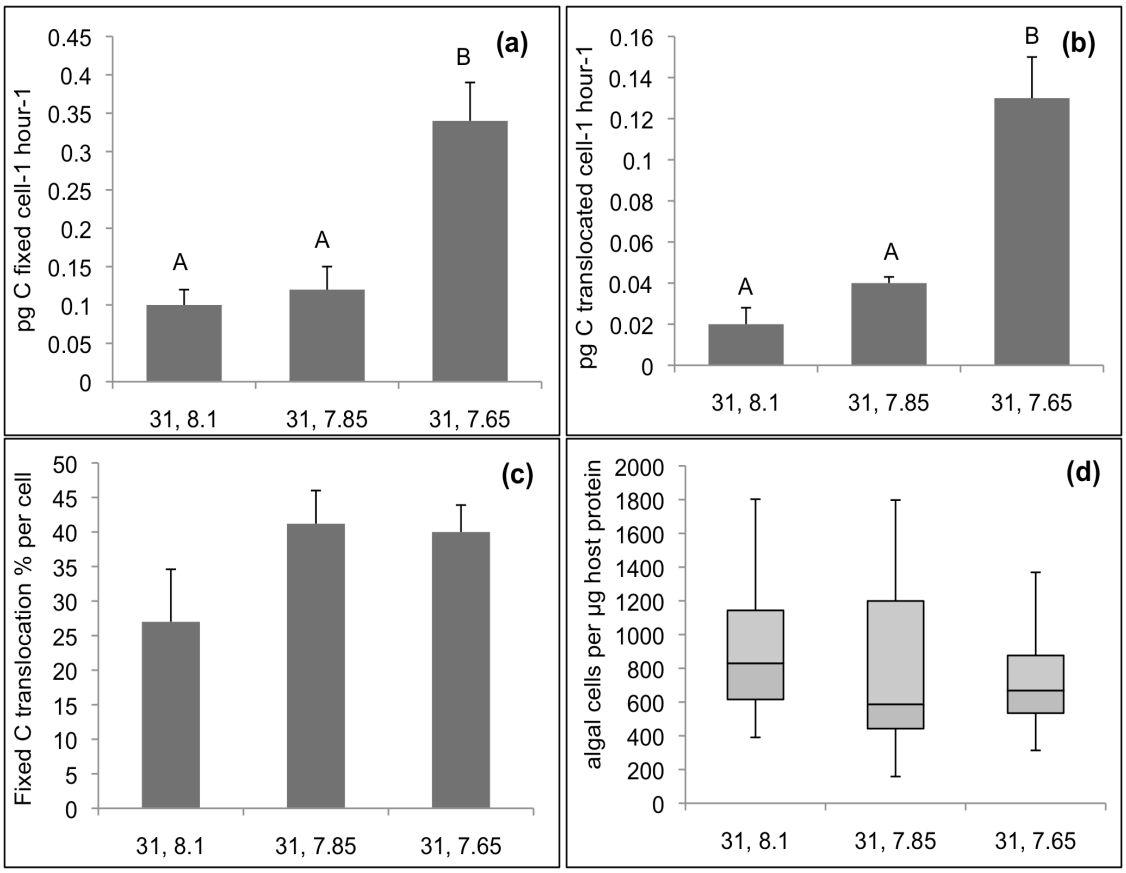


Figure 3.9. The effects of pH on C1 *Symbiodinium* in *Palythoa* sp. maintained at 31°C, and different pH levels. Panels show carbon fixation per symbiont (a), fixed carbon translocated per symbiont (b), percent of total fixed carbon translocated from symbiont to host (c), and symbiont population density (d) at the control pH (8.1) and two treatment pH values (7.85 and 7.65). Symbols and statistical indicators are the same as in Figure 3.1. Sample sizes for panels a, b, and c are n=9 (31°, 8.1), n=10 (31°, 7.85) and n= 11 (31°, 7.65). Samples sizes for panel d are n=17 for each.

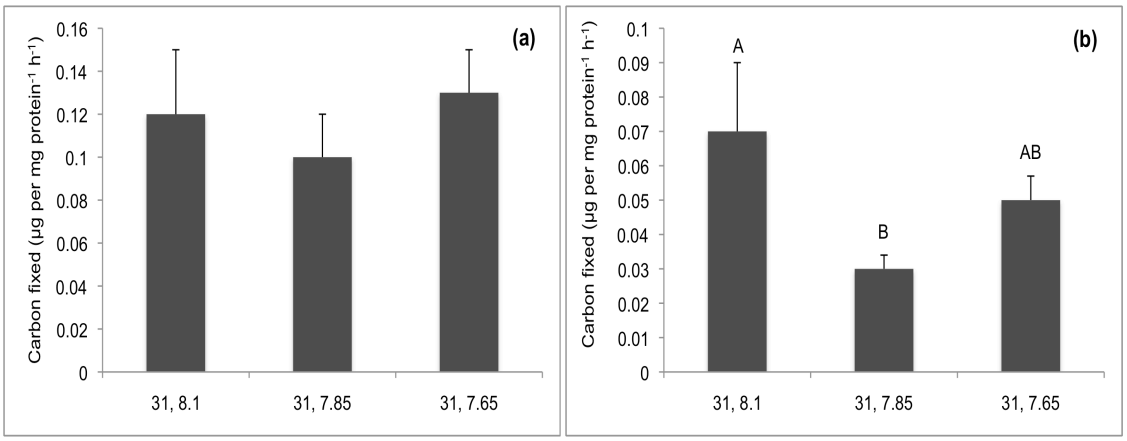


Figure 3.10. The effects of pH on C1 carbon fixation and translocation maintained at 31°C, and different pH levels, normalized to host protein. Panels show carbon fixation per host protein (a) and fixed carbon translocated per host protein (b). Symbols and statistical indicators are the same as in Figure 3.1. Sample sizes are n=9 (31°, 8.1), n=10 (31°, 7.85) and n= 11 (31°, 7.65).

3.4.7 Symbiont population density and host protein

The highest temperature treatment (33°C) caused considerable mortality in both species. Approximately 38% of *Palythoa* sp. and 35% of *Zoanthus* sp. polyps died before the end of the three-month trials (data not shown). Therefore, population density and protein measurements were conducted only on surviving polyps. There was a significant positive correlation between symbiont population density and host protein concentration for both species and all treatments (0.2294, p = 0.0086). Symbiont population density was also negatively correlated with fixed carbon per cell (-0.3413, p=0.0002) and fixed carbon translocated per cell (-0.2821, p = 0.0031).

Host protein in *Palythoa* sp. polyps was similar among all of the temperature and pCO_2 treatments, with the exception that protein at 31°C and 33°C (pH 8.1) was significantly lower than the control (27°C, pH 8.1) (Table 3.2). *Zoanthus* sp. polyps had significantly less protein at pH 7.65 compared to the other treatments; protein did not differ among all other treatments (Table 3.2).

Only C1 symbionts associated with *Palythoa* sp. had changes in symbiont population density in both high temperature and high pCO_2/low pH treatments. Interestingly, these decreases were not seen in the harshest treatments, but rather in the intermediate temperature and pH regimes—31°C and pH 7.85 (Fig. 3.1D, 3.3D). In addition, combination temperature and pH treatments did not affect C1 population densities (Figure 3.9D). There was no change in population density of A4 symbionts in *Zoanthus* sp. in either the pH or temperature treatments when normalized to host protein (Figure 3.5D, 3.7D); however, because host protein was significantly lower for *Zoanthus* sp. at pH 7.65, cell population density per µg protein is inflated. On a basis of cells per ml, cell density was significantly lower for A4 at pH 7.65 (data not shown), therefore, pH 7.65 negatively affect A4 population density (Figure 3.7D) and *Zoanthus* sp. protein (Table 3.2).

Table 3.2: Host protein concentrations for *Palythoa* sp. and *Zoanthus* sp. in the various pH and temperature treatments. Protein concentrations were compared within but not between species, by a means comparison using Dunnett's Method with the control designated as 27°, pH 8.1 for each species.

Species	Treatment	Mean protein ($\mu g\ ml^{-1}$) + S.E.M.	*p* value	*N*
Palythoa sp.	27° pH 8.1	310.47 ± 34.70	Na	19
	31° pH 8.1	209.68 ± 16.67	0.0365*	21
	33° pH 8.1	172.9 ± 24.59	0.0276*	8
	27° pH 7.85	366.53 ± 37.18	0.4797	19
	27° pH 7.65	254.62 ± 25.98	0.4364	23
	31° pH 7.85	240.8 ± 20.63	0.3047	17
	31° pH 7.65	233.71 ± 15.16	0.1636	21
Zoanthus sp.	27° pH 8.1	224.58 ± 20.07	na	22
	31° pH 8.1	182.63 ± 15.45	0.2948	18
	33° pH 8.1	153.97 ± 19.56	0.0727	10
	27° pH 7.85	223.37 ± 21.68	1.00	18
	27° pH 7.65	109.23 ± 13.78	0.0003*	13

3.4.8 Chlorophyll a concentrations

Larger differences in chlorophyll *a* concentrations were observed between *Symbiodinium* C1 and A4 in temperature treatments compared to pCO_2 treatments. Overall, chlorophyll *a* was higher in C1 than A4 symbionts for all treatments, however, this difference was significant only at 27°C and 33°C, pH 8.1 (Figure 3.11A) and 27°C, pH 7.85 (Figure 3.11B). Chlorophyll *a* increased in C1 symbionts as temperature increased, however, this trend was not significant (Figure 3.11A). High pCO_2/low pH did not affect chlorophyll *a* concentrations in C1 symbionts (Figure 3.11B). For A4 symbionts, chlorophyll *a* was significantly higher at 31°C compared to 27°C and 33°C

(Figure 3.11A). There were no differences among the pH treatments (Figure 3.11B). In the combination treatments, chlorophyll *a* was significantly lower in C1 symbionts at 31°C, pH 7.85, compared to the control and 7.65 treatment (Figure 3.11C). For all species and treatment conditions, there was a negative correlation between symbiont population density and chlorophyll *a* concentration (-0.2269, $p = 0.0254$).

