A COMPARATIVE APPROACH TO ELUCIDATING THE PHYSIOLOGICAL RESPONSE IN *SYMBIODINIUM* TO CHANGES IN TEMPERATURE

by

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ABSTRACT

A COMPARATIVE APPROACH TO ELUCIDATING THE PHYSIOLOGICAL RESPONSE IN *SYMBIODINIUM* TO CHANGES IN TEMPERATURE

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Coral reefs are one of the most biodiverse ecosystems, and play a large role in the functioning of oceanic and coastal ecosystems. They are currently declining around the world, largely due to the effects of climate change and local anthropogenic issues. Temperature stress in particular has been associated with significant coral mortality over the last 30 years due to adverse effects on the relationship between reef-building corals and their algal endosymbionts from the genus *Symbiodinium.* Periods of elevated temperature have led to the phenomenon of coral bleaching, a disruption of the symbiosis that is characterized by the loss of the algal symbionts or their pigments. Variations in bleaching responses have been observed that correlate with the identity of the algal symbiont, but the mechanisms behind these differences remain unresolved. To examine how *Symbiodinium* physiology may be contributing to these observed differences, eight *Symbiodinium* cultures, or *Symbiodinium* types, were exposed to elevated temperature stress, and several parameters that impact the bleaching response were measured. These included the production of reactive oxygen species (ROS), the activity of antioxidants responsible for scavenging ROS, oxygen uptake rates ($\dot{V}O_2$) as a measure of metabolism, growth rates, and loss of the photosynthetic chlorophyll *a* (Chl *a*)

pigment. Each *Symbiodinium* type was found to display unique responses to elevated temperature stress, demonstrating a gradient of sensitivity. *Symbiodinium* types that were sensitive to elevated temperatures had increased production of ROS without a corresponding increase in antioxidants to scavenge them, increases in $\dot{V}O_2$, and decreases in growth rate concomitant with a loss in Chl *a* at relatively low temperatures. Tolerant attributes were found in other *Symbiodinium* types including low production of reactive oxygen, sometimes coupled with higher activity of antioxidants, stable $\dot{V}O_2$ and continued growth at relatively higher temperatures, and resistance to loss of the Chl *a*.

In addition, the functional differences of intact associations from four coral species collected in La Parguera, Puerto Rico, were examined with regard to host and symbiont factors linked to resistance to bleaching and disease. Components of the melanin cascade in host tissue can confer resistance to bleaching as well as being important factors in the immune response, and antioxidant activity of both the host and symbiont have a role in preventing damage due to ROS produced by symbionts and by the host during the melanin cascade. Significant differences were found among different coral-*Symbiodinium* associations, and correlations were found in coral-*Symbiodinium* responses, including symbiont antioxidant activity and host melanin cascade. This indicates that each partner in the relationship can significantly influence the response of the other to environmental stress and pathogens.

The culmination of this research demonstrates that the *Symbiodinium* stress response is a complex interaction of the stability of their metabolism, disruption to their photosynthetic apparatus, and influences from their host when in symbiosis. As temperatures increase, some types of *Symbiodinium* are physiologically robust, some are able to compensate, and others are negatively affected. These results suggest that stressors such as elevated temperature may also be influencing the relationship between the host and symbiont, potentially shifting the relationship from a mutualism to a parasitism.

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CHAPTER 1

INTRODUCTION

1.1 Introduction to Coral Reefs

Coral reefs are highly diverse and complex ecosystems that influence both oceanic and terrestrial ecosystems. They consist of a dynamic array of interactions among plants, animals and microbes to achieve complex structures and functions. Central to this are the sessile invertebrates, particularly the reef building scleractinian coral colonies that create the underlying reef structure for all other organisms (Connell 1978, Moberg & Folke 1999, Knowlton et al. 2010).

Globally, coral reefs are important for both ecological and anthropocentric reasons. Ecologically, their extremely high biodiversity is matched only by tropical rainforests (Connell 1978). They are vital to ocean functioning by creating habitat and providing breeding grounds and nurseries for other organisms, many of which contribute significantly to the different trophic levels of ocean food webs (Wild et al. 2011). Their proximity to land directly contributes to human welfare, as many coastal communities rely on reefs as a food source and acquire most of their protein intake from fish and invertebrates, and many communities also rely on the tourism industry they generate for a source of economic income (Birkeland 1997, Bell et al. 2006). They also provide indirect services by acting as a barrier to diminish the strength of wave action during inclement weather before it makes landfall, thus protecting coastlines and human developments (Moberg & Folke 1999, Wild et al. 2011). All of these benefits are inherently linked to the diversity contained within these ecosystems.

1.1.1 The Coral-Symbiodinium *relationship*

All of the services provided by coral reefs are made possible by the symbioses established between many animals, such as corals, sponge, and tridachnid clams, and

endosymbiotic unicellular dinoflagellate algae in the genus *Symbiodinium*. While corals are heterotrophs, many rely on translocated photosynthetic products from their symbionts for greater than 90% of their nutritional requirements (Muscatine & Porter 1977). The *Symbiodinium* also aid in skeletal deposition by the coral, enabling the coral to create the underlying solid reef structure and increasing the rate of habitat creation (Muller-Parker & D'Elia 1997). Because of these contributions to the functioning unit, it can be argued that the relationship between the scleractinian coral host and the algal symbiont is the most crucial aspect of a functioning coral reef.

Explorations of this symbiosis over the last 60 years have revealed a complex relationship between host and symbiont. While *Symbiodinium* has existed in culture since the 1950's (McLaughlin & Zahl 1957), it was thought that all cnidarian symbionts were the same species, *Symbiodinium microadriaticum,* until the 1980's (Blank & Trench 1985). Advances in systematics have since provided a resolution that divides the genus into nine different clades, lettered A-I (Pochon & Gates 2010). Within each clade, further clarification is sought to classify the massive genetic diversity. One commonly used method is to further subdivide each clade into symbiont "types" by sequencing a portion of ribosomal DNA, the internal transcribed spacer region 2 (ITS-2) (LaJeunesse 2001, Baker 2003, Coffroth & Santos 2005). This region, combined with additional genetic markers, is revealing an extensive amount of diversity that may lead to further restructuring of *Symbiodinium* taxonomy, particularly at the species level (Sampayo et al. 2009, Finney et al. 2010, LaJeunesse & Thornhill 2011).

Scleractinian corals host the greatest diversity of *Symbiodinium*, and are known to commonly associate with members of clades A, B, C, and D, and occasionally with members of clades F and G (Baker 2003, LaJeunesse et al. 2010). The specificity between host and symbiont varies; some coral species only host one specific type of symbiont, while others can be found with different symbiont types depending on varying environmental conditions such as light exposure and temperature (LaJeunesse 2002, Baker 2003, Finney et al. 2010). Some

coral are also able to form associations with multiple symbionts at a given time, with one generally dominating while the others exist at low density in the background (Mieog et al. 2007, Silverstein et al. 2012). These associations can be flexible, with symbiont populations changing with seasonal changes, so that the dominant symbiont population varies as temperatures rise over the summer months and fall during the winter (Chen et al. 2005). This variability can also enable some corals to utilize different habitat types; studies that have identified and compared the symbionts of coral species found in outer reefs to lagoon reefs that are typically 1-2°C warmer have demonstrated differing symbiont communities (Oliver & Palumbi 2011). This diversity of strategies results in coral 'holobionts' that are made up of different host-symbiont combinations, and hosting different symbionts may result in significant functional differences (Buddemeier et al. 2004, Stat et al. 2008). For example, studies have found that tradeoffs may exist between hosting different *Symbiodinium* types; some symbionts convey higher growth rates to the host, but at the cost of being less tolerant to fluctuations in temperature (Rowan et al. 1997, Baker 2003, Little et al. 2004, Cantin et al. 2009).

1.2 Threats to Coral Reefs

A healthy coral reef is dependent on optimum functioning of all of the participants, which can make these ecosystems incredibly fragile and susceptible to environmental change. Coral reefs are currently declining because of habitat exploitation, overfishing, pollution, and climate change. In particular, increases in ocean temperatures, changes in weather patterns associated with El Niño Southern Oscillations that lead to doldrum conditions (hot, still days with high irradiance), and ocean acidification due to increases in atmospheric CO_2 concentrations are influencing the health and composition of coral reefs (Harvell et al. 2007, Hoegh-Guldberg et al. 2007). These stressors have been linked to dramatic declines in coral reef cover and increases in coral disease (Bruno & Selig 2007, Carpenter et al. 2008, Mydlarz et al. 2010).

One of the largest contributors to coral decline is the phenomenon known as coral bleaching. A bleached coral is characterized by the loss of most of the algal symbionts and/or their associated pigments (Figure 1.1) (Brown 1997, Hoegh-Guldberg 1999), and when severe and pervasive this compromised state can lead to starvation, increased disease susceptibility, decreased reproductive capacities, and mortality (Wilkinson 2004, Venn et al. 2008, Mydlarz et al. 2010). Coral bleaching can be induced by changes in temperature, salinity, pH, and light levels, as well as by the presence of pathogens and pollutants (Mydlarz et al. 2010). Recent decades have seen an increase in large scale bleaching events induced by periods where temperatures rise 1-2°C above the daily average for several weeks, often concurrent with periods of increased solar exposure. Severe global bleaching events have occurred in 1998, 2005 and 2010 (Coles & Brown 2003, Buddemeier et al. 2011, Guest et al. 2012, Monaco et al. 2012). The 1998 event was the most wide spread bleaching on record, and destroyed 16% of the world's coral reefs (Wilkinson 2004). Bleaching events are predicted to become increasingly common, with conservative estimates predicting bi-annual occurrence by the year 2050 (Hoegh-Guldberg 1999, Donner et al. 2005, Hoegh-Guldberg et al. 2007). High coral mortality can result in community shifts on coral reefs to algal dominated, animal depauperate ecosystems that are extremely difficult to recover from, especially in areas where herbivorous fish have been overharvested (Knowlton 1992, Mumby 2009, Hoegh-Guldberg & Bruno 2010). The increased frequency of these events will therefore result in massive alterations of coral reef ecosystems, which will have severe ramifications on a global scale due to loss of their ecosystem services (Birkeland 1997, Downs et al. 2002, Smith et al. 2005, Bell et al. 2006, Hoegh-Guldberg et al. 2007).

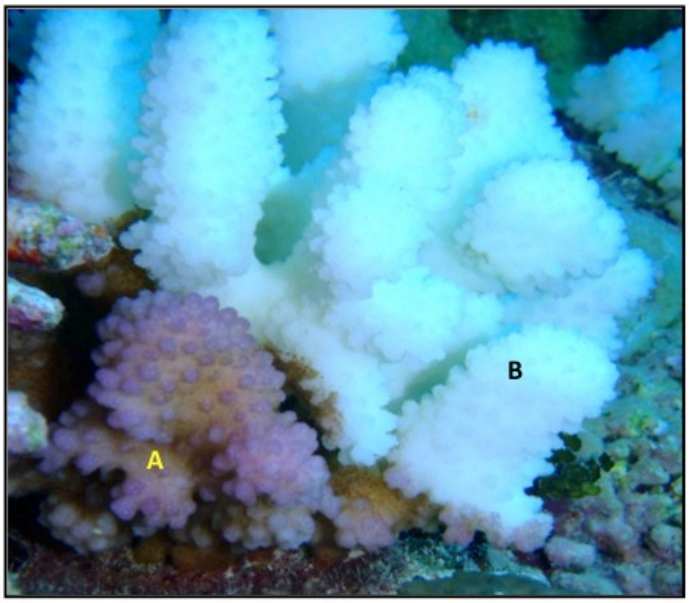

Figure 1.1 Bleached *Pocillopora* sp. A) Healthy, pigmented portion of colony. B) Bleached portion of colony.

1.2.1 Symbiodinium *and Coral Bleaching*

The mechanisms behind coral bleaching are poorly understood, but are tied to the physiology of the algal symbiont. While the symbiosis allows the host to benefit from the *Symbiodinium*'s photosynthetic product, it also makes them susceptible to stressors that disrupt photosynthesis, such as elevated temperatures and irradiance (Venn et al. 2008). The current explanatory model for the underlying mechanisms behind environmentally induced bleaching events posits that accumulation of reactive oxygen species (ROS) during exposure to stress results in cellular damage to the symbiont and host, resulting in the temporary degradation of the symbiosis through a multitude of different cellular mechanisms that ultimately culminates in the expulsion of the symbionts (Figure 1.2) (Downs et al. 2002, Franklin et al. 2004, Smith et al. 2005, Lesser 2006, Weis 2008).

ROS are naturally produced during the passage of electrons through electron transport chains in photosynthesis and cellular respiration, and include the hydroxyl radical (OH^-), superoxide (O_2^-), and hydrogen peroxide (H_2O_2) (Lesser 2006). These molecules can cause

damage that includes mutating and oxidizing DNA, denaturing and carbonylating proteins, oxidizing membranes, and inducing apoptosis (Neill et al. 2002, Lesser 2006, Venn et al. 2008, Wong et al. 2010). Hydrogen peroxide is of particular note because it is permeable to cellular membranes (Lesser 1996, Brown 1997, Lesser 2006, Suggett et al. 2008), allowing it to move from its point of origin into other areas of both the algal and host cells (Figure 1.2).

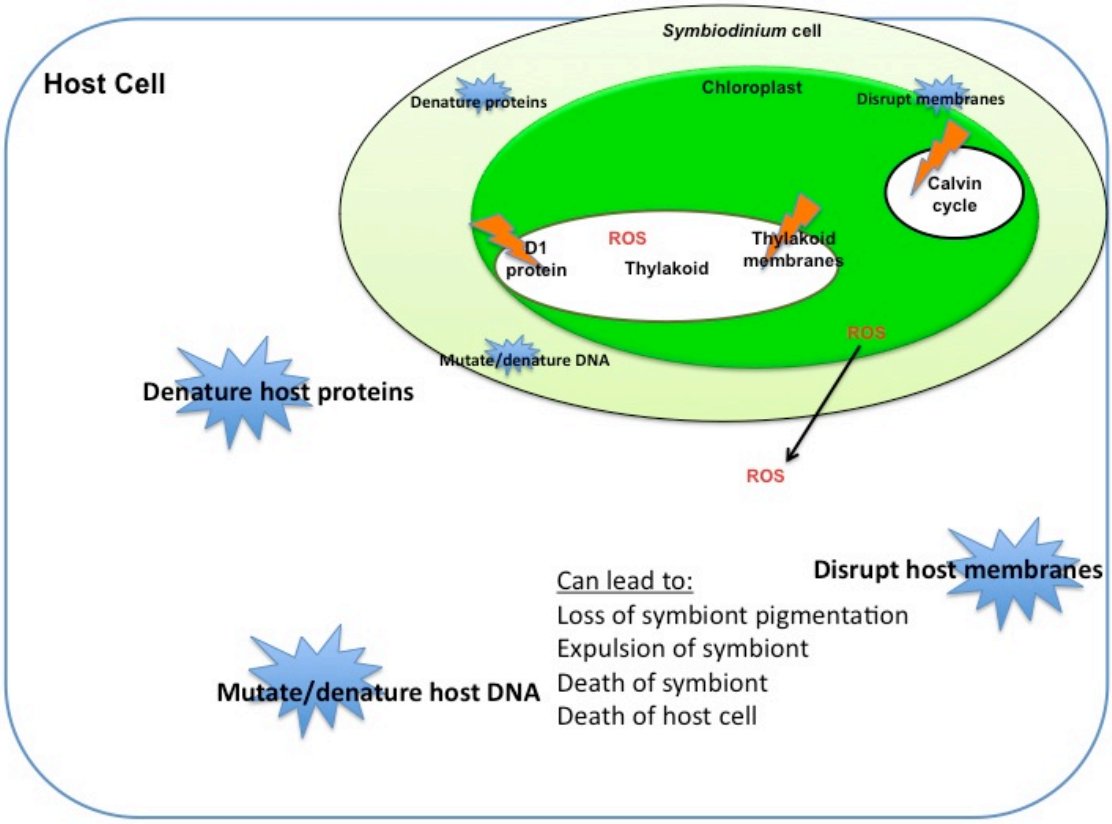

Figure 1.2 Working model of ROS generation, sites of cellular targets within *Symbiodinium* and host cells, and potential consequences of prolonged ROS exposure.

Because ROS are a ubiquitous component of cellular functioning, cells have multiple strategies to prevent them from causing unintentional damage. They can be utilized, as signaling or defensive molecules, or neutralized or scavenged by molecules like antioxidants

(Neill et al. 2002, Asada 2006, Lesser 2006, Wong et al. 2010). Exposure to stress, such as elevated temperature, salinity, UV exposure, pollution, and disease, has been shown to elicit an increase in the production of ROS from the symbiont that overwhelms the alga's natural defenses. The main sources of ROS production in *Symbiodinium* are under debate, but appear to be predominantly associated with disruption of one of the three main components of the photosynthetic machinery, Photosystem I (PSI), Photosystem II (PSII) and/or the Calvin Cycle (Weis 2008). It has been postulated that under stressful conditions damage to antennae proteins, decreased protein repair mechanisms (particularly the replacement of the D1 protein in PSII), and destabilization of the thylakoid membrane result in the photosystems being overwhelmed and a failure to transmit electrons through to the Calvin cycle (Warner et al. 1999). Alternatively, it has been suggested that initial site of damage relates to decreases in the activity of RUBISCO, leading to a decrease in the CO_2 being delivered to the Calvin cycle, and that damage to PSI and PSII is a consequence of the subsequent build-up of electrons failing to move on to the Calvin cycle (Jones et al. 1998). In either case, the backlog of excess electrons will result in an increase in ROS production. Unless the subsequent increased levels of ROS are neutralized, these molecules have the potential to cause damage to a variety of cellular components, and if damage to the host is extensive enough, bleaching will result.

1.2.2 The Role of Symbiodinium *Identity*

A great amount of variation exists in bleaching patterns; some coral bleach rapidly under periods of stress while others retain pigmentation, and some suffer high rates of bleaching related mortality while others recover from bleaching. These observations have made it clear that not all holobionts respond the same way to stress, and studies indicate that in some instances variability is due to the identity of the symbiont, with certain *Symbiodinium* types consistently found associating with corals that were able to tolerate the stress. For example, studies in *Symbiodinium* distribution on reefs before and after bleaching events have revealed a dynamic fluctuation from a high prevalence of certain clade C types prior to bleaching to a

higher prevalence of clade D in post-bleaching survivors (Baker et al. 2004, Buddemeier et al. 2004, van Oppen et al. 2005, Jones et al. 2008).

The source of this variation in *Symbiodinium* tolerance remains unclear, and is attributed to many factors including ROS production (Suggett et al. 2008, McGinty et al. 2012), antioxidant production (Lesser 1996, Yakovleva et al. 2004, McGinty et al. 2012), membrane stabilizing compounds (Mydlarz & Jacobs 2004, Tchernov et al. 2004), and cellular repair mechanisms (Lesser 1997, Warner et al. 1999, Lesser 2006). While many studies have focused on correlating *Symbiodinium* identity with field observations, fewer have examined the *Symbiodinium* response to stressors. This can be done by isolating *Symbiodinium* from a coral collected in the field, or by using cultures of *Symbiodinium*. In particular, the use of cultured *Symbiodinium* has allowed for the development of a comparative approach directly examining physiological variability. This area of research has significant gaps, as there is a significant bias towards examining only the short-term (< 24 hr) photosynthetic response of the algae, without considering additional properties that may influence the long-term tolerance to stress (Iglesias-Prieto et al. 1992, Frade et al. 2008a, Hennige et al. 2009, Ragni et al. 2010). In addition, studies that use cultured isolates have often compared a limited number of types (Lesser 1997, Robison & Warner 2006, Suggett et al. 2008), and many of the field studies that have measured responses of the *Symbiodinium* have not identified the symbiont (Ralph et al. 2001, Richier et al. 2006, Haslun et al. 2011).

The central role the symbiont plays in coral bleaching and coral decline, combined with rapidly expanding insights into *Symbiodinium* diversity, clearly illustrates the necessity to understand how *Symbiodinium* will respond to stressors like elevated temperatures and disease, so that we will then be better informed to understand and ultimately predict the effect that climate change will have on coral reef ecosystems (Iglesias-Prieto et al. 1992, Baker 2003, Chen et al. 2005).

1.3 Dissertation Aims

The overarching aim of this body of work is to further our understanding of variability in *Symbiodinium* physiology, particularly in response to elevated temperatures, by using an extensive comparative approach to examine *Symbiodinium* physiology both *in vitro,* using *Symbiodinium* cultures*,* as well as *in hospite,* using algae extracted from multiple coral species collected from the field. The specific aims and hypotheses are expounded below.

Aims addressed by culture studies:

Aim 1: To characterize the production of ROS and the antioxidants catalase and superoxide dismutase among seven different *Symbiodinium* types at elevated temperatures.

