Environmental Controls on the Reassembly of Symbiodinium Communities in Reef Corals Following Perturbation: Implications for Reef Futures under Climate Change

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ENVIRONMENTAL CONTROLS ON THE REASSEMBLY OF Symbiodinium COMMUNITIES IN REEF CORALS FOLLOWING PERTURBATION: IMPLICATIONS FOR REEF FUTURES UNDER CLIMATE CHANGE

By

Rivah Norwood Winter

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ENVIRONMENTAL CONTROLS ON THE REASSEMBLY OF
SYMBIODINIUM COMMUNITIES IN REEF CORALS
FOLLOWING PERTURBATION: IMPLICATIONS FOR REEF
FUTURES UNDER CLIMATE CHANGE

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The persistence of coral reef ecosystems, valued in the hundreds of billions of dollars annually, relies on the survival and growth of the coral colonies that build the habitat. However, these ecosystem engineers are threatened by a host of stressors, including warming ocean temperatures due to global climate change. Anomalously high sea surface temperatures can result in coral bleaching, the breakdown in the obligate symbiosis between the coral host and its community of single-celled, dinoflagellate algae in the genus *Symbiodinium*. Bleaching stress that is too prolonged or too severe can result in coral mortality, and increasingly frequent and severe bleaching is one of the most pressing threats to coral reef systems today. However, corals can recover from bleaching, and this recovery can include associations with thermally tolerant symbiont types that can help corals resist future heat stress. Thus, studying the way corals recover from bleaching stress is critical to our understanding of the way reefs will respond to global climate change. This dissertation aims to elucidate the factors that influence the way symbiont communities recover, with a particular focus on the thermally tolerant
symbiont type *Symbiodinium* D1a (*S. trenchii*), which has been shown to increase corals’ bleaching thresholds. By using highly sensitive, quantitative PCR assays to assess symbiont community structure, this work reveals dynamic community recovery following disturbance. First, I used the bleaching events in 2014 and 2015 as a natural experiment to examine the response of *Orbicella faveolata* colonies to back-to-back bleaching. By sampling colonies from two sites with differing thermal histories, I show that even small differences in thermal regime may drive dramatic differences in symbiont community structure. Further, examining the relationship between symbiont community structure and spawning revealed that the commonly accepted tradeoffs to hosting thermally tolerant clade D symbionts are context dependent. Next, I show that $pCO_2$ elevated to end-of-century levels (900ppm) had no effect on the trajectories of recovering symbiont communities in *Montastraea cavernosa* corals. Then, I examined the effect of mild thermal stress and recovery temperature on symbiont communities in *Acropora cervicornis*. I show that even short-term thermal perturbations can result in dramatic dynamism in the symbiont to host cell ratio. Finally, I used repeated acute thermal stress and gradually warming baseline temperatures to experimentally explore a mechanism by which clade D symbionts may rise to dominate a coral’s algal community. I show that together, punctuated thermal stress and gradual warming acted to increase the level of clade D in a stepwise fashion, which affected the response of the coral to subsequent stress. These findings help to explain how even minor differences in thermal regime can lead to dramatically different symbiont community structures. In summary, this dissertation explored which factors significantly influence the reassembly of symbiont communities in bleached corals and presented an explanation for how clade D symbionts
can become dominant. These findings illustrate the dynamic reassembly of coral symbiont communities and demonstrate that the costs and benefits of hosting thermally tolerant D1a are context dependent.
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Chapter 1

Introduction

Coral reefs are critically important ecosystems, valued at an estimated $375 billion annually (Costanza et al. 1997), providing protein and livelihoods to millions of people and a home to a diversity of species rivaling that of tropical rainforests. These ecosystems are built by scleractinian corals, which, like the trees in a rainforest, are the ecosystem engineers that provide the physical structure of the habitat. The ecological success of corals has been attributed to the obligate symbiotic relationship between corals and their communities of single-celled dinoflagellate algae in the genus Symbiodinium, a mutualism that involves the symbionts’ provision of photosynthate to the coral host in exchange for nitrogenous wastes and a relatively protected, stable place in the light. The continued existence of this valuable ecosystem therefore depends on coral health and the state of the obligate coral-algal symbiosis.

Unfortunately, corals are increasingly threatened by a myriad of stressors, including declining water quality, coastal development, overfishing, and disease. However, the most significant threats to coral reefs globally are rising sea surface temperatures and ocean acidification due to anthropogenic carbon dioxide (CO₂) emissions (Hoegh-Guldberg et al. 2007). These local and global stressors have already contributed to an estimated loss of 19% of reefs worldwide (Wilkinson 2008) and a decline in coral cover of 80% since the 1970s in the Caribbean alone (Gardner et al. 2003). Back-to-back bleaching events in Florida in 2014 and 2015 and the devastating 2016 bleaching event in the Pacific are stark reminders of the increasing frequency and severity of thermal stress events. It has been estimated that limiting greenhouse gas
emissions to avoid the worst effects of climate change would save $22 billion in economic benefits through 2100 in the US alone as a result of prolonged reef health (Lane et al. 2013, 2015). But, predictive models have indicated that unless greenhouse gas emissions are curbed drastically, correcting the course of climate change, most coral reefs will not be able to persist into the next century (van Hooidonk et al. 2013; 2014).

*Coral bleaching*

The obligate relationship between the coral host and its symbiotic algae allows for more rapid coral growth in oligotrophic waters due to the tight recycling of nutrients (Falkowski et al. 1984), but this asset can also be a critical weakness. Much of these reef declines are attributed to coral morbidity and mortality due to coral bleaching (e.g., Baker et al. 2008), which is the stress-mediated breakdown in the coral-algal symbiosis. While the mechanistic details of bleaching are not fully understood, it is generally well accepted that bleaching occurs as a result of the release of reactive oxygen species (ROS) from damaged symbionts leading to a cascading response from the host once antioxidant defense systems are overwhelmed (Weis 2008), resulting in the loss of algal cells and/or pigment from coral tissues. Anomalously high sea surface temperatures as little as 1-2°C above the average local summertime maximum can cause corals to bleach (Glynn 1991), and such temperature excursions are becoming more frequent and more severe (e.g., Hoegh-Guldberg et al. 2007).

There are a number of factors that influence bleaching susceptibility, including coral species (Loya et al. 2001), heterotrophic feeding (Grottoli et al. 2006), and host acclimatory responses (Bellantuono et al. 2012; Guest et al. 2012; Bay & Palumbi 2015). However, one of the most critical factors determining a colony’s thermal stress tolerance
is the type of symbiont hosted by the coral (Glynn et al. 2001; T. C. LaJeunesse et al. 2010). The symbiosis results in a “holobiont,” which is defined as the coral host, its community of symbiotic algae, and all of the associated microbes and viral elements that live in and on the coral colony (Rohwer et al. 2002). Here, the term holobiont will be used to refer to the coral and algal partners. Because the environmental tolerance of the holobiont is the envelope of the overlapping tolerances of each partner, exceeding one partner’s stress threshold disturbs the holobiont.

Symbiodinium diversity

Importantly, the genus *Symbiodinium* is highly diverse, with nine currently described clades (A-I, Pochon & Gates 2010) and numerous sub-clade types (e.g., LaJeunesse 2001; Rodriguez-Lanetty 2003; Van Oppen et al. 2005; Coffroth & Santos 2005). This genetic diversity is accompanied by significant physiological diversity, such that different *Symbiodinium* types have differing environmental tolerances (Tchernov et al. 2004; McGinty et al. 2012; Wang et al. 2012). These physiological traits can cause them to be distributed predictably along physical gradients such as light or temperature on a reef (Rowan et al. 1997; Iglesias-Prieto et al. 2004; Fabricius et al. 2004; Baker et al. 2013) and sometimes within a coral colony (Rowan & Knowlton 1997; Kemp et al. 2008, 2014). The differing physiological tolerances of different types have been well demonstrated during bleaching events, in which corals that host different types may respond very differently to the elevated temperature, such that one appears unaffected while another immediately adjacent bleaches and dies (Glynn et al. 2001). This patchy bleaching distribution can also be seen on the scale of a single colony in corals that host multiple symbiont types, where areas with the more sensitive symbiont bleach more
readily than the tissue hosting a more tolerant type (e.g., Rowan et al. 1997; Kemp et al. 2014).

Some *Symbiodinium*, such as some members of clade D, have been shown to have such elevated stress tolerance compared to other types, experiencing photoinhibition at higher light (Rowan 2004) and temperature (Wang et al. 2012), and showing no change in ROS or antioxidant production in response to heat (McGinty et al. 2012). Dominance by clade D often is associated with higher or more variable temperature environments (Oliver & Palumbi 2009; Baker et al. 2013) and is found on reefs recovering from recent bleaching (Baker et al. 2004; LaJeunesse et al. 2009; Kemp et al. 2014). Hosting clade D has been found to be accompanied by a 1.5-2°C increase in the bleaching threshold in both Pacific (Berkelmans & van Oppen 2006) and Caribbean (Silverstein et al. 2015) corals, leading to great interest in the role of thermally tolerant clade D symbionts on future reefs.

**Tradeoffs**

Despite these elevations in thermal tolerance, it has been argued that hosting clade D ultimately may not be beneficial to corals. Symbionts in clade D have been found to translocate less photosynthate to their hosts (Cantin et al. 2009), resulting in slowed coral growth and less allocation of energy toward reproduction (Little et al. 2004; Jones & Berkelmans 2011). Furthermore, it has been suggested that symbiont type D1a (*Symbiodinium trenchii*, LaJeunesse et al. 2014) is invasive in the Caribbean (Pettay et al. 2015). Thus, clade D symbionts are often described as opportunists with parasitic characteristics (Stat & Gates 2010; Lesser et al. 2013), outcompeting other, more beneficial types following perturbation to the community (but see Baker et al. 2013b).
Accordingly, some models have predicted declines in Caribbean coral cover as a result of the spread and dominance of D1a, concluding that reefs will persist longer if corals maintain associations with ‘wild type’ symbionts that are assumed to confer greater direct physiological benefits, such as faster growth rates (Ortiz et al. 2013a; 2013b). However, Cunning et al. (2014) found that the growth tradeoff to hosting clade D disappeared when corals were grown at higher temperatures, indicating that tradeoffs to hosting thermally tolerant symbionts may be highly context-dependent (Cunning & Baker 2014b).