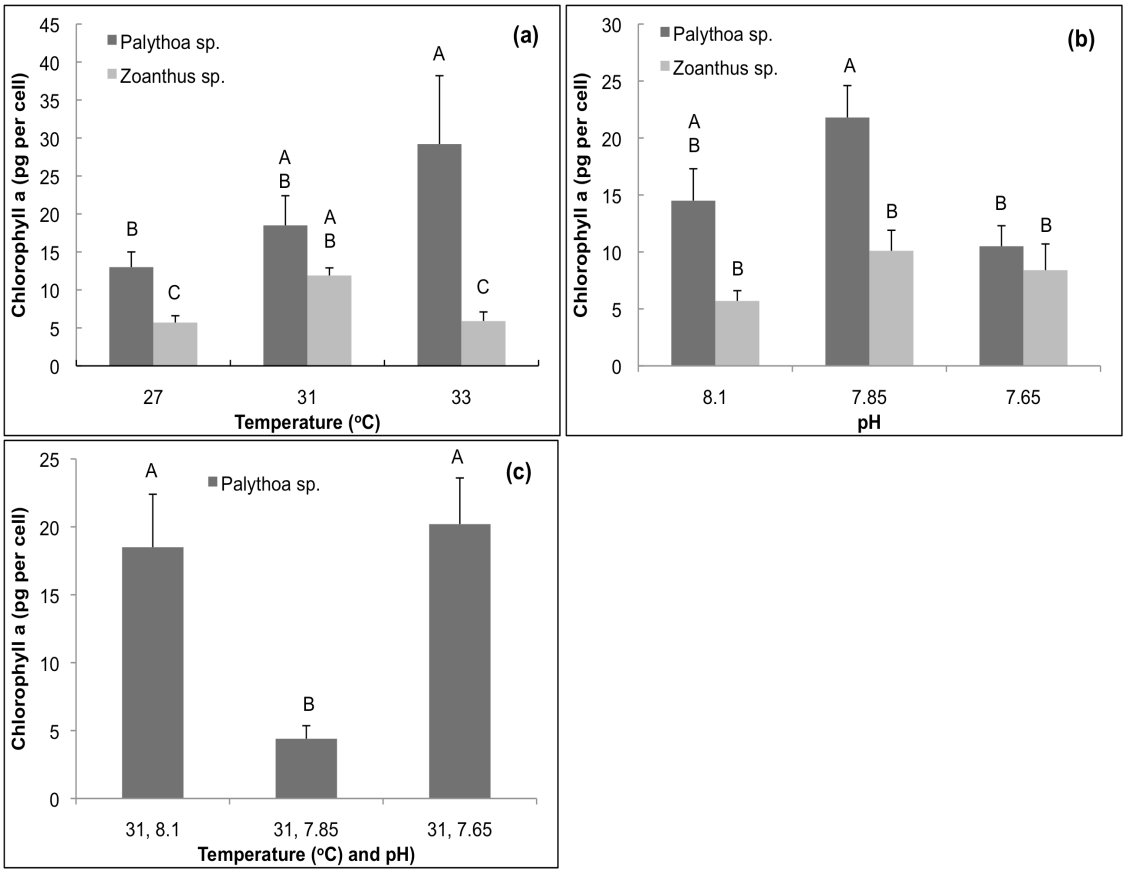


Figure 3.11. Concentrations of chlorophyll *a* in *Palythoa* sp. and *Zoanthus* sp. symbionts (C1 and A4, respectively) following pH and temperature treatments. Bars represent means with S.E.M. For each panel, matching letters indicate means that are not significantly different, while non-matching letters indicate significant differences (ANOVA with Tukey post-hoc, $p < 0.05$). Sample sizes are indicated on each bar.

3.5 Discussion

Rising temperature and pCO_2 are predicted to have devastating effects on marine ecosystems, especially in tropical and subtropical regions where temperature already reaches the thermal tolerance limit of some species. However, previous studies have shown that these effects are not universal across taxa, nor are they always negative. The current study shows that *Palythoa* sp. and *Zoanthus* sp. associations have discrete physiological responses to OA and thermal stress. Further, I demonstrate that high temperature and high pCO_2/low pH concurrently can produce effects unlike those of the individual treatments.

3.5.1 Temperature and *p*CO2 effects on carbon fixation and translocation

Photosynthesis varied greatly between *Symbiodinium* types A4 and C1 under ocean acidification and thermal stress. In control conditions (27°C, pH 8.1), C1 symbionts fixed and translocated 10 times more carbon per cell to *Palythoa* sp. than A4 symbionts translocated to *Zoanthus* sp. hosts. Carbon fixation and translocation have only been reported previously for one *Palythoa* species (*P. variabilis*) using similar methods as this study (Steen and Muscatine, 1984). These rates were approximately ten-fold lower than our results, however, symbiont type was not reported. Clade C symbionts, under non-stressful environmental conditions, were shown to translocate more carbon to coral hosts than clade D (Baker et al. 2013b), or clade A symbionts (Stat et al. 2008). In the current study, higher carbon fixation and translocation of C1 symbionts (relative to A4) were not simply a result of differences in cell population size, as symbiont densities were similar between the two types. However, C1 symbionts had more chlorophyll *a* per cell,

which may have contributed to greater carbon fixation, and hence, greater translocation. Conversely, rates of carbon fixation for *Zoanthus* sp. in this study were similar to results reported previously for *Zoanthus sociatus* (Steen and Muscatine 1984), but again with unknown symbiont type.

Temperatures above 30°C have been shown to inhibit photosynthesis in several *Symbiodinium*-cnidarian symbioses (Goulet et al. 2005; Jones et al. 1998; Takahashi et al. 2009). In the current study, temperature > 30°C also significantly impaired carbon fixation and translocation in C1 symbionts (Figure 3.1). This response is similar to results by Baker *et al*. (2013), who showed impaired carbon fixation by C1 symbionts in the coral *Acropora tenuis* at 30°C, that was significantly lower than fixation by the thermally-tolerant clade D. The percentage of fixed carbon translocated by C1 symbionts to *Palythoa* sp. was significantly higher at 33°C compared to 27°C, however, this did not compensate for the sharp decline in carbon fixation, and thus, the total amount of fixed carbon translocated was much lower than the control (Figure 3.1B). In contrast to C1, A4 symbionts maintained similar rates of carbon fixation and translocation at all temperatures (Figure 3.5A-B). This is consistent with other studies that reported thermal tolerance in clade A symbionts with respect to photosynthesis. In *Aipastia pallida,* clade A maintained photosynthesis at temperatures up to 34°C, although rates were reduced (Goulet et al. 2005), and in the coral *Porites astreoides,* A4 and A4a symbionts maintained higher levels of photosystem II (PSII) activity *in situ* than multiple symbiont types in surrounding coral species, indicating lower thermal sensitivity (Warner and Berry-Lowe 2006). Indeed, the ability of clade A symbionts to maintain photosynthesis in a variety of hosts, including *Zoanthus sp.*, when ambient temperature exceeds 30°C

supports their role as shallow-water specialists associating exclusively with hosts in the upper three meters of the ocean (LaJeunesse et al. 2003).

Similar to thermal stress, high pCO_2/ low pH significantly inhibited photosynthesis and carbon translocation in C1 symbionts *in hospite* with *Palythoa* sp. In contrast, carbon fixation and translocation by A4 symbionts increased in *Zoanthus* sp. as pH declined. Previous studies examining the effects of pCO_2 on photosynthesis in *Symbiodinium* associations have reported mixed responses: positive (Anthony et al. 2008; Herfort et al. 2008; Langdon and Atkinson 2005), negative (Anthony et al. 2008; Reynaud et al. 2003), and neutral (Rodolfo-Metalpa et al. 2010; Schneider and Erez 2006). Unfortunately, symbiont type was not reported in any of these studies, so, relevant comparisons to our results cannot be made. In one study of C3 symbionts in *Acropora formosa,* photosynthesis increased at pCO_2 of 550 ppm compared to 380 ppm, however, this experiment lasted only four days, and results may have changed following a longer incubation (Crawley et al. 2010). Gross photosynthesis also increased with rising pCO_2 in A19 symbionts of *Anemone viridis* inhabiting a natural pH gradient, although this increase was attributed to higher symbiont population densities (Suggett et al. 2008). In the current study, trends in fixation and translocation represent actual increases (or decreases) per symbiont cell. Finally, clade A symbionts in *Stylophora pistillata* had lower gross photosynthesis but higher translocation at pH 7.2 compared to 8.1, resulting in similar amounts of fixed carbon received by the host (Tremblay et al. 2013).

Thermal stress can affect photosynthesis of *Symbiodinium* by inducing photoinhibition of PSII. This occurs when the rate of PSII repair does not keep up with the accumulation of excess energy (Suggett et al. 2008; Takahashi et al. 2009; Warner et

al. 1999). In certain *Symbiodinium* types, high temperature (>30°C) reduces the efficiency of PSII, increases electron transfer rates between photosystems, changes rates of photochemical and non-photochemical quenching, and decreases PSII reaction centers; all of which inhibit effective energy dissipation (Robison and Warner 2006; Suggett et al. 2008; Takahashi et al. 2004; Warner et al. 1996; Warner et al. 1999). Although photoacclimation can prevent damage to PSII, not all *Symbiodinium* can modify their photosynthetic apparatus to adapt to periods of thermal stress (see Hennige et al. 2008), and symbiont types differ in their ability to repair damage to PSII (Takahashi et al. 2004; Takahashi et al. 2009). Furthermore, some repair mechanisms may depend on thylakoid lipid composition, which also varies among *Symbiodinium* types (Tchernov et al. 2004). Additional studies examining photobiology and photo repair mechanisms are needed to identify the cause of thermal sensitivity in C1 symbionts.

The host also plays a role in the thermal tolerance of the holobiont. First, host pigments regulate the amount of ambient light reaching the symbionts, which could reduce symbiont photoinhibition (Dove et al. 2006). Indeed, symbiont chlorophyll absorption may be regulated more by host pigments and the internal light environment than by the external light regime (Hennige et al. 2008). Second, Bellantuono *et al*. (2012) recently showed that following pre-exposure to high temperature, cnidarians placed in high temperature environments did not bleach or shift symbiont type. This suggests that cnidarian hosts themselves have adaptive responses to thermal stress (Bellantuono et al. 2012). Finally, thermal stress has been shown to downregulate carbonic anhydrase genes in symbiotic *Anemone viridis* (Moya et al. 2012a), which are needed to transport dissolved inorganic carbon (DIC) into the holobiont (see below).