Hypothesis 1: Differences in ROS and antioxidant production among *Symbiodinium* types may explain some of the variation observed in coral bleaching patterns.

Hypothesis 2: Differences in ROS and antioxidant production exist at the inter-cladal level between types B1 and B2.

This aim and associated data are presented in Chapter 2.

Aim 2: To explore the relationship between symbiont identity, oxygen consumption and growth rates, and bleaching susceptibilities from six *Symbiodinium* types over a range of temperatures.

Hypothesis 1: There is variability in metabolic responses across a wide temperature gradient between *Symbiodinium* types in culture

Hypothesis 2: The thermal threshold and bleaching response differs between these *Symbiodinium* types in culture.

Hypothesis 3: Differences exist between symbionts from the same clade (B1 and B2), as well as two identified as the same type (A1) but isolated from different hosts and geographic locations.

This aim and associated data are presented in Chapter 3.

Aims addressed by field studies

Aim 3: To examine the functional differences among different coral host:*Symbiodinium* combinations, the potential ways that one member of the symbiosis can influence the other, and expand the current knowledge of how hosting different symbionts can lead to functional differences of the entire unit

Hypothesis: Corals hosting different *Symbiodinium* types will be functionally different, and *Symbiodinium* types found in more than one coral host will differ in their antioxidant production.

Aim 4: To explore potential host and symbiont responses that may contribute to coral health, using parameters from the host and symbiont associated with susceptibility or resistance to coral bleaching and disease, and relate these factors to actual patterns in the field.

Hypothesis: Coral species that are more susceptible to bleaching and disease will have lower levels of melanin cascade and antioxidant activity and less stable symbioses.

These aims and associated data are presented in Chapter 4.

These projects expand the current understanding of *Symbiodinium* diversity by providing physiological data that complement the recent expansions by molecular work, as well as provide direct evidence that genetic diversity goes beyond the popular ITS-2 identification technique. The findings of these experiments will aid in resolving some of the differences in symbiont physiology that contribute to their relative tolerance or sensitivity to temperature stress, contribute to the understanding of how cnidarian-*Symbiodinium* units will respond to climate change, and provide a framework that can ultimately contribute to the development and implementation of effective conservation and management programs.

CHAPTER 2

VARIATIONS IN REACTIVE OXYGEN RELEASE AND ANTIOXIDANT ACTIVITY IN MULTIPLE *SYMBIODINIUM* TYPES IN RESPONSE TO ELEVATED TEMPERATURE

2.1 Introduction

Photosynthetic microorganisms experience unique challenges in the face of global climate change, and rising sea surface temperatures are a particular concern for marine microalgae (Davison 1991, Beardall & Raven 2004, Hallegraeff 2010, Huertas et al. 2011). Temperature stress can result in changes to the ecology of these organisms through a variety of direct and indirect factors affecting physiological parameters including photosynthetic, metabolic and growth rates (Davison 1991, Beardall & Raven 2004, Hallegraeff 2010). Microalgae are the foundation of ocean ecosystems, and these changes pose a significant problem to the many organisms that rely on or are in close association with them (Venn et al. 2008). Coral reefs are of particular concern as many of the organisms present, particularly the reef-building scleractinian corals, form a mutualistic relationship with dinoflagellates in the genus *Symbiodinium* enabling them to thrive in oligotrophic tropical habitats (Muscatine 1973). Elevated temperatures have been linked to a loss of the algal symbiont and/or its pigments, causing the phenomenon of coral bleaching (Brown 1997). While corals regularly expel their symbionts during stress associated with the peak summer months without discernable long-term adverse affects, extreme cases can be detrimental to coral survival, potentially leading to increased incidence of disease and mortality (Mydlarz et al. 2010, Suggett & Smith 2011). Coral bleaching has become a focus of attention over the last three decades (Glynn 1984, Baker et al. 2008), as events leading to mass mortality and significant die offs are increasing in frequency, and have become a significant factor in worldwide decline of these ecosystems

(Hoegh-Guldberg 1999, Wilkinson 2004, Bruno & Selig 2007, Carpenter et al. 2008, van Oppen et al. 2009, Veron et al. 2009).

A contributing factor in the bleaching phenomenon is the algal production of damaging reactive oxygen molecules (ROS) (Downs et al. 2002, Lesser 2006). These molecules are a by-product of photosynthesis and cellular respiration, and their highly reactive properties can mutate DNA, denature proteins, and oxidize lipids and cellular membranes (Neill et al. 2002, Lesser 2006, Venn et al. 2008). As ROS are produced under normal conditions by the chloroplasts, mitochondria and peroxisosmes (Apel & Hirt 2004, Weis 2008) cells apply various strategies to mitigate their detrimental effects. ROS produced by the cell can be used as signals for cellular defensive responses as well as apoptosis (Neill et al. 2002, Apel & Hirt 2004, Asada 2006, Lesser 2006, Wong et al. 2010) or eliminated or scavenged by various cellular products, such as antioxidants including catalase (CAT) and superoxide dismutase (SOD) (Yakovleva et al. 2004, Asada 2006, Lesser 2006, Levy et al. 2006, Merle et al. 2007). Exposure to elevated temperatures can disrupt the photosynthetic apparatus, leading to a build-up of electrons that may react with O_2 molecules and increase the production of ROS such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) (Downs et al. 2002, Tchernov et al. 2004, Smith et al. 2005, Asada 2006). During prolonged periods of stress, ROS are generated in quantities large enough that the algal cells cannot mitigate their production and damage is sustained by cellular components. This damage is not constrained to the algal cell as one of the ROS', H_2O_2, is permeable to cellular membranes, and can move out of the symbiont and into host cells, and potentially trigger the host to expel the algal symbiont (Downs et al. 2002, Smith et al. 2005, Asada 2006, Lesser 2006, Tchernov et al. 2011).

Investigations of the symbiont have discovered a great amount of genetic variability within the *Symbiodinium* genus, which is now classified into 9 different clades, lettered A-I (Pochon & Gates 2010). Further identification within each clade can be determined, for example by sequencing of the internal transcribed spacer (ITS) region, into what will be referred

to as “types” in this paper (LaJeunesse 2001, Coffroth & Santos 2005). Research into *Symbiodinium* physiology using *in hospite* and culture techniques has led to the understanding that physiological variability exists both between and within *Symbiodinium* clades, and this variability extends to their tolerance of stress (Rowan et al. 1997, Tchernov et al. 2004, Goulet et al. 2005, Robison & Warner 2006, Hennige et al. 2009). For example, previous work with cultured *Symbiodinium* has identified types A1, E1, and F2 as tolerant to elevated temperature stress, and type B1 as sensitive (Robison & Warner 2006, Suggett et al. 2008, McBride et al. 2009). Work by Thornhill et al. (Thornhill et al. 2008) found type B2 to be tolerant of lowered temperatures, contributing to its distribution in sub-tropical waters. It is clear that differences exist in the thermal tolerance of *Symbiodinium* types, but the extent of this and the factors conveying tolerance or sensitivity remain unclear.

This study addresses the need for more knowledge from empirical data concerning physiological differences among *Symbiodinium* types in the context of elevated temperatures as an environmental stressor. As such, seven different types of cultured *Symbiodinium* (A1, B1, B2, C1, D, E1, and F2) were exposed to temperatures between 26° and 31°C, and the production of ROS, and the antioxidants CAT and SOD were measured. *Symbiodinium* types found to be tolerant in previous studies, such as A1, were predicted to produce more antioxidants at elevated temperatures, and/or less ROS than more sensitive types, such as B1.

2.2 Methods

2.2.1 Materials

Chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise noted, and all assays read with a Bio-Tek Synergy 2 Multi-detection microplate reader using the Gen-5 software package (Bio-Tek Instruments, Inc. Winooski, VT, USA). Assay plates were manufactured by Corning, Inc. (Lowell, MA, USA).

2.2.2 Symbiodinium Cultures

Symbiodinium (Freudenthal 1962) cultures are identified based on the ITS-2 region as A1, B1, B2, C1, D (ITS type unknown), E1, and F2, and were generous gifts from the collections of Todd LaJeunesse (Pennsylvania State University) and Scott Santos (Auburn University). For a list of host species and geographic origins of all types see Table 2.1. Once received, all *Symbiodinium* cultures were grown in 250 mL polycarbonate culture flasks with 0.2 µm filter tops (IscBioExpress, Kaysville, UT, USA) in ASP-8A media (Chang et al. 1983), and 10 mL were used to seed new subcultures every 3 to 4 weeks. Cultures were maintained in a diurnal growth chamber (Powers Scientific, Inc, Pipersville, PA, USA) at 25°C with 20-watt fluorescent plant growth bulbs under a 14:10 h photoperiod at approximately 55µmol quanta·m^{-2}·s^{-1} irradiance (measured with a LI-COR model LI-250 light meter, LI-COR Environmental, Lincoln, NE, USA).

All experiments used *Symbiodinium* cells in stationary phase that were three to four weeks of age to minimize the effects of senescence (Chang et al. 1983). To normalize algal cells per experiment, cells were pelleted by centrifugation for 18 min at 2530 x *g* on an Eppendorf 5810R centrifuge (Eppendorf AG, Hamburg, Germany) and then standardized to specific concentrations using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA, USA).

2.2.3 Temperature Stress Experiments

All experiments were run in water baths in a temperature and light controlled room. One bath was maintained as a control environment while the temperature of a second bath was manipulated. Water temperatures were maintained or manipulated using Techne Tempette Junior TE-8J (Cambridge, UK) circulating water heaters and monitored with a total immersion thermometer. Irradiance levels were the same as the culture conditions and provided by the same bulb-type as in the growth chamber.

For ROS and antioxidant experiments each *Symbiodinium* type was standardized to 500,000 cells·mL^{-1} by dilution in fresh sterile media, and 15 mL were transferred into clean and sterile polypropylene test tubes (BD Biosciences, San Jose, CA, USA). For ROS studies, three replicate test tubes of each *Symbiodinium* type were placed randomly in each water bath and were rotated every other day to minimize the effect of position. Control baths were maintained at 26°C ±0.5 throughout the experiment. Treatment baths started at 26°C ±0.5, and then were raised by 1°C every 48 h until 31°C was reached to avoid acute temperature stress. After 48 h of exposure to each temperature, 1.3 mL of samples were taken from each test tube on days 8, 10, and 12, at treatment temperatures of 29°, 30°, and 31°C. To prevent interference with the DCFH-DA assay (see below) and immediate scavenging of ROS, cells were maintained in ASP-8A media lacking trace minerals, metals and vitamins. Cell concentration, viability and ROS production were established from cells in each aliquot. For antioxidant studies the design was the same, except that there were 5 replicate test tubes of each *Symbiodinium* type and the entire tube contents were sampled (see below) only on day 12 after 48 h at 31°C.

2.2.4 ROS Analysis

Prior to the assay, samples were incubated in the dark for 20 min to account for normal ROS production during photosynthesis, as cells from different *Symbiodinium* types may photosynthesize at different rates and contain different numbers of chloroplasts. ROS production was quantified after a 5 min incubation of 1 mL of sample, 75 µL of 10 mM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in DMSO and 50 µL of esterase (1 mg·mL^{-1}). DCFH-DA is permeable to cellular membranes and fluoresces in the presence of esterase and ROS (except singlet oxygen), allowing the quantification of extra- and intra-cellular ROS concentrations (Franklin et al. 2004, Mydlarz & Jacobs 2004). The fluorescence of the samples was measured (485 nm excitation, 528 nm emission) in a Costar 12 well plate, and concentration of ROS was determined from calibration to a standard curve of 30% high purity H_2O_2. To adjust for the effect of time each culture had spent in the water baths, net ROS

production was calculated by subtracting the amount of ROS produced in the cells maintained at the control temperature (26°C ±0.5) measured that same day from ROS produced by the cells in the treatment tubes.

2.2.5 Antioxidant Assays

All *Symbiodinium* cells in each replicate were harvested at the end of the experiment (12 d) by centrifugal pelleting and immediately flash freezing in liquid nitrogen. Pellets were then freeze-dried for 24 h on a VirTis Benchtop 6K freeze drier (SP Industries, Stone Ridge, NY, USA). To break open the cells and extract the soluble protein, phosphate buffer (50 mM, pH 7.8) and a spatula tip of borosilicate glass beads (1 mm) were added to each freeze-dried pellet. Pellets were then vortexed on high for 5 min in 20 s intervals. They were maintained on ice during each interval. Samples were then centrifuged for 2-3 min at 4300 x *g* in a microcentrifuge (Baxter Scientific Products, Deerfield, IL, USA) to remove cellular debris. Supernatants were removed and transferred to clean 0.5 mL microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -80°C until analyzed. Total protein for all samples was quantified using the Quick Start™ Bradford Protein Assay kit (Bio-Rad, Hercules, CA, USA). Absorbance was read at 595 nm and standardized to BSA. For all antioxidant assays, activity was normalized to the total protein concentration of each sample. CAT activity was determined using the Amplex Red catalase kit (Invitrogen Corporation, Carlsbad, CA, USA, excitation 540 nm, emission 590 nm), which fluorescently detects the presence of H_2O_2. SOD activity was determined using the SOD Assay kit (Fluka BioChemika, Buchsm, Switzerland, absorbance at 450 nm), which colorimetrically detects the scavenging of O_2^-.

2.2.6 Statistical Analysis

Baseline ROS production for each *Symbiodinium* type was calculated by averaging the control (26°C) sample's ROS production on days 8, 10 and 12 (n=9); comparisons among *Symbiodinium* types were then made using a one-way ANOVA. To calculate net ROS production during temperature stress, ROS values were averaged for all control (26°C)

replicates at that time period, and this averaged value subtracted from each temperature stress replicate of corresponding *Symbiodinium* type (n=3). The effect of *Symbiodinium* type and temperature on net ROS production was then evaluated using repeated-measures ANOVA. Further comparisons within and among *Symbiodinium* types were made using univariate ANOVA and Tukey-Kramer *post-hoc* tests with $\alpha=0.05$. SOD and CAT (n=5) were analyzed using two-way ANOVA with *Symbiodinium* type and temperature as factors. For all analyses, data were first tested for normality and homoscedasticity using Shapiro-Wilks and Levene's tests, respectively. Data that did not conform were power transformed using the Box-Cox Y method to meet the parametric criteria of normality and equal variances. All statistical analyses were performed using JMP Statistical Discovery Software version 8.0 (SAS Institute, Cary, NC, USA).

2.3 Results

2.3.1 Effect of Temperature on ROS Production

There was a significant difference in baseline ROS production among *Symbiodinium* types ($df = 6$, $F = 12.48$, $p < 0.001$, Figure 2.1). Within type, there was no effect of temperature alone ($df = 2$, $F = 2.88$, $p = 0.092$), but there was a significant interaction between temperature and type ($df = 12$, $F = 7.28$, $p < 0.0001$, Table 2.2). Significant differences were observed among *Symbiodinium* types for each temperature (Table 2.2). At 29°C the net ROS production of type F2 was significantly lowest compared to the rest. Those in A1, C1 and E1 types also produced relatively lower ROS. At 30°C A1 produced the most net ROS and was significantly higher than B1 and F2. The greatest differences in net ROS production among types occurred at 31°C (Table 2.3, Figure 2.2). Type C1 showed a four-fold increase in ROS production over all other *Symbiodinium* types, while A1, B2 and E1 had a negative net production of ROS (Figure 2.2).

There was a significant affect of elevated temperature on ROS production in *Symbiodinium* types B2, C1 and E1 (Figure 2.2). C1 was the only type to have a higher net production at 31°C than at lower temperatures and produced four times the amount of ROS than at 29°C (df = 2, F = 24.41, p = 0.0013, Figure 2.2). *Symbiodinium* types B2 and E1 had lower net levels of ROS at 31°C compared to 29°C (B2: df = 2, F = 6.77, p = 0.029 and E1: df = 2, F = 8.68, p = 0.017, Figure 2.2) and produced less ROS than ambient (26°C) controls at 31°C making their net ROS production negative. *Symbiodinium* types A1, B1, D, and F2 showed no significant change at any of the three test temperatures (Figure 2.2).

2.3.2 Effect of Temperature on Antioxidant Activity

Both CAT and SOD were significantly affected by temperature (2-way ANOVA, CAT: df = 1, F = 40.25, p = <0.0001 and SOD: df = 1, F= 7.74, 0.0074) and *Symbiodinium* type (CAT: df = 6, F = 22.84, p <0.0001 and SOD: df = 6, F=32.29, p <0.0001), while the interaction of temperature and type was not significant for either CAT (df = 6, F = 1.76, p = 0.125) or SOD (df = 6, F = 0.951, p = 0.467). CAT activity was the same for all types except E1, which displayed the lowest activity at 26°C and 31°C, and F2, which was highest at 26°C and 31°C (Figure 2.3-A). Greater intertype differences were observed in SOD activity (Figure 2.3-B) with F2 again displaying high values of SOD activity units at 26°C and at 31°C, and E1, the lowest values of at 26°C and at 31°C.

Antioxidant activity was elevated in response to temperature stress for only a few *Symbiodinium* types. Types A1, B2 and F2 all induced their CAT activity with an increase in temperature from 26°C to 31°C (Figure 2.3-A). For types A1 and F2, SOD activity was positively correlated to elevated temperature as both types elevated their activity of SOD in response to increasing temperatures (Figure 2.3-B).

2.4 Discussion

Microalgal stress responses have the potential to influence ecosystems through organismal level processes, especially in regards to global climate change. This is especially true in microalgae that live symbiotically with ecologically important hosts, such as the *Symbiodinium*-cnidarian relationship. The genetic diversity within the *Symbiodinium* genus has led to questions concerning how variable their physiology may be, particularly in response to factors associated with climate change such as elevated temperatures. Investigations have begun to tease apart the complex responses, and have led to the determination that responses are not uniform either within or among clades. Increased production of ROS is an indicator of algal stress (Lesser 1996, Suggett et al. 2008, Saragosti et al. 2010), while increased antioxidant activity may provide the algae with some level of tolerance to increased ROS output (Lesser 1996, Yakovleva et al. 2004). Our results support previous findings by demonstrating that each *Symbiodinium* type displays a distinct stress response, and the sensitivity to elevated temperatures varies between types.

At ambient temperature and without stress, ROS production and release differed significantly among *Symbiodinium* types. Types C1 and D were among the highest producers of ROS under basal conditions of 26°C, while types B1 and F2 were among the lowest. This pattern changed as temperatures were raised, and at 29° and 30°C net ROS production was similar among all *Symbiodinium* types. Differences emerged at 31°C, with type C1 still among the highest ROS producers. Type B1 was also among the highest, in contrast to being among the lowest at 26°C. Within each *Symbiodinium* type little significant variation was observed, suggesting that under these conditions, these cultures may have a higher threshold of tolerance that exceeds 31°C. *Symbiodinium* type C1 had the greatest response, increasing ROS production four-fold at 31°C, while types B2 and E1 had surprisingly significant decreases in net ROS production.

The activity of the antioxidants CAT and SOD also varied between algae. CAT activity was the same among types A1, B1, B2, C1, and D. Type E1 had the lowest production of CAT while type F2 had the highest. SOD activity was more variable, with types C1 and F2 having the highest activity and types A1 and E1 the lowest. We predicted that as a mechanism to offset increased ROS production during exposure to elevated temperatures some *Symbiodinium* types would increase antioxidant activity to scavenge ROS shortly after its generation. *Symbiodinium* type B2 did up-regulate the production of CAT and produced less ROS at 31°C than at 26°C, while types A1 and F2, which showed no change in ROS production with temperature stress, increased the production of both CAT and SOD. No increases in antioxidants were observed for types B1, C1, D or E1, and it is possible that a longer exposure to elevated temperatures may be necessary to induce a more discernable response within these *Symbiodinium* types (Lesser 2006). Alternatively, for types B1, D and E1 it is possible that at elevated temperatures the kinetics of the antioxidants was sufficiently increased so that the same amount of antioxidant was sufficient to keep ROS levels from inducing further upregulation. In either case, it may be that the mechanisms responsible for the observed tolerance of some *Symbiodinium* types, like A1 and F2, is due to a rapid active response, preventing or at least delaying the onset of oxidative damage from ROS that can lead to cell death. If this is true, there may be tradeoffs in genetic traits or expression of genetic traits *i.e.,* phenotypes, that were beyond the scope of this study such as growth rates or photosynthetic repair rates (Robison & Warner 2006).