*Bleaching recovery*

Corals can recover from bleaching if the proximate stressor is not too severe or prolonged (Baker et al. 2008), and recovery can sometimes involve the dominance of new symbiont types (e.g., Berkelmans & van Oppen 2006; Jones et al. 2008; Cunning et al. 2015; Silverstein et al. 2015). While bleaching is generally thought of as a detrimental phenomenon, leading to depressed lipid reserves (Porter et al. 1989; Fitt et al. 1993; Rodrigues & Grottoli 2007; Grottoli et al. 2015), reduced growth rates (e.g., Goreau & Macfarlane 1990; Baird & Marshall 2002), lowered fecundity (Szmant & Gassman 1990; Jones & Berkelmans 2011), and increased susceptibility to disease (e.g., Randall & van Woesik 2015; Precht et al. 2016), the Adaptive Bleaching Hypothesis (ABH, Buddemeier & Fautin 1993) suggests that post-bleaching recovery with different symbiont types that are more suited to new environmental conditions could be a rapid mechanism by which sessile corals can adapt to changing conditions (Baker 2001). This hypothesis has been met with some skepticism, because it has been argued that most corals are inflexible in their symbiotic associations, tending to associate stably with only one algal type (e.g.,
Goulet & Coffroth 2003; Goulet 2006; Thornhill et al. 2009; Coffroth et al. 2010; LaJeunesse et al. 2010), thereby limiting the general applicability of the ABH.

However, much of this work has used low sensitivity molecular techniques such as Denaturing Gradient Gel Electrophoresis (DGGE), which fails to detect symbionts at abundances below even 5-20% of the community (e.g., Thornhill et al. 2006; LaJeunesse et al. 2009). More recently, highly sensitive assays for Quantitative PCR (qPCR) have been developed that are able to detect symbionts with up to 1000-fold greater sensitivity (Mieg et al. 2007). Furthermore, qPCR assays have enabled the calculation of the symbiont to host cell ratio (S:H), a metric of symbiont abundance (Cunning & Baker 2013). These tools have revealed that most corals are in fact able to associate with multiple symbiont types (Baker and Romanski 2007; Loram et al. 2007; Correa et al. 2009; Cunning et al. 2015; Silverstein et al. 2012, 2015). For example, Silverstein et al. (2012) found that all of the 39 coral species tested using these assays associated with more than one clade of *Symbiodinium*.

This demonstrated level of flexibility in symbiotic association suggests that the ABH may in fact be much more generally applicable than was previously assumed. Indeed, corals have been found to recover from bleaching with thermally tolerant members of clade D (Berkelmans & van Oppen 2006; Jones et al. 2008; Jones & Berkelmans 2011; Kemp et al. 2014; Cunning et al. 2015; Silverstein et al. 2015), although some corals have been shown to bleach and recover with no changes to the composition of the symbiont community (e.g., Sampayo et al. 2008; Thornhill et al. 2009; LaJeunesse et al. 2010). In addition, novel associations are not always stable through time (e.g., Thornhill et al. 2006; Coffroth et al. 2010). However, Cunning et al. (2015)
found that more severe bleaching and higher recovery temperatures resulted in more symbiont community change during recovery and showed that even incremental changes in the abundance of stress tolerant types affected community function. Importantly, it has been demonstrated that hosting clade D, not previous exposure to thermal stress, conferred subsequent thermal tolerance to corals, elevating the bleaching threshold (Silverstein et al. 2015).

**Reef futures**

In the context of annual bleaching, which predictive models indicate may occur near the middle of the current century (van Hooidonk et al. 2014), the ability of corals to withstand repeated thermal stress will be critical to the survival of reefs long term. Because the community with which a bleached coral recovers will impact the response of the holobiont to future stress, it is a major goal of this dissertation to better understand the factors that shape the trajectory of a community during bleaching recovery. In Chapter 2, I examine recovery from a natural bleaching event at two locations in the field and examine the impact of thermal history on symbiont community structure. Unprecedented back-to-back bleaching events provided a natural experiment on the effects of repeated annual bleaching on symbiont community structure and coral reproduction. In Chapter 3, I examine the effect of elevated $pCO_2$ on bleaching recovery. While ocean acidification has been shown to interact with high light to cause bleaching (Anthony et al. 2008; but see Wall et al. 2014), the effect of elevated $pCO_2$ specifically on the recovery process had not yet been assessed. In Chapter 4, I examine bleaching and recovery on a fine time scale in the threatened staghorn coral, looking at the effect of stress duration and recovery temperature on the structure and function of the symbiont community in this sensitive,
thin-tissued species. In Chapter 5, I examine the effects of repeated, acute thermal stress and gradually warming baseline temperatures on symbiont community structure and function and assess whether exposure to these conditions affects bleaching thresholds. Finally, in Chapter 6 I synthesize my findings, identify areas for future research, highlight the implications of my studies for reef futures under climate change, and discuss some practical applications of this research for real-world reef conservation efforts.

Summary

The symbiont community with which a bleached coral recovers affects future holobiont fitness and responses to environmental change. As the effects of global climate change increasingly threaten the long-term existence of coral reefs, it is critical that we understand the coral stress response and the factors that shape the structure of the symbiont community during recovery. This work uses highly sensitive qPCR assays to examine the symbiont community in corals during bleaching recovery under different environmental conditions, with a particular focus on symbiont type D1a. Thermally tolerant symbionts such as D1a may prove to be a lifeline for corals under siege from increasingly frequent and severe thermal stress events, and restoration efforts will benefit from understanding the factors that influence the spread and dominance of this stress resistant symbiont. Whether clade D ultimately proves beneficial to the long-term health of reefs, it is important to understand the environmental factors that contribute to shaping the trajectory of the recovering symbiont community.
Chapter 2

Increased abundance of heat-tolerant symbionts following repeat thermal bleaching events increases thermal tolerance and reproductive output in the threatened Caribbean coral *Orcella faveolata*

Summary

Coral reefs are threatened by increasingly frequent and severe episodes of coral bleaching due to global climate change, evidenced by the unprecedented bleaching events seen in 2014 and 2015. In Florida, back-to-back bleaching events in 2014 and 2015 provided a natural experiment to investigate the role of the environment in shaping community dynamics following perturbation and the consequences of these dynamics for the coral host. We tagged and sampled colonies of the threatened Caribbean coral *Orcella faveolata* at two locations in the northern Florida Keys: Emerald Reef, off Key Biscayne (ER, >6m depth, N=22 colonies, sampled from colony tops ~monthly from Aug 2014 – Sept 2015), and Horseshoe Reef off Key Largo (HS, < 6m and >6m depths, N=31 colonies, sampled from colony tops and bottom edges ~bimonthly from Oct 2014 – Nov 2015). We applied highly sensitive, actin-based qPCR assays to characterize the symbiont communities over time at both locations. We also analyzed HS colonies for *Symbiodinium* cell count, visually scored them for percent bleaching, and assessed their reproductive output (as the percent of the colony that spawned in the summer of 2015). Hourly water temperature data were obtained from two buoys from the National Data Buoy Center: Fowey Rocks Lighthouse, ~9.5km south of ER, and Molasses Reef Lighthouse, ~16.5km south of HS.
Environment played a significant role in symbiont community response to stress as well as in the structure of recovering communities, with thermal history a strong determinant of clade abundance and dominance on the two reefs. HS was significantly warmer and had significantly more positive thermal anomalies than ER. While bleaching in 2014 caused significant increases in the proportion of clade D dominance at both reefs, D dominance in ER colonies subsequently declined, while levels of D were maintained on warmer HS. Deeper HS colonies hosted more clade D than shallow colonies and did not significantly bleach in 2015. At HS, differences in the symbiont communities within colony microhabitats and across depths on the reef can be explained by changing light levels, with significant differences found in the abundance and dominance of clades A, B, C, and D. These differing community structures affected 2015 reproductive output, with colonies hosting greater proportions of clade D bleaching less and spawning from greater proportions of the colony surface area. Thus, hosting thermally tolerant clade D symbionts had a significant positive impact on stress response, resource allocation, and recovery rates in response to warming. This natural experiment is a valuable case study examining the impact of environment on symbiont community structure and emphasizes the continued importance of considering the coral-algal symbiosis contextually when analyzing partner costs and benefits.

**Background**

The ecological success of coral reefs can be attributed to their obligate symbiotic relationship with single celled dinoflagellate algae in the highly diverse genus *Symbiodinium*, which has nine currently described clades (A-I, Pochon & Gates 2010) and contains levels of diversity equivalent to some other Orders of dinoflagellates.
Bleaching is the stress-mediated breakdown of this relationship, but if the bleaching stressor, such as high temperature or light, is not too severe or prolonged, corals are able to recover their communities of algae. Bleaching recovery can sometimes favor symbiont types more suited to new environmental conditions (Buddemeier & Fautin 1993; Berkelmans & van Oppen 2006; Jones et al. 2008), including types that were at very low background levels or were previously undetectable in the community (e.g., LaJeunesse et al. 2009; Kemp et al. 2014).

*Symbiodinium* are physiologically as well as phylogenetically diverse, and some types have been found to confer greater stress tolerance to their coral hosts (Rowan et al. 1997; Rowan 2004; Berkelmans & van Oppen 2006; Jones et al. 2008; Fitt et al. 2009). For example, some symbionts in clade D are more thermally tolerant (e.g., Rowan 2004) and have been shown to confer a 1.5-2°C increase in the bleaching threshold to their coral hosts (Berkelmans & van Oppen 2006). Certain types of *Symbiodinium* in clade D (such as D1a/*S. trenchi*, LaJeunesse et al. 2005) have been found to dominate recovering bleached corals (Baker 2001; Baker 2003; Baker 2004; Jones et al. 2008; Silverstein et al. 2015). The threatened Caribbean coral *O. faveolata* is found routinely to associate with multiple *Symbiodinium* types (Rowan & Knowlton 1995; Rowan et al. 1997; Toller et al. 2001) including clade D (Kemp et al. 2008; Kemp et al. 2014) and is therefore an ideal species in which to examine the factors that structure mixed symbiont communities and that promote the rise of a background type to dominance (e.g., LaJeunesse et al. 2009; Kemp et al. 2014).

Novel algal-host associations following perturbation are not always stable (Thornhill et al. 2005; Goulet 2006; Coffroth et al. 2010; but see Toller et al. 2001), and
as a result, corals are often presumed to host temporally stable symbiont communities (e.g., Goulet & Coffroth 2003; Goulet 2006). Some field surveys have demonstrated such stability over time, finding minimal or no symbiont community change and rapid reversion to pre-bleaching communities in *O. faveolata* following perturbation by thermal stress (Thornhill et al. 2006; Thornhill et al. 2009). However, other, more recent field monitoring work has shown that symbiont communities can change significantly following bleaching and that these changes can persist over time. In Kenya, Baker et al. (2013) found that increases in clade D prevalence post-bleaching depended on coral species and the temperature regime at a given site, with clade D more prominent in warmer, more variable locations. In the Florida Keys, Kemp et al. (2014) found significant changes in the symbiont communities in *O. faveolata* following a bleaching event, including significant acquisition of D1a, which was previously undetected.