Both host and symbiont contribute to changes in carbon fixation under different pCO_2 conditions. *In hospite*, the host uses a carbon concentrating mechanism (CCM) to regulate DIC transport for photosynthesis (Allemand et al. 1998a). In *Symbiodinium*-cnidarian associations, HCO_3^- is the primary form of DIC transported to the holobiont to supply carbon for photosynthesis, however *Symbiodinium* fix CO_2 (Furla et al. 2000). The enzyme carbonic anhydrase (CA) interconverts HCO_3^- and CO_2 multiple times as DIC passes through host membranes, ultimately arriving as CO_2 to the symbiont. Multiple CA isoforms are present throughout the host, and in/on the symbiont (Furla et al. 2000; Furla et al. 2005). CAs are essential for delivery of CO_2 to symbionts, and can be affected by several abiotic factors such as ambient CO_2 concentrations and temperature (Beardall et al. 1998; Bertucci et al. 2013; Giordano et al. 2005). High pCO_2 has been shown to both upregulate and downregulate CA gene expression in corals, which may indicate species-specific differences in CA performance (see Bertucci et al. 2013; Moya et al. 2012b). Indeed, multiple CA isoforms, presumably with different efficiencies, have been identified in anthozoan hosts (Bertucci et al. 2013). In addition, *Symbiodinium* types in culture utilize CA to different extents depending on their mechanism for acquiring DIC (Brading et al. 2011; Brading et al. 2013). Given these data, it is reasonable to assume that *in hospite*, both host and symbiont possess CAs with discrete biochemical characteristics. The overall response of the holobiont depends on the efficiency and response of all host and symbiont CAs. Therefore, increased production in *Zoanthus* sp. at low pH could be due to up-regulation of CA genes in the host, symbiont, or both, while inhibited production in *Palythoa* sp. suggests down-regulation of CA genes and/or reduced enzyme efficiency in the host and symbiont.

Changes in fixed carbon translocation rates and/or percentages without concomitant increases in fixation (as seen in *Palythoa* sp. at 33°) could indicate increased energy demands by the host and increased respiration. However, host and symbiont respiration rates cannot be determined by the methods used in this study.

3.5.2 Temperature and pCO_2 interactions

In stark contrast to the results of the temperature and pCO_2 single treatments, carbon fixation and translocation *increased* in C1 symbionts when maintained at combined high temperature (31°C) and low pH. The percentage of fixed carbon translocated also increased. These results are somewhat perplexing, considering the negative effects that each factor had independently on C1 photosynthesis. It is possible that the combination of thermal stress and high pCO_2 inhibited *Palythoa* sp. to the extent that host regulation of DIC uptake was greatly reduced, and symbiont genes (e.g. CA and photomachinery genes) were upregulated. Another possibility is that the combined treatment affected membrane permeability of *Palythoa* sp., such that passive diffusion of CO_2 occurred and allowed for greater photosynthesis by the symbiont.

3.5.3 Symbiont population density

In the absence of environmental stress, zooxanthellae population density is stable and population equilibrium is maintained by cell division at a rate that replaces dead or expelled cells within the host (Drew 1972; Jones and Yellowlees 1997; Muscatine et al. 1989). Following bleaching events, often caused by thermal stress, hosts regain peak algal densities by stimulating division in the remaining symbionts (Jones and Yellowlees

1997). In this study, C1 population density was lowest in the intermediate treatments (31°C, pH 8.1 and 27°C, pH 7.85) as opposed to the harshest treatments (33°C, pH 8.1 and 27°C, pH 7.65). One possible explanation for this result is that *Palythoa* sp. experienced extensive bleaching in the most extreme treatments; but the remaining symbionts eventually divided and reestablished a population density similar to the control. In the intermediate treatments, bleaching reduced the number of *Symbiodinium*, but maintained a population density high enough that division was not stimulated. A similar pattern has been reported in corals *Stylophora pistillata* and *Porites* sp., where zooxanthellae population density dropped significantly from pH 8.09 to pH 7.49, but then increased from pH 7.49 to pH 7.19 (Krief et al. 2010). C1 population densities did not change in combination high temperature/ low pH treatments.

Although DGGE analysis indicated only one primary symbiont type in each zoanthid species, DGGE may fail to detect symbiont types that make up less then 7% of the population (Thornhill et al. 2006). If a second, small population of a thermally-resistant symbionts remained in *Palythoa* sp. following bleaching of C1, the remaining clade could have reestablished a population over the three-month long experiments. A change in the dominant symbiont type in *Palythoa* sp. would also explain why carbon fixation and translocation increased in the combination treatments, as opposed to the strong suppression in the individual temperature and pCO_2 experiments. Unfortunately, genetic analyses that could have tested this hypothesis were not performed post-experiment.

Population density of A4 symbionts in *Zoanthus* sp. was maintained in all of the temperature treatments. These results agree with other reports of clade A population

densities remaining the same or even increasing with high temperature (Robison and Warner 2006). However, at the lowest pH (7.65), A4 population density decreased on a per mL (but not per µg protein) basis. This contradicts a report of population densities of clade A19 symbionts in *Anemone viridis* increasing three fold from pH 8.18 to pH 7.66 (Suggett et al. 2012).

3.5.4 Chlorophyll and protein levels

Chlorophyll *a* was higher in C1 than A4 symbionts for all treatments. This is not surprising considering the overall higher rates of carbon fixation for C1, even when fixation was impaired. Despite the large reduction in carbon fixation and translocation, chlorophyll *a* increased in C1 symbionts as temperature increased. A similar response was reported in a few previous studies that showed elevated chlorophyll *a* following thermal stress (Jones 1997; Jones and Yellowlees 1997). Thermal stress did not affect chlorophyll *a* concentrations in cultured A1 symbionts (Suggett et al. 2008), or in A4 symbionts *in hospite* in the current study (Figure 3.11A). High pCO_2 also had no affect on chlorophyll *a* in A4 symbionts. Chlorophyll *a* in C1 symbionts was similar at pH 7.65 compared to the control in both the independent pCO_2 treatments and the combined temperature/pCO_2 treatments.

3.5.5 Host-symbiont combinations and environmental tolerance

Overall, my results show that the effects of high pCO_2/low pH on photosynthesis in *Symbiodinium*-zoanthid associations are holobiont-specific. Differences in the physiological responses of holobionts to climate change and OA may depend on a variety

factors: photoacclimation, photoinhibition, regulation of genes involved in photobiology and DIC transport, mechanisms of DIC uptake, carbonic anhydrase efficiency, and cell repair. The extent to which each partner in the symbiosis is responsible for the overall response remains unknown. However, this study reiterates the point that environmental tolerance cannot be generalized across taxa, particularily in a symbiosis where adaptability crosses two species, and the interactive effects of a particular host-symbiont combination may outweight responses of the individual partners. Environmentally-sensitive associations, such as *Palythoa* sp./C1, will not necessarily perish in the environment predicted for the upcoming centuries if they are able to shuffle or replace their symbiont type to one that increases the tolerance of the holobiont (see Baker et al. 2013a). This will depend on geographic distributions of *Symbiodinium*, and the availability of types in the host's environment. Clade-diverse communities will be more resilient to climate change than communities with a limited number of environmentally-tolerant symbionts (LaJeunesse et al. 2010). Fortunately, there is indication that in many regions of the world (including the Florida Keys), *Symbiodinium* diversity is extensive (Baker et al. 2013a; LaJeunesse et al. 2003; LaJeunesse et al. 2010; Pochon et al. 2001; Santos et al. 2004; Silverstein et al. 2011). However, the extent to which *Symbiodinium* diversity allows for more resilient holobionts remains to be seen.

CHAPTER 4

CARBONIC ANHYDRASE ACTIVITY IN SYMBIOTIC ZOANTHIDS UNDER CLIMATE CHANGE AND OCEAN ACIDIFICATION

4.1 Abstract

Carbon dioxide makes up less than 1% of dissolved inorganic carbon (DIC) in the ocean. To acquire carbon dioxide for photosynthesis, many marine photoautotrophs rely on the enzyme carbonic anhydrase (CA) to catalyze the conversion of HCO_3^- to CO_2. In zoanthids and other cnidarians with *Symbiodinium* sp. endosymbionts, CA is essential for transporting CO_2 to zooxanthellae for photosynthesis. Temperature and ambient DIC affect CA activity, therefore, climate change and ocean acidification will alter CO_2 transport in symbiotic cnidarians. However, these effects are likely to be species specific for both host and symbiont, as different cnidarians and *Symbiodinium* sp. types vary in their mechanisms of DIC transport and utilization of CA. In this study I show that host and symbiont CA activity in the zoanthids *Palythoa* sp. and *Zoanthus* sp. had very different responses to thermal stress and ocean acidification. Increased temperature inhibited algal, but not host CA activity in *Zoanthus* sp. polyps with A4 symbionts, while temperature had no affect on CA activity in *Palythoa* sp. with C1 symbionts. High pCO_2/low pH affected CA activity in both zoanthid species, however, synchronous increases in temperature and pCO_2 only affected algal CA activity in *Palythoa* sp. This study shows that environmental conditions induce species-specific changes in DIC transport in symbiotic zoanthids. My observations suggest that carbonic anhydrase

activity in symbiotic cnidarians will be altered by climate conditions predicted for the future, and for some cnidarians, changes in CA activity may reduce photosynthesis.

4.2 Introduction

Carbon dioxide is the substrate for photosynthesis, however, its availability in the ocean is minimal, and environmental conditions can reduce it even further. Consequently, many marine photoautotrophs have evolved carbon concentrating mechanisms (CCMs) to compensate for the low level of dissolved CO_2 in the ocean accessible for photosynthesis (see Badger and Price 2003; Giordano et al. 2005). CCMs vary widely among species, but usually involve active transport of CO_2 and/or HCO_3^- and conversion between HCO_3^- and CO_2 catalyzed by the enzyme carbonic anhydrase (CA). CA has multiple functions, and in addition to facilitating the reversible conversion of HCO_3^- to CO_2, it transports CO_2 and HCO_3^- across cell membranes, increases the flux of CO_2 to Rubisco, reduces CO_2 leakage, regulates cell pH, and is involved in ion exchange (Berman-Frank et al. 1994; Kaplan and Reinhold 1999; Sültemeyer 1998). In organisms with photoautotrophic endosymbionts, such as in cnidarian-*Symbiodinium* sp. associations, both the host and symbiont posses CAs that aid in delivering CO_2 for photosynthesis. Host CAs play a major role in acquiring dissolved inorganic carbon (DIC), primarily as HCO_3^-, moving DIC through host tissues, and delivering DIC to *Symbiodinium* for photosynthesis. Symbiont CAs may also be involved in ensuring that HCO_3^- is converted to CO_2 at the site of carboxylation (Al-Moghrabi et al. 1996; Allemand et al. 1998a; Weis 1993).