After synthesis of these responses to higher thermal stress, types A1, D, E1, and F2 display attributes of tolerance, while B1 and C1 seem to be sensitive. *Symbiodinium* type C1 demonstrated a distinct sensitivity to thermal stress with the greatest ROS release but no significant increases in antioxidants to mitigate oxidative stress. While the within type results showed a non-significant increase in net ROS production for type B1, it did produce more ROS among types at 31°C than 26°C, and also did not increase the activity of CAT or SOD. The

antioxidant activity of both B1 and C1 was not significantly different than most of the other *Symbiodinium* types, suggesting that they may not be capable of sufficiently scavenging the excess ROS, particularly the four-fold higher ROS production in C1. Thus, it is likely that significant oxidative damage is occurring to the algal cells. It can be further hypothesized that if compromised cellular integrity cannot be repaired, acclimatization of these *Symbiodinium* types to high temperature may not be possible.

In contrast, *Symbiodinium* types A1, B2, and F2 all displayed responses that appeared to indicate active responses to mitigate damage due to thermal stress. Type B2 showed a significant decrease in net ROS production in addition to a significant increase in CAT activity, while *Symbiodinium* types A1 and F2 had no change in net ROS production and an increase in CAT and SOD activity. This upregulation may be sufficient to scavenge ROS as it is produced and prevent oxidative damage to the cell. *Symbiodinium* type E1 also had a significant decrease in ROS production, but without a change in antioxidant activity, suggesting effective scavenging of ROS by a different antioxidant such as ascorbate peroxidase (Lesser 1996, Shigeoka et al. 2002).

It is notable that *Symbiodinium* type D was the only type where all three physiological measures were unaffected by elevated temperatures. This suggests alternative mechanisms that prevent ROS up-regulation in the first place, such as a more stable photosynthetic apparatus. If these factors are inherent to the *Symbiodinium* type, such as in the lipid composition of their thylakoid membranes (Tchernov et al. 2004), energy and resources may be utilized for growth instead of diverted to the production of compounds for protection or repair.

These responses are consistent with previous findings (Robison & Warner 2006, Suggett et al. 2008, McBride et al. 2009) and expand the knowledge base to a broader comparison of *Symbiodinium* types. The variable responses observed among *Symbiodinium* types indicate a range of sensitivity to elevated temperatures unique to each type. In addition to the factors studied here, many other responses, such as production of mycosporine-like amino

acids (Lesser 1996, Banaszak et al. 2006), composition of cellular membranes (Mydlarz & Jacobs 2004, Tchernov et al. 2004), photosynthetic repair mechanisms (Takahashi et al. 2009, Ragni et al. 2010), and growth rates (Kinzie et al. 2001, McBride et al. 2009) may contribute to understanding the *Symbiodinium* stress response. It is unlikely that any one of these factors will explain the stress response of all *Symbiodinium*; thus an understanding of how each one contributes as well as the synergy between temperature and increased irradiance (Lesser 1996, Rowan et al. 1997, Bhagooli & Hidaka 2003, Robison & Warner 2006, Suggett et al. 2008) is essential. Placing these responses in an ecological context requires a greater understanding of how the stress response is affected by an intact symbiosis, incorporating algal symbiont identity beyond the cladal level and host genotype (Goulet et al. 2005, Baird et al. 2009, Tchernov et al. 2011). Fully characterizing *Symbiodinium* physiology, both in culture and *in hospite*, is necessary to gain insight into the effects of climate change *e.g.*, higher temperature, on coral reefs.

Table 2.1 The host species and geographic origins for each type of *Symbiodinium* used in these studies. For all clades but D, information is found in LaJeunesse (2001). For clade D, see the *Symbiodinium* Cultures database hosted by Dr. Scott Santos at http://www.auburn.edu/~santosr/phplabware.htm.

Identification number	Clade	ITS-2 Type	Geographic Origin	Host Origin
61	A	1	Caribbean, FL	*Cassiopeia xamachana*
147	B	1	Caribbean, Jamaica	*Pseudoterogorgia bipinnata*
141	B	2.1	Western Atlantic, Bermuda	*Oculina diffusa*
152	C	1	Caribbean, Jamaica	*Discosoma sancti-thomae*
10.8A	D	[a]	Caribbean, FL	*Montastraea faveolata*
383	E	1	East Pacific, CA	*Anthopleura elegantissima*
133	F	2	Caribbean, Jamaica	*Meandrina meandrites*

([a]) Unknown ITS type

Table 2.2 Repeated measures ANOVA results for temperature effects on *Symbiodinium* type's reactive oxygen species (ROS) production. Data are for the net production of ROS, i.e. ROS production at elevated temperature – ROS production at basal, 26°C. Underlined p values are statistically different (α= 0.05).

Repeated Measures ANOVA	F	df	p
Within Symbiont Type			
Wilks-Lambda	7.28	12	< 0.0001
Temperature	2.88	2	0.092
Type*Temperature	7.28	12	< 0.0001
Among Symbiont Types			
F-Test	8.08	6	0.0007
Univariate Test			
29°C	4.22	6	0.013
30°C	4.42	6	0.010
31°C	20.26	6	< 0.0001

Table 2.3 Net production of reactive oxygen species (ROS), i.e. ROS production at elevated temperature – ROS production at basal, 26°C among types at each treatment temperature (29°, 30° or 31°C). Significant differences between types within each temperature are denoted by different letters (α=0.05).

	Net ROS production (ρmol cell^{-1})		
Symbiodinium type	29°C	30°C	31°C
A1	1.67 ± 0.21^{y}	3.05 ± 1.24^{yz}	-0.68 ± 0.84^{xyz}
B1	0.38 ± 0.23^{yz}	-1.05 ± 0.51^{yz}	2.22 ± 0.56^{x}
B2	1.19 ± 0.30^{yz}	0.033 ± 0.83^{z}	-2.65 ± 0.97^{yz}
C1	1.69 ± 0.51^{y}	0.40 ± 0.60^{y}	6.99 ± 0.96^{w}
D	0.46 ± 0.25^{yz}	0.32 ± 0.16^{yz}	0.66 ± 0.14^{xy}
E1	1.64 ± 0.74^{y}	0.70 ± 0.66^{yz}	-2.98 ± 1.03^{z}
F2	-0.43 ± 0.21^{z}	-1.41 ± 0.10^{z}	1.54 ± 0.24^{x}

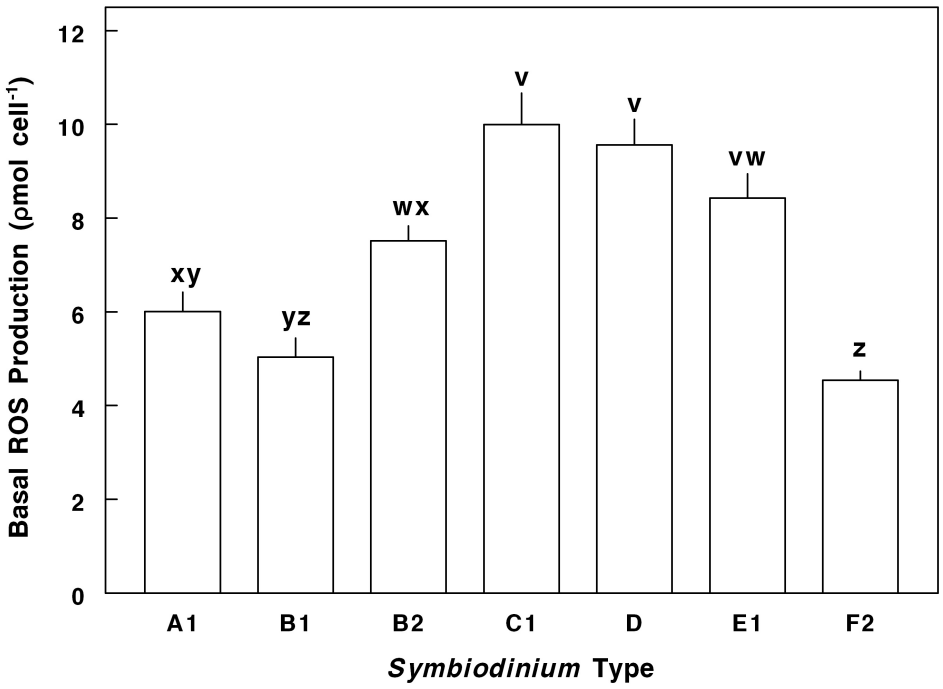

Figure 2.1 Baseline production of reactive oxygen species (ROS) (mean ± s.e, n=9) for all *Symbiodinium* types at 26°C. Letters v, w, x, y or z denote significant differences (α=0.05) in baseline ROS production; values which do not share a letter are statistically different.

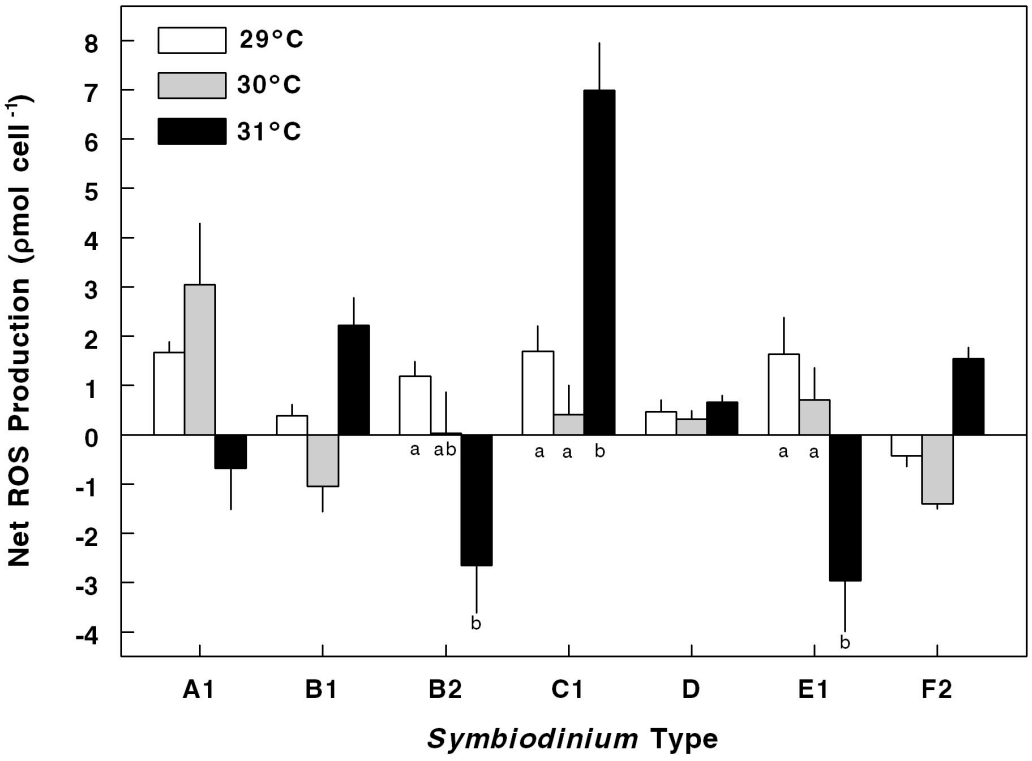

Figure 2.2 Differences in net production of reactive oxygen species (ROS) i.e. ROS production at elevated temperature – ROS production at basal, 26°C within *Symbiodinium* types at 29°, 30°, and 31°C (mean ± s.e., n=3). For those types (B2, C1 and E1) that had significant differences in ROS net production with temperature, letters a or b below the values indicate significant differences among temperature treatments. For significant differences (α=0.05) in net ROS production among types at each treatment temperature (29°, 30° or 31°C) see Table 2.3.

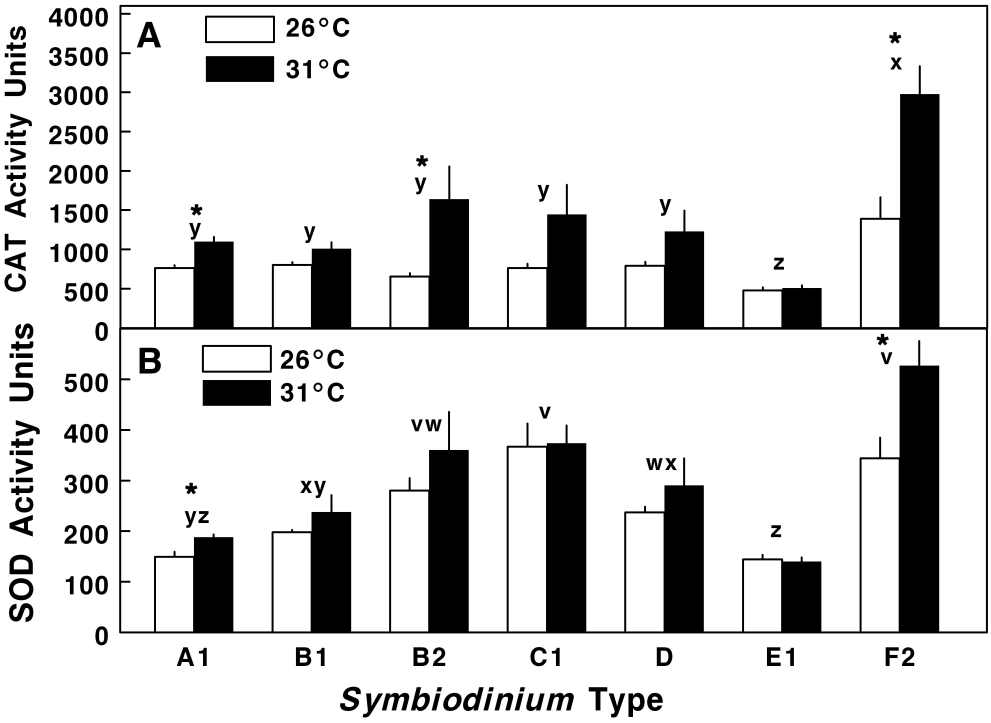

Figure 2.3 A) Catalase (CAT) activity units (mean ± s.e., n=5) and B) Superoxide dismutase (SOD) activity units (mean ± s.e., n=5). Since the interaction between *Symbiodinium* type and temperature was not significant, the between *Symbiodinium* type significant differences (α=0.05) in CAT or SOD activity are indicated by the letters v, w, x, y or z. In both panels, asterisks above paired bars for any one type indicate that there was a significant difference (α=0.05) in CAT or SOD activity between the 27° and 32°C treatment temperatures.

CHAPTER 3

SOME DON'T MIND IT HOT - ELEVATED TEMPERATURES DIFFERENTIALLY AFFECT *SYMBIODINIUM* TYPES METABOLIC AND BLEACHING RESPONSES

3.1 Introduction

Warming trends associated with climate change have already led to a 0.74°C rise in global temperatures over the last 100 years, and with conservative models predicting another 2°C increase by the next century (Solomon et al. 2007), understanding how organisms respond to temperature stress is a critical aspect of predicting ecosystem responses (Davison 1991, Walther et al. 2002, Pötner & Farrell 2008, Hofmann & Todgham 2010, Hale et al. 2011). The physiological changes organisms will experience due to rising temperatures will range, and be determined by their breath of thermal tolerance. Within their tolerance limits, exposure of organisms to increasing environmental temperatures could result in increased metabolic rates, enzymatic reaction rates, accumulation of protein isoforms that are more stable at higher temperatures, and production of compounds that stabilize, repair or replace damaged proteins. It could also lead to reductions in the fluidity of cellular membranes (Davison 1991, Beardall & Raven 2004, Hofmann & Todgham 2010). Once organisms pass their tolerance limits negative consequences due to elevated temperatures will quickly accumulate, such as build up of damaged proteins, impaired enzymatic reactions (Hofmann & Todgham 2010) and disruption of membranes (Tchernov et al. 2004, Hofmann & Todgham 2010), diminishing their capacity to survive.

For primary producers in any system, the interplay between metabolic rates and photosynthetic rates adds a level of complexity in the response to climate changes not present

in heterotrophic organisms due to effects on photosynthetic machinery (Berry & Björkman 1980, Davison 1991, Beardall & Raven 2004). Elevated temperatures can disrupt the photosynthetic apparatus (Berry & Björkman 1980, Lesser 1996), leading to a degradation of photosynthetic pigments (Berry & Björkman 1980, Dove et al. 2006, Venn et al. 2006), which in turn will lower photosynthate production. Ultimately, this may create a situation where the organism cannot meet its own metabolic demands.

Organisms that have evolved in the tropics where temperatures are relatively stable, particularly in marine environments, tend to have narrow thermal tolerance limits (Hoegh-Guldberg et al. 2007, Hofmann & Todgham 2010, Sunday et al. 2010, Hale et al. 2011), which creates unique problems for organisms that form symbiotic relationships with photosynthesizers (Venn et al. 2008, Yellowlees et al. 2008, Hofmann & Todgham 2010). This is particularly true of reef-building corals which obtain more than 90% of the energy required to support their metabolic demands from their algal endosymbionts, dinoflagellates belonging to the genus *Symbiodinium* (Muscatine & Porter 1977). Already, elevated temperature anomalies, especially when in conjunction with increased irradiance, have been linked with worldwide "bleaching events," conditions where the host loses most of its algal symbionts or their associated pigments, particularly chlorophyll *a* (Chl *a*) (Brown 1997). When this condition persists, it can have severe ramifications for the host, such as increased susceptibility to disease, diminished reproduction, and increased likelihood of mortality. Severe bleaching events have led to significant global declines in coral reef cover (Carpenter et al. 2008, Mydlarz et al. 2010).

The bleaching response is not uniform for all coral species, and differences have been correlated with genetic differences found in *Symbiodinium* communities (Rowan et al. 1997, Baker et al. 2004, Jones et al. 2008). The genus is very diverse, consisting of 9 different clades that are lettered A-I (Pochon & Gates 2010), with an unknown number of unique species within each clade (Sampayo et al. 2009, LaJeunesse & Thornhill 2011). *Symbiodinium* are commonly identified using the sequence of the internal transcribed spacer-2 (ITS-2) rDNA region and

referred to as “types” (LaJeunesse 2001, Coffroth & Santos 2005). Studies examining the relationship between *Symbiodinium* identity and potential mechanisms behind this variability have found variation in a number of physiological responses as indicators of thermal tolerance or sensitivity. These include the repair of photosystem proteins (Warner et al. 1999, Takahashi et al. 2009), production of antioxidants (Lesser 1996, McGinty et al. 2012), production of photoprotective accessory pigments such as β-carotene (Lesser 2006, Venn et al. 2006, Apprill et al. 2007, Lee et al. 2012), production of protective molecules like heat shock proteins and mycosporine-like amino acids (Lesser 1996, Dunlap & Shick 1998, Banaszak et al. 2000, Rosic et al. 2011), and saturation of thylakoid membranes (Tchernov et al. 2004). This leaves little doubt that the fitness of some algal species under future climate models will vary, but how those variations translate into the performance of these algae at elevated temperatures, and how that may vary across algal species, is still unclear.

As important primary producers in the coral reef ecosystem, understanding *Symbiodinium* physiology is important for understanding how coral reefs will respond to increasing ocean temperatures. The cascade of factors driven by increasing temperatures will potentially have dramatic effects for the survival of both the symbionts and their hosts. To examine a potential range of algal responses, this study measured parameters of survival and growth across a range of temperatures from genetically distinct algal cultures representing four different clades and isolated from various cnidarian hosts and geographic origins. The dark oxygen uptake rates ($\dot{V}o_2$) as an indirect measure of metabolic rate in six *Symbiodinium* types were recorded in response to acute temperature stress to determine the temperature at which maximum $\dot{V}o_2$ was attained. A subset of these *Symbiodinium* types was then further examined with a measurement of growth rates at three temperatures representing a non-stressful temperature (26°C), a moderately elevated sub-lethal temperature (30°C), and an elevated and potentially lethal temperature (34°C). The fluorescence of Chl *a* and β-carotene was also monitored at these three temperatures over time to identify potential bleaching responses

(Apprill et al. 2007, Lee et al. 2012). I predicted that *Symbiodinium* species previously found to be "sensitive" to elevated temperatures (Robison & Warner 2006, Suggett et al. 2008, McGinty et al. 2012) would attain maximum $\dot{V}o_2$ at a lower relative temperature and have diminished growth rates and rapid loss of Chl *a* at 34°C. In contrast, types that have previously demonstrated relatively higher temperature tolerance (Robison & Warner 2006, Suggett et al. 2008, McGinty et al. 2012) were predicted to attain maximum $\dot{V}o_2$ at greater temperatures, and have rates of growth and Chl *a* loss less adversely affected by elevated temperatures.