Various factors can influence the relative abundances of different algal types and their rates of reassembly during bleaching recovery. For example, Cunning & Baker (2013) found that the algal abundance at the start of thermal stress affected the severity of symbiont loss during bleaching. Cunning et al. (2015) then showed that bleaching severity and recovery conditions interacted to affect the final symbiont community structure (e.g., Toller et al. 2001), with more severe bleaching and higher recovery temperatures promoting greater recovery with clade D. Reversion to pre-bleaching communities following recovery has been hypothesized to occur because the “native” symbionts are more beneficial partners under normal, non-stressful conditions (e.g., Little et al. 2004; Thornhill et al. 2006), leading to the characterization of novel symbionts recovering in bleached tissue, such as clade D, as weedy, opportunistic, or parasitic (e.g.,
Stat & Gates 2010). Indeed, trade-offs to hosting clade D have been documented for the coral host, including slower growth (Little et al. 2004) and reduced reproductive capacity (Jones & Berkelmans 2011). However, there is evidence that these trade-offs may be eliminated at higher temperatures, indicating that they are context dependent (Cunning et al. 2014) and presenting interesting implications for the stability of novel associations in a warming climate.

Most field monitoring of symbiont communities to date have used Denaturing Gradient Gel Electrophoresis (DGGE) (Thornhill et al. 2006; Kemp et al. 2008; LaJeunesse et al. 2009; A. Baker et al. 2013; Kemp et al. 2014), a non-quantitative technique that can fail to detect symbionts that make up even as much as 20% of the community (e.g., LaJeunesse et al. 2009). Few field studies have examined symbiont communities using highly sensitive molecular tools such as qPCR (but see e.g., Silverstein et al. 2012; Kennedy et al. 2015), which enables the detection of symbionts present even at very low abundances (Mieog et al. 2007) and the quantification of relative abundances of symbionts in mixed communities (e.g., Cunning & Baker 2013).

The goal of the current study was to use highly sensitive qPCR assays to examine symbiont community dynamics at two locations as they recovered from thermal stress in 2014. This natural experiment enabled the comparison of symbiont community responses to perturbation in environments with differing thermal histories and light levels. The bleaching event in the summer of 2015 then provided an unprecedented opportunity to examine the effects of back-to-back thermal stress events on symbiont community dynamics. As reefs continue to experience such frequent and increasingly severe bleaching events due to global climate change (Hoegh-Guldberg et al. 2007), it is
becoming more important to understand what factors contribute to shaping symbiont community structure, which affects the physiology of the coral host and the susceptibility of the holobiont to future stress.

**Materials and Methods**

*Field sample collection*

Colonies of *Orcella faveolata* were sampled at two offshore reefs in southern Florida: Emerald Reef (ER, N=22 colonies), ~1.6 km east of Key Biscayne at >6m depth (range: 6-7.5m), and Horseshoe Reef (HS, N=31 colonies) in the upper Florida Keys (Figure 1). The colonies at HS ranged from ~2.5 to ~7.3m, and the bathymetry of the reef is such that colonies were in one of two distinct sections of the reef: < or >6m (n=23, n=8, respectively), over a very small spatial scale (<0.1km). Tissue samples were collected from the top-facing surfaces (TS; ER and HS) and the bottom edges of the colonies (BE; HS only) following the bleaching event in summer 2014. 8mm diameter cores were taken from ER colonies approximately monthly between late August 2014 and early September 2015, with each colony sampled at least 5 times, while 13mm diameter cores were taken approximately bimonthly from each HS colony from October 2014 to November 2015.

At HS, each colony was visually assessed for bleaching status at the time of sampling, with a subset of the <6m colonies surveyed in the month before sampling began. Each was assigned to one of 6 bins denoting percent colony surface bleached (1-6: 0%, >0-10%, 10-25%, 25-50%, 50-75%, 75-100%). Each % bleaching bin was then centered for use as a continuous variable. Additionally, spawning observations were made at HS in August 2015 between 4 and 7 nights after the new moon. Colonies were
scored as having spawned or not; colonies that were not observed were not scored. If a colony spawned, the percentage of the colony that released gametes was recorded (%SSA – percent spawning surface area); to reduce inter-observer variability, values were binned into more or less than 50%SSA.

**Environmental data**

Hourly water temperature data were obtained from the National Data Buoy Center ([http://www.ndbc.noaa.gov](http://www.ndbc.noaa.gov)) from the Fowey Rocks Lighthouse (1991-2014; ~9.5 km south of ER) and the Molasses Reef Lighthouse (1991-2015; ~16.5 km south of HS), which were used to approximate each reef’s respective temperature regime (Figure 1). Average monthly temperatures were plotted over time. Temperature loggers were deployed at HS at 2m and at 7m, respectively, to assess whether temperature regimes differed by depth.

In addition, Degree Heating Weeks (DHW) were calculated for each site from 1991 to 2014 based on the monthly maximum mean temperature (MMM) for the nearest pixel at 4km resolution in the Pathfinder climatology model. DHW were calculated in two ways: the traditional Coral Reef Watch (CRW) methodology, which sums anomalies of 1°C or more above the MMM, and a method in which all positive anomalies above the MMM are summed in order to amplify the temperature anomaly signal. The data were smoothed with a 7-day running average before calculating anomalies, and the positive anomalies were summed for each 84-day period. The resulting values were then divided by 7 in order to convert from Degree Heating Days to DHW. While the method summing all positive anomalies overestimates the DHW values compared to the CRW method, the latter often will not reveal a signal and is therefore not as useful for
comparing smaller, but still potentially meaningful, differences between different
locations (R. van Hooidonk, *pers. communication*). Although no bleaching thresholds
are being derived from these data, using both methods allows a more detailed comparison
of the positive thermal anomalies between sites.

*Laboratory molecular analyses*

Whole ER cores were preserved in 1%SDS in DNA buffer heated to 65°C for 1.5
hours (Rowan & Powers 1991). Coral tissue was removed from HS cores with an air-
brush following Szmant & Gassman (1990), the resulting slurry was well homogenized,
and a 1mL aliquot of the blastate was preserved in 1%SDS in DNAB as above. DNA
was extracted from the tissue samples using an organic extraction protocol modified from

TaqMan-MGB (Life Technologies) assays targeting the actin gene region of both
the coral host and symbiont clades A, B, C, and D were used to analyze DNA samples in
duplicate through quantitative PCR (qPCR) (primers and conditions for *O. faveolata* and
clade B given in Cunning, et al. 2015). The clade A assay included 300nM Aact_F (5’-
AT GAAGTGCGACGTGGAC AT-3’), 200nM Aact_R (5’-GGAGGACAGGATGG
AGCCT-3’), and 300nM AactPrbe (5’-VIC-CGTTGGAGTAGAGGGTC-MGB-3’). Clade
C and D assays were multiplexed, using the primers and reaction conditions described in
Cunning & Baker (2012). 10µL reaction volumes with 5µL TaqMan Genotyping
MasterMix and 1µL DNA template were run on a StepOnePlus Real-Time PCR System
(Applied Biosystems, Foster City, CA). The StepOnePlus software package calculated
cycle threshold (C_T) values with a ΔR_n=0.01 threshold fluorescence.
The target DNA was considered present when amplification occurred in both technical replicates with $C_T$ values no more than 1.5 cycles apart and when no target was detected in negative control reactions. $C_T$ values were reduced by -0.064, 4.197, 3.798, and 7.416 cycles for *Symbiodinium* clades A, B, and C, and *O. faveolata*, respectively, to correct for differences in fluorescent signal intensity between the TaqMan-MGB fluorophores used for these targets and clade D. These fluorescence correction factors were calculated based on standard curves generated following Cunning and Baker (2013).

Adjusted $C_T$ values were used to calculate symbiont to host cell ratios (S:H) using the formula $2^{C_T(\text{host})-C_T(\text{symbiont})}$. Total S:H was calculated by summing the S:H for each clade present in a sample. The proportion of each clade within a community was calculated by dividing the clade S:H by the total S:H. A sample was considered to be dominated by a given clade if it made up $>$50% of the sample’s total S:H.

**Statistical analyses**

Mean monthly temperature data from the two sites were analyzed using a Student’s t test to assess differences between the two locations. Calculated DHW values were not normally distributed, so a nonparametric Kolmogorov-Smirnov two-sample test was used to examine differences in the magnitude of the thermal anomalies at each reef. Because of the large number of observations and the consequently high degree of power, $\alpha = 0.01$ was chosen to minimize Type I error.

Statistical analyses used log-transformed S:H values, which normalized the data and suppressed the influence of outlying values. Discriminant Analysis of Principle Components (DAPC) was used to visualize the differentiation in the symbiont community structures between the two reefs and among the different combinations of
sampling position and depth (<6m, TS; <6m, BE; >6m, TS; >6m, BE) within the samples from HS. The visualizations informed further testing, and ANOVA was used to determine whether the variables contributing to the axis variation were significantly different between and among groups or over time, testing the pooled samples at each time point. Levene’s test was used to determine equality of variances, and Welch’s ANOVA was used when appropriate. In addition, the frequency of samples dominated by each clade of *Symbiodinium* was calculated for each time point and used as the response in generalized linear models that included date, sample position (TS or BE), depth (<6m or >6m), and all interactions. The statistical package JMP v.12.0 was used for all analyses.

**Results**

*Temperature regime*

Mean monthly temperatures at HS were significantly higher overall than those at ER (p<0.0001; Figure 2.2A). August was the hottest month of each year, and HS was warmer than ER by 0.18±0.17°C from 1991-2014 (p<0.0001; Figure 2.2B). From 2010-2014, HS was warmer by 0.28±0.23°C (p<0.0001). When DHW were calculated using MMM+1°C, HS was not found to have significantly higher accumulated bleaching stress than ER overall from 1991-2014. However, some years showed significant differences by location, and HS did have a DHW signal in 2014 that was absent at ER (p<0.0001; Figure 2.2C). Additionally, HS had significantly more positive thermal anomalies overall from 1991-2014 relative to its own baseline temperature than had ER (p<0.0001; Figure 2.2D).
Emerald Reef

Symbiont community structure

Sampling of the top-facing surfaces of *Orbicella faveolata* colonies at ER began in late August 2014, after bleaching on the reef was already underway. There were significant differences in the mean total S:H over time (p=0.0168;), with colonies tending to increase total symbiont abundance during post-bleaching recovery until October 2014, after which the total S:H oscillated before declining significantly in June at the start of the 2015 bleaching event (Figure 2.3).

A high overall abundance of clade B symbionts was found at ER, with a majority of colonies dominated by B across the sampling period (Figure 2.4). Mean clade B S:H varied significantly over time (p=0.0204), but highly variable mean levels of clades A, B, and D S:H caused no significant differences to be found over time at $\alpha = 0.05$ (Figure 2.3). Mean proportion of clade B remained high throughout the sampling period, with minor, non-significant oscillations mirrored almost exactly by mean proportion clade D (Figure 2.4).

While clade D S:H dynamics over the course of the whole time series were not statistically significant, clade D tended to become more abundant on average during post bleaching recovery (Figure 2.3). During the months immediately following bleaching in 2014, the proportion of colonies dominated by clade D significantly increased (p=0.0488, Figure 2.4), before beginning to decline again throughout the following year.