Climate change is altering ocean carbonate chemistry in profound ways. Along with a rise in sea surface temperatures, increased atmospheric carbon dioxide is causing

DIC enrichment and pH decline, or ocean acidification (Guinotte and Fabry 2008; Meehl 2007). Additional DIC could potentially increase photosynthesis in CO_2-limited photoautotrophs, particularly *Symbiodinium* sp. *in hospite* that rely on their hosts for DIC acquisition and transport. Indeed, research has shown that increased CO_2 enhances primary production in several phytoplankton species (see Rost et al. 2008), however the effects of ocean acidification on photosynthesis in symbiotic cnidarians have been mixed (Anthony et al. 2008; Herfort et al. 2008; Langdon and Atkinson 2005; Reynaud et al. 2003; Rodolfo-Metalpa et al. 2010; Schneider and Erez 2006). One reason for this may be that the benefit of increased DIC associated with ocean acidification may be outweighed by the concomitant decrease in pH, which affects most physiological processes. Moreover, rising temperature combined with changing carbonate chemistry can shift mineral and nutrient composition in the ocean, imposing additional constraints on primary production in photoautotrophs, particularly endosymbionts.

Symbiodinium sp. fix CO_2 during photosynthesis, yet, CO_2 makes up less than 0.5% of DIC in the ocean, making CA critical in delivering CO_2 for photosynthesis. CA activity is regulated by ambient DIC concentrations, whereby increased DIC (and lower pH) inhibits CA activity, and DIC limitation stimulates CA expression (see Beardall et al. 1998; Giordano et al. 2005). Changes in ocean carbonate chemistry alter CA activity, and thus, directly affect primary production in symbiotic cnidarians.

Temperature also affects ocean carbonate chemistry and CCMs in marine photoautotrophs. Warmer water decreases the solubility of CO_2 and favors speciation of inorganic carbon into more carbonate ions (CO_3^{2-}) and less HCO_3^- and CO_2, increasing the need for CA activity (see Beardall et al. 1998). Experimental evidence has shown that

CA activity increases in microalgae as temperature increases (Shiraiwa and Miyachi 1985). How temperature and ocean acidification affect CA activity in cnidarian-*Symbiodinium* associations remains largely unknown, as few studies have measured CA activity in symbiotic cnidarians (see Bertucci et al. 2013).

The efficiency of DIC acquisition in cnidarian-*Symbiodinium* symbioses can also be affected by species-specific differences in the mechanisms of DIC uptake and transport, as well as differences in CA isoforms. For example, the genus *Symbiodinium* consists of numerous phylotypes that vary in their environmental tolerance, physiology, and photobiology (Hennige et al. 2008; Pochon et al. 2006; Stat et al. 2006), and in their modes of DIC uptake and utilization of internal and external CAs (Brading et al. 2013). In addition, multiple CA isoforms, with different roles and activities, have been identified in host cnidarians (reviewed in Bertucci et al. 2013). Under normal environmental conditions, DIC acquisition and transport can vary among cnidarians due to differences in either (or both) host and symbiont CCMs and CAs. Indeed, host CA activity varies widely among tropical zooxanthellate cnidarians (Weis 1993), and changes in temperature and DIC concentrations due to climate change may amplify these differences.

Changes in CA activity can have dramatic effects on photosynthesis and calcification in symbiotic cnidarians (Al-Horani et al. 2003; Goreau 1959; Isa and Yamazato 1984; Tambutté et al. 2007; Weis et al. 1989; Weis 1993). However, to my knowledge, CA activity has not been compared among *Symbiodinium* sp. phylotypes freshly isolated from hosts, nor have the effects of temperature and ocean acidification on both host and symbiont CA been examined. In the present study, I tested the hypothesis

that total carbonic anhydrase activity differs between zoanthids *Palythoa* sp. and *Zoanthus* sp. harboring *Symbiodinium* sp. types C1 and A4, respectively. Further, I predicted that changes in symbiont CA activity due to increased temperature and ocean acidification would be distinct between C1 and A4 symbionts.

4.3 Materials and methods

Zoanthids were collected and maintained in laboratory aquaria as described in chapter three. Target temperatures and pH values were the same as in chapter three, and all tank conditions were reported in chapter three. To determine the effects of bleaching on CA activity, I bleached some zoanthids using a cold shock method modified from Steen and Muscatine (1987): polyps were placed in 4°C ASW in the dark for four hours, then transferred immediately to light-inhibited tanks. One week following cold shock, symbiont population densities were counted for bleached polyps and I confirmed that >90% of symbionts were expelled. Bleached polyps were maintained in dark aquaria at the three target temperatures (27˚C, 31˚C or 33˚C).

4.3.1 Assays for CA activity

For experimental assays, no two polyps from the same cluster were used to avoid pseudo-replication. Zoanthids were homogenized individually in 3.5 mL fresh ASW to disrupt the host cell membrane and release the algal cells. The homogenate was centrifuged for 10 minutes at 1600*g* to separate host material from zooxanthellae. 200 μL of host supernatant was reserved and frozen for later protein analysis. The remaining supernatent was decanted and stored on ice. The host supernatent was diluted 1:1 with

chilled (4°C) veronal buffer containing 25 mM barbitol, 5 mM EDTA, 5mM dithiothreitol (DDT) and 10mM $MgSO_4$, adjusted to pH 8.3 (Weis et al. 1989).

The algal pellet was rinsed twice in fresh ASW and resuspended in 3.5 mL fresh ASW. 100 μL was frozen for later cell counts. To disrupt the algal cells and release CA, the remaining resuspended cells were sonicated at 45 watts (Branson Sonifer 250, Branson Ultrasonics Corp. Danbury, CT) on ice for five, thirty-second intervals, with one minute breaks between cycles. Microscopy verified that this method broke >90% of algal cells. Sonicated algal cells were then diluted 1:1 with the same veronal buffer and stored on ice until the assay.

Carbonic anhydrase assays were performed following the methods of Weis et al. (1989) as modified from Graham and Smillie (1976). For each assay, 1 mL of the buffered homogenate was placed in a 7 mL scintillation vial and an additional 1 mL of veronal buffer was added to the sample. The sample was equipped with a magnetic stir bar and dual temperature-pH electrode (Accumet®, Cole-Parmer, Vernon Hills, IL). When the pH stabilized and the sample temperature reached 13°C, one mL of CO_2-saturated water (pH 3.5) was injected into the mixture. Upon injection of CO_2-saturated water, pH was recorded every 10 seconds for two minutes. As a control, the same assay was conducted with homogenate or algal samples that were boiled to denature the enzyme. Changes in pH followed Michaelis–Menten kinetics. CA activity was determined as ΔpH of the homogenate or algal mixture over the first 60 seconds (approximately ½ V_{max}) minus ΔpH of the denatured control over the first 60 seconds. CA activity was normalized to host protein concentration. Each assay was performed in triplicate.

4.3.2 Host protein and symbiont population density

Host protein concentrations and symbiont population densities were determined using the same methods as described in chapter three.

4.3.3 Statistical analysis

Carbonate chemistry in the experimental tanks was compared using a two-way ANOVA with treatment as a fixed effect, and tank a random effect nested within treatment (see chapter three). CA data were transformed to meet the assumptions of normality and equal variance using log transformations. To determine the effects of pH and temperature on host and algal CA activity, a MANOVA was conducted for each host species using an identity matrix and Wilks' lambda distributions. CA activity within temperature and pH treatments was also analyzed, as well as host protein concentrations and symbiont population densities, using one-way ANOVAs with Tukey post-hoc tests. Finally, relationships between *Symbiodinium* population densities and host CA activity were examined using pairwise correlations estimated by the REML method. All statistical tests were performed using JMP 10.0.0 statistical software (SAS, Cary, NC).

4.4 Results

4.4.1 Host and symbiont identification

As indicated in chapter three, zoanthids were identified as *Palythoa* aff. *clavata* and *Zoanthus* sp.. Symbionts were identified as C1 (in *Palythoa* sp.) and A4 (in *Zoanthus* sp.).

4.4.2 Carbonic anhydrase activity

At the control temperature, 27°C, CA activity in both host and symbiont fractions was not different between *Zoanthus* sp. and *Palythoa* sp. (Figures 4.1A and 4.2A). However, algal CA activity on a per cell basis was significantly higher in *Palythoa* sp. than *Zoanthus* sp. (p=0.0269) (Figures 4.1B and 4.2B). Increased temperatures caused differences in both host and algal CA activity between the two zoanthid species. At 31°C, host CA activity did not vary between *Palythoa* sp. and *Zoanthus* sp., but algal CA activity per mg host protein and per algal cell was significantly higher in *Palythoa* sp. (p=0.0184 and p=0.0012, respectively). Host CA activity was also higher in *Palythoa* sp. than *Zoanthus* sp. at 33°C (p=0.0037). Unfortunately, due to high polyp mortality and reduced *Symbiodinium* population density, algal CA activity could not be determined for *Palythoa* sp. at 33°C; therefore, total CA activity and algal CA activity could not be compared between the two species.

Temperature affected algal CA activity per mg protein in *Zoanthus* sp. (Wilks' lambda=0.426, $F_{(2,36)}$ =7.659, p<0.0017), but not host CA activity (Wilks' lambda=0.067, $F_{(2,36)}$ =1.199, p<0.3133) (Figure 4.1A). Algal CA activity per mg host protein was significantly lower in A4 symbionts at 33°C compared to 27°C (p= 0.0026) (Figure 4.1A). However, CA activity per algal cell was not different among the three temperatures (Figure 4.1B). At the control temperature (27°C), mean algal CA activity was 3.04 per mg host protein, and contributed to 35% of the total holobiont CA. This percentage decreased as temperature increased, and was only 18% at 33°C (Figure 4.1A). Host and algal CA activity were also partially correlated (r= 0.2301).

Multivariate analysis of CA activity for *Palythoa* sp., all treatments, revealed no effect of temperature on either host or algal CA activity (Figure 4.2). At 33°C, *Palythoa* sp. suffered extreme bleaching and polyp mortality (≈ 40%), and as noted above, CA activity in C1 symbionts could not be determined. Symbiont CA activity per mg host protein and per algal cell was not significantly different at 27°C and 31°C (Figure 4.2). C1 symbionts contributed approximately 28% and 50% to total CA activity for the holobiont at 27°C and 31°C, respectively (Figure 4.2A). Host CA and algal CA were also partially correlated for *Palythoa* sp. (r= 0.3682).

Host CA activity in experimentally bleached *Palythoa* sp. polyps was not significantly different than in unbleached polyps across all temperatures (Figure 4.2A and 4.3). For bleached *Zoanthus* sp. at 27°C, host CA activity was significantly lower than in non-bleached polyps (p=0.0004; Figures 4.1A and 4.3). Host CA activity increased with increasing temperature in bleached *Zoanthus* sp., such that CA activity at 33°C was significantly higher than at 27°C (p=0.0016; Figure 4.3). There were no significant differences, however, between bleached and non-bleached *Zoanthus* sp. at 31°C and 33°C (Figures 4.1A and 4.3).