3.2 Methods

3.2.1 Symbiodinium *culture conditions*

Symbiodinium (Freudenthal 1962) cultures were generous gifts from the collections of Todd LaJeunesse (Pennsylvania State University). Algal identification was based on the ITS-2 region as A1 (*Symbiodinium microadriaticum,* Trench and Blank, 1987), A1 (designated A1* to provide clarity for the purposes of this study), B1, B2 (*Symbiodinium psygmophilium*, (LaJeunesse et al. 2012)), E1, and F2, following the methods of LaJeunesse (2001). The host species, geographic origins and average cell size of all types are listed in Table 3.1. Stock *Symbiodinium* cultures were maintained in a diurnal growth chamber (Powers Scientific®, Inc, Pipersville, PA, USA) at 25.5°C under 20-watt fluorescent plant growth bulbs with a 14:10 h photoperiod at approximately 55 µmol quanta·m^{-2}·s^{-1} irradiance (measured with a LI-COR® model LI-250 light meter, LI-COR® Environmental, Lincoln, NE, USA). Cultures were grown in 500 mL polycarbonate culture flasks with 0.2 µm filter tops (IscBioExpress, Kaysville, UT, USA) in ASP-8A media (Chang et al. 1983), and new subcultures were seeded every 3 to 4 weeks.

Table 3.1 *Symbiodinium* clade, type as designated by the ITS-2 region, host and geographic origins, and average cell diameter (µm). Two types are identified as A1 but have different origins, so one will be distinguished with a (*) for clarity.

Clade	ITS-2 type	Host origin	Geographic origin	Average cell diameter (µm)
A	1	*Cassiopeia xamachana*	Caribbean, FL	8.91 µm ± 0.095
A	1(*)	*Stylophora pistillata*	Red Sea, G of Aqaba	9.09 µm ± 0.038
B	1	*Pseudoterogorgia bipinnata*	Caribbean, Jamaica	7.84 µm ± 0.037
B	2.1	*Oculina diffusa*	W. Atlantic, Bermuda	7.70 µm ± 0.15
E	1	*Anthopleura elegantissima*	East Pacific, CA	12.31 µm ± 0.059
F	2	*Meandrina meandrites*	Caribbean, Jamaica	8.74 µm ± 0.048

3.2.2 Oxygen consumption rates

Volume-specific algal oxygen consumption rates (Vo_2) were measured between 25°-37°C at one degree intervals for all types except A1* and F2 for which it was measured up to 39°C and 42°C, respectively. All cultures were between weeks 3 and 4 of growth to remove the effect of age. Because *Symbiodinium* cultures are not axenic, initial *Symbiodinium* samples from types A1, A1*, B1, B2, E1, and F2 were spun down at 2530 x *g* for 18 min, and the supernatant was discarded to minimize the bacterial contribution to O_2 uptake. The remaining pellet was then resuspended in sterile ASP-8A media, and a small aliquot (< 500 µl) was removed and visually inspected for bacterial presence and to determine cell concentration and size. Cell concentration for each sample was established by replicate counts (n = 8 counts) on a Bright-Line® hemacytometer (Hausser Scientific®, Horsham, PA, USA). Average cell volume was established from pictures (n=10 cells per temperature) that were taken using a Nikon Optiphot® microscope equipped with a Nikon DXM1200F® digital camera through a 40X objective lens using the Nikon NIS-Elements® software package (Nikon Instruments®, Melville, NY, USA).

$\dot{V}o_2$ was measured using Clark-type oxygen electrodes connected to a YSI® (Yellow Springs Instruments®, Yellow Springs, OH, USA) Model 5300 Biological Oxygen Monitor, and changes in O_2 concentration were recorded using a Kip and Zonen®, Model BD-41, dual channel strip-chart recorder. Test temperatures (± 0.5°C) were maintained with a Lauda K-2/R® refrigerated constant temperature circulator (Brinkman Instruments®, Delran, NJ, USA). At each test temperature, 3 ml of media containing *Symbiodinium* types (n = 4) were placed in respiration chambers which were held open to the atmosphere for ~20 min. Magnetic stirrers on the bottom of each chamber brought the media to experimental temperature and aerated the samples to near full air saturation with atmospheric oxygen prior to measuring $\dot{V}o_2$. Simultaneously, electrodes were also brought to the experimental temperature. Chambers containing 3 ml of sterile media were concurrently brought to full air O_2 saturation by ~20 min stirring while open to the atmosphere. The electrodes were then inserted into chambers containing sterile media and *Symbiodinium* type cultures creating a seal, and chamber O_2 concentration was continuously monitored for ~20 min (McMahon & Russell-Hunter 1981, Iglesias-Prieto et al. 1992, Karako-Lampert et al. 2005). Oxygen electrodes were equilibrated to 100% air O_2 saturation in blank chambers containing 3 ml of continuously stirred deionized water between algal medium and blank medium oxygen uptake determinations. Barometric pressure was determined during testing to allow adjustment of $\dot{V}o_2$ rates to a standard atmospheric pressure at sea level of 101.3 kPa (760 mmHg). All $\dot{V}o_2$ determinations were recorded in the dark to prevent O_2 generation by photosynthesis. The $\dot{V}o_2$ of test cultures was adjusted to account for any electrode drift or O_2 consumption recorded in media blanks. Algal respiration rates were standardized using cell concentrations, average cell volume, and O_2 solubility at the experimental temperature to calculate final respiration rates as µl O_2•µl cell volume^{-1}•hr^{-1} at a standard temperature (0°) and atmospheric pressure (101.3 kPa).

3.2.3 Growth rates and bleaching response

Types A1, B1 and F2 were chosen for the growth study. Types A1 and F2 had previously been demonstrated to be tolerant of, and type B1 sensitive to, elevated temperature and irradiance stress (Robison & Warner 2006, Suggett et al. 2008, McGinty et al. 2012). Cultures in the exponential growth phase were allowed to settle and media was removed; cells were then resuspended in new media to approximately $3x10^5$ cells ml^{-1}. Forty milliliter aliquots were then distributed into replicate flasks, with 12 replicate flasks for each *Symbiodinium* type distributed randomly throughout 12 water baths. Replicate water baths (n = 4) at 26°, 30° and 34°C ± 1°C were set up with two Rio® 200 (TAAM inc®, Camarillo, CA, USA) submersible water pumps and one submersible water heater (Hydor USA Inc®, Sacramento, CA, USA) to insure constant water circulation and even temperature distribution within the baths. Light levels were similar to those in the incubator (i.e., under 40-watt fluorescent plant growth bulbs with a 14:10 h photoperiod at approximately 55 μmol quanta·m^{-2}·s^{-1} irradiance as measured with a LI-COR® model LI-250 light meter, (LI-COR Environmental®, Lincoln, NE, USA). Cultures were grown in 26°C baths for at least one month prior to the beginning of this study. All baths started at 26°C, and elevated temperature baths were increased by 2°C per day until experimental temperature was reached. Culture aliquots of 500 μl were taken 36 hours after all baths had reached their experimental temperature (day 2), and then daily from day 4 to day 16. All samples were collected between 09:00 and 10:00 to minimize any variation in pigment fluorescence due to diel cycle. Aliquots were briefly sonicated for 7 s at 3W (Misonix Microson Ultrasonic Cell Disruptor, Misonix Inc®, Farmingdale, NY, USA) to disrupt clumps of cells. Previous studies confirmed that this did not decrease cell concentrations or pigment fluorescence.

Cell density was established using a BD LSR-II Flow Cytometer® (BD Biosciences®, San Jose, CA, USA) using a 488 nm argon laser (20 mW), and gated based on size and Chl *a* and β-carotene fluorescence. Any object less than 4 μm in diameter was excluded using

forward scatter standardized to 3, 7, 10 and 14 µm polystyrene beads (Spherotech Inc.®, Lake Forest, IL, USA). Red fluorescence from Chl *a* was detected with a 695/40 nm band-pass filter, and green fluorescence from β-carotene with a 530/30 nm band-pass filter to identify healthy and bleached *Symbiodinium* cells (Apprill et al. 2007, Lee et al. 2012). Plots of red versus green fluorescence allowed the identification of healthy populations exhibiting high fluorescent signals from both red and green wavelengths (P1, Figure 3.1), while cells with diminished Chl *a* pigments but retaining β-carotene were identified as bleached (P2, Figure 3.1). Data was analyzed using FACS DiVa software (BD Biosciences®, San Jose, CA, USA), and growth rates (µ) calculated as $\mu = (\ln N_{t2}/N_{t1})/(t_2-t_1)$, where t is the time of sampling (day) and N_t is the cell concentration on that day. Samples were periodically monitored by parallel hemacytometer counts to confirm flow cytometer counts and bleaching analysis. On day 16, cell viability was confirmed, especially for the visually bleached cells, using the stain Trypan Blue.

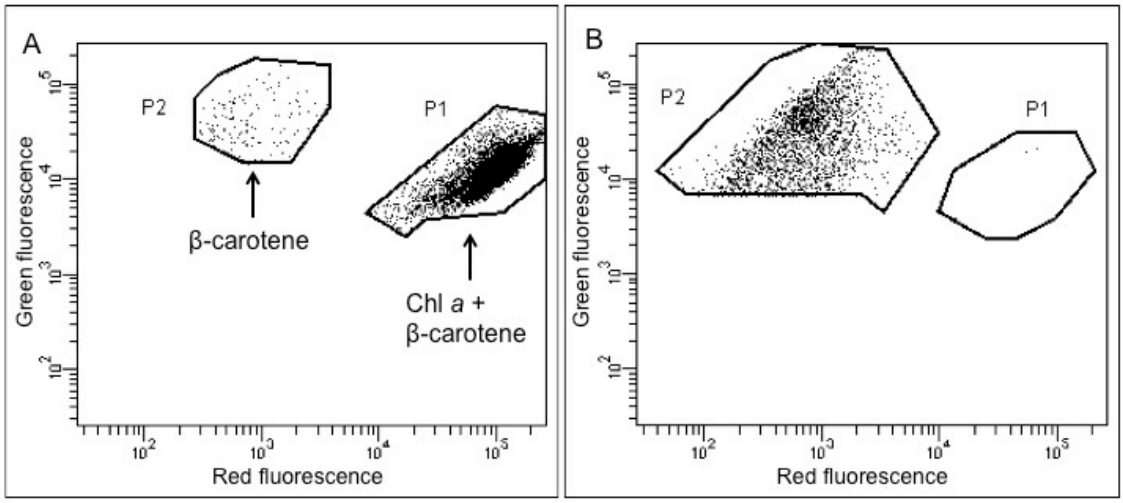

Figure 3.1 Normally pigmented *Symbiodinium* cells (P1) were distinguished from bleached cells (P2) based on Chl *a* fluorescence. A - Healthy population of *Symbiodinium* cells. B - Bleached population of *Symbiodinium* cells.

3.2.4 Statistical Analyses

Algal $\dot{V}o_2$ at each test temperature were calculated as mean µl O_2 consumed per h (n = 4) standardized to a total cell volume of 1 µl at a standard temperature of 0°C and standard atmospheric pressure of 101.3 kPa (i.e., µl O_2•µl cell volume^{-1}•hr^{-1}). Q_{10} values estimating response to temperature over a 10°C temperature increase were estimated for each of the six *Symbiodinium* types. These values indicate how responsive an organism is to changes in temperature, and higher relative Q_{10} values indicate that increases in temperature induce larger effects. Q_{10} values were estimated from a least squares exponential regression of mean $\dot{V}o_2$ values as the dependent variable and test temperature as the independent variable [$Y = a(b^X)$] where Y = estimated $\dot{V}o_2$, b = test temperature in °C, and a and X are constants determined by exponential regression equations (Table 3.2). The exponential regressions were used to estimate $\dot{V}o_2$ for each *Symbiodinium* type at the starting test temperature of 25°C and at the test temperature at which peak $\dot{V}o_2$ was attained. These $\dot{V}o_2$ values were then used to compute Q_{10} values for each species as follows $Q_{10} = (R_2/R_1)^{(10/(T2-T1))}$ where R_1 and R_2 were $\dot{V}o_2$ at the lower and higher test temperatures, respectively, and T1 and T2 were the lower and higher test temperatures, respectively. Q_{10} values were also determined across a temperature range of 25-32°C for all *Symbiodinium* types except for type B1 which reached maximum $\dot{V}o_2$ at 31°C for which Q_{10} was computed over 25-31°C.

Growth data were analyzed using a two-way ANOVA, with *Symbiodinium* type and temperature as the main effects, and Tukey-Kramer *post-hoc* analyses were used to examine differences within and among *Symbiodinium* types. The bleaching responses among types were analyzed using a repeated measures ANOVA with type and temperature as the main effects. The amount of bleaching among temperatures within each *Symbiodinium* type was compared using Student's *t-test*. All data were first checked for normality and unequal variances with the Shapiro-Wilks and Levene's tests respectively. All $\alpha = 0.05$, and analyses

were performed using JMP Statistical Discovery Software version 9.0 (SAS Institute, Cary, NC, USA).

3.3 Results

3.3.1 Oxygen consumption rates

Each *Symbiodinium* type demonstrated unique patterns of $\dot{V}o_2$ across temperature (Figure 3.2, Figure 3.3), and achieved maximum $\dot{V}o_2$ at different temperatures (Table 3.2, Figure 3.3). Q_{10} values for maximum $\dot{V}o_2$ were varied, ranging from 2.66 for type E1 to 6.06 for type F2 (Table 3.2). For all Symbiodinium types, O_2 consumption occurred at all temperatures, indicating that acute exposure to elevated temperatures up to 37°C (39°C for A1*, 42°C for type F2) did not result in cell death. Because some of the maximum respiration rates occurred outside of the temperature range these algae would experience under natural conditions, Q_{10} values were also calculated based on respiration rates to 32°C (except for type B1, which reached maximum respiration rates at 31°C) to compare their responsiveness under ecologically relevant temperatures (Table 3.3, Figure 3.4). Types A1* and F2 demonstrated the lowest values, 1.06 and 0.73 respectively (Table 3.3).

Table 3.2 *Symbiodinium* oxygen consumption response to elevated temperatures. Maximum oxygen consumption rates ($\dot{V}o_2$, mean ± s.e., n = 4), the temperature where maximum $\dot{V}o_2$ was attained, exponential regression equations for oxygen consumption rates (25° to maximum $\dot{V}o_2$ temperature) and Q_{10} values

Symbiodinium type	Maximum $\dot{V}o_2$ ($\mu l\ O_2 \bullet \mu l\ cell\ volume^{-1} \bullet hr^{-1}$)	Maximum $\dot{V}o_2$ temperature (°C)	Exponential regression equation	Q_{10} at maximum temperature
F2	0.013 ±0.0012	40	$y = 7^{E-05}e^{0.1431x}$	6.06
A1*	0.020 ±0.00057	35	$y = 0.0002e^{0.116x}$	3.19
E1	0.0049 ±0.00023	34	$y = 3^{E-05}e^{0.1677x}$	2.66
A1	0.010 ±0.00071	33	$y = 3^{E-05}e^{0.1759x}$	4.18
B2	0.0072 ±0.00016	32	$y = 0.0001e^{0.0978}$	5.81
B1	0.0058 ±0.00043	31	$y = 0.0011e^{0.0374x}$	5.35

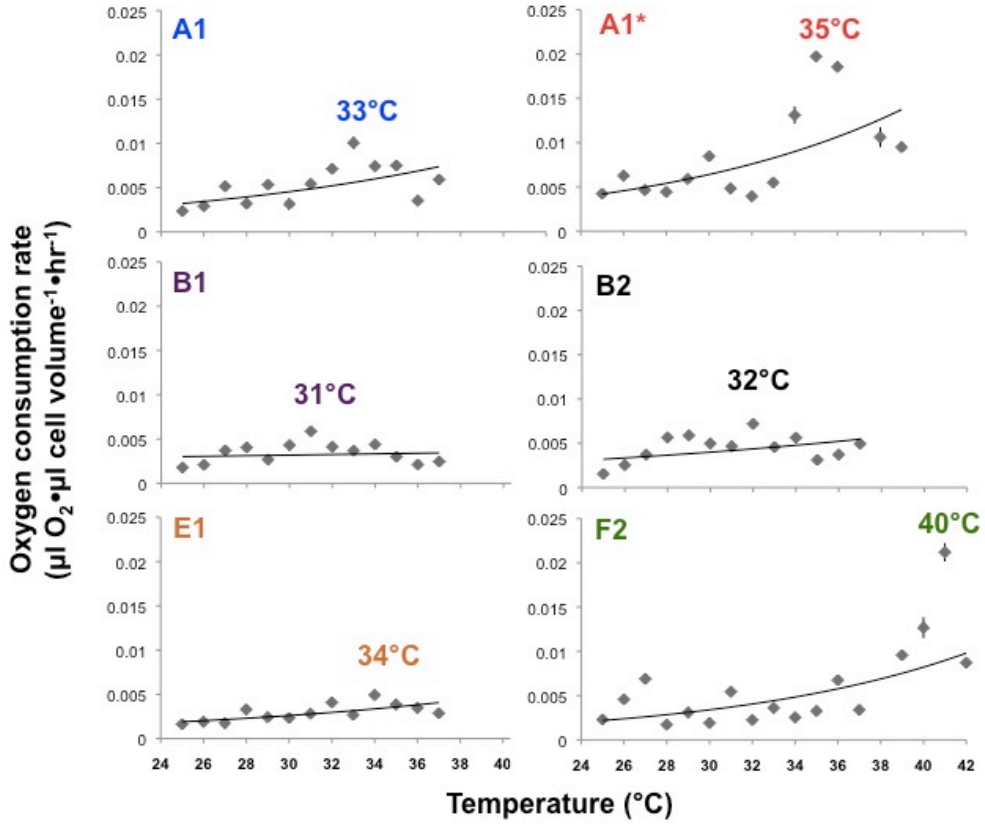

Figure 3.2 Mean oxygen consumption rates ($\dot{V}O_2$, ± s.e.) (µl O_2•µl cell volume^{-1}•hr^{-1}) of *Symbiodinium* cultures during acute exposure to treatment temperature (A1, B1, B2, E1: 25°-37°C; A1*: 25°- 39°C; F2: 25°-42°C) (n = 4). Temperature where each type reached maximum $\dot{V}O_2$ is listed on each graph.

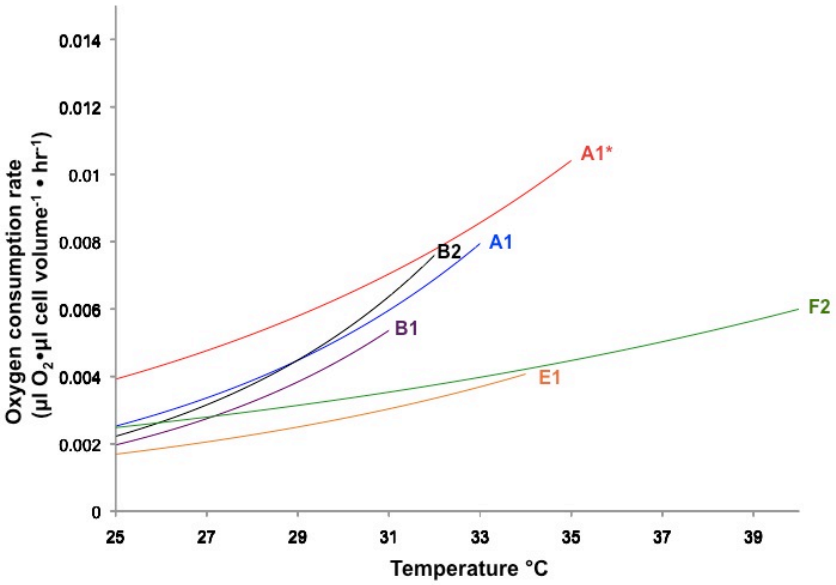

Figure 3.3 Fitted exponential curves for *Symbiodinium* oxygen consumption rates from 25°C until maximum temperature (A1 - 33°C, A1* - 35°C, B1 - 31°C, B2 - 32°C, E1 - 34°C, F2 - 40°C)

Table 3.3 Oxygen consumption rates ($\dot{V}o_2$) and Q_{10} values for *Symbiodinium* types across 25° to 32°C, except for type B1 which reached maximum $\dot{V}o_2$ at 31°C.

Symbiodinium type	Respiration rates at 32°C (µl O_2•µl cell volume^{-1}•hr^{-1})	Q_{10} at 32°C
F2	0.0022 ±0.000098	0.73
A1*	0.0040 ±0.00023	1.03
E1	0.0041 ±0.00021	2.91
A1	0.0071 ±0.00014	3.25
B2	0.0047 ±0.00018	5.81
B1	0.0059 ±0.00043	5.35

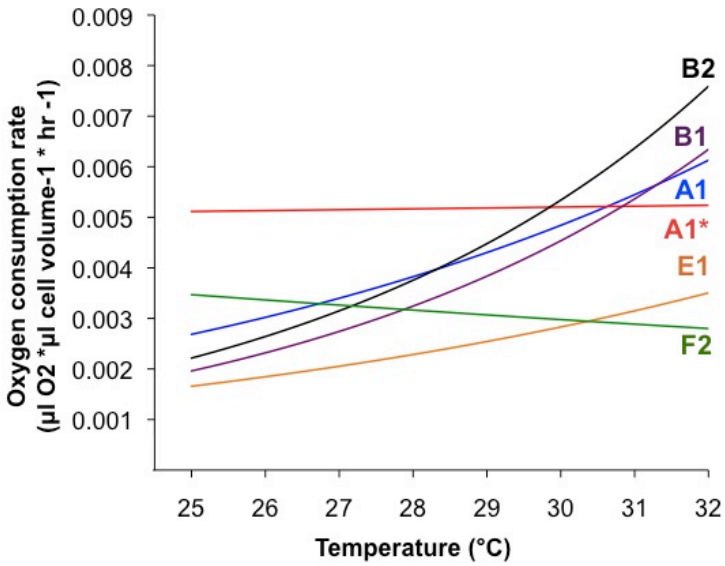

Figure 3.4 Fitted exponential curves for *Symbiodinium* oxygen consumption rates (25°-32°C, n = 4 per temperature).