Conversely, clade C symbionts showed the opposite pattern, with colonies dominated by clade C only during and immediately post disturbance in 2014 and 2015 (quadratic AICc -22.5, $R^2=0.87$, Figure 2.4), and the mean abundance of clade C
following a similar relationship over time (quadratic AICc 424.02, R²=0.03, Figure 2.3).

The mean proportion of clade C within the algal communities varied significantly over the year (p=0.0407), elevated during early bleaching recovery in 2015 (Figure 2.4).

In contrast, clade A was undetected in samples until April 2015, when it was detected at significantly higher levels (p=0.0147, Figure 2.3). While it remained a background symbiont (achieving a maximum proportion of just ~6.6% of the total community), its proliferation followed a logistic growth curve (logistic 5P AICc 269.02, R²=0.136), reaching approximately 1% of the community on average and then maintaining those background levels.

**Horseshoe Reef**

**Bleaching severity**

The ten colonies <6m surveyed at HS in September 2014 before sampling began were scored an average % bleaching bin of 4.3±1.7 (~50% bleached when scaled, Figure 2.5). Deeper colonies were not scored in that month, but were visually less bleached than colonies <6m. Over the course of the sampling period, shallower colonies had significantly higher bleaching scores than deeper colonies (p<0.0001). During bleaching recovery, binned scores declined steadily (p<0.0001), and there was a significant interaction with depth, as the initially more severely bleached, <6m colonies recovered to the same level as >6m colonies in the same time (p=0.0034). The 2015 bleaching event caused a significant spike in bleaching score of colonies <6m, while deeper colonies not bleaching significantly (p<0.0001; Figure 2.5).

Binned bleaching scores also significantly predicted mean total S:H, with symbiont abundance significantly declining only at higher bleaching scores (p<0.0001;
quadratic AICc 416.85, $R^2=0.129$; Figure 2.5), as well as predicted total symbiont cell count, which declined linearly with bleaching score ($p=0.0003$; Figure 2.6). Total S:H and total cell count were positively correlated ($p=0.0447$; Figure S3).

**Symbiont community structure**

The colonies at HS exhibited significantly different symbiont communities based on sample position and depth (Figure 2.6). DAPC revealed that the overall abundances of clades A, C, and D and the total S:H drove the separation of top surface (TS) and bottom edge (BE) samples, while deeper samples from both positions (>6 m) had greater levels of clade D than their shallower counterparts (<6 m) (Figure 2.7). TS contained significantly higher clade A S:H ($p<0.0001$; Figure 2.8), were more likely to be dominated by A than were BE samples ($p<0.0001$; Figure 2.9), and contained higher proportions of B ($p=0.0123$; Figure 2.9). Samples <6m were more dominated by A than deeper samples ($p<0.0001$), and there was a significant interaction between sample position and depth, with shallow, TS samples dominated by clade A more frequently than any other group ($p<0.0001$; Figure 2.9).

BE hosted greater overall abundances of clades C and D ($p<0.0001, p=0.0072$, respectively) and a larger total S:H ($p=0.0009$) than TS (Figure 2.8). Further, BE samples were significantly more frequently dominated by clades C ($p=0.0006$) and D ($p=0.0016$) than TS (Figure 2.9). Deeper colonies had significantly higher total S:H ($p=0.0103$; Figure 2.8), more abundant clade D ($p<0.0001$; Figure 2.8), and were dominated by clade D more often than shallow colonies ($p<0.0001$; Figure 2.9).

The symbiont communities were dynamic over time, with total S:H differing significantly at all position and depth combinations (<6m/TS $p=0.0416$; <6m/BE
p=0.0323; >6m/BTE p=0.0054), except >6m/TS (p=0.1309) (Figure 2.8). <6m/TS samples showed no difference in the abundances of clades A and B over time, but exhibited significant differences in the proportions of both (clade A p=0.0254; clade B p=0.0084), increasing clade A during 2014 bleaching recovery and proportionally losing clade B during 2015 bleaching recovery (Figure 2.9). The overall proportion of TS samples dominated by clade B declined linearly throughout the sampling period (p=0.0059; Figure 2.9).

In contrast, the overall proportion of samples dominated by clade D increased over time (p<0.0001; Figure 2.9). <6m/TS samples showed significant increases in both the abundance and proportion of clade D symbionts (p<0.0001; Figures 2.8, 2.9), and the proportion dominated by D increased linearly (p=0.0412, Figure 2.9). By the summer of 2015, all >6m/BTE samples were dominated by clade D (Figure 2.9) and showed significant increases in D abundance into the fall (p=0.0201; Figure 2.8).

< 6m/BTE samples showed a significant difference in clade A S:H over time (p=0.0478), increasing in late April 2015 (Figure 2.8), but the proportion of clade A in those samples did not change significantly over time (Figures 2.8, 2.9), indicating that the bloom in the symbiont community did not disproportionately favor clade A. The frequency of clade C dominance in these samples initially increased during post-bleaching recovery, then declined linearly throughout the year (p=0.0283; Figure 2.9).

2015 Spawning observations

Seven of the 31 colonies at HS did not spawn during the August 2015 spawning event, 5 of which were <6m. Twenty-two colonies were scored on what percentage of the colony surface area released gametes (% spawning surface area: %SSA), with half
releasing gametes from <50% SSA. Colonies were tissue sampled approximately one week prior to spawning. Binned bleaching score and the symbiont community were significantly correlated with %SSA in colonies <6m, with increased %SSA associated with greater total S:H \((p=0.0153)\) and significantly less bleaching \((p=0.0408; \text{Figure 2.10})\). Importantly, %SSA significantly increased not only with increasing clade D S:H \((p<0.0001)\), but also with proportion D \((p=0.0002; \text{Figure 2.10})\).

**Between-site comparisons**

**Symbiont community structure**

The *O. faveolata* colonies at the two reefs hosted very different symbiont communities, with DAPC showing strong separation between ER and HS (Figure 2.11). Overall, colonies at HS had a higher abundance of clade D symbionts than those at ER \((p=0.0002)\), and this comparison held true when comparing ER samples against only their depth and sample position counterparts (>6m/TS) from HS \((p=0.001)\). HS >6m/TS samples also contained significantly higher S:H of clades A \((p=0.0025)\) and C \((p=0.0034)\), with lower levels of clade B \((p<0.0001)\) compared to ER. ER and HS >6m/TS samples each showed mirrored changes in the proportions of clades B and D, indicating potential competition, but the dominant symbiont was reversed at the two locations. ER had significantly elevated proportions of clade B \((p<0.0001)\), while HS hosted proportionately greater levels of clade D symbionts \((p<0.0001)\). Only the total S:H at the two locations showed a significant location X time interaction \((p=0.0367)\), where total S:H at ER tended to decline, while >6m/TS samples from HS saw no significant changes.
Discussion

Environment structures symbiont communities

Temperature

While the two reefs examined in this study are similar distances offshore and have *Orbicella faveolata* colonies at comparable depths, the corals’ symbiont communities are structured significantly differently, with HS corals not only containing greater abundances of *Symbiodinium* D1a (a.k.a. *Symbiodinium trenchii*), but also more frequently dominated by this algal type. I hypothesize that these structural differences are being driven by the significant differences in the thermal histories of the two locations (see Baker et al. 2013).

Temperature is an environmental factor of critical importance to the health of coral reefs as a whole, limiting their growth on a regional scale to between the northern and southern 18°C winter minimum isotherms, while thermal anomalies as little as 1°C above the local summertime maximum can result in the breakdown of the relationship between corals and their symbiotic algae. It is well established that different symbiont types vary in their thermal tolerance (Rowan 2004; McGinty et al. 2012), conferring differing stress tolerances to their coral hosts (Glynn et al. 2001; Baker 2004; Jones et al. 2008; Fitt et al. 2009), and performing optimally in different environments (e.g., Cunning & Baker 2014; Cunning et al. 2014).

HS was significantly warmer than ER from 1991-2014; August was consistently the warmest month of each year on average for both sites, with HS warmer than ER by 0.18°C on average. More recently, from 2010-2014, HS was warmer on average by 0.28°C in August. That HS is the consistently warmer site and contains more clade D
symbionts is congruent with field observations showing that clade D is more abundant in warmer environments (e.g., Baker 2004; Fabricius et al. 2004; Ghavam Mostafavi et al. 2007; Oliver & Palumbi 2009).

The difference in average monthly temperatures between the two sites, while significant, is nevertheless quite small and alone is perhaps insufficient to explain the observed differences in symbiont community structure. Clade D is often found at higher abundances on reefs following bleaching events (e.g., Baker 2004; Jones et al. 2008; LaJeunesse et al. 2009), and both ER and the most heavily bleached samples at HS, <6m/TS, saw significant increases in the proportions of colonies dominated by clade D during bleaching recovery. When DHW are calculated using the CRW method, HS accumulated significantly more bleaching stress since 1991. Indeed, HS saw a DHW signal in 2014 that ER did not, and total S:H in TS samples indicate that HS was more severely bleached than ER. Moreover, corals at HS have also been subjected to more frequent and more extreme excursions above their own baseline MMM than have ER corals, even when those excursions are not severe enough to cause a bleaching event. Therefore, HS is not only warmer on average, it is also more often anomalously warmer than is ER, at both sub-bleaching as well as stressful intensities.

The claim that HS’s warmer, more thermally variable environment is driving the high abundance of clade D relative to ER is in keeping with established literature showing that thermally tolerant members of clade D consistently are found to be more abundant in warmer and more variable environments (e.g., Oliver & Palumbi 2009; Oliver & Palumbi 2011; Baker et al. 2013). Therefore, even if corals at ER and HS had bleached to the same degree in the past, providing the same foothold to ‘opportunistic’
clade D symbionts (Stat & Gates 2010), the differing thermal regimes at the two locations would be expected to drive different community structures over time (Oliver & Palumbi 2009).

Thornhill et al. (2005) found that colonies of *O. faveolata* from multiple sites in the Florida Keys quickly lost the clade D symbionts they had gained following the 1997-98 bleaching event or did not gain D1a at all, concluding that corals with such stable associations with particular symbiont types are unlikely to switch to alternate symbionts in response to environmental changes. However, that study examined the symbiont communities from 2000-2004, years during which there were very few positive thermal anomalies found at the sites examined in this study. Extrapolating these temperature patterns more widely to the Florida Keys, it is not surprising, therefore, that wholesale changes in symbiont community structure were not observed in that study. Importantly, the current study strongly suggests that the combination of minor elevations in mean temperature and sub-bleaching thermal anomalies in conjunction with punctuated bleaching events can drive the proliferation and potentially the persistence of clade D *Symbiodinium* in the algal communities hosted by reef-building corals.