Both host and algal CA activity per mg protein were similar between *Palythoa* sp. and *Zoanthus* sp. at control pH 8.1 (27°C) (Figures 4.4A and 4.5A). Similar to effects for increased temperatures, however, increased pCO_2/ low pH caused differences in CA activity between the two zoanthid species. Total CA activity was significantly higher in *Palythoa* sp. than *Zoanthus* sp. at pH 7.85 and pH 7.65 (p= 0.0046 and p<0.0001, respectively). At pH 7.85, algal CA activity per cell was higher in *Palythoa* sp. than *Zoanthus* sp. (p<0.0001). At pH 7.65, both *Palythoa* sp. host CA activity (p<0.0001) and

algal CA activity were higher per mg host protein (p=0.0442) and per algal cell (p=0.0312) than in *Zoanthus* sp..

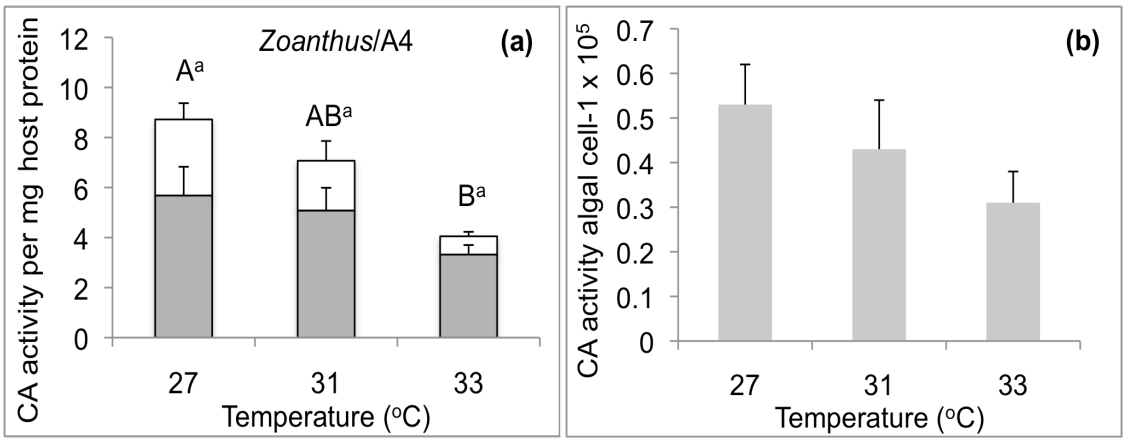


Figure 4.1. Effects of temperature on carbonic anhydrase activity in *Zoanthus* sp. **a.** Host and algal CA activity normalized to host protein. The shaded portion of the bars represent mean plus standard error of *host* CA activity, while the white portion represents mean plus standard error of *algal* CA activity. Tops of the stacked bars equal the sum of host and algal CA activity. **b.** Symbiont CA activity normalized to *Symbiodinium* population density. Bars represent mean plus standard error. Sample sizes for both panels are n=10, 9, and 7, respectively. For each panel, matching letters indicate means that are not significantly different, while non-matching letters indicate significant differences (ANOVA with Tukey post-hoc, $p < 0.05$). Superscripts indicate whether the statistical significance is for *host* CA activity (superscript h) or *algal* CA activity (superscript a).

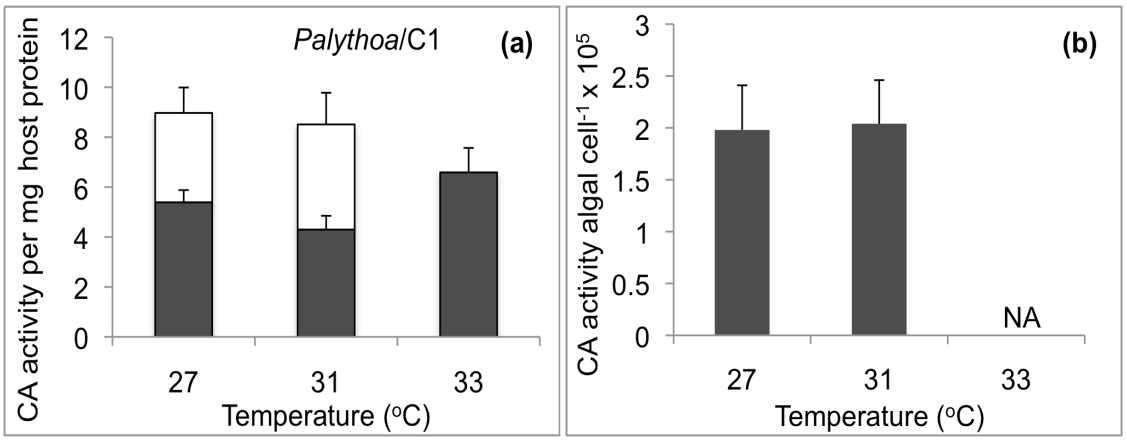


Figure 4.2. Effects of temperature on carbonic anhydrase activity in *Palythoa* sp. **a.** Host and algal CA activity normalized to host protein. **b.** Symbiont CA activity normalized to *Symbiodinium* population density. Bars represent mean plus standard error. Sample sizes for both panels are n=10, 8, and 8, respectively. Shading and statistical indicators are the same as in Figure 4.1.

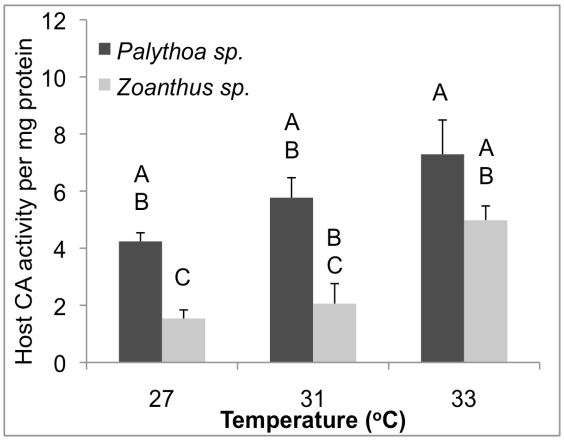


Figure 4.3 Effects of temperature on *host* carbonic anhydrase activity in **bleached** zoanthids. Bars represent mean plus standard error of *host* CA activity. Sample sizes for *Zoanthus* sp. are n=8, 6, and 6, respectively, and sample sizes for Palythoa sp. is n=10 for each bar. Matching letters indicate means that are not significantly different, while non-matching letters indicate significant differences (ANOVA with Tukey post-hoc, $p < 0.05$).

Increased pCO_2/ low pH reduced both host CA activity (Wilks' lambda=0.294, $F_{(2,36)}$ =5.288, p<0.0097) and algal CA activity (Wilks' lambda=0.228, $F_{(2,36)}$ =4.109, p<0.0247) in *Zoanthus* sp. (Figure 4.4A). Host CA activity was significantly lower at pH 7.65 compared to pH 8.1 (p= 0.0138). CA activity per mg host protein of A4 symbionts decreased significantly at pH 7.85 compared to pH 8.1 (p=0.0076), however CA activity at pH 7.65 was not significantly different from either pH 8.1 or pH 7.85 (Figure 4.4A). Moreover, algal CA activity per cell was not significantly different among the pH values (Figure 4.4B).

Increased pCO_2/ low pH also had significant effects on symbiont CA activity in *Palythoa* sp. (Wilks' lambda=0.359, $F_{(2,53)}$ =9.524, p<0.0003), but not on host CA activity (Wilks' lambda=0.060, $F_{(2,53)}$ =1.5997, p<0.2116). Host CA activity did not vary among the pH treatments (Figure 4.5A), however, C1 symbiont CA activity per mg protein decreased significantly at pH 7.85 compared to pH 8.1 (p=0.0107). Algal CA activity per mg protein at pH 7.65 was not significantly different from either pH 8.1 or pH 7.85 (Figure 4.5A). In contrast, algal CA activity per cell was significantly *higher* at pH 7.85 compared to pH 8.1 (p=0.0105) (Figure 4.5B).

Exposure of *Palythoa* sp. to a combination of high temperature (31°C) and high pCO_2/ low pH did not significantly affect host or symbiont CA activity per mg host protein (Figure 4.6A). Mean host CA activity ranged from 4.3 to 5.9 per mg host protein, while symbiont CA activity was more variable (mean range 2.5 to 4.2 per mg host protein), but was still not significantly different among treatments (Figure 4.6A). Symbiont CA activity per algal cell was not significantly different at pH 7.85 or pH 7.65

compared to the control (pH 8.1), however, activity per cell was higher at pH 7.65 than pH 7.85 (Figure 4.6B).

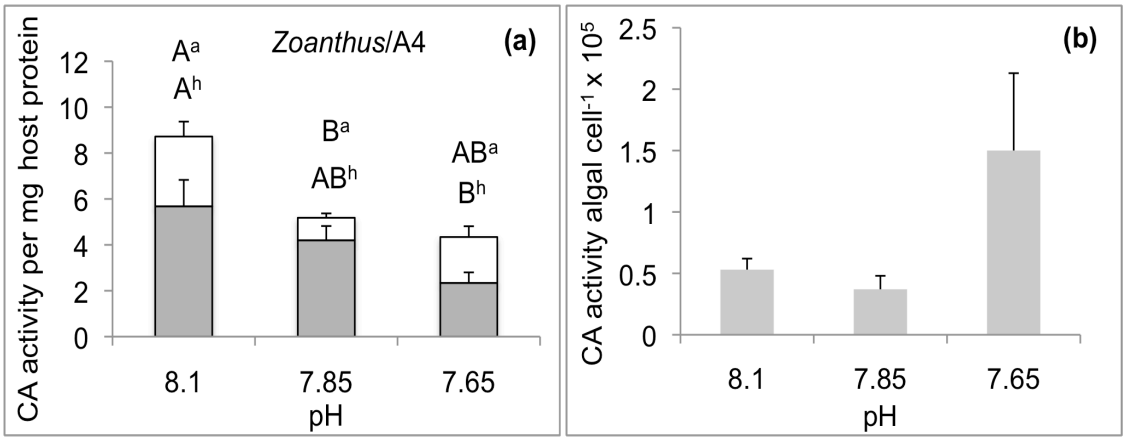


Figure 4.4. Effects of pH on carbonic anhydrase activity in *Zoanthus* sp. **a.** Host and algal CA activity normalized to host protein. **b.** Symbiont CA activity normalized to *Symbiodinium* population density. Bars represent mean plus standard error. Sample sizes for both panels are *n*=10, 8, and 8, respectively. Shading and statistical indicators are the same as in Fig. 4.1.