3.3.2 Growth rates and bleaching response

There were significant effects of type and temperature on *Symbiodinium* growth rates (Two-way ANOVA, df = 8,25, F = 3.44, p = 0.023). Growth rates for all *Symbiodinium* types were similar at 26°C and 30°C and showed no significant decrease in growth at 30°C (Table 3.4, Figure 3.5). All types had significantly lower growth rates at 34°C, with types A1 and B1 having negative and type F2 positive growth at this elevated temperature (Table 3.4, Figure 3.5).

Bleaching response varied among types (repeated measures ANOVA, Type*Temperature df= 4,21, F = 46.45, p <0.0001). After 16 d, bleached cells made up less than 15% of the population for all types at 26° and 30°C (Table 3.4). Cultures were considered fully bleached after >97% of the cells fell into P2 (low Chl *a* fluorescence). Type B1 was fully bleached after 7 days of exposure to 34°C, and type A1 after 10 days (Figure 3.6). After 16

days at 34°C, 7.09 % ± 0.71 of type F2 cells were bleached, which was significantly more than those grown at 26°C and 30°C (ANOVA, df = 2,9, *t*-ratio 12.912, *p* = 0.0115, Table 3.4).

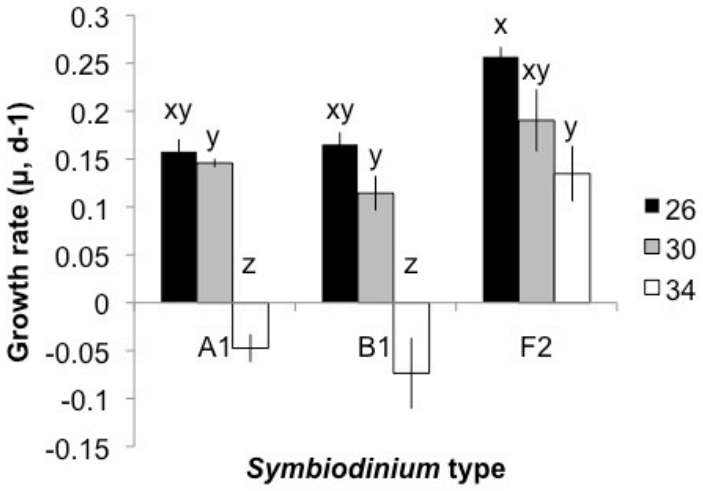

Figure 3.5 Mean growth rates (± s.e.) (μ) after 6 d of exposure to treatment temperature (n = 4). Letters above the graph indicate statistical differences both within and among groups.

Table 3.4 Growth rates (μ , mean ± s.e.) after 6 d of growth and percent of the population showing loss of Chl *a* signal (mean ± s.e.) after 16 d of exposure to each treatment (n=4).

	26°C		30°C		34°C	
Symbiodinium type	Growth rates	% Bleached	Growth rates	% Bleached	Growth rates	% Bleached
A1	0.16 ± 0.013	1.1 ± 0.19	0.15 ± 0.013	11.65 ± 4.28	-0.047 ± 0.01	99.08 ± 0.85
B1	0.17 ± 0.0045	1.17 ± 0.02	0.11 ± 0.018	3.79 ± 0.30	-0.073 ± 0.032	99.83 ± 0.078
F2	0.26 ± 0.015	3.91 ± 0.57	0.19 ± 0.037	3.22 ± 0.33	0.13 ± 0.029	7.09 ± 0.71

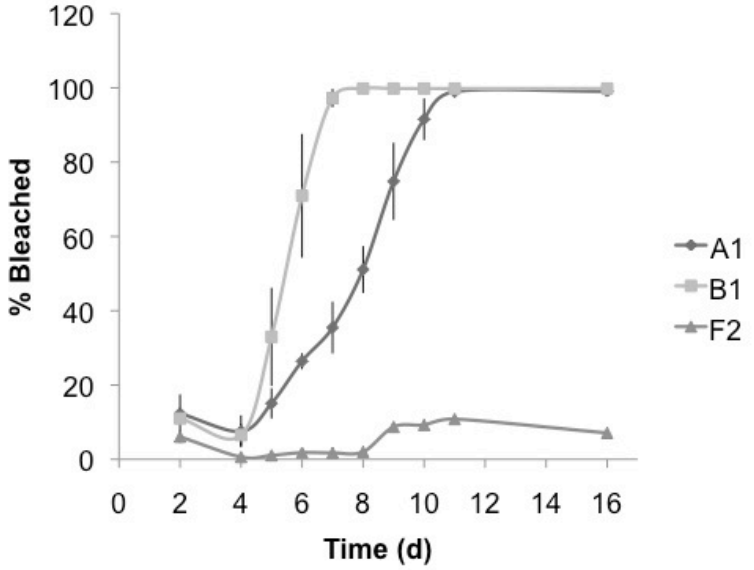

Figure 3.6 Bleaching response (mean ± s.e.) of *Symbiodinium* type A1, B1 and F2 cultures at 34°C over 16 d of exposure to treatment temperatures (mean ± s.e., n = 4).

3.4 Discussion

Concerns over the decline in coral reefs have been driving research into the varying responses of coral and *Symbiodinium* to factors associated with climate change, namely temperature. Understanding *Symbiodinium* thermal limits will inform our understanding of physiological variation seen in coral reef bleaching events. Using oxygen consumption ($\dot{V}o_2$) and growth rates as indicators of sensitivity or tolerance to increasing temperatures, we found a wide range in the tolerance to thermal temperatures.

Maximum $\dot{V}o_2$ rates were different for all *Symbiodinium* types measured, and ranged from 31°C (type B1) to 40°C (type F2). The maximum ($\dot{V}o_2$) and Q_{10} rates to 32°C were similar for types B1 and B2 (31°C and 5.35, 32°C and 5.81 respectively), A1 and E1 (33 °C and 3.25, 34°C and 2.91 respectively), and A1* and F2 (35°C and 1.03, 40°C and 0.73 respectively). This indicates that B1 and B2 were most sensitive to increases in temperature, A1 and E1 were

moderately sensitive in comparison, and A1* and F2 were most tolerant to increases in temperature.

The growth rates and bleaching response, as defined as the loss of chlorophyll *a* (Chl *a*), of one member of each of these groups (B1 from the most sensitive, A1 from the moderately sensitive, and F2 from the tolerant group) at 26°, 30° and 34°C were consistent with these findings. For *Symbiodinium* type B1 both parameters indicated susceptibility to elevated temperatures, showing a negative growth rate at 34°C, and a relatively rapid loss of Chl *a* fluorescence, with cultures fully bleached (no Chl *a* fluorescence in >97% of the population) after 7 days at 34°C. Previous work with this *Symbiodinium* found relative sensitivity with an inability to increase antioxidant activity (McGinty et al. 2012), which can be essential in protecting the photosynthetic apparatus (Asada 2006). These results suggest that once past its maximum $\dot{V}o_2$, type B1 does not invest in protective mechanisms and is unable to sufficiently repair its photosynthetic machinery, resulting in a loss of photosynthetic function and an inability to meet metabolic demands.

Symbiodinium type A1 also had negative growth rates after 6 days at 34°C, but retained Chl *a* fluorescence until 10 days of exposure to 34°C. These responses were consistent with previous studies, which found it to be able to increase the activity of the antioxidants catalase and superoxide dismutase at 31°C (McGinty et al. 2012), as well as photoacclimate during high temperature and increased irradiance, but with a decrease in growth rates (Robison & Warner 2006). These results support the suggestion of Robison and Warner (2006) that this type may be investing more in repair of damaged proteins or the production of protective mechanisms, such as antioxidants, allowing it to remain photosynthetically active but with a tradeoff in growth rates.

The growth rates and bleaching response of *Symbiodinium* type F2 demonstrated a tolerance to elevated temperatures of 34°C with a reduced but still positive growth rate and a resistance to bleaching. This type has been shown to photoacclimate at 32°C (Robison &

Warner 2006) and increase antioxidant activity at 31°C (McGinty et al. 2012), suggesting that the photosynthetic apparatus for this type may be more stable, and that this type is able to sufficiently scavenge any excess ROS (Tchernov et al. 2004, Robison & Warner 2006, McGinty et al. 2012). As a result, this type may not divert as many resources to repairing the photosystem and can maintain positive growth.

These *Symbiodinium* responses demonstrated a very narrow tolerance threshold, consistent with findings for other tropical marine organisms (Hofmann & Todgham 2010, Sunday et al. 2010). For type B1 grown at 30°C, just 1°C below their maximum $\dot{V}o_2$ temperature, there was no significant reduction in growth rate and only 3% of the population bleached. *Symbiodinium* type A1 grown at 34°C, just 1°C above their maximum $\dot{V}o_2$ temperature, had negative growth and bleached within 10 days. In contrast, types A1* and F2* demonstrated surprisingly high maximum temperatures (35° and 40°C), and a large change between Q_{10} values at 32°C and their maximum $\dot{V}o_2$ temperature (A1* Q_{10} to 32° - 1.03, Q_{10} to 35°C - 3.19; F2 Q_{10} to 32° - 0.73, Q_{10} to 35°C - 6.06) potentially demonstrating an ability to regulate their metabolism until a critical threshold near their maximum $\dot{V}o_2$ temperature.

These responses are consistent with several other studies that show reduced photosynthetic efficiency for some *Symbiodinium* and maintained photosynthetic competency for others over short term (45 min to 24 hr) and longer (5 d) exposure to temperatures greater than 32°C (Iglesias-Prieto et al. 1992, Ralph et al. 2001, Robison & Warner 2006, Suggett et al. 2008, Takahashi et al. 2008). By examining the *Symbiodinium* response over a range of temperatures and several weeks, this study expands our understanding of metabolic and Chl *a* responses, demonstrating variation in the temperature and the amount of time necessary to induce responses in different *Symbiodinium* types. In addition, the finding that *Symbiodinium* type F2 can maintain positive growth after longer exposures to 34°C was surprising, and indicates that the photosynthetic apparatus is still functional. Investigations into the photobiology of these symbionts may be informative as to how their photosynthetic efficiency

performs after chronic temperature stress. Further studies of what factors influence their continued photosynthetic function, such as higher repair or replacement rates of photosystem proteins or higher concentrations of saturated lipids in thylakoid membranes, may elucidate why other *Symbiodinium* types cannot tolerate elevated temperatures (Warner et al. 1999, Tchernov et al. 2004, Suggett et al. 2008, Takahashi et al. 2008).

These results suggest that the physiology of corals hosting *Symbiodinium* types like B1 can change dramatically in a very narrow range, while corals hosting *Symbiodinium* like F2 may not experience any physiological changes within the temperature variation experienced in a natural reef setting. While the differences in the $\dot{V}o_2$ and growth rates were highly variable among *Symbiodinium* types, it is important to note that intact associations and the responses of the cnidarian host have the potential to mediate or exacerbate the *Symbiodinium* responses. For example, some coral products such as antioxidants may have the ability to mediate the detrimental effects of elevated temperatures (Lesser 1997, Levy et al. 2006). Coral bleaching among coral genera is also variable, and can occur seasonally with minimal lethality (Suggett & Smith 2011). In many cases bleaching is the near complete loss of *Symbiodinium* cells instead of a loss of *Symbiodinium* pigmentation (Ralph et al. 2001, Dove et al. 2006). Further investigations into differences between seasonal sub-lethal bleaching and lethal bleaching, and how loss of symbionts themselves versus pigmentation demonstrated in this study will clarify how *Symbiodinium* physiology contributes to persistence or loss of coral species.

This study also examined two A1 *Symbiodinium* originally isolated from different hosts and geographic locations. Despite having the same ITS-2 sequence, the response in $\dot{V}o_2$ to elevated temperatures was quite different. This supports the need for further clarification into the genetic identification of *Symbiodinium*, and demonstrates that physiological and ecological characterization of *Symbiodinium* should be taken into account in addition to their ITS-2 region (Sampayo et al. 2009, LaJeunesse & Thornhill 2011). In particular, applying characterizations of *Symbiodinium* types as thermally tolerant or sensitive based on ITS-2 regions may confound

ecological patterns when examining intact associations with cnidarian hosts. This may explain contradictions in responses of types identified as the same ITS-2 region but from different geographic locations or cnidarian hosts (Abrego et al. 2008).

These changes in $\dot{V}o_2$, growth rates and loss of Chl *a* suggest that the symbiosis between cnidarian hosts and algal symbionts may change under future climate scenarios. Combined with the other detrimental effects of increased temperatures, such as the symbiont's generation of reactive oxygen species that have the potential to damage the host (Downs et al. 2002, Franklin et al. 2004, Hennige et al. 2009, McGinty et al. 2012), the relationship may shift from a mutualism to a parasitism (Stat et al. 2008, Kiers et al. 2010, Wooldridge 2010). This variation in physiological response among *Symbiodinium* types may have significant implications for their abundance on coral reefs. In a future with a warmer ocean, types like B1 will have a difficult time meeting both their own metabolic demands as well as the demands of the host, leading to a restriction in their distributions. Types like F2, with lower metabolic demands and higher temperature limits, may be able to persist if their hosts are also tolerant to elevated temperatures. Further studies exploring how common *Symbiodinium* thermal tolerance is, and whether diminished performance at elevated temperatures can still maintain mutualisms, will inform predictions into coral reef futures.

CHAPTER 4

THE HOST AND SYMBIONT BOTH MATTER: A COMPARATIVE STUDY OF THE FUNCTIONAL DIFFERENCES IN MELANIN SYNTHESIS AND ANTIOXIDANT ACTIVITY AMONG DIFFERENT CORAL AND SYMBIONT COMBINATIONS.

4.1 Introduction

Reef-building coral are a myriad of symbioses between the coral animal and microbial organisms, collectively called the coral holobiont (Rohwer et al. 2002). Because of the obligate nature of these symbioses, responses to environmental changes are influenced by all members (Rohwer et al. 2002). This includes the unicellular algal endosymbionts that are critical to the functioning of a coral reef by providing the host with more than 90% of its nutritional requirement (Muscatine & Porter 1977) and increasing skeletal deposition (Muller-Parker & D'Elia 1997). These algae are members of the extremely diverse *Symbiodinium* genus, and are classified into nine different clades, A-I (Pochon & Gates 2010). Within the genus they are commonly identified by their ITS-2 region and often referred to as “types” (LaJeunesse 2001, Coffroth & Santos 2005), and these types have been shown to demonstrate distinct physiologies (Robison & Warner 2006, Suggett et al. 2008, Hennige et al. 2009, McGinty et al. 2012).

Reef-building corals have been found hosting members of A-D, F and G, and the symbioses have varying amount of specificity (LaJeunesse 2002, Pochon & Gates 2010). Some corals will only host one type of *Symbiodinium*, while some are more flexible in their associations, capable of forming symbioses with multiple partners, and some corals are capable of hosting multiple symbionts at the same time (Baker 2003, Baker & Romanski 2007, Finney et al. 2010, Oliver & Palumbi 2011, Putnam et al. 2012, Silverstein et al. 2012). These associations are often structured by abiotic factors such as temperature and light exposure

(Iglesias-Prieto et al. 2004, Frade et al. 2008b). There is debate concerning how extensive the flexibility between host and symbiont is (Goulet 2006, Baker & Romanski 2007, Cooper et al. 2011b) and whether or not this flexibility conveys any advantage or disadvantage to the unit (Putnam et al. 2012). Regardless, it has been shown that different members in a holobiont will lead to functional differences of the entire unit (Perez et al. 2001, Little et al. 2004, Goulet et al. 2005, Loram et al. 2007, Correa et al. 2009, Mieog et al. 2009, Littman et al. 2010, Cooper et al. 2011a).

Environmental stressors, such as changes in temperature and light, can lead to a loss of these algal symbionts or their pigments, a phenomenon called coral bleaching (Buddemeier & Fautin 1993, Brown 1997). The severity of coral bleaching can range from a normal reaction to seasonal variability to a symptom of extreme distress (McClanahan 2004, Suggett & Smith 2011). Bleaching is attributed to the *Symbiodinium* production of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide radicals (Lesser 1996, Downs et al. 2002, McGinty et al. 2012). These ROS are a natural by-product of photosynthesis and cellular respiration (Asada 2006), and can be scavenged by antioxidants or utilized as a signaling molecule or in defense responses to a perceived pathogen (Thannickal & Fanburg 2000, Neill et al. 2002, Apel & Hirt 2004). ROS production can increase due to stress (Warner et al. 1999, Downs et al. 2002, Tchernov et al. 2004, Smith et al. 2005). When in excess, ROS can cause damage to both the symbiont and host, including mutating DNA and damaging cellular membranes, culminating in a breakdown of the symbiosis (Downs et al. 2002, Weis 2008). If this state persists it can have negative effects such as decreased growth, reproduction and death (Glynn 1993, Brown 1997, Baker et al. 2008).

In addition to bleaching, rates of coral disease caused by bacterial, fungal and viral pathogens are also on the rise globally, but particularly in the Caribbean (Weil et al. 2006, Harvell et al. 2007). Some of these pathogens attack the host (Weil et al. 2006), while others may be specific to the algal symbiont (Cervino et al. 2004). Disease and bleaching are

correlated (Muller et al. 2008, Brandt & McManus 2009, Miller et al. 2009), with one often following the other, and the synergy of both of these factors contributes significantly to the global decline of coral reefs (Carpenter et al. 2008, Miller et al. 2009, Mydlarz et al. 2010, Wild et al. 2011). Observations of bleaching events and disease outbreaks have shown variability that correlates with coral species and *Symbiodinium* identity, but the mechanisms that lead to this variation remain unresolved (Loya et al. 2001, Douglas 2003, Rowan 2004, Green et al. 2008, Correa et al. 2009, LaJeunesse et al. 2010, van Woesik et al. 2011).

Susceptibility to bleaching and disease is due to a variety of compounds produced by both the host and symbiont, many of which overlap in function. Antioxidants, mycosporine-like amino acids, and fluorescent pigments have roles in either preventing or mediating the detrimental effects of elevated light and temperatures (Lesser 1996, Dunlap & Shick 1998, Franklin et al. 2004, Banaszak et al. 2006, Merle et al. 2007, Palmer et al. 2009, McGinty et al. 2012). It has recently been shown that differing levels of baseline immunity and inducible immune factors including components of the melanin cascade, common among invertebrates, play a role in the observed variation among coral susceptibility to both bleaching and disease (Nappi & Christensen 2005, Levy et al. 2006, Palmer et al. 2009, Palmer et al. 2010, Mydlarz & Palmer 2011). One of the first components of this pathway is prophenoloxidase (PPO), the precursor of the active protein phenoloxidase, and high levels of PPO have been correlated with several invertebrate diseases (Loker et al. 2004, Newton et al. 2004, Butt & Raftos 2008). The pigment melanin is the end point of this cascade, which can provide a physical barrier that can prevent the spread of pathogens and provide protection against excess light (Schmitz et al. 1995, Shick et al. 1996, Nappi & Christensen 2005). This pathway also produces ROS intermediates, and as such the production of antioxidants is important to prevent self-harm (Nappi & Christensen 2005, Costantini & Møller 2009). Studies in scleractinian corals have found positive correlations between basal levels of phenoloxidase activity and antioxidant activity levels (Mydlarz & Palmer 2011).

Despite the understanding that both host and symbiont factors influence the holobiont response to stressors and susceptibility to bleaching and disease (Brown et al. 2002, Yakovleva et al. 2004, Banaszak et al. 2006, Ferrier-Pages et al. 2007, Fitt et al. 2009, DeSalvo et al. 2010), our understanding of the functional properties of different host-symbiont combinations remains limited (Bourne et al. 2009). To investigate this, we sampled 4 coral species (*Diploria strigosa, Montastraea faveolata, Porites astreoides* and *Porites porites*) that have all been reported to have flexibility in their symbiont populations (LaJeunesse 2002, Finney et al. 2010). Because differnt geographic locations and light conditions can lead to different symbiont populations (Iglesias-Prieto et al. 2004, Frade et al. 2008b) all corals were collected from two different reefs, and two depths, 1 meter and 5 meters, at each reef in an attempt to obtain samples of the same host species but with different symbiont populations. Host tissue and symbiont cells were separated, symbionts were identified by their ITS-2 region, and, because of their dual role in bleaching and immune responses, the melanin cascade activity of the host and antioxidant capabilities from both host and symbiont were measured (Figure 4.1). We aimed to assess 1) if the same coral species hosting different symbiont types would have the same melanin cascade activity and antioxidant capabilities, 2) if the same symbiont type would have the same antioxidant capabilities in different coral hosts, and 3) what interactive effects host identity and symbiont identity may have on basal physiological parameters associated with both bleaching and disease susceptibility as a whole.