**Light**

The symbiont communities in the HS corals showed strong structuring by both the sample position (TS v. BE) and depth (<6m v. >6m). Because temperature loggers deployed at both depths showed no significant differences between the two areas of the reef, light is the most parsimonious environmental factor driving the differences observed in these communities. Light has been found to strongly influence coral symbiont performance (e.g, Falkowski & Dubinsky 1981; Dustan 1982; Iglesias-Prieto & Trench
1994) and is an important structuring force in shaping symbiont communities in corals, both across depth gradients (e.g., Rowan & Knowlton 1995; Baker et al. 1997; Iglesias-Prieto et al. 2004) and within different microhabitats in a coral colony (e.g., Rowan et al. 1997; Kemp et al. 2008). High light, especially in combination with elevated temperature, has been found consistently to stress *Symbiodinium* (e.g., Fitt et al. 2001; Weis 2008) and can lead to coral bleaching even in the absence of elevated temperature (e.g., Anthony et al. 2008).

The distributions of the symbionts in the colonies at HS are highly consistent with early work on the symbiosis ecology of *O. faveolata*. Rowan and Knowlton (1995) found that colonies in Panama hosted three distinct taxa of *Symbiodinium* – A, B, and C – and that the distributions of these symbionts were structured strongly with depth (i.e., light). A and B types were found more commonly in shallower, high light environments, and only dominated colonies <6m, while C types were found to dominate only deeper colonies. Further, Rowan, et al. (1997) describe the landscape ecology of algal types within colonies of *O. faveolata*, with clades A and B dominating in microhabitats experiencing higher down-welling light and clade C predominant in more shaded areas (e.g., Kemp et al. 2014). The current study also finds that clades A and C showed strong, reciprocal structuring with depth and that the position within a colony influenced the symbiont types found.

While clade D was not detected in the early studies on symbiont community partitioning in *O. faveolata*, this study provides strong evidence that clade D is also structured by light. D was found to dominate some samples from all position and depth combinations at HS, but was significantly more abundant and more frequently dominant
in lower light environments: at depth and in the bottom edges of colonies. These patterns in D abundance are consistent with other work on the distribution of this lineage, because in addition to being found in warmer waters, clade D is also found in more turbid environments (e.g., Toller et al. 2001; Garren et al. 2006; LaJeunesse et al. 2010).

The light environment clearly is an important driver of symbiont community composition, shaping the structure both within a colony as well as across the reefscape. This differential community structure has important implications for resilience. HS colonies >6m were strongly dominated by clade D during the 2015 bleaching event and displayed no significant visual bleaching. It is likely that a combination of lower light levels at depth and significantly higher levels of clade D in these deeper colonies resulted in higher resilience to thermal stress.

**Potential symbiont competition**

This study suggests that despite its characterization as a weedy opportunist, D symbionts compete with other symbiont types with varying degrees of success, depending on the environmental conditions. Symbiont competition and subsequent community structuring could occur through (i) competitive exclusion through more effective resource use (e.g., Hardin 1960), (ii) niche partitioning along an environmental gradient (e.g., Iglesias-Prieto et al. 2004) (iii) out-performance leading to host selection of the more beneficial partner (e.g., Cunning et al. 2015), or (iv) some combination of the above.

At ER and in HS TS samples, mean proportions of clades D and B mirror each other throughout the sampling period, while proportions of clades C and D are mirrored in HS BE samples. However, at ER, clade D may not have an overwhelming competitive
advantage over clade B, likely because of the temperature regime discussed above. These patterns may also be a result of host selection (e.g., Coffroth et al. 2001; Coffroth et al. 2010). Further, the mean abundances of clades B and D oscillate throughout the year, with D always reaching peak S:H values after B (see Figure 4). These oscillations and delayed peaks resemble models examining resource competition among plankton, in which overall biomass remains relatively constant, but the abundances of different species vary (Huisman & Weissing 1999). In contrast, the warmer, more variable environment at HS may have enabled D to dominate corals much more frequently.

Similarly, D has been able to proliferate successfully in coral tissue historically found to be dominated by clade C (Rowan et al. 1997), supporting the claim that clade D is a generalist given appropriate environmental conditions. Interestingly, while some BE samples at HS commonly were dominated by clade C, TS samples from both ER and HS were dominated by clade C only immediately following bleaching disturbance in both 2014 and 2015. These patterns may be related to the intrinsic growth rates of C vs D symbionts, with clade C characterized by a higher mitotic index than the more slowly proliferating clade D algae (M.J.H van Oppen, unpublished data; Wooldridge & Done 2009). Remnant C populations in bleached coral tissue may be able to proliferate more quickly initially, before later being displaced.

However, clade D may not be able to compete as effectively in pigment-denuded, bleached tissue under high light against *Symbiodinium* types that are adapted to high irradiance. Clade A could be favored over D1a in the high light environment of shallow, top surfaces by incurring less photodamage (e.g., Warner et al. 1999), leading to niche partitioning along the light gradient. Whatever the mechanism(s), this study provides
further evidence that potential competitive interactions and the outcome of symbiont community dynamics are highly dependent on the environment.

*Symbiont community composition influences reproductive output*

Coral reproduction is critical to the persistence of reefs (e.g., Richmond 1997). Thermal stress has been shown to negatively impact the success of coral spawning events (e.g., Szmant & Gassman 1990; Omori et al. 2001; Ward et al. 2002; Hagedorn et al. 2016), with important downstream implications for recruitment and subsequent coral population structure (Mcclanahan 2000; Zahir 2002). Knowlton (2001) discusses how declining coral population sizes can result in the Allee effect, in which lowered concentrations of spawn lead to reduced fertilization success. Importantly, the effective population size of a colonial animal such as a coral is inversely proportional to individual variance in reproductive success (Hughes et al. 1992). Thus, a stressor that decreases an individual colony’s gamete output will increase the Allee effect and is likely to decrease the effective population size. Therefore, while reproductive output in colonial animals is closely tied to individual size (e.g., Babcock 1984), the proportion of a colony that is able to allocate sufficient resources to participate in a spawning event also has important implications for the reproductive fitness of the individual as well as the success of the population’s spawning event.

In this study, symbiont community composition was strongly correlated with the reproductive output of the coral host in colonies <6m. Those corals with higher %SSA had significantly lower visual bleaching scores at the time of spawning, as well as increased total S:H and a greater abundance of clade D. Importantly, corals that had higher %SSA hosted communities with greater proportions of clade D at the time of
spawning. These patterns were not found in colonies occupying the less stressful, lower light, >6m habitat, likely due to the relative lack of variation in the symbiont communities at depth in combination with the smaller sample size. These data suggest that hosting more clade D symbionts and maintaining symbiont communities more dominated by D can be beneficial to corals’ reproductive output in high-stress environments by limiting the degree of bleaching.

Members of clade D are often characterized as selfish, sub-optimal partners within the symbiosis (Stat & Gates 2010), translocating relatively less photosynthate than other types (Cantin et al. 2009), enabling a higher rate of photosystem repair (Tchernov et al. 2004) and maintaining higher performance under stress (McGinty et al. 2012) at the expense of the coral host under normal conditions (e.g., Little et al. 2004). It is becoming clear, however, that a blanket characterization of coral partnership with clade D is inappropriate, as the trade-offs to hosting thermally tolerant algae are context dependent. For example, Cunning et al. (2014) found that the negative growth trade-off often associated with hosting D disappeared when corals were grown at higher temperatures. While Jones & Berkelmans (2011) found that hosting D diminished the reproductive capacity of Acropora millepora, bleaching and reproduction did not co-occur in that study. In contrast, O. faveolata colonies in Florida spawn in August and September, during the warmest time of the year. The current study suggests that hosting clade D in the context of coincident thermal anomalies and spawning allows less stressed colonies to allocate more resources to reproduction. This finding has significant implications for modeling reef reproduction under climate change scenarios and highlights the continued
importance of considering the coral-algal symbiosis within an environmental context when analyzing partner costs and benefits.