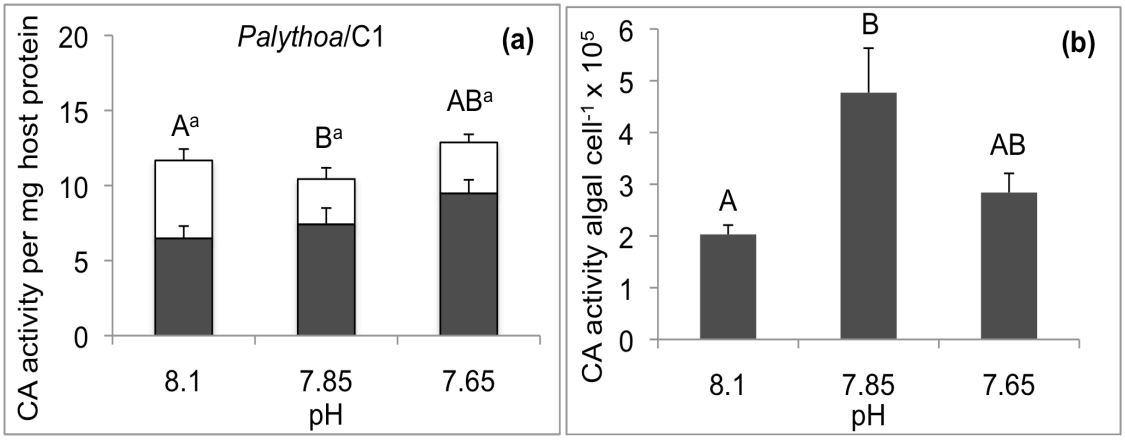


Figure 4.5. Effects of pH on carbonic anhydrase activity in *Palythoa* sp. **a.** Host and algal CA activity normalized to host protein. **b.** Symbiont CA activity normalized to *Symbiodinium* population density (x 10^5). Bars represent mean plus standard error. Sample sizes for both panels are *n*=7, 7, and 8, respectively. Shading and statistical indicators are the same as in Fig. 4.1.

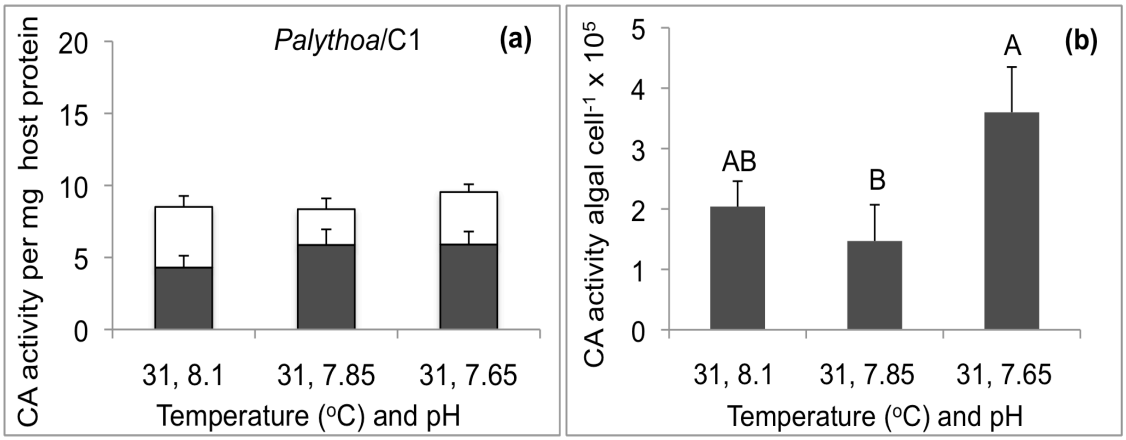


Figure 4.6. Carbonic anhydrase activity in *Palythoa* sp. maintained at 31°C, and different pH levels. **a.** Host and algal CA activity normalized to host protein. **b.** Symbiont CA activity normalized to *Symbiodinium* population density. Bars represent mean plus standard error. Sample sizes for both panels are *n*=8, 8, and 11, respectively. Shading and statistical indicators are the same as in Fig. 4.1.

4.4.3 *Symbiodinium* population density and host protein

Temperature did not affect algal cells per µg host protein of A4 symbionts in *Zoanthus* sp. or of C1 symbionts in *Palythoa* sp. (Figure 4.7B-C). Also, there was no change in host protein among the temperature treatments for *Zoanthus* sp. Host protein in *Palythoa* sp. decreased at 31°C and 33°C, compared to the control (27°C) (Figure 4.7A).

Host protein did not change for either species among the pCO_2/pH treatments, but symbiont population densities were affected by pH (Figure 4.8). A4 symbionts in *Zoanthus* sp. decreased as pCO_2 increased, and population density at pH 7.65 was significantly lower than at pH 8.1 (p=0.0387; Figure 4.8C). Similarly, population densities of C1 symbionts in *Palythoa* sp. decreased as pCO_2 increased, and were significantly lower at pH 7.85 compared to pH 8.1 (p=0.013). However, population density at pH 7.65 was not significantly different than pH 8.1 or pH 7.85 (Figure 4.8B). Elevated temperature combined with high *p*CO2/ low pH negatively affected C1 population densities as well (Figure 4.9), but increased host protein concentrations. For all treatments, there were no significant correlations between algal population density and host CA activity for either *Zoanthus* sp. (r = 0.2719, p=0.1087) or *Palythoa* sp. (r =0.0591, p=0.7284).

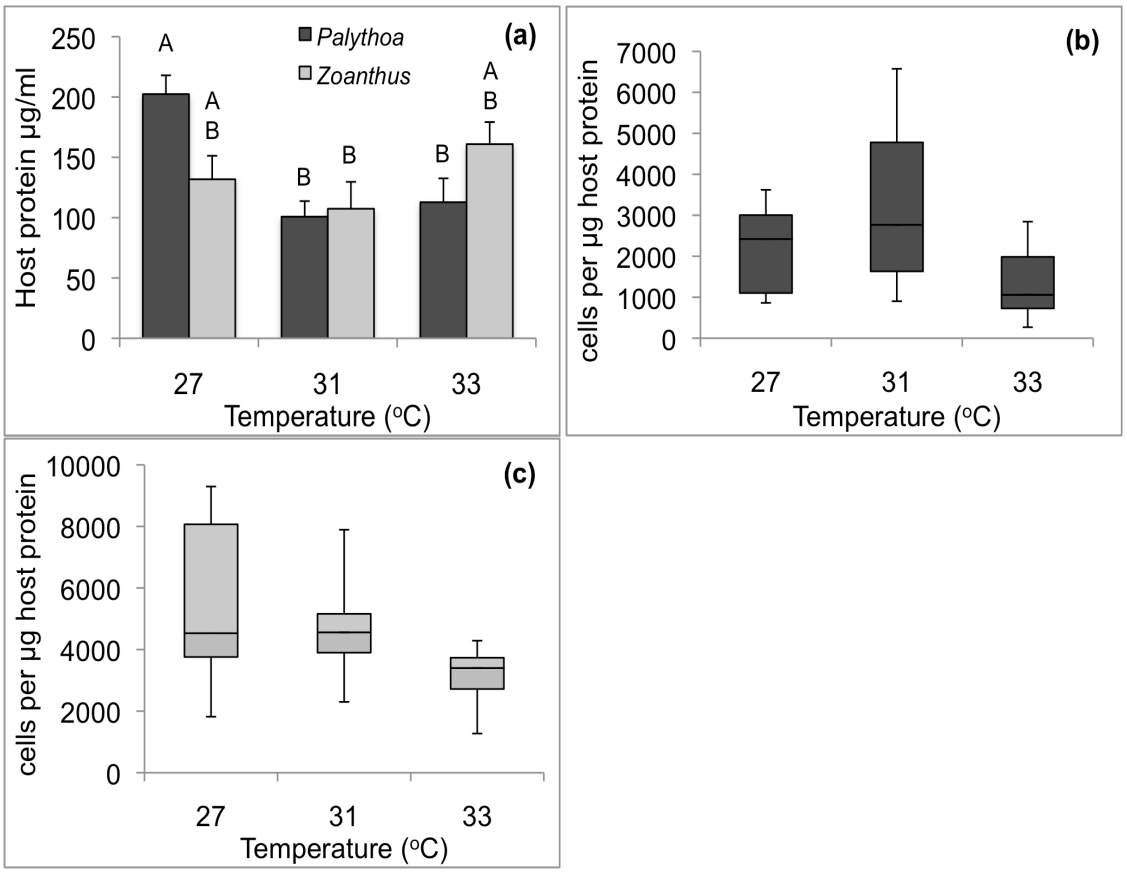


Figure 4.7. Effects of temperature on host protein concentrations and symbiont population density. **a.** Host protein concentrations for *Zoanthus* sp. and *Palythoa* sp. Bars represent means plus standard error. **b** and **c.** *Symbiodinium* population densities for *Palythoa* sp. **(b)** and *Zoanthus* sp. **(c)**. For each panel, matching letters indicate means that are not significantly different, while non-matching letters show significant differences (ANOVA with Tukey post-hoc, $p < 0.05$). Sample sizes for each species and treatment are indicated in Figures 4.1 and 4.2.