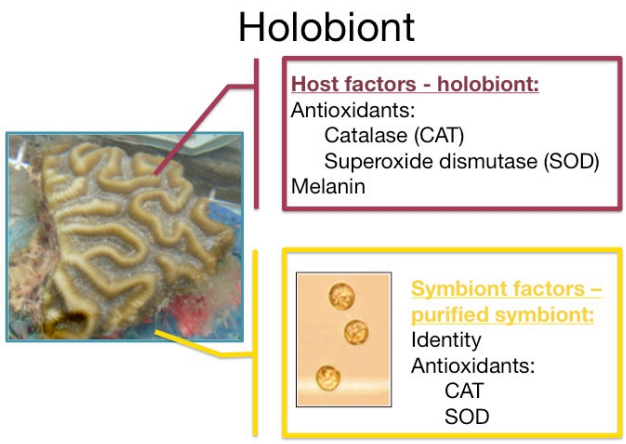

Figure 4.1 Schematic for experimental analysis.

4.2 Methods

4.2.1 Study area

Collections occurred in May 2010 at in the Natural Reserve of La Parguera, Puerto Rico, at Media Luna (17°56.091'N, 67°02.577'W (Pinzon & Weil 2011) and San Cristobal (17°56.501'N, 67°04.509'W, Pinzon and Weil, 2011) reefs. Both are fringing reefs along the mid-reef shelf region off the southwest coast of Puerto Rico with similar orientation and exposure to environmental conditions, and experience low levels of rainfall, sedimentation and wave action (Almy & Carrion-Torres 1963, Acevedo & Morelock 1988, Weil et al. 2009). Monthly Secchi disk readings have been recorded in the area since 1993, and the average of their data yields a light extinction coefficient of 0.19, equating to a 61.3% reduction in light from 1 m to 5 m depth. Although not exactly the same range we used in this study, sea water temperature data collected at 3 m and 15 m depth show a difference of 0.2°C across the seasons. Therefore, the temperature difference between 1 m and 5 m in May are likely to be extremely minor.

4.2.2 Coral collections

The four coral species collected included two representatives of the family Favidae, *Diploria strigosa and Montastraea faveolata,* and two from the family Poritidae, *Porites*

astreoides and *Porites porites.* Five different colonies per coral species were collected at each reef from 1 m and 5 m of depth in an attempt to acquire different host-symbiont combinations. While these depths have previously been considered relatively shallow, differences in symbiont communities have been observed (LaJeunesse 2002, Finney et al. 2010). Colonies were clearly distinct and separated by several other corals to insure genetic variance in the host. Samples were taken from similar location and orientation within each individual colony. Fragments were collected with a hammer and chisel, placed in Whirl-pak® bags and immediately snap frozen in liquid nitrogen. The frozen samples were shipped on dry ice to UTA, and stored at -80°C until processing.

4.2.3 Extract preparation

4.2.3.1 Sample processing

Tissue was removed from the coral skeleton by airbrushing with a Paansche® air brush (Chicago, IL, USA) using an extraction buffer (Tris-HCl buffer, 100mM, pH 7.8 with 0.5 mM dithiothreitol) over ice. Only the tissue in the center of each fragment was used to minimize the effect of the collection process.

4.2.3.2 Host extract preparation

The first 5 ml of tissue slurry airbrushed form the coral skeleton was retained for host analyses. Host tissue slurries were homogenized using a Power Gen 125 tissue homogenizer® with medium sawtooth generator (Fisher Scientific®, Pittsburg, PA, USA) for 20 s, and 0.5 ml of the slurry was snap-frozen in liquid nitrogen and freeze-dried for melanin analysis (see below). To extract host proteins from the remaining portion for biochemical assays, the remaining was vortexed with a spatula of glass beads for 20 s, and left on ice to extract for an additional 5 min. They were then centrifuged at 4°C for 5 min at 2400 x *g* to remove cellular debris, and the supernatant was transferred to new microcentrifuge tubes and stored at -80°C until analysis.

4.2.3.3 Symbiont extract preparation

The algal symbionts were isolated from the rest of the tissue slurry airbrushed off the skeleton and centrifuged at 4°C for 8 min at 200 x *g*. The cells were isolated from the host tissue by a series of washes with artificial seawater (35 g L^{-1}) with 5 mM EDTA. Washes and centrifugation continued (approximately 7x) until algal pellets were confirmed to be 99% free of host cells by visual inspection on a Bright-Line hemacytometer (Hausser Scientific®, Horsham, PA, USA). One portion of the pellet was stored in DMSO and EDTA for ITS-2 identification, and the rest was stored at -80°C in ASW + EDTA for biochemical protein assays. To extract symbiont proteins, pellets were freeze-dried for 24 h on a VirTis Benchtop 6K freeze drier® (SP Industries®, Stone Ridge, NY, USA). Phosphate buffered saline (50 mM, pH 7.8) and a spatula tip of borosilicate glass beads (1 mm diameter) were then added, and cells were ruptured by 5 min of vortexing on high in 20 s intervals to extract the soluble protein. Sample tubes were kept on ice in between intervals. Samples were then spun down for 2-3 min at 4300 x *g* in a microcentrifuge (Baxter Scientific Products®, Deerfield, IL, USA) to remove cellular debris, and the supernatant was transferred to a new 0.5 mL microcentrifuge tube, flash frozen in liquid nitrogen, and stored at -80°C until analyzed.

4.2.4 Symbiont identification

DNA extractions from *Symbiodinium* were performed using a modified Promega Wizard® genomic DNA extraction protocol (LaJeunesse 2002). Types were identified with PCR-DGGE using the ITS-2 region of rDNA. The ITS was amplified with a touch-down thermal cycle profile using the primers “ITS2clamp” and “ITSintfor2”, as described in LaJenesse (2002). Positive PCR amplifications were confirmed in 1% agarose gel and then run on a 45-80% formamide-urea denature gradient overnight at 110 volts. Distinct banding patterns on the DGGE gel were used to identify the symbiont by comparing our patterns with those observed in other Caribbean species (LaJeunesse 2002). When necessary, bands were excised, re-

amplified and sequenced to confirm the identity. Matching sequences were found by blasting our sequences on GenBank.

4.2.5 Biochemical assays

All assays were performed with a Bio-Tek Synergy 2 Multi-detection microplate reader® using the Gen-5® software package (Bio-Tek Instruments®, Inc. Winooski, VT, USA), using assay plates manufactured by Greiner Bio-one® (Monroe, NC, USA). Total protein concentration for all coral host and *Symbiodinium* samples was quantified using the Red 660® (G Biosciences®, St. Louis, MO) protein assay (absorbance 660 nm and standardized to bovine serum albumin).

4.2.5.1 Melanin cascade activity

PPO activity was determined following the protocol of Palmer et al., 2011. Briefly, host protein extract, phosphate buffer (50 mmol, pH 7.5), and trypsin (0.1 mg ml^{-1} in deionized water, Sigma Aldrich®, St Loius, MO, USA) were aliquoted in duplicate to clear 96-well microtiter plates. The reaction was initiated by the addition of L-1,3-dihydroxyphenylalanine (10 mmol, Sigma-Aldrich®), and the absorbance (490 nm) was monitored over the linear range of the reaction that occurred between 0 and 10 min. Change in sample absorbance was normalized to mg protein for each sample. Host melanin concentration was determined following the protocol of Palmer et al., 2011. Briefly, aliquots of host tissue slurry were freeze dried, and then gently vortexed with sodium hydroxide (10 M) and left to extract for 48 hrs. Samples centrifuged at 7000 x *g* for 5 min, and then duplicate aliquots of the supernatant containing the melanin were placed into a 96-well microtiter plate. The absorbance at 410 nm was compared to a standard curve of commercial melanin (in 10 M NaOH, Sigma-Aldrich®). Data presented are mg melanin normalized to g tissue.

4.2.5.2 Antioxidant activity

Antioxidant activities were determined using the SOD assay kit (Fluka BioChemika®, Buchsm, Switzerland, absorbance at 450 nm), which colorimetrically detects the scavenging of

O^{2-} and the Amplex Red® catalase kits (Invitrogen Corporation®, Carlsbad, CA, USA, excitation 540 nm, emission 590 nm), which fluorescently detects the presence of H_2O_2 (following McGinty et al., 2012). Antioxidant activity was normalized to the total protein concentration of each sample.

4.2.6 Statistical Analyses

A permutational multivariate analysis of variance (Anderson 2001) was initially used to examine the dataset because missing data due to failed symbiont extractions would have removed a number of host data. The host and symbiont parameters (melanin concentrations, PPO activity, and antioxidant activity) were each examined with regard to coral host species, *Symbiodinium* identity, holobiont (coral x symbiont) identity, reef where they were collected, and depth of collection to identify significant interactions. The coral host factors and *Symbiodinium* factors were analyzed independently as there were less *Symbiodinium* data points and the data were collected from each extraction independently. One-way multivariate analysis of variance (MANOVA) was then used to examine interactions between host factors and depth, host factors and holobiont identity, and symbiont factors and holobiont identity. Data were then further analyzed using one-way ANOVAs and Tukey *post-hoc* tests to explore within coral species and holobiont relationships, and among *Symbiodinium* relationships. Regression analyses were used to identify relationships among host and symbiont factors for the samples where all the data points were collected (n = 63). The PERMANOVA was implemented using the Primer® version 6.0 software with the PERMANOVA + add on package (Clark & Gorley 2006); all other analyses were performed using the JMP Statistical Discovery Software® version 9.0 (SAS Institute®, Cary, NC, USA).

4.3 Results

4.3.1 Symbiont identification

Symbiodinium ITS-2 types were successfully established from 78 colonies. Six genetically distinct ITS-2 types were identified, culminating in six different holobiont (coral-symbiont) combinations (Table 4.1). The difference in water conditions between 1 m and 5 m was enough to induce changes in the symbiont composition of *Montastraea faveolata* and *Porites porites*, while the symbionts in *Diploria strigosa* and *Porites astreoides* did not change (Table 4.1). *Symbiodinium* type A4 was the only type found in more than one host (*P. astreoides* at both depths and *P. porites* at 1 m, Table 4.1).

Table 4.1 *Symbiodinium* types identified by ITS-2 region from corals at 1 m and 5 m depths. Numbers in parentheses indicate the number of colonies found hosting the *Symbiodinium* type.

Coral host	*Symbiodinium* types identified	
	1 m	5 m
D. strigosa	B1 (10)	B1 (10)
M. faveolata	A3 (5), D1a (4)	D1a (9)
P. astreoides	A4 (10)	A4 (10)
P. porites	A4 (9), C1 (1)	C1 (10)

4.3.2 Parameters by host and symbiont

Analysis of host and *Symbiodinium* parameters by PERMANOVA found no significant effect of reef where the corals were collected (Table 4.2, $P = 0.109$ and 0.92 respectively), and all further analyses combined data from each reef. The effect of collection depth on the host factors was significant (Table 4.2, $P = 0.02$).

Table 4.2 Full output from the PERMANOVA examining coral host parameters and *Symbiodinium* parameters in response to coral host species, *Symbiodinium* identity, holobiont (host x symbiont) identity, reef where samples were collected, and the depth the samples were collected. Underlined values of *P* indicate significance at α = 0.05.

PERMANOVA results	Factor	df	*F*	*P*
Coral host factors	Coral host species	3	6.9	0.001
	Symbiodnium identity	5	3.6	0.009
	Holobiont (Host x Symbiont)	7	3.08	0.01
	Reef collection site	1	2.85	0.109
	Depth of collection	1	5.9	0.02
Symbiodinium factors	Coral host species	3	2.9	0.028
	Symbiodnium identity	6	3.8	0.001
	Holobiont (Host x Symbiont)	7	4.3	0.001
	Reef collection site	1	2.62	0.092
	Depth of collection	1	1.7	0.184

Analysis by one-way MANOVAs showed an overall significant effect of collection depth on host parameters for *M. faveolata* (Table 4.3). One-way ANOVAs of each host factor showed different effects on each coral species. None of the *P. astreoides* factors were affected by depth, and in *P. porites* depth only affected the PPO activity ($F = 6.21$, $p = 0.0226$, Figure 4.2), demonstrating higher activity at 1 m depth. In *M. faveolata,* PPO ($F = 22.15$, $p = 0.0003$, Figure 4.2), CAT ($F = 6.29$, $p = 0.0225$, Figure 4.4) and SOD ($F = 5.3$, $p = 0.0341$, Figure 4.5) activities were all higher in corals collected from 5 m. For *D. strigosa,* the CAT ($F = 8.05$, $p = 0.0014$, Figure 4.4) and SOD activities ($F = 8.48$, $p = 0.0097$, Figure 4.5) differed with depth, with higher activity in corals collected at 5 m.

Table 4.3 MANOVA results of the effect of collection depth on coral host factors. Underlined values of *P* indicate significance at α = 0.05.

MANOVA	Whole Model		
Wilks-Lambda	df	*F*	*P*
D. strigosa	4	2.07	0.139
M. faveolata	4	8.15	<u>0.0019</u>
P. astreoides	4	2.32	0.107
P. porites	4	1.48	0.257

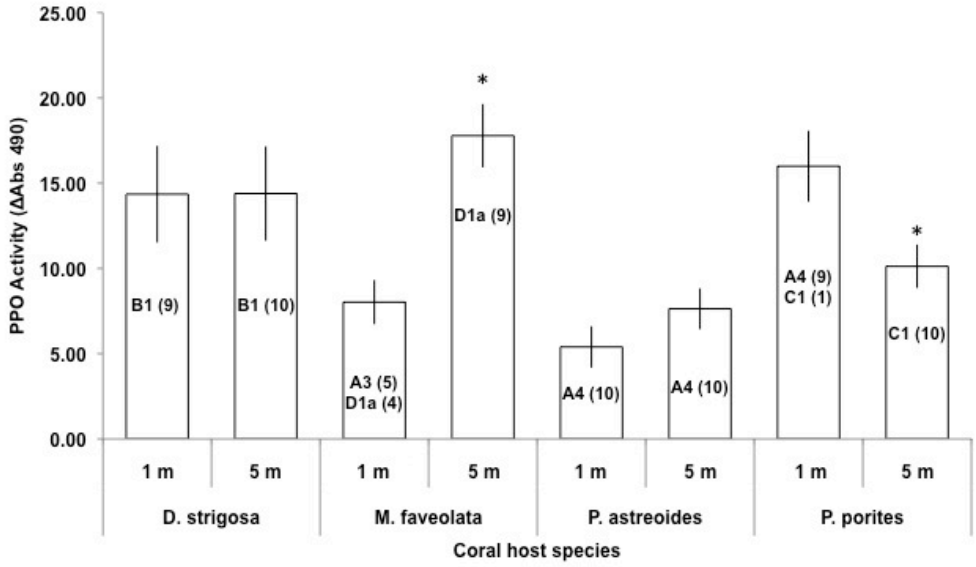

Figure 4.2 Host coral PPO activity (mean, ± s.e.). The *Symbiodinium* types identified from each host are listed within the bar, and numbers within parentheses indicate how many colonies hosted that symbiont. *Asterisks* above bars indicate significant differences (α = 0.05) within coral species collected from 1 m and 5 m.

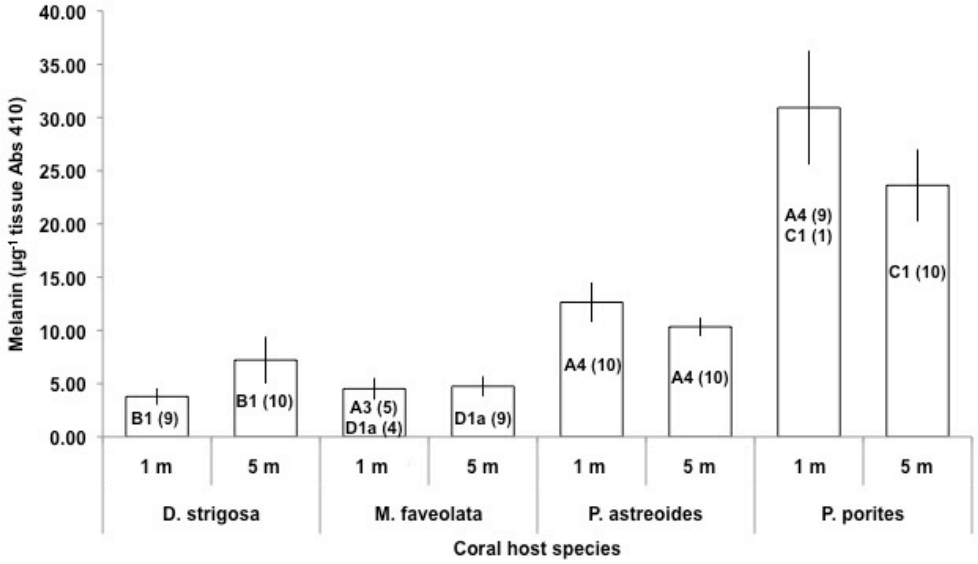

Figure 4.3 Host coral melanin concentrations (µg^{-1} tissue, mean, ± s.e.). The *Symbiodinium* types identified from each host are listed within the bar, and numbers within parentheses indicate how many colonies hosted that symbiont. There were no significant differences (α = 0.05) within a coral species between sample collected from 1 m and 5 m.

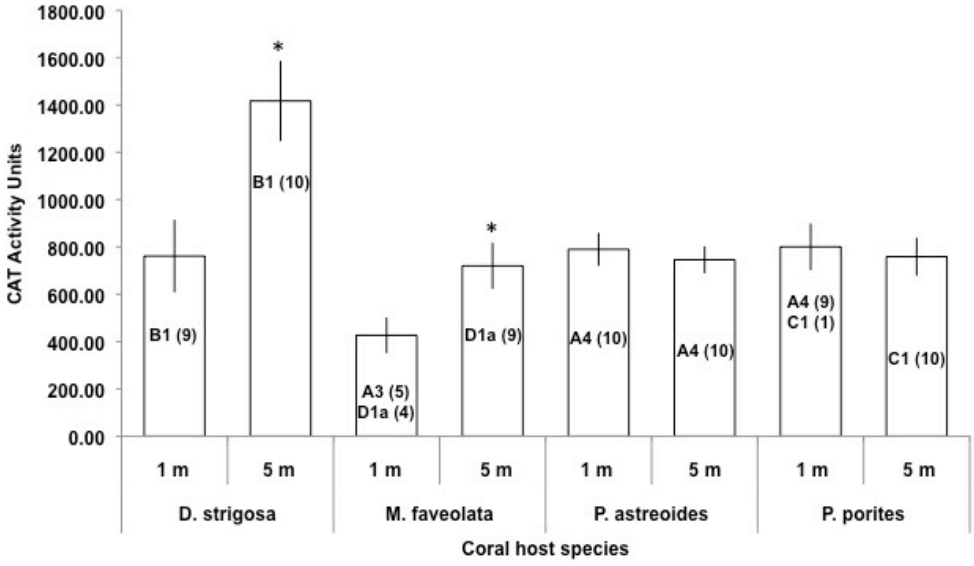

Figure 4.4 Host coral CAT activity (mean, ± s.e.). The *Symbiodinium* types identified from each host are listed within the bar, and numbers within parentheses indicate how many colonies hosted that symbiont. *Asterisks* above bars indicate significant differences (α = 0.05) within coral species collected from 1 m and 5 m.