**Conclusions and Significance**

This study emphasizes the importance of the environment and environmental history in shaping symbiont community structure, and is the first to use highly sensitive molecular tools to examine symbiont community dynamics across different depths and two reef sites over time. We found that variations in thermal history help to explain the patchy dominance of clade D on Caribbean reefs, and that light may have been a driving force in these symbiont community dynamics, with different light environments potentially leading to altered competitive outcomes among different symbiont types. Hosting thermally tolerant clade D symbionts had positive impacts on the coral host, associated with more rapid bleaching recovery and greater spawning activity. These data inform our understanding of how symbiont communities can impact host processes like reproduction and have important implications for the health and survival of reefs in an era of global climate change.
**Figure 2.1 Map of sampling and temperature logging sites.** Sampling sites are indicated by a white diamond, and National Data Buoy Center sea surface temperature logging stations used to assess each site’s thermal history are indicated in blue.
Figure 2.2  Temperature differences by location. (A) Mean monthly temperatures at HS are significantly higher than those at ER (p<0.0001). (B) Mean August temperatures (the warmest month of each year) to illustrate significant site differences in greater detail (p<0.0001). On average, HS is warmer than ER in August by 0.18±0.17°C from 1991-2014. (C) DHW calculated using the CRW method (MMM+1°C). HS saw significantly more positive thermal anomalies, but while there are significant differences by year (p-value indicated with asterisks: 0.001 > ** > 0.0001 > ***), the calculated bleaching stress is minimal (note the y-axis scale). (D) DHW calculated using MMM+0°C. A value of 1 indicates that for one week, temperatures at a given location exceeded the baseline MMM for that site that year. HS has significantly more positive anomalies than ER overall (p<0.0001) and in 2014 specifically (p<0.0001). Note that data for ER in 2015 are missing due to buoy malfunction.
Figure 2.3 Symbiont community dynamism following perturbation. Total S:H significantly increased during 2014 bleaching recovery ($p=0.0168$), and clade B S:H varied significantly over time ($p=0.0204$). Clade D was undetected until bleaching recovery was underway, and all clades were highly variable over time, with clade C more abundant immediately following bleaching disturbances in both years.
**Figure 2.4 Thermal stress promotes clade D.** The pie charts display the proportions of ER colonies dominated (>50% of the total S:H) by clade B, D, or C symbionts, respectively, over time. During recovery from bleaching in 2014 (August-November), the proportion of colonies dominated by clade D significantly increased (p<0.0001). The proportion of colonies dominated by clade C significantly increased immediately following perturbation in both years (p<0.0001). The mean proportions of each clade within ER colonies over time are displayed in the line graph. Note that clades B and D are almost completely mirrored, while the proportion of clade C hosted increases significantly following the 2015 bleaching event (p=0.0407). Colonies at ER were never dominated by clade A symbionts.
Figure 2.5 Colonies >6m bleached significantly less than those <6m. Binned % bleaching scores were scaled to create a continuous variable and are shown here over time for HS colonies at both depths. Colonies <6m experienced significantly more visual bleaching during the sampling period (p<0.0001), with deeper colonies not significantly bleaching during 2015. Sampling points with significant differences between depths are indicated with symbols (p-value= 0.05 > ● > 0.01 > * > 0.001 > ** > 0.0001 > ***).
Figure 2.6 Symbiont zonation with depth and colony microhabitat. Mean proportion of clades A, B, C, and D hosted by colony microhabitat and depth. Letters indicate significant, within-group differences at $\alpha = 0.05$. All clades show significant differences by sampling location.
Figure 2.7 Symbiont communities are structured by sample position and depth. HS samples were visualized by a canonical plot of DAPC, separated by combined sample position and depth on levels of clades A, B, C, and D, and Total S:H. Top surface samples separate with more A, less C, and lower total S:H, while deep samples separate based primarily on higher levels of clade D symbionts.
Shallow colonies bleach more severely on top surfaces, promoting symbiont community change. Total S:H was significantly elevated in samples >6m (p=0.0103), and significantly more severe visual bleaching in 2014 in colonies <6m is evidenced by significantly lower total S:H in those samples in early recovery (p=0.0007). <6m/TS samples also significantly increased the abundance of clade D hosted over time (p<0.0001). TS samples contained significantly more clade A overall than did BE samples (p<0.0001), while clade C was significantly more abundant in BE samples (p<0.0001). Clade D was hosted at significantly higher abundances in colonies >6m (p<0.0001) and in BE samples (p=0.0072).
Figure 2.9 Bleaching promotes symbiont community change. Pie charts show the proportion of HS colonies dominated (>50% of total S:H) by each clade over time, by sampling position and depth, while line charts display the mean proportion of each clade over time. The most heavily bleached samples (<6m/TS) experienced the greatest changes in clade proportions over time, significantly increasing the proportion of clade D hosted (p<0.0001) and decreasing the proportion of clade B (p<0.0001). Clade A occurred at higher mean proportions and was more dominant in TS samples (p<0.0001) and exhibited a significant interaction between sample position and depth, with <6m/TS samples hosting communities with significantly greater proportions of A (p<0.0001). TS samples contained higher proportions of clade B than did BE samples (p=0.0123), but experienced linear declines in dominance and proportion of B over time (p=0.0059, p=0.0084, respectively) while concurrently exhibiting increases in the proportion of samples dominated by clade D (p<0.0001). BE samples were more frequently dominated by clades C (p=0.0006) and D (p=0.0016). Clade C was dominant in TS samples primarily following perturbation in both 2014 and 2015.
Figure 2.10 HS corals hosting greater total S:H and a higher proportion of clade D bleach less and spawn more. Mean total S:H, clade D S:H, proportion D, and binned bleaching score in colonies <6m that spawned with varying %SSA. There is a significant positive association between greater spawning and higher total S:H ratio (p=0.0153), higher clade D S:H (p<0.0001), and greater proportion of clade D symbionts (p=0.0002). With increasing mean proportion D, mean binned % bleaching score decreased (p=0.0408).
Figure 2.11  Symbiont communities are structured differently by location. Canonical plot of DAPC to visualize separation of HS and ER samples by levels of clades A, B, C, and D, and Total S:H. HS samples separate with more A, C, and D symbionts, while ER samples pull out with more clade B and higher total S:H.
Figure 2.S1  Bleaching scores predict total symbiont to host cell ratio.  Scaled, binned % bleaching scores significantly predict the total S:H (p<0.0001), supporting the use of S:H as a metric of symbiont abundance.
Figure 2.S2 Bleaching scores predict total areal symbiont cell count. Binned % bleaching scores significantly predict the total symbiont cell count per cm$^2$ ($p=0.0003$), supporting the use of the visual bleaching score metric.
Figure 2.S3 Correlation of symbiont to host cell ratio and cell counts. High variability results in a rather weak correlation ($R^2=0.039$) between total S:H and total cell count, likely due to the fact that the S:H metric is a ratio and can vary with changing host cells even if symbiont counts remain constant. Nevertheless, S:H and cell counts are significantly correlated ($p=0.0447$), with increasing S:H indicating higher cell counts.
Chapter 3

**Elevated pCO₂ does not affect algal symbiont community dynamics in reef corals following disturbance**

**Summary**

Symbiont community dynamics in response to perturbation have critical implications for the coral holobiont’s stress response and long-term health. As coral reefs are subjected to increasingly frequent thermal stress events and intensifying ocean acidification, it is becoming more important for managers to understand how simultaneous stressors will affect the reassembly of *Symbiodinium* communities in corals recovering from bleaching. It has been hypothesized that elevated pCO₂ will interact with thermal stress to have synergistic effects on corals, including shaping the structure of symbiont communities. We examined the effect of elevated pCO₂ (900ppm) on the symbiont community dynamics and calcification rates of bleached and unbleached *Montastraea cavernosa* cores exposed to natural light. Elevated pCO₂ played no role in either (1) the restructuring of symbiont communities in response to high light in the absence of thermal stress or (2) the reassembly of bleached communities following thermal stress. While both bleaching and elevated pCO₂ negatively affected skeletal calcification, the combination of stressors was additive rather than synergistic. Thus, while the combined stressors of elevated pCO₂ and temperature will each negatively affect reef growth, this study indicates that acidification will not play a strong role in restructuring symbiont communities following episodes of climate change related coral reef bleaching and suggests that the ability of some corals to recover from bleaching events with thermally tolerant symbionts will not be hindered by rising atmospheric CO₂.
Background

Coral reef systems increasingly are experiencing global declines due to climate change caused by the emission of greenhouse gases like carbon dioxide (CO₂) (Hughes et al. 2003; Aronson & Precht 2006; Hoegh-Guldberg et al. 2007). Rising levels of CO₂ result in ocean acidification when seawater absorbs CO₂ from the atmosphere. The majority of the CO₂ forms carbonic acid, which dissociates into hydrogen ions and bicarbonate (H⁺ and HCO₃⁻, respectively), leading to a decrease in seawater pH and driving carbonate ions (CO₃²⁻) to combine with H⁺ to form more HCO₃⁻. This shift in seawater chemistry results in steeply falling concentrations of CO₃²⁻, simultaneously decreasing the aragonite saturation state (Ωₛ), which affects the chemical precipitation of aragonite, the mineral from which reef corals and many other marine organisms build their skeletons.

As external pH falls, corals must exert more metabolic energy to raise the pH of their calcifying medium, causing calcification to become more costly to maintain (Venn et al. 2011; 2013). The declines in Ωₛ associated with ocean acidification have been found to negatively, though variably (Chan & Connolly 2013), affect coral calcification (e.g., Langdon & Atkinson 2005; Castillo et al. 2014; Enochs et al. 2014). But beyond calcification declines, there is a laundry list of documented impacts of elevated pCO₂ on corals, including changes in fertilization and recruitment success (Albright et al. 2010), altered competitive outcomes between corals and macroalgae (Díaz-Pulido et al. 2011), and changes in gene expression (Kaniewska et al. 2012). Further, as the effects of global climate change continue to intensify, reefs will experience multiple stressors simultaneously, including increasingly frequent thermal anomalies, which can lead to the
phenomenon known as coral bleaching, in conjunction with worsening acidification pressure.

Bleaching is the stress-mediated breakdown of the obligate mutualism between the coral host and its community of symbiotic dinoflagellate algae in the genus *Symbiodinium*, which has nine currently described clades (A-I, Pochon & Gates 2010) and contains levels of diversity equivalent to some other Orders of dinoflagellates (Rowan & Powers 1992). If the bleaching stressor, such as high temperature or light, is not too severe or prolonged, corals are able to recover their communities of algae, and this recovery can sometimes include dominance of symbiont types that were at very low background levels or were previously undetectable in the community (e.g., LaJeunesse et al. 2009; Silverstein et al. 2015). *Symbiodinium* are physiologically as well as phylogenetically diverse, and some types have been found to confer greater stress tolerance to their coral hosts (Rowan et al. 1997; Rowan 2004; Berkelmans & van Oppen 2006; Jones et al. 2008; Fitt et al. 2009).

Some symbionts in clade D are more thermally tolerant (e.g., (Rowan 2004) and have been shown to confer a 1.5-2°C increase in the bleaching threshold to their coral hosts (Berkelmans & van Oppen 2006). *Symbiodinium* type D1a, also known as *Symbiodinium trenchi* (Lajeunesse et al. 2005), will often dominate recovering bleached corals (Baker 2001; Baker 2003; Baker 2004; Jones et al. 2008; Silverstein et al. 2015). However, the association is not always stable (Thornhill et al. 2006), likely because other symbionts can be more beneficial partners under normal, non-stressful conditions (Little et al. 2004; Loram et al. 2007).
It is becoming clear that various factors can influence the relative abundances of different algal types and their rates of reassembly during bleaching recovery. Cunning & Baker (2013) found that the algal abundance at the start of thermal stress affected the severity of symbiont loss during bleaching, and Cunning et al. (2015) showed that bleaching severity and recovery conditions interacted to affect the final symbiont community structure. Further, hosting D1a, not experiencing previous heat stress, conferred subsequent thermal tolerance to corals (Silverstein et al. 2015), demonstrating the importance of the composition of the symbiont community itself to later stress tolerance. Thus, factors that alter the trajectory of symbiont community repopulation in bleached corals and that change the relative abundances of different symbiont types can have critical implications for the physiology of the coral host and the susceptibility of the holobiont to future stress.

It has been suggested that increasing $p$CO$_2$ may improve the photosynthetic efficiency of *Symbiodinium* (e.g., Crawley et al. 2010; Brading et al. 2011), because the form II Rubisco in *Symbiodinium* (Rowan et al. 1996) has a lower affinity for CO$_2$ (Tortell 2000), which potentially could lead to changes in symbiont community dynamics under ocean acidification. Elevated $p$CO$_2$ has been found to have varying effects on bleaching thresholds, but can intensify coral bleaching under high light conditions (Anthony et al. 2008; but see Wall et al. 2014). Therefore, because acidification can affect the bleaching process itself, and because the recovery trajectory depends on how severely a coral bleaches, it is difficult to address the effect of elevated $p$CO$_2$ itself on community reassembly after bleaching. The purpose of this study was to examine how elevated $p$CO$_2$ specifically affected the recovery trajectory of heat stressed
symbiont communities in the Caribbean coral species *Montastraea cavernosa*. Thus, elevated $pCO_2$ was not applied during heat stress in order to isolate the effect of ocean acidification to bleaching recovery under high light.

**Materials and Methods**

*Field collection and generation of replicate cores*

Six parent colonies of *Montastraea cavernosa* were collected in January 2015 at ~8m depth from Emerald Reef, off Key Biscayne, FL. Replicate cores (N=32) were generated from each colony (total N=192) using a 2.5cm diameter drill press, trimmed to standard height (2 cm), and glued to 2.5cm diameter plaster reef plugs (Boston Aquatics). Cores were allowed to recover from coring for ~2 months indoors at 29°C in a semi-recirculating seawater system filtered to 10um under custom spectrum artificial lights (BuildMyLED) on a 12h:12h light:dark cycle at approximately 200μmol quanta m$^{-2}$ sec$^{-2}$. Cores were considered recovered when tissue growth was observed on the core edges and polyp tentacles were regularly extended for feeding.