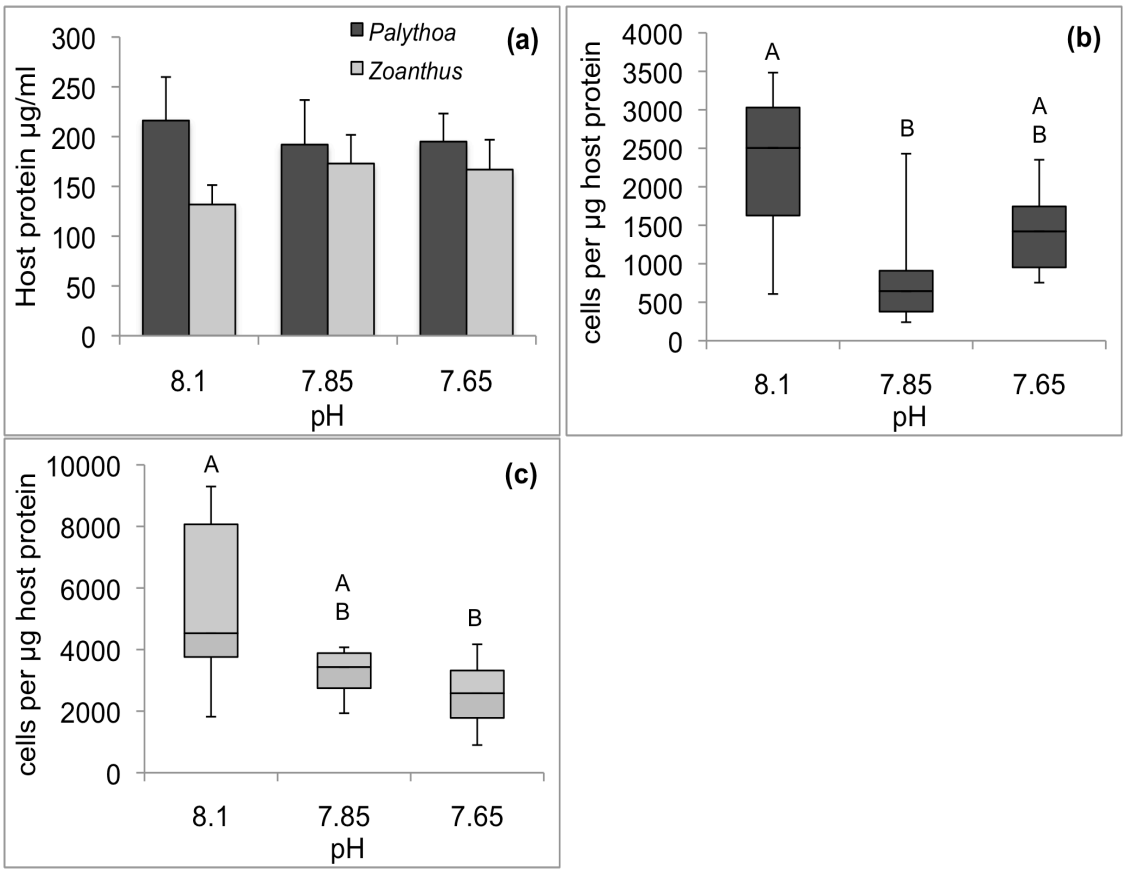


Figure 4.8. Effects of pH on host protein concentrations and symbiont population density. **a.** Host protein concentrations for *Zoanthus* sp. and *Palythoa* sp. Bars represent means plus standard error. **b** and **c.** *Symbiodinium* population densities for *Palythoa* sp. **(b)** and *Zoanthus* sp. **(c)**. For each panel, matching letters indicate means that are not significantly different, while non-matching letters show significant differences (ANOVA with Tukey post-hoc, $p < 0.05$). Sample sizes for each species and treatment are indicated in Figures 4.4 and 4.5.

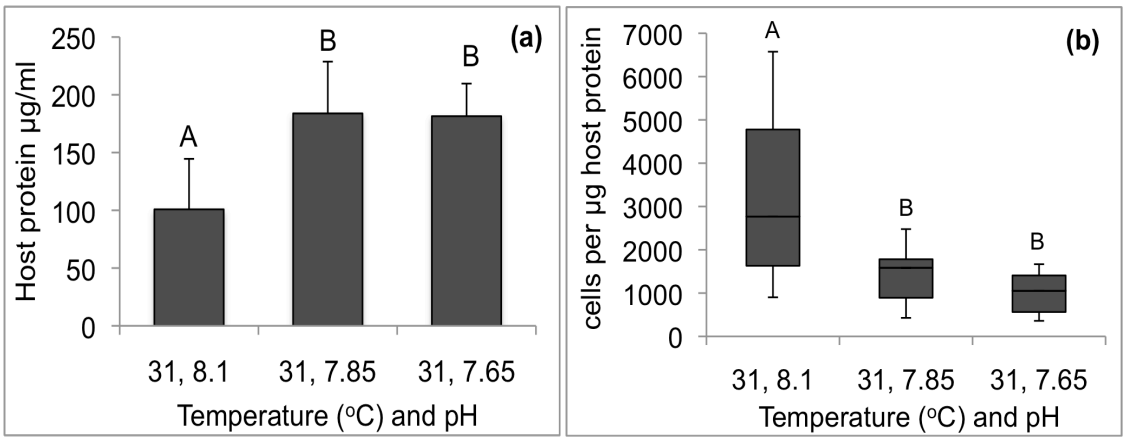


Figure 4.9. Effects of elevated temperature (31°C) and pH on host protein concentrations and symbiont population density in *Palythoa* sp.. **a.** Host protein concentrations *Palythoa* sp. Bars represent means plus standard error. **b.** *Symbiodinium* population densities for *Palythoa* sp.. For each panel, matching letters indicate means that are not significantly different, while non-matching letters show significant differences (ANOVA with Tukey post-hoc, $p < 0.05$). Sample sizes for each species and treatment are indicated in Figure 4.6.

4.5 Discussion

Carbonic anhydrase is essential for delivering CO_2 to *Symbiodinium* sp. for photosynthesis *in hospite*, and changes in CA activity can alter primary production in symbiosis (see Bertucci et al. 2013). In chapter three, I reported that changes in photosynthesis due to high temperature and high pCO_2/ low pH were very different between *Palythoa* sp. and *Zoanthus* sp. zoanthids with C1 and A4 symbionts, respectively. In *Palythoa*-C1 associations, photosynthesis and fixed carbon translocation were greatly inhibited by both increased temperature (31°C and 33°) and increased

pCO_2/low pH (pH 7.85 and 7.65), while photosynthesis and carbon translocation in *Zoanthus*-A4 associations were not affected by high temperature and *increased* with high pCO_2 (see chapter three). In the present study, I tested the hypothesis that differences in carbonic anhydrase activity between the two host zoanthids, and variable changes in CA activity in response to high temperature and high pCO_2/ low pH may have caused differences in primary production between *Palythoa*-C1 and *Zoanthus*-A4. The present study shows that under normal environmental conditions, CA activity is not different between these two species, and therefore, is not the primary reason for higher carbon fixation and translocation in *Palythoa*-C1 zoanthids under control conditions (27°C, pH 8.1). However, this study does show that high temperature and high pCO_2/ low pH cause species-specific changes in CA activity between the two zoanthids, which may contribute to differences in photosynthesis reported previously.

Theoretically, higher temperatures increase the need for CA, due to shifting equilibrium of DIC into less pCO_2 and HCO_3^-; in microalgae, CA activity increases concomitantly with temperature up to 37°C (Shiraiwa and Miyachi 1985). However, temperature-induced *decreases* in CA activity were reported in a recent study that showed downregulation of CA genes in symbiotic *Anemone viridis* exposed to thermal stress (Moya et al. 2012a). The present study shows that total CA activity does not increase or decrease with temperature in symbiotic zoanthids, as was noted for microalgae and anemones, respectively. Instead, total CA activity and host CA activity in both *Palythoa* sp. and *Zoanthus* sp. was not significantly different among the temperature treatments (Figures 4.1A and 4.2A).

Chapter three reported massive declines of photosynthesis and fixed carbon translocation in *Palythoa*-C1 associations at 31°C and 33°C. In the present study, the lack of change in both host and symbiont CA activity at high temperatures suggests that reduced photosynthesis in *Palythoa*-C1 was caused by photoinhibition and not inadequate CO_2 delivery. This hypothesis is supported by previous reports of low thermal tolerance in clade C symbionts (Baird and Marshall 2002; Baker et al. 2013a; Dove et al. 2006), as well as extensive polyp bleaching at 33°C seen in this study.

Although host CA activity was not affected by temperature in either zoanthid species, A4 symbionts in *Zoanthus* sp. had significantly lower CA activity at 33°C than at 27°C (Figure 4.1A). This is unusual considering that host CA activity did not change, but could be explained by several hypotheses. First, CA in A4 symbionts may operate more efficiently at higher temperatures. As seen in this study, *Zoanthus* sp. host CA activity was stable among all temperature treatments; therefore, it is likely that a similar amount of DIC was delivered to symbionts at all temperatures. If the same amount of DIC reached the symbionts, and the efficiency of symbiont CA increased, then, reduced symbiont CA activity could still deliver the same amount of CO_2 to the site of carboxylation. Another possibility is that, despite less solubility of CO_2 at higher temperatures, *Zoanthus* sp. still relies on some passive diffusion of CO_2 into the host tissues, and more so than *Palythoa* sp.. If this were true, algal CA activity would presumably decrease because DIC would be reaching the symbiont as CO_2 and would not need to be reconverted at the site of the symbiont. Furla et al. (2005) suggested that even though the bulk of DIC (85%) is taken up by symbiotic cnidarians via active transport (Allemand et al. 1998b), passive transport of DIC can also occur. Previous studies have

showed transepithelial flux of DIC by passive diffusion (Benazet-Tambutte 1996; Furla et al. 1998), although it can only account for 15% of DIC uptake (Allemand et al. 1998b). Finally, it is possible is that some other mechanism facilitates the final conversion of HCO_3^- to CO_2 at higher temperatures. In chapter three, carbon fixation by A4 symbionts in *Zoanthus* sp. was shown to remain stable at 27°C, 31°C and 33°C, which suggests that despite lower algal CA activity at higher temperatures, similar amounts of CO_2 reached *Symbiodinium* at all three temperatures.

In symbiotic cnidarians, high pCO_2 has been shown to both upregulate and downregulate CA gene expression in corals (see Bertucci et al. 2013; Moya et al. 2012b). This may point to species-specific differences in CA efficiencies, and /or different mechanisms of DIC uptake and transport. Indeed, multiple CA isoforms have been identified in anthozoan hosts (Bertucci et al. 2013), and studies have suggested that the mechanisms of DIC assimilation vary among cnidarian species (see Allemand et al. 1998a). In the current study, host CA activity was unaffected by high pCO_2/ low pH in *Palythoa* sp., however, host CA activity in *Zoanthus* sp. was significantly reduced at pH 7.65. As described in chapter three, photosynthesis increases as pH declines in *Zoanthus*-A4 associations; therefore, adequate levels of CO_2 are reaching A4 symbionts at pH 7.65. The results of the previous study and this study suggests that under more saturating DIC conditions, *Zoanthus* sp. downregulates CA genes, and perhaps relies on more passive diffusion of DIC.

For both species, symbiont CA activity per mg protein was significantly lower at pH 7.85 than pH 8.1, but algal CA activity per mg protein at pH 7.65 was not significantly different than at pH 7.85 or pH 8.1. It is possible that at pH 7.85, host

endodermal cells started to become saturated with DIC (despite no change in host CA activity), and symbiont CA activity per mg protein declined in response to excess DIC. At pH 7.65, however, host cells became super saturated with DIC, which triggered algal CA activity to increase to utilize some of the accumulating DIC for photosynthesis. In other words, pH 7.85 may have increased DIC flux at a level that suppressed algal CA activity, while DIC flux at pH 7.65 increased enough to stimulate algal CA activity.