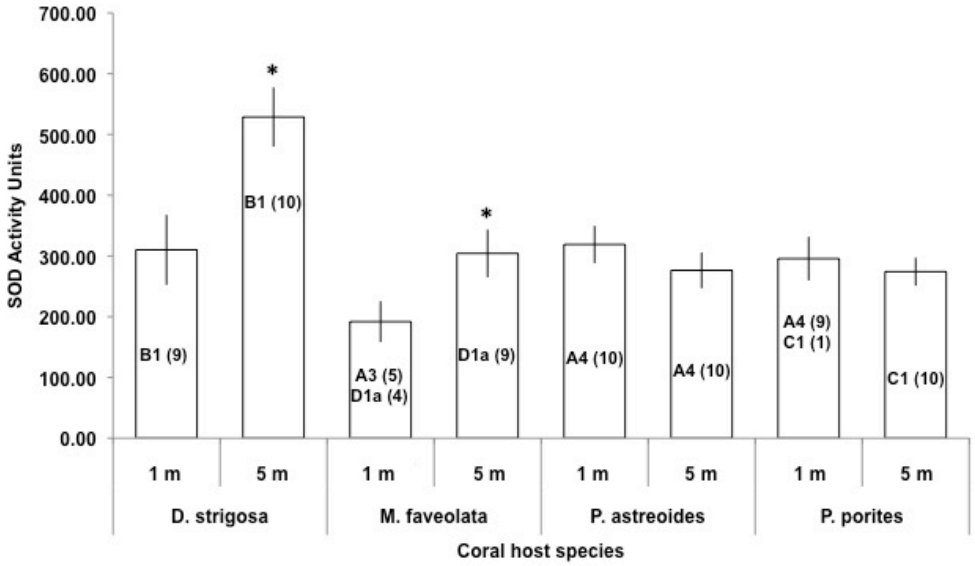

Figure 4.5 Host coral SOD activity (mean, ± s.e.). The *Symbiodinium* types identified from each host are listed within the bar, and numbers within parentheses indicate how many colonies hosted that symbiont. *Asterisks* above bars indicate significant differences (α = 0.05) within coral species collected from 1 m and 5 m of depth.

Because there was no effect of depth on *Symbiodinium* factors, data were pooled for each *Symbiodinium* type (A3 n = 4; A4 n = 24; B1 n = 12; C1 n = 10; D1a n = 13) and one-way ANOVAs were run to examine differences among *Symbiodinium* types. There was a significant effect of *Symbiodinium* identity on CAT activity, but no significant differences were observed among types ($F = 3.156$, $p = 0.02$, Figure 4.6). SOD activity was also affected by *Symbiodinium* identity, and there were significant differences among *Symbiodinium* types ($F = 6.085$, $p = 0.0004$, Figure 4.7).

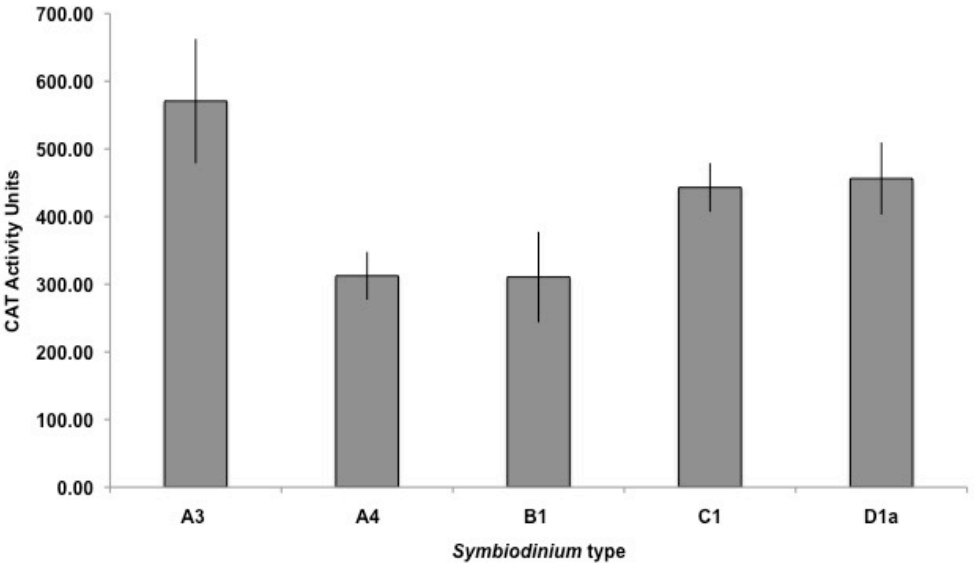

Figure 4.6 *Symbiodinium* CAT activity (mean, ± s.e.). There were no significant differences (α = 0.05) within a *Symbiodinium* type collected from 1 m and 5 m. No significant differences were observed among *Symbiodinium* types.

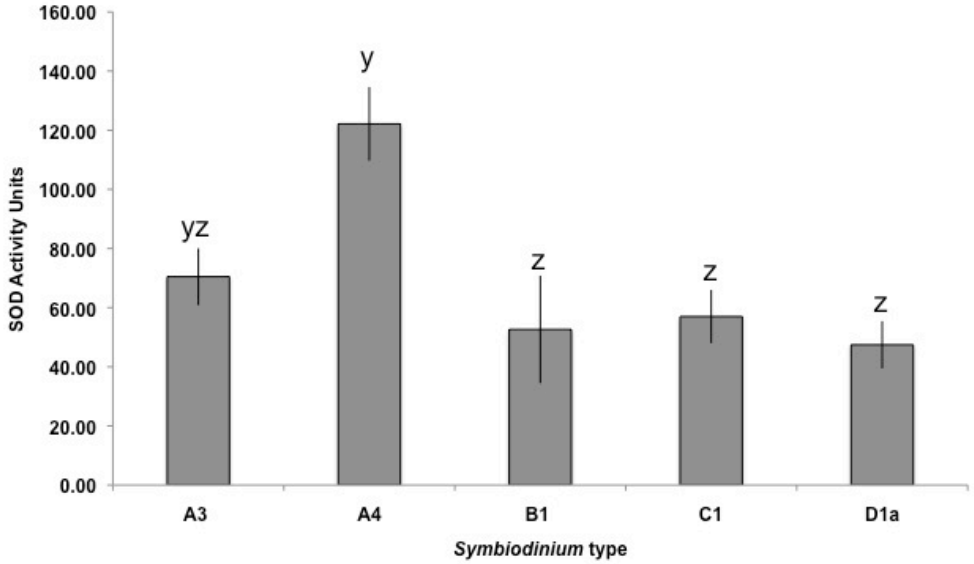

Figure 4.7 *Symbiodinium* SOD activity (mean, ± s.e.). There were no significant differences (α = 0.05) within a *Symbiodinium* type collected from 1 m and 5 m. Letters above the bars indicate differences among *Symbiodinium* types, and those sharing a letter are not significantly different (α = 0.05).

4.3.3 Holobiont factors

There was a significant effect of symbiont identity on overall host parameters (There were significant differences among the holobionts (coral x symbiont identity) in both host (Table 4.5, Figure 4.8 - Figure 4.11) and symbiont factors (Table 4.6, Figure 4.12 and Figure 4.13). Table 4.4, *P* = <0.0001; *D. strigosa* B1 n = 20; *M. faveolata* A3 n =5; *M. faveolata* D1a n = 13; *P. astreoides* A4 n = 20; *P. porites* A4 n = 9; *P. porites* C1 n = 11). Melanin concentrations, host CAT activity and host SOD activity were all influenced by the *Symbiodinium* type (There were significant differences among the holobionts (coral x symbiont identity) in both host (Table 4.5, Figure 4.8 - Figure 4.11) and symbiont factors (Table 4.6, Figure 4.12 and Figure 4.13). Table 4.4, *p* = <0.0001, 0.0094 and 0.0023 respectively). There were significant differences among the holobionts (coral x symbiont identity) in both host (Table 4.5, Figure 4.8 - Figure 4.11) and symbiont factors (Table 4.6, Figure 4.12 and Figure 4.13).

Table 4.4 MANOVA results of host factors by *Symbiodinium* identity. Underlined values of *P* indicate significance at α = 0.05.

MANOVA	df	*F*	*P*
Whole Model			
Wilks-Lambda	16	6.129	<0.0001
Univariate Tests			
PPO activity	4	2.11	0.07
Melanin concentration	4	17.88	<0.0001
Host CAT activity	4	3.63	0.0094
Host SOD activity	4	3.02	0.0233

Table 4.5 MANOVA results of host factors by holobiont (coral x symbiont identity). For Tukey *post-hoc* results, see Figure 4.8 - Figure 4.11. Underlined v alues of *P* indicate significance at α = 0.05.

MANOVA	df	*F*	*P*
Whole Model			
Wilks-Lambda	20	6.94	<0.0001
Univariate Tests			
PPO activity	7	4.27	0.0019
Melanin concentration	7	21.91	<0.0001
Host CAT activity	7	3.99	0.003
Host SOD activity	7	3.89	0.0036

Table 4.6 MANOVA results of symbiont factors by holobiont (coral x symbiont identity). For Tukey *post-hoc* results see Figure 4.12 and Figure 4.13. Underlined values of *P* indicate significance at α = 0.05.

MANOVA	df	*F*	*P*
Whole Model			
Wilks-Lambda	10	6.36	<0.0001
Univariate Tests			
Symbiont CAT activity	5	4.3	0.0022
Symbiont SOD activity	5	6.03	<0.0001

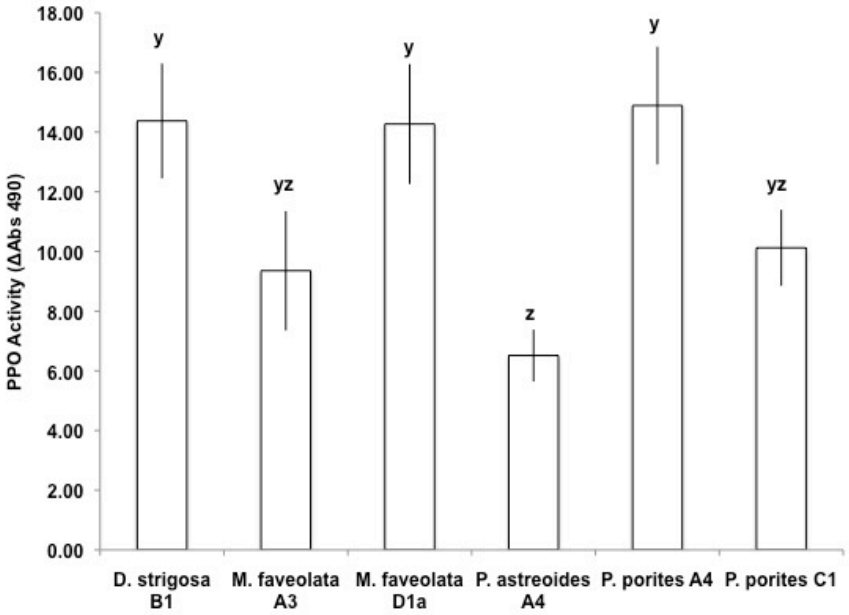

Figure 4.8 Coral PPO activity (mean, ± s.e.) by holobiont (coral x symbiont identity). Letters above each bar indicate among holobiont differences (α = 0.05, *p* = 0.0019).

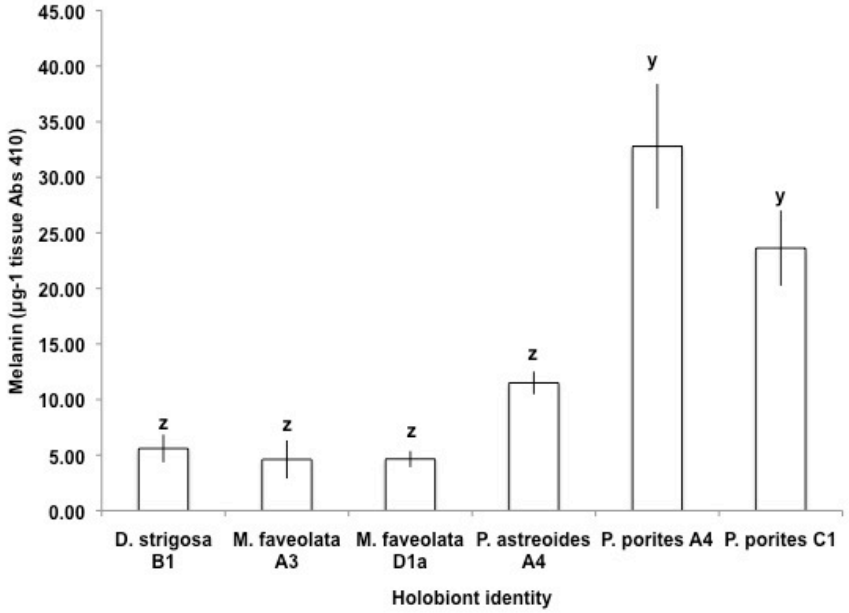

Figure 4.9 Coral melanin concentration (μg^{-1} tissue, mean, ± s.e.) by holobionts (coral x symbiont identity). Letters above each bar indicate among holobiont differences (α = 0.05, *p* = <0.0001).

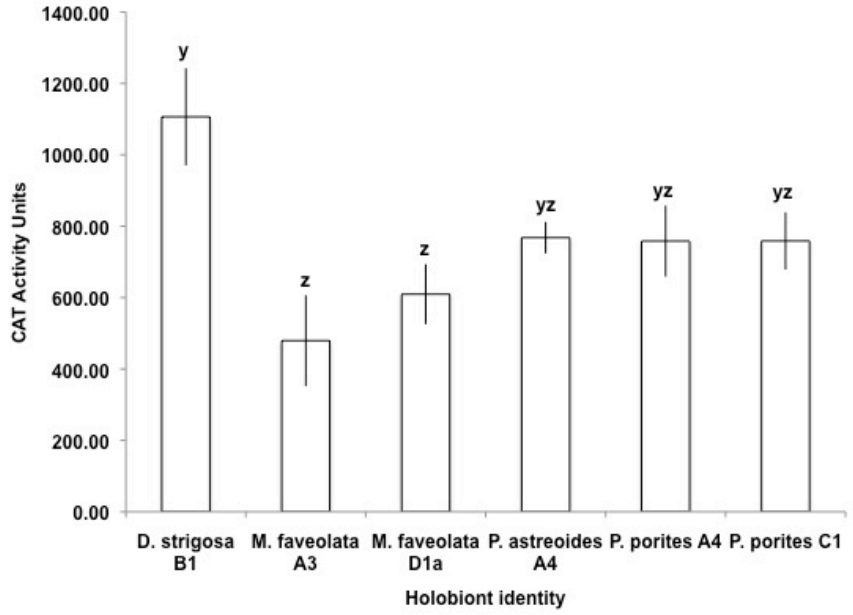

Figure 4.10 Coral CAT activity (mean, ± s.e.) by holobionts (coral x symbiont identity). Letters above each bar indicate among holobiont differences (α = 0.05, p = 0.003).

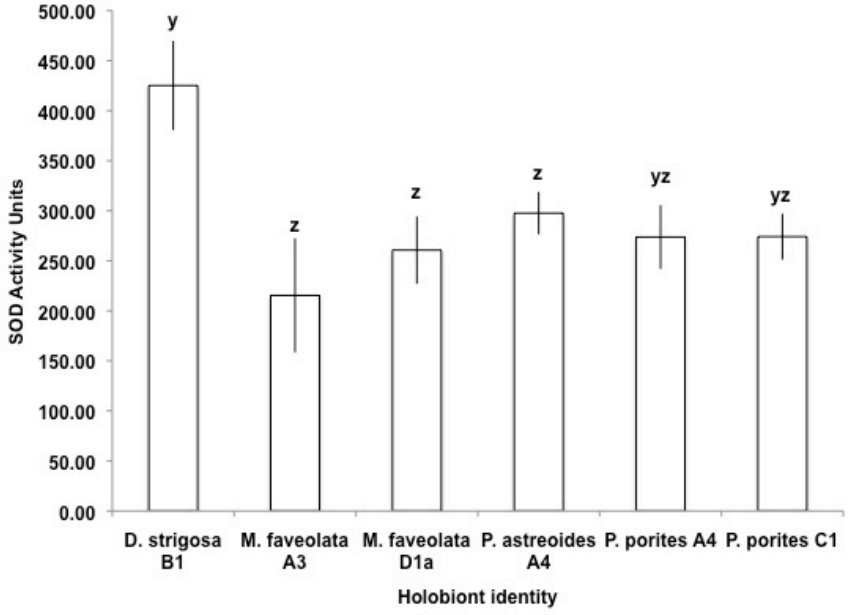

Figure 4.11 Coral SOD activity (mean, ± s.e.) by holobionts (coral x symbiont identity). Letters above each bar indicate among holobiont differences (α = 0.05, p = 0.0036).

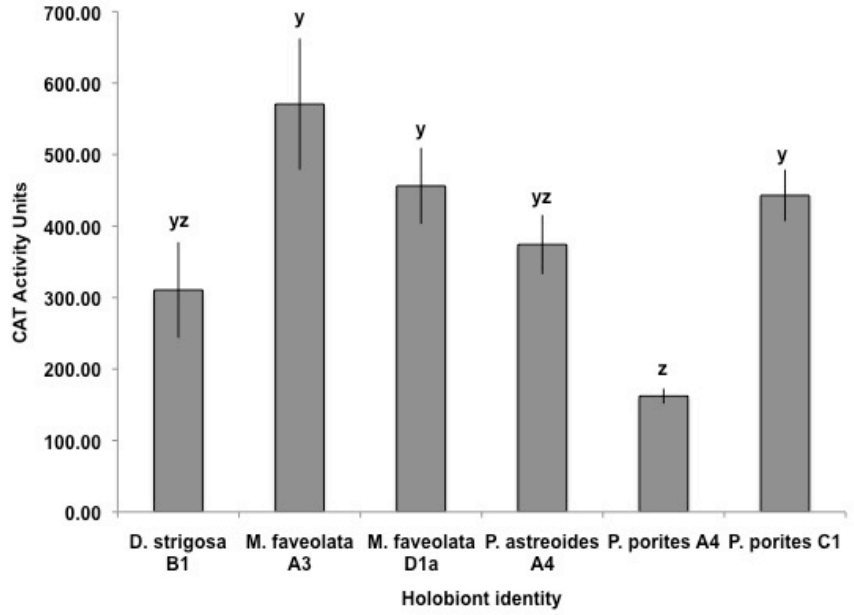

Figure 4.12 Symbiont CAT activity (mean, ± s.e.) by holobionts (coral x symbiont identity). Letters above each bar indicate among holobiont differences (α = 0.05, *p* = 0.0022).

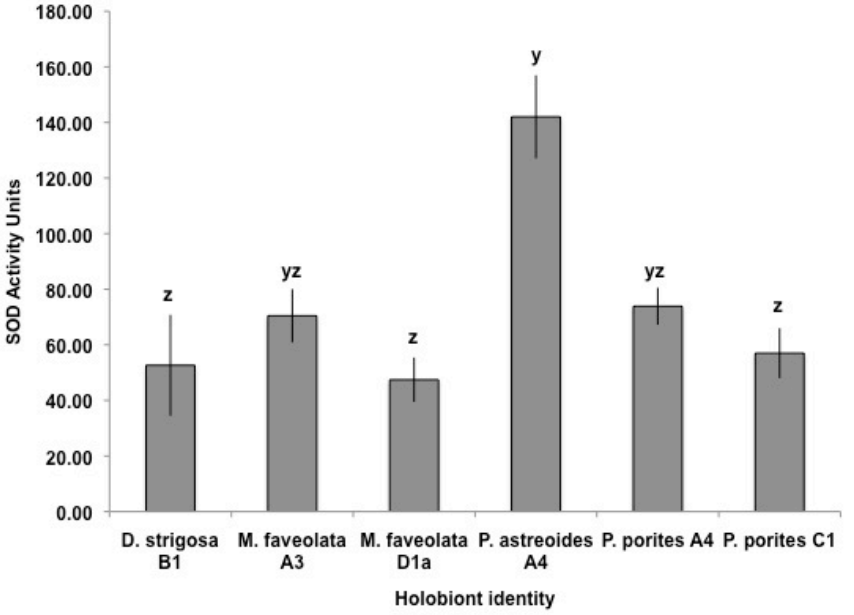

Figure 4.13 Symbiont SOD activity (mean, ± s.e.) by holobionts (coral x symbiont identity). Letters above each bar indicate among holobiont differences (α = 0.05, *p* = < 0.0001).

Both *P. astreoides* and *P. porites* were found to host *Symbiodinium* type A4 (*P. astreoides* n = 17, *P. porites* n = 7). The host coral species did make a difference in the antioxidant activity of A4, with symbionts being hosted by *P. astreoides* showing significantly more CAT and SOD activity (Table 4.7, Figure 4.14).

Table 4.7 One-way MANOVA results for the effect of host on *Symbiodinium* type A4 antioxidant activity. Underlined values of *P* indicate significance at α = 0.05.