*Experimental design*

**Phase 1: Heat stress**

Half of the cores from each parent colony were heated to 32°C over a 3-day period, with control cores maintained at 29°C. These temperatures were maintained for ~4 weeks indoors before the cores were moved to 4 shaded outdoor tanks with independent temperature and $pCO_2$ control at the University of Miami Experimental Hatchery to expose the cores to bright, natural light (~1000μmol quanta m$^{-2}$ sec$^{-2}$). Two of the outdoor tanks were maintained at 32°C, while the other two were maintained as
controls at 29°C. During this ~2 week period, all cores were maintained under control $pCO_2$ (~400ppm). Layers of shade cloth were periodically removed over the first week, until all cores were unshaded for the final week of temperature stress.

**Phase 2: Recovery under elevated $pCO_2$**

After two weeks outside, the temperature of the heated treatment was decreased to 29°C over three days. All cores were then distributed haphazardly into one of 8 tanks at either control (400ppm, n=4) or high (900ppm, n=4) $pCO_2$ at 29°C. This elevated $pCO_2$ corresponds to projected end-of-century levels. CO$_2$-enriched air was produced using mass flow controllers and bubbled into tank sumps to achieve treatment levels. The cores were maintained under these experimental conditions for 12 weeks during bleaching recovery.

**Data collection**

Initial tissue, calcification, and photochemical efficiency measurements were taken in April 2015, before the start of bleaching. Tissue samples were taken using the corner of a new single-edged razor blade to scrape tissue from approximately one third of the circumference of a single calyx wall. Samples were preserved in 1% SDS in DNA buffer heated to 65°C for 1.5 hours (Rowan & Powers 1991). Coral calcification was assessed using the buoyant weight method, and measurements were made before and after tissue sampling to account for potential skeletal loss during razor blade scraping. Tissue samples and buoyant weight measurements were taken from all cores at the end of the bleaching stress, as well as 3, 6, and 12 weeks into recovery. Daily calcification rate (g CaCO3 day$^{-1}$) was calculated as the difference between initial and final buoyant
weights, divided by number of days elapsed between each time point.

Photochemical efficiency (F_v/F_m) was measured using an Imaging Pulse Amplitude Modulated fluorometer (I-PAM, Walz, Germany) following dark adaptation for 30min after sundown. I-PAM measurements were taken approximately weekly during the heat stress period, and monthly during the recovery phase.

Water chemistry

Water samples were taken weekly at noon and fixed using mercuric chloride for later total alkalinity (TA) analysis. pCO_2 was measured at this time directly from each tank using an equilibrator.

Laboratory molecular analyses

DNA was extracted from the tissue samples using an organic extraction protocol modified from Baker, et al. 1997. TaqMan-MGB (Life Technologies) assays targeting the actin gene region of both the coral host and symbiont clades A, B, C, and D were used to analyze DNA samples in duplicate through quantitative PCR (qPCR). 10µL reaction volumes with 5µL TaqMan Genotyping MasterMix and 1µL DNA template were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Clade C and D assays were multiplexed, using the primers and reaction conditions described in Cunning & Baker (2013). Clade B was analyzed using the primers and conditions described in Cunning, et al. 2015, while M. cavernosa was assayed as described in Silverstein, et al. 2015. Clade A was assayed using the primers and conditions described in Chapter 2. The StepOnePlus software package calculated cycle threshold (CT) values with a ∆R_n=0.01 threshold fluorescence.
The target DNA was considered present when amplification occurred in both technical replicates with $C_T$ values no more than 1.5 cycles apart and when no target was detected in negative control reactions. $C_T$ values for *Symbiodinium* clade C were reduced by 3.798 cycles to correct for differences in fluorescent signal intensity between the TaqMan-MGB fluorophores used for clades C and D. This fluorescence correction factor was calculated based on standard curves generated following Cunning and Baker (2013). Adjusted $C_T$ values were then used to calculate symbiont to host cell ratios using the formula $2^{(C_T(\text{host})-C_T(\text{symbiont}))}$.

**Statistical analyses**

The software package JMP v12.0 was used to run all statistical analyses. Models used log-transformed symbiont to host cell ratios (clade C S:H, clade D S:H, or total S:H = clade C S:H + clade D S:H), proportion clade D (clade D S:H/total S:H), or calcification rate (gCaCO3/day) as the response variables. No tank effect was observed (p-values ranging from 0.14 to 0.42), so pCO2 replicates were pooled for all tests.

**Results**

qPCR of initial samples revealed that all 6 parent colonies of *Montastraea cavernosa* used in this experiment were dominated by clade C symbionts (native type C3, as determined by DGGE), with 3 colonies containing detectable clade D symbionts (D1a, a.k.a. *Symbiodinium trenchii*) at mean levels of 23.23%, 1.52%, and 0.01%, respectively (Figure 3.1).
Phase 1: Heat stress

Both bleached and control cores experienced declines in photochemical efficiency (Fv/Fm) values (p<0.0001), though there was a significant effect of bleaching on the degree of symbiont function loss (p<0.0001; Figure 3.2). As a result of this thermal stress, total bleached core calcification was significantly reduced (0.129±0.181 g CaCO3) compared to the controls (0.303±0.165 g CaCO3) (p<0.0001; Figure 3.3).

Cores with higher symbiont to host cell ratios lost proportionately more symbionts during bleaching (p<0.0001; Figure 3.4). Similarly, control cores with lower symbiont to host cell ratios gained significantly more symbionts during Phase 1 (p<0.0001; Figure 3.4). There is a significant relationship between the proportional change in the total symbiont community during Phase 1 and the change in the proportion of D1a hosted (p=0.0368; Figure 3.5); while there is not a significant effect of bleaching treatment in the slope of this relationship, bleached cores tended to lose symbionts overall but proportionally increase their relative abundance of D1a. Control cores tended in the reverse, gaining symbionts overall during the same time period but proportionally losing clade D (Figure 3.5). Bleached cores thus preferentially lost clade C symbionts during heat stress, while control corals preferentially gained clade C symbionts during Phase 1 (Figure 3.6 C, D).

Phase 2: Recovery under elevated pCO₂

Experimental tanks achieved pCO₂ levels approaching targets, with control tanks averaging 374ppm, and elevated pCO₂ tanks averaging 998ppm (Figure 3.7). As observed during Phase 1, a significant relationship was found between the total initial symbiont community and the proportional change in the symbiont community during
Phase 2 (Figure 3.8). Linear regressions revealed significantly negative slopes (p<0.0001), but elevated $pCO_2$ had no significant effect on this relationship.

Total S:H in bleached cores remained statistically unchanged during Phase 2, while control cores continued to experience S:H losses. Bleached cores recovered from thermal stress over time with significantly increasing levels of thermally tolerant clade D symbionts (p<0.0001; Figure 3.6 E, F), while control cores showed no significant change in D1a symbionts (p=0.5781; Figure 3.6 E, F). Both bleached and control cores significantly decreased their levels of clade C symbionts during Phase 2 (p<0.0001; Figure 3.6 C, D). The proportion of D1a in recovering bleached corals increased significantly during Phase 2 at both $pCO_2$ levels (p<0.0001), but did not change in control cores (Figure 3.10 C, D). Elevated $pCO_2$ had no significant effect on these community dynamics.

Heat stressed cores showed significant recovery of photochemical efficiency over time (p<0.0001; Figure 3.2). In contrast, control cores continued to exhibit declines in symbiont community function (p<0.0001; Figure 3.2). The peak observed in Fv/Fm on July 17 is likely a result of lower PAR values the previous two days due to thunderstorms (see supplementary Figure 3.S1). Both bleached and control cores showed a plateauing in Fv/Fm at the end of Phase 2, with control cores still significantly higher (p<0.0001), but there was no significant effect of $pCO_2$ level on changes in photochemical efficiency for bleached or control corals.

Bleaching and elevated $pCO_2$ each had a significant effect on total calcification (p<0.0001, Figure 3.9) and calcification rates (p<0.0001, Figure 3.10 A, B) during Phase 2, with bleached cores at elevated $pCO_2$ showing net dissolution during the ~14.5 weeks
of recovery. No significant interaction was observed between the two factors for either total calcification or calcification rate. The rates of calcification changed significantly over time for both bleached and control cores at each $pCO_2$ level ($p<0.0001$, Figure 3.10 A, B), declining first before recovering. Recovery of calcification also appeared to occur more quickly in control cores than in recovering bleached cores (Figure 3.10 A, B).

**Discussion**

*Thermal stress and recovery under elevated $pCO_2$*

**Symbiont community dynamics**

Photochemical efficiency ($Fv/Fm$) declined precipitously in bleached cores during heat stress as symbionts underwent photo-damage (e.g., Iglesias-Prieto et al. 1992). The brief increase in $Fv/Fm$ observed on May 7, 2015, is a result of the shade cloth used when the cores were initially moved outside. As layers of shade cloth were removed over the next week, photochemical efficiency continued to collapse. Cores with higher initial symbiont abundances lost proportionally more symbionts during bleaching, and this community response to heat stress further supports previously observed patterns (e.g., Cunning & Baker 2013). This relationship has been found in this study species before (Silverstein et al. 2015) and has been generally hypothesized to occur as a result of higher symbiont abundances leading to greater cumulative ROS production, causing more severe bleaching relative to the initial symbiont density (Cunning et al. 2014). In addition, corals that bleached more severely in this study showed greater increases in the proportion of D1a hosted at the end of the bleaching stress, indicating that bleached corals were preferentially losing symbionts in clade C. These data are consistent with
previous work showing that thermally tolerant symbionts in clade D are more abundant following heat stress (e.g., Baker 2004; Berkelmans & van Oppen 2006).

Increasing $pCO_2$ has been hypothesized to improve the photosynthetic efficiency of *Symbiodinium* (e.g., Crawley et al. 2010; Brading et al. 2011), potentially causing ocean acidification to affect symbiont community dynamics. However, elevated $pCO_2$ had no significant effect on the symbiont community function or dynamics measured in this study. The recovery of photochemical efficiency (Fv/Fm), the reassembly of symbiont communities, and the uptake of thermally tolerant D1a algae in heat stressed corals were unchanged under acidified conditions.

Elevated $pCO_2$ has been found to significantly accelerate recovery in bleached *Orbicella faveolata* colonies (Jones 2013). However, this effect was seen only in *O. faveolata* hosting solely D1a symbionts and was not observed in colonies hosting mixed communities. This question could not be examined in the current study because all of the *M. cavernosa* cores initially hosted C3 only or mixtures of C3 and D1a.