Another possible explanation for lower algal CA activity per mg protein in both species at pH 7.85 is that higher pCO_2 caused a change in the mechanism utilized for the final dehydration of HCO_3^- to CO_2 at the site of the symbiont, such as an H+-ATPase (Allemand et al. 1998). This, in turn, could reduce algal CA activity. Then, with a further decline in pH (to 7.65), excess DIC caused algal CA activity to increase again to supplement dehydration by the alternative mechanism. In addition, as pCO_2 increases, the final dehydration of HCO_3^- to CO_2 could be occurring in host tissue, and not in or on the surface of the symbiont (see Allemand et al. 1998). Despite differences in algal CA activity per mg host protein for both species, algal CA activity per algal cell was not significantly different for A4 symbionts among all pH treatments. However, CA activity per cell was significantly higher in C1 symbionts in *Palythoa* sp. at pH 7.85 compared to pH 8.1 (Figure 4.5B). This could be because algal population density was lowest at pH 7.85 compared to the other pH treatments. Thus, at pH 7.85, some CA may have remained in algal cells that were disintegrated and not counted as viable cells when determining *Symbiodinium* population density, resulting in higher CA activity on a per cell basis.

Studies to date have reported that the majority of CA in *Symbiodinium*-cnidarian associations is located in the host, and host CA activity is approximately 2.5 times higher than algal CA activity under normal conditions (Weis, 1989). In the present study, host CA activity was 2.5 times higher in *Palythoa* sp. and 1.8 times higher in *Zoanthus* sp. than algal CA activity at control conditions (27°C and pH 8.1). This ratio increased for *Zoanthus* sp. in the elevated temperature treatments, where host CA activity was as much as 4.5 times higher than symbiont CA activity at 33°C. *Zoanthus* sp. host CA activity was also 4.3 times higher than algal CA activity when pH was reduced to 7.85. These values indicate that, as environmental conditions change, the proportion of host CA and algal CA utilized in DIC acquisition may shift. As discussed above, these shifts may be due to changes in the efficiencies of particular CAs, and/or in the mechanism of DIC uptake, and as seen in this study, the extent of changes in proportion are species specific.

In previous studies, host CA activity was correlated with algal density (Estes et al. 2003; Weis 1991). In this study, however, host CA activity in *Palythoa* sp. was not different in bleached versus non-bleached polyps, and bleached *Zoanthus* sp. polyps had significantly lower CA activity than non-bleached at 27°C, but not at 31°C or 33°C. Moreover, I found no correlation between host CA activity and symbiont population density. In the present study, I used a cold shock method to beach zoanthid polyps. Although I verified that >90% of symbionts were expelled from both *Palythoa* sp. and *Zoanthus* sp. polyps one week following cold shock and maintenance in dark aquaria, it is possible that a few symbionts remaining in the polyps reestablished population densities over the three-month experiment. If symbionts remained in the host over the three-month experiment, then host CA activity would remain at a level representative of the symbiotic

state, as seen in these results. However, there was still no correlation between symbiont population density and host CA activity for all of our non-bleached trials.

Lower CA activity can inhibit primary production in symbiosis, reducing the nutritional contribution of the symbiont. In the present study, both host CA and algal CA activity per mg protein were reduced in *Zoanthus* sp. as pCO_2 increased, yet as reported in chapter three, photosynthesis in *Zoanthus* sp. significantly *increased* at pH 7.65 compared to pH 8.1. These results suggest that CAs in *Zoanthus*-A4 associations operate efficiently enough that CA genes are downregulated in the presence of additional DIC. Also, *Zoanthus*-A4 may acquire a greater portion of DIC through passive diffusion as pCO_2 increases (possibly aided by increased membrane permeability). Thermal stress did not have an effect on carbon fixation or translocation in *Zoanthus* sp. (see chapter three), nor did it change host CA activity in this study, but it did reduce algal CA activity. Again, these data suggest that *Symbiodinium* were not CO_2 limited, and that greater passive diffusion and/or greater CA efficiencies allow for lower CA activity at higher temperatures. In contrast, high *p*CO2 did not affect *Palythoa* sp. host CA activity in either individual or combination temperature/pCO_2 treatments. Both high temperature and high pCO_2 greatly inhibited photosynthesis by C1 symbionts in *Palythoa* sp. (see chapter three), however, given the results this study, it is more likely that photoinhibition in C1 symbionts, and not CO_2 limitation, is the cause of decreased photosynthesis in *Palythoa* sp. at high temperatures and high pCO_2.

This study shows that changing environmental conditions have variable effects on CA activity among both zoanthid species and *Symbiodinium* types. Further, this study supports the hypothesis that climate-induced changes in DIC transport in symbiotic

cnidarians will be highly holobiont dependent. *Symbiodinium*-cnidarian associations likely utilize different mechanisms for DIC uptake and transport, relying on CA to different degrees, and they may alter these mechanisms in response to environmental stimuli such as temperature and DIC. Moreover, CA isofoms in both host and symbiont fractions may respond differently to certain environmental conditions. Genetic analyses to identify CA isoforms may help to determine the source of variability in CA activity among zoanthid species and *Symbiodinium* types, and molecular and pharmacological methods can be used to target specific host and symbiont CAs and delineate holobiont-specific pathways of DIC transport. Additional studies to characterize CA isoforms and measure their activity in different host-symbiont combinations are critical to predicting changes in DIC flux as climate change and ocean acidification continue to shift ocean carbonate chemistry.

CHAPTER 5

CONCLUSIONS

This body of work has revealed several major findings about carbon flux in algal mutualisms. First, this work was the first to experimentally demonstrate translocation of fixed carbon in the *Oophila*-salamander symbiosis, which is the first example of carbon translocation to a vertebrate host. This finding has enormous implications for symbiosis research and suggests several things: 1) vertebrates may have evolved physiological and morphological features to allow for symbiosis during some point in their life 2) symbiosis between algae and/or cyanobacteria and vertebrates is likely more prevalent than thought and 3) symbiosis with photoautotrophs may place some vertebrates at an evolutionary advantage by accelerating development and reducing mortality of their young.

The cnidarian-*Symbiodinium* symbiosis is, by far, one of the most commonly studied mutualisms, largely because of the importance of coral reef ecosystems. Despite this, many aspects of this symbiosis remain unclear, particularly in terms of carbon acquisition and transport in the holobiont. Moreover, only in recent years has it emerged that *Symbiodinium* sp. represents hundreds of phylotypes with different photobiological efficiencies, environmental tolerances, geographic distributions, and host specificities. Only in the past decade have researchers recognized that cnidarian-*Symbiodinium* assocations have discrete, species-specific physiological responses to abiotic factors. Despite a large body of existing research on cnidarian-*Symbiodinium* symbiosis, broader conclusions and predictions cannot be made among many of these studies because the host and/or symbiont were not properly identified using molecular techniques. Another shortcoming of existing research is that most has focused on coral and calcification, even

though calcification and photosynthesis are coupled, there are a large number of non-calcifying organisms hosting *Symbiodinium*, and future climate conditions may favor soft-bodied cnidarians.

This body of work contributed several important findings to cnidarian-*Symbiodinium* research. First, it confirmed that C1 symbionts are sensitive to both thermal stress and high pCO_2. Temperatures exceeding 31°C cause extensive bleaching and mortality in C1 symbionts, and temperatures higher than 27°C result in significantly reduced photosynthesis and symbiont population densities, as well as reduced host protein concentrations. High pCO_2 also greatly inhibits photosynthesis and causes loss of C1 symbionts. Throughout both thermal stress and high pCO_2, however, *Palythoa* sp. hosts maintained steady carbonic anhydrase activity. This suggests that the cause of breakdown in this association may be the environmentally- sensitive C1 symbionts, and that other organisms hosting C1 symbionts are not likely to persist in future ocean conditions. Considering the prevalence and large geographic distribution of C1 *Symbiodinium* sp., a large number of cnidarian hosts may require symbiont shuffling to survive (as discussed in Chapter 3). Second, this study also shows that A4 symbionts are tolerant of high temperatures and high pCO_2. The *Zoanthus*-A4 association maintains photosynthesis and carbon translocation in both high temperature and high pCO_2, and the mechanisms for acquiring DIC appear to operate highly efficiently. These results support the hypothesis that hosting A4 symbionts may benefit cnidarians during extreme temperature events, and as the oceans continue to change.

This study is also one of only few studies that examined the combined effects of high temperature and high pCO_2/ low pH. As summarized above, temperatures >27°C

and pH below 8.1 caused significant negative effects on photosynthesis, symbiont population density, and host protein in *Palythoa* sp./C1. Despite these results, when *Palythoa* sp. was maintained at 31°C and different pH values, symbiont population density and host protein remained the same, and carbon fixation and translocation *increased* at the lowest pH value (7.65). The results of these experiments are perplexing, and indicate that the effects of synergistic factors cannot always be predicted from the effects of individual treatments. Therefore, additional studies examining combined effects are needed to form better conclusions about the fate of cnidarian-*Symbiodinium* associations under future climate conditions. Finally, this work supports the new body cnidarian-*Symbiodinium* literature that considers the unique species-specific responses of different hosts and symbionts.

In conclusion, fixed carbon from photosynthesis serves a variety of functions in algal mutualisms. For some host organisms, such as *A. maculatum*, photosynthetic carbon provides a nutritional supplement during a critical time in development. For other host organisms like zoanthids, fixed carbon is essential for the survival of the host, and changes in photosynthetic rates have immediate effects on host health. As research on mutualisms between photoautotrophic symbionts and both invertebrate and vertebrate hosts continues, it will likely reveal additional roles of photosynthetic carbon in symbiosis. At the same time, while we are unraveling carbon flux in symbiosis and discovering new algal mutualisms, global climate change and ocean acidification will continue to impact carbon flux in symbiotic associations. Indeed, predicting the stability of algal mutualisms in future climate conditions is a challenging task that we have only begun to address.

NOTES

Chapter 2 was originally published in The Journal of Experimental Biology in February 2013. Adam Davey, Scott Fay and Robert Sanders were co-authors on this study. All of the experimental work was conducted by Erin Graham, and the manuscript was written by Erin Graham, with the exception of the statistical methods section. Adam Davey helped with statistical analysis and wrote a portion of the statistical methods section. Scott Fay taught me how to perform molecular techniques to identify the algal species, assisted in the genetic analysis of the algal species, and contributed to editing the original manuscript. Robert Sanders also contributed to editing the original manuscript.

All other work in chapters 3 and 4 was conducted entirely by Erin Graham and written by Erin Graham.

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