MANOVA	df	F	*P*
Whole model	2	11.016	0.0005
Univariate tests			
Symbiont CAT activity	1	10.47	0.0038
Symbiont SOD activity	1	8.088	0.0094

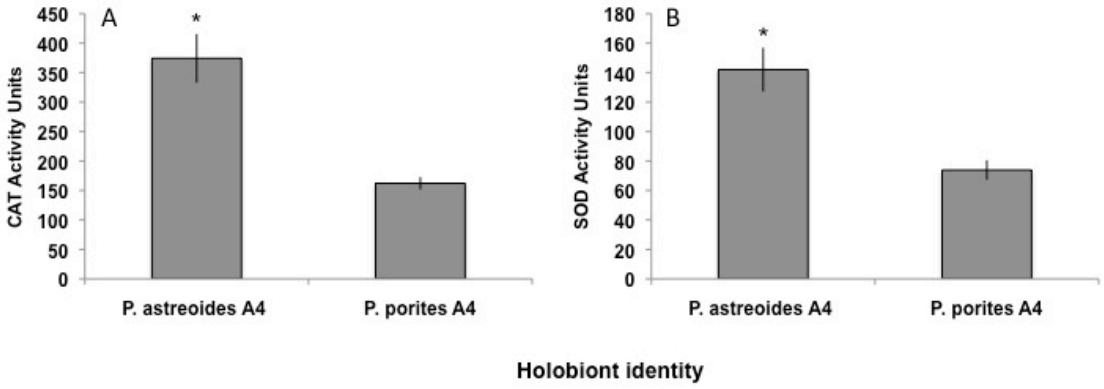

Figure 4.14 A) CAT and B) SOD activity (mean, ± s.e.) for *Symbiodinium* type A4 hosted by *P. astreoides* and *P. porites. Asterisks* indicate significant differences (α = 0.05, see Table 4.7, CAT *p* = 0.0038, SOD *p* = 0.0094).

4.3.4 Relationship between host and symbiont factors

There were strong significant correlations between coral CAT and SOD activity, as well as weak correlations between coral antioxidant activity and PPO activity (Table 4.8, Figure 4.15). In addition, weak correlations were present between symbiont CAT activity and melanin

concentrations, as well as symbiont SOD activity and PPO activity (Table 4.8, Figure 4.15). Although coral CAT and SOD activities were correlated, symbiont CAT and SOD activities did not follow the same trend (r = -0.195, r2 = 0038, *p* = 0.666, Figure 4.15).

Table 4.8 Regression analysis of significant correlations between host and symbiont factors. See Figure 4.15 for all comparisons. Underlined values of *p* indicate significance at α = 0.05.

Factors			*r*	r^2	*p*
Host CAT activity	x	Host SOD activity	0.96	0.93	<0.0001
Host CAT activity	x	PPO activity	0.30	0.089	0.0083
Host SOD activity	x	PPO activity	0.39	0.15	0.0005
Symbiont CAT activity	x	Melanin concentration	- 0.32	0.10	0.0108
Symbiont SOD activity	x	PPO activity	- 0.43	0.18	0.0005

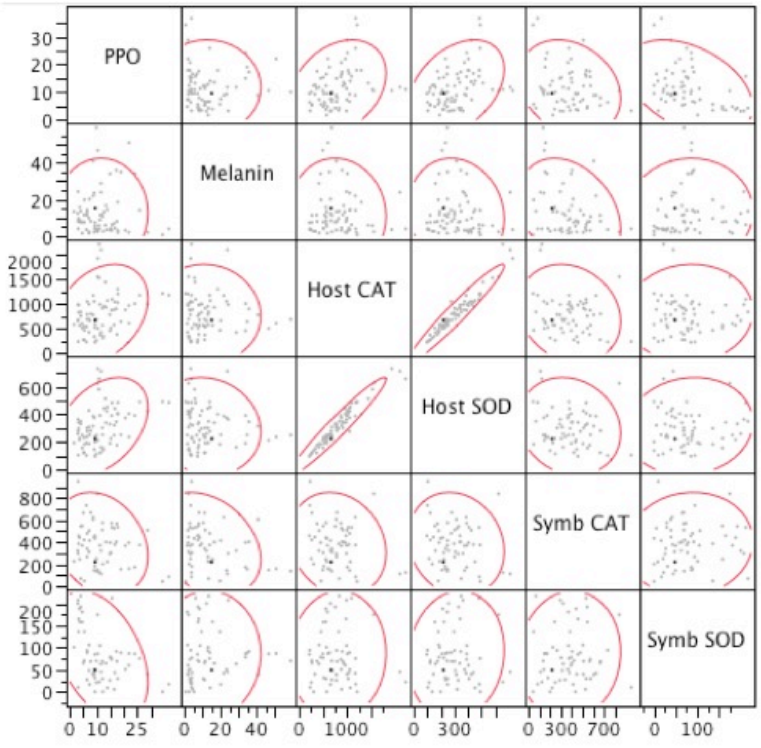

Figure 4.15 Bivariate analysis of all factors. See Table 4.8 for details of significant correlations.

4.4 Discussion

Understanding the factors driving coral reef decline has become a priority for conservation of these ecosystems. Examining how different coral host-symbiont combinations vary as a unit can provide insight into changing coral reef communities (Bourne et al. 2009, Correa et al. 2009, Veron et al. 2009). Differences in the prevalence of bleaching and disease have become apparent; corals such as *Montastraea faveolata* are disproportionately susceptible to bleaching and disease, while others, like *Porites astreoides*, have been much more resistant (Green et al. 2008, Cróquer & Weil 2009, Weil et al. 2009, Edmunds 2010). While the mechanisms underlying these differences are yet unresolved (Mydlarz et al. 2009, Palmer et al. 2011), this disparity can be correlated with components of the melanin cascade and activity of the antioxidant catalase and superoxide dismutase (Mydlarz et al. 2009, Palmer et al. 2011). Previous studies have shown that factors such as growth rates and bleaching can be affected by differences in *Symbiodinium* communities (Rowan et al. 1997, Little et al. 2004, Abrego et al. 2008). This study investigated differences in specific enzyme activity in different coral-*Symbiodinium* combinations. We hypothesized that hosting different symbionts could affect components of the host melanin synthesis pathway and antioxidant activity, and that antioxidant activity in the symbiont would be different if hosted by different coral species. Evidence from this study supports both of these hypotheses.

4.4.1 Holobiont factors

Of the four coral species collected, only *M. faveolata* and *Porites porites* associated with more than one *Symbiodinium* type. Five *M. faveolata* colonies at 1 m hosted *Symbiodinium* type A3 and four hosted type D1a, but all nine colonies at 5 m hosted type D1a. Nine *P. porites* colonies hosted *Symbiodinium* type A4 and one colony hosted type C1 at 1 m, but all 10 colonies at 5 m hosted type C1. *M. faveolata* also had higher levels of PPO activity and higher levels of coral host antioxidant activity at 5 m, while *P. porites* had higher levels of PPO activity at 1 m. The changes in these factors may be due to the changes in symbiont

identity alone, or due to differences in factors that change with depth such as light intensity or water motion, or a combination of these factors. In contrast, *Diploria strigosa* had significantly higher levels of coral host antioxidant activity at 5 m, but only hosted *Symbiodinium* type B1, so this is most likely due to an environmental affect. *Porites astreoides,* which only hosted type A4, displayed no difference with depth in any of the factors measured, suggesting that not all holobionts respond to changes in environmental conditions in the same way.

These differences among host and symbiont factors may contribute to observations of changes on the ecosystem level. The abundances of these coral species on Caribbean reefs are changing, with a loss in the major reef builders such as *M. favelolata* and increasing abundances of *P. astreoides*, due primarily to the differences in their life history traits (Green et al. 2008, Edmunds 2010). The members of the Favidae family, *D. strigosa* and *M. faveolata,* are large, slow growing boulder corals and important reef builders, and are more susceptible to bleaching and disease (Aeby & Santavy 2006, Cróquer & Weil 2009, Weil et al. 2009). Both *Porites* species have a faster growth rate, but the smaller size of the mounding *P. astreoides* and the branching morphology of *P. porites* have historically limited the contributions of these corals to the construction of reef frameworks. The loss of bleaching and disease susceptible reef framebuilder corals has allowed an increase in the relative abundance of these species (Sutherland et al. 2004, Weil et al. 2009, Edmunds 2010).

The fact that *D. strigosa* and *M. faveolata* showed significant changes in several host factors (*D. strigosa* in two of the four factors and *M. faveolata* in three of the four factors) with depth may indicate that they are more sensitive to changes in their environment, such as increases in temperature. *Porites astreoides* and *P. porites* showed little to no change in any of the host factors with depth, indicating that they are likely less responsive to changes in the environment and more robust, as observations from the field suggest (Santavy et al. 1999, Stimson et al. 2002, Green et al. 2008, Edmunds 2010). In addition, *M. faveolata* consistently had low to moderate levels of the melanin cascade and antioxidants, suggesting that it is less

resistant to an environmental or pathogenic stressor. Interestingly, this was the only coral species to host *Symbiodinium* type D1a, which has been associated with thermal tolerance (Little et al. 2004, Rowan 2004, Cooper et al. 2011b), and has also been identified in healthy genotypes of *M. faveolata* but not in diseased colonies, leading to the hypothesis that hosting D1a may confer resistance to bleaching and disease (Rowan 2004, Correa et al. 2009). This disconnect between the sensitivity of the host and the tolerance of the symbiont to both bleaching and disease highlights how complex the functional responses of coral holobionts can be, and further examinations of specific host genotype in regards to their resident symbiont populations would be a worthwhile endeavor.

The levels of *Symbiodinium* antioxidant activity from *P. astreoides* A4 and *P. porites* A4 holobionts were significantly different, providing evidence that the host is influencing the symbiont. We found *Symbiodinium* type A4 had significantly different antioxidant activity depending on if the host was *P. astreoides* or *P. porites*. This is possibly due to differences in host investment in its own biochemical processes, such as other antioxidants, differences in host demands on symbiont photosynthate, or different host tissue thickness, which may affect factors such as internal light refraction and availability (Loya et al. 2001, Stimson et al. 2002, Enríquez et al. 2005). It is also possible that this is due to symbiont genetic variation at a level that we did not examine, as it has recently been shown that combining ITS-2 markers with additional genetic markers provides a greater resolution of *Symbiodinium* genetic diversity (LaJeunesse & Thornhill 2011).

4.4.2 Relationship between host and symbiont factors

Examinations of the relationships between the functional differences between host and symbiont factors above that of specific coral species and symbiont identities patterns that may be more informative on an ecological scale. Weak, but positive, correlations existed between coral host antioxidant production and PPO activity, and are consistent with the hypothesis that high levels of elements of the melanin cascade come with some cytotoxicity (Cornet et al.

2007). This pattern has also previously been demonstrated in Caribbean corals (Mydlarz & Palmer 2011), and suggests that in order to maintain high levels of immune proteins that have reactive oxygen byproducts, antioxidants need to be elevated as well.

The relationship between the coral host functional factors and symbiont antioxidants may be more informative to what a coral may gain by hosting symbionts that vary in antioxidant levels. There was no relationship between coral and symbiont antioxidants, however, suggesting that each can behave independently, or that there is no pattern across different species. In contrast, the *Symbiodinium* antioxidant activities were significantly negatively correlated with the host melanin cascade, potentially due to available light levels within the host tissue. Because the host skeleton refracts light and increases the internal light environment (Enríquez et al. 2005) and high light levels can overexcite algal photosynthetic machinery, a higher light environment will lead to increased production of damaging reactive oxygen species (ROS) (Lesser 1996, Asada 2006). Symbionts residing in hosts with lower melanin concentrations, such as *M. faveolata* or *P. astreoides*, may therefore have an increased need for antioxidants. In contrast, corals with high levels of melanin, such as *P. porites,* would experience less excitation pressure on their photosystems, leading to less ROS and a lower need for antioxidants.

Understanding the functional differences between different host-symbiont combinations may lead to further clarification of the "winners and losers" on coral reefs (Loya et al. 2001, van Woesik et al. 2011). The loss of certain holobionts may shift coral ecosystems to new assemblages (Yakob & Mumby 2011). This study has demonstrated links between *Symbiodinium* identity and antioxidant parameters and important coral parameters implicated in tolerance or sensitivity to bleaching and disease. These results also showed relationships that may lead to trade-offs in how much a *Symbiodinium* type needs to invest in antioxidants depending on its host. The significant differences in the same symbiont type hosted by different corals, and the lack of differences in corals that host different symbionts, help define this

important symbiosis, and supports this multilayered approach when examining coral in regards to tolerance or sensitivity to environmental stressors. Further work is necessary to incorporate a larger scheme of holobiont combinations, as well as examining parameters over a wider geographic area. In addition, understanding the role of other members of the holobiont, such as the bacterial and fungal participants (Wegley et al. 2007, Ainsworth et al. 2010), is important to fully understand the coral response under future climate scenarios.

CHAPTER 5

CONCLUDING REMARKS

This dissertation has shown remarkable differences among *Symbiodinium* types. In chapters 2 and 3, I have demonstrated a wide range in the physiological responses to elevated temperatures (summarized in Table 5.1), with sensitive types such as B1 exhibiting diminished capacity at much lower temperatures than tolerant types such as F2. We found that of the eight *Symbiodinium* types examined, types C1 and B1 displayed characteristics of sensitivity to elevated temperatures, such as increased production of reactive oxygen species (ROS) and rapid loss of chlorophyll *a* when exposed to elevated temperatures. Types A1 and B2 displayed characteristics of being moderately tolerant to elevated temperatures, with increased antioxidant activity, possibly leading to their slightly higher maximum oxygen consumption rates. Types A1*, E1 and F2 were most thermotolerant, with A1* and F2 both able to regulate their metabolism within ecologically relevant temperatures, and reaching their maximum oxygen consumption rates outside of temperatures normally experienced on coral reefs. Type E1 had a significant decline in ROS production without any increase in antioxidant activity, suggesting that it may rely on mechanisms not measured here, such as other antioxidants or scavenging molecules like mycosporine-like amino acids, for protection during stressful conditions.

In addition, in Chapter 4 I have also shown functional differences in different coral-symbiont combinations, and that each partner in the relationship can significantly affect the other. The same symbiont was shown to have different antioxidant activity when hosted by a different coral species. Symbiont antioxidant activity and the host PPO activity and production of melanin were significantly correlated, demonstrating that each participant can influence the response of the holobiont to stress and pathogens.

Interestingly, the patterns from the culture studies of *Symbiodinium* type B1 were seen in the *Symbiodinium* type B1 collected in the field study. The activities of its antioxidants were consistently among the lowest for both field samples and cultures. In comparison, the antioxidant activities of its host, *Diploria strigosa*, were among the highest of the corals, and demonstrate how host and symbiont responses may be related (Figure 5.1). Potentially, if hosts invest more in antioxidants, their symbionts may be able to produce more photosynthate that can be transferred. In contrast, if the symbiont preferentially invests in growth instead of basal antioxidant activity levels, this may induce the host to upregulate its own antioxidant defenses. This provides evidence of how culture studies can inform and relate to studies of the intact symbiosis.

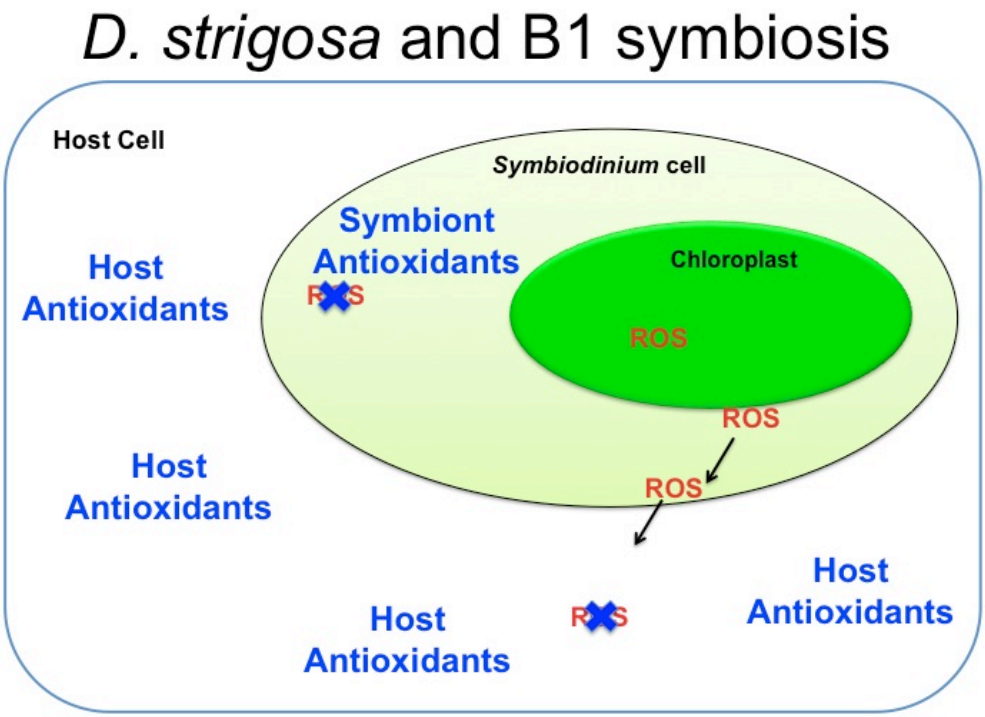

Figure 5.1 Potential model of the *Diploria strigosa* symbiosis with *Symbiodinium* type B1.

All of this data taken together suggests that the relationship between the algal symbiont and its host may exist as a dynamic fluctuation between mutualism and parasitism based on environmental conditions. The prevailing model is that when the coral holobiont experiences stress, the production of damaging reactive oxygen models triggers the host to expel the symbiont. Types such as C1 support this model, and this type may become toxic at elevated temperatures, shifting the relationship to a parasitism. In contrast, types such as B1 may simply become non-functional at elevated temperatures due to a loss of photosynthetic capacity. By receiving nutrients from the host without contributing photosynthate for the host metabolic needs, the relationship is no longer mutually beneficial, but it is unknown if this alone will lead to a disassociation of the symbiosis. Alternatively, if the rate of photosynthesis is diminished and the host can indeed demand photosynthate to the point of it being detrimental to the symbiont, the host will become a parasite. The range in temperatures where this shift may occur also appears to depend on the specific *Symbiodinium*, suggesting that this shift is determined by the symbiont's thermal tolerance threshold and metabolic response to increasing temperature.

This dissertation provides the foundation for future studies into the dynamics of this relationship. This could possibly include establishing symbioses with aposymbiotic *Aiptasia* sp. anemones using *Symbiodinium* types that vary in their thermal tolerance and exploring the response of both members of the unit to elevated temperature stress. Evidence is also accumulating that many coral species can host multiple symbionts simultaneously, so explorations into competition between algal symbionts may inform how symbiotc dynamics change when more than one symbiont is being hosted. Finally, experimentally manipulating different host-same symbiont and same host-different symbiont combinations will contribute to our understanding of how influential each member of the symbiosis may be.

Table 5.1 Summary of cultured *Symbiodinium* responses to elevated temperature stress. ROS = Reactive oxygen species production. AOX = Antioxidant activity. $\dot{V}o_2$ and Q_{10} = Temperature where maximum oxygen consumption rates were reached and Q_{10} values at 32°C (or temperature of maximum $\dot{V}o_2$ if less than 32°C). Growth = changes in growth rate from 26° to 34°C. Bleaching response = days before cultures lost Chl *a* fluorescence. Values were assigned to each category based on relative tolerance or sensitivity to thermal stress. +1 was assigned when responses indicated thermal tolerance, 0 when no indication of thermal tolerance, and -1 when sensitive to thermal stress. Empty cells indicate no data. The total value of summed responses was compared in relation to how many responses were present to determine relative sensitivity or tolerance to thermal stress. Values < 0 were sensitive to thermal stress. Values 0 – + 2 were moderately tolerant. Values > + 2 were tolerant to thermal stress.

Symbiodinium type	ROS	AOX	$\dot{V}o_2$ and Q_{10}	Growth	Bleaching response	Sum of responses	Sensitive vs Tolerant
A1	0	+1	0	-1	0	0	Moderately tolerant
A1*			+2			+2	
B1	0	0	-2	-1	-1	- 4	Sensitive
B2	+1	+1	-2			0	
C1	-1	0				-1	
D	0	0				0	
E1	+1	0	+2			+3	
F2	0	+1	+2	+1	+1	+5	Tolerant

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BIOGRAPHICAL INFORMATION

Elizabeth S. McGinty was born in State College, Pennsylvania, in 1982 to Albert E. Stolte, Jr. and Patricia E. Burns. She received her Bachelors of Science in Animal Bioscience, with minors in Microbiology and English, from the Pennsylvania State University in 2004. After her undergraduate degree she held positions at zoos in Texas and Florida, but during that time discovered a passion for coral. After joining the laboratory of Dr. Laura Mydlarz in 2007, her research interests focused on the comparative thermal physiology of the coral algal symbiosis. During her graduate career she conducted laboratory experiments and field-work in the Florida Keys, Puerto Rico and Panama. She has co-authored publications in peer-reviewed journals such as *Microbial Ecology* and the *Journal of Experimental Marine Biology and Ecology*. She received multiple grants and awards, including the Outstanding Young Scientist Award during the 8th International Symbiosis Symposium. Her future plans include investigating the shifting relationships between parasitism and mutualism through continuations of physiological studies on coral reef organisms.