While no fertilization effect of elevated $pCO_2$ was demonstrated, there was also no inhibition of bleached coral recovery with D1a. This lack of constraint on community reassembly with thermally tolerant symbionts could have important implications for corals’ ability to recover from bleaching in the future, when thermal stress events and more severe ocean acidification occur in tandem.

**Calcification**

Six weeks of thermal stress decreased calcification in bleached cores by ~57%. These data are consistent with numerous other studies that have found calcification to decline as a result of thermal stress (e.g., Suzuki et al. 2000; Abramovitch-Gottlib et al.
2002; Mendes & Woodley 2002; Suzuki et al. 2003), and similar reductions in growth rate have been found in adult *Montipora capitata* immediately following one month of bleaching stress (-65%: Rodrigues & Grottoli 2006).

The current experiment further adds to the growing list of studies demonstrating calcification declines as a result of elevated $pCO_2$ in both field and laboratory settings (Langdon et al. 2000; Langdon & Atkinson 2005; Okazaki et al. 2013; but see e.g., Reynaud et al. 2003). However, despite the dramatic depressions in mean total calcification in bleached cores at high $pCO_2$, which resulted in net skeletal dissolution, there was no synergistic effect of elevated $pCO_2$ and 6 weeks of thermal stress.

*High light and $pCO_2$*

**Symbiont community dynamics in control cores**

Control cores experienced significant changes in the structure of their symbiont communities in response to the dramatic increase in light levels during Phase 1. These novel environmental conditions demanded a different optimal S:H (see Cunning & Baker 2014), and I hypothesize that the coral perceived the high light as a mild stressor, causing them to approach a new optimal S:H. However, the control corals approach the equilibrium S:H much like a pendulum, overshooting before correcting. The control corals tended initially to gain symbionts, with cores hosting lower S:H gaining proportionally more *Symbiodinium*. These increases in total S:H were driven by the proliferation of clade C symbionts, with no significant acquisition or proliferation of D1a, reaching a peak S:H approximately one month after exposure to natural light. Over the subsequent three weeks, the controls exhibited a significant decline in S:H, with symbiont losses beginning to level off by the end of the study at a significantly lower mean
symbiont abundance. Following acclimatization to higher light levels, corals can maximize the capacity for light absorption with a low pigment investment (Enriquez et al. 2005), allowing symbiont communities to be pruned (e.g., Fitt et al. 2000). Overcorrections in the abundance of symbionts following severe thermal perturbations have been observed in the field (Kemp et al. 2014), and the dynamics observed here could be a more subdued example of this process mediated by high light.

I hypothesize that the change in S:H in the control cores effectively mitigated the stress of increased light levels, converging toward a new optimum S:H (Cunning & Baker 2014). The control corals with higher symbiont abundances at the start of Phase 2 experienced greater proportional declines in S:H, as has been observed in bleached cores following thermal stress in this study and others (Cunning & Baker 2013; Silverstein et al. 2015). However, while the control cores exhibited significant declines in Fv/Fm over the course of the experiment before stabilizing, unlike the bleached cores, they did not experience a wholesale collapse in photochemical efficiency. This symbiont loss may represent a restructuring of the community in response to changed conditions, rather than stress-mediated breakdown in coral-algal symbiosis (“bleaching”). The fact that control cores with higher S:H lost proportionally more symbionts during their community restructuring is simply a fact that they had more to lose to arrive at the new S:H equilibrium.

Control coral calcification

The changes in the calcification rates of control corals over time are also best explained as a response to high light. At both $pCO_2$ levels, the growth rates of the control cores decline precipitously to the same level as the bleached cores, followed by rapid
recovery and stabilization. However, elevated $p$CO$_2$ significantly exacerbated the decline in growth rate likely caused by high light stress, leading to initial net skeletal dissolution, and continued to depress the growth rate once the control corals were acclimatized to the new light levels, reducing total Phase 2 calcification by $\sim$78% and calcification rate at the final time point by $\sim$42% with respect to control corals at low $p$CO$_2$. This growth rate decline is highly consistent with the values observed in Anthony et al. (2008), in which the calcification rates of Acropora and Porites corals under high light (~1000 mmol photon m$^{-2}$ day$^{-1}$) and elevated $p$CO$_2$ (1000-1300 ppm) were each found to drop by $\sim$40%.

**Dynamics of D1a**

The effects of high light are also apparent in the reassembly of symbiont communities in bleached corals. Despite thermal stress having been lifted at the start of Phase 2, the remnant symbiont communities within the bleached cores likely experienced what has been called “photic hell” as a result of the bleaching-induced reductions in pigment in combination with high natural light levels (Hoegh-Guldberg et al. 2005). This combination can result in greater irradiance levels within the coral tissue itself than are incident on the coral surface (Kuhl et al. 1995), termed multiple scattering, and creates conditions that can be stressful for Symbiodinium (Enriquez et al. 2005) and potentially inhibit recovery from bleaching (Hoegh-Guldberg et al. 2005).

In this study, D1a symbionts supplanted remnant clade C algae in the recovering bleached symbiont communities at both $p$CO$_2$ levels; clade C abundance continued to decline in bleached cores while they simultaneously gained D1a symbionts, leading to no significant changes in total S:H over time. Bleached coral recovery with thermally
tolerant D1a is well documented (e.g., Rowan et al. 1997; Baker 2001; Baker 2003; Thornhill et al. 2005; Berkelmans & van Oppen 2006; Jones et al. 2008). These community dynamics are in keeping with the characterization of D1a as a stress tolerant symbiont (Stat & Gates 2010 for review), which is hypothesized to be a result of less photosynthate translocation to the host (Cantin et al. 2009) leading to a greater capacity for photosystem repair (Tchernov et al. 2004). I hypothesize that the clade D symbionts proliferating in the bleached cores are thus better able to withstand the “photic hell” of the bleached coral tissue than the clade C symbionts. In Chapter 2, clade A symbionts in *Oribicella faveolata* appeared to be better competitors in the high light environment of the top surfaces of shallow colonies, with clade D occupying lower light niches. Here, in the absence of a strong competitor, clade D appears able to proliferate even in high light. Indeed, D1a has been shown to experience photoinhibition at higher light levels than other *Symbiodinium* (Rowan 2004).

As a result of this increase in clade D symbionts, the bleached cores have a higher total S:H at the final time point than the control cores, which are approaching a lower equilibril S:H in response to high light. This higher recovery S:H may be a result of the varying costs and benefits associated with different symbiont types, leading to differing optimal S:H for clades C and D even while under the same experimental conditions (see Cunning & Baker 2014). While D1a symbionts have the advantage of being able to proliferate in the “photic hell” of bleached coral tissue, the corals may require a larger standing stock of clade D to maintain growth, due to the translocation of less photosynthate per symbiont.
Bleached coral calcification

Corals hosting symbionts in clade D have been shown to calcify more slowly than conspecifics dominated by other algal types (Little et al. 2004; Jones & Berkelmans 2011), which has led to dire predictions about the negative long-term consequences of reefs’ becoming dominated by D1a following bleaching events (Ortiz, González-Rivero & Peter J Mumby 2013). Here, the mean calcification rates of bleached cores were significantly lower than those of control cores at both $pCO_2$ levels. However, the current study does not support the hypothesis that corals’ recovery with D1a negatively affects growth. During Phase 2, bleached cores significantly gain clade D symbionts, both in abundance and proportionally within the community. Growth tradeoffs associated with hosting thermally tolerant symbionts have been shown to be context dependent (Cunning et al. 2014). Recovery from bleaching with increasing levels of clade D did not prevent the corals in this study from recover calcification at either $pCO_2$ level. As clade D proliferated in bleached corals, their calcification rates recovered and at the final sampling point still appeared to be increasing to meet the rate of the control cores.

The initial calcification declines in recovering bleached cores can be explained as a response and acclimatization to high light. While it is possible that these declines are a result of proliferating D1a, the patterns of bleached coral calcification rates over time are similar to the controls, first declining during the beginning of Phase 2 before returning to initial values by the final time point. Instead, high light stress in combination with an already reduced symbiont population due to thermal bleaching likely resulted in less energy allocation to growth. Unsurprisingly, acclimatization to high light thus appears to occur more slowly in bleached cores than in controls. By the end of the study, bleached
corals had not achieved a stable calcification rate, indicating that their recovery was still ongoing. While elevated $pCO_2$ caused slower growth rates in bleached cores overall, depressing calcification rates by ~48% at the end of Phase 2, it does not appear to have hindered high light acclimatization, as the bleached cores in both $pCO_2$ levels recover growth rates at the same rate during the final two time points.

**Conclusions and Significance**

As global climate change continues, it will be increasingly important to understand the complicated ways in which different stressors will interact. Bleaching stress and elevated $pCO_2$ negatively impacted the calcification of *M. cavernosa*, resulting in net skeletal dissolution over ~14.5 weeks. However, elevated $pCO_2$ did not influence the restructuring of symbiont communities in response to high light or the reassembly of thermally bleached symbiont assemblages. Bleached corals recovered with increasing levels of thermally tolerant D1a and had initially suppressed growth rates. However, the slow initial recovery of calcification rate in bleached corals compared to controls could be a result of reduced symbiont abundance and is not necessarily due to the proliferation of D1a. Indeed, bleached coral calcification rates continued to approach those of control corals, suggesting that increasing levels of D1a in recovering corals were not preventing the recovery of calcification rates. These data further support the idea that tradeoffs to hosting stress tolerant symbiont types are context-dependent rather than absolute. While this study indicates that bleaching stress and ocean acidification are likely to additively negatively influence coral growth rates under global climate change scenarios, there is no evidence that elevated $pCO_2$ will play a strong role in shaping the structure of symbiont communities as they respond to environmental perturbation.
Figure 3.1 Mean initial symbiont to host cell ratio. Mean symbiont to host cell ratios for clade C (blue) and clade D (red) symbionts detected in each colony. The mean proportions of D1a symbionts are 23.23%, 1.52%, and 0.01% in colonies 1, 2, and 3, respectively, with no detectable clade D symbionts found in colonies 4, 5, and 6.
Figure 3.2 Mean photochemical efficiency ($Fv/Fm$) over time. Mean photochemical efficiency ($Fv/Fm$) for bleached (red) and control (blue) cores at control (left) or high (right) $pCO_2$ levels. Cores were moved outside to shaded tanks on May 4, 2015, with layers of shade cloth removed over the next week, indicated by the shaded bars. The vertical line indicates transition from Phase 1 to Phase 2. After the initial measurement, all differences between bleached and control cores are significant at each time point. Letters indicate significant within-group differences in the color corresponding to the heat stress treatment. Error bars are one standard error.
Figure 3.3 Mean total calcification during bleaching stress. Total calcification (g CaCO3) is significantly lower in between bleached (red) than in control (blue) cores (p<0.0001).
Figure 3.4 Symbiont abundance correlates with symbiont loss during bleaching. Relationship between initial total symbiont density and the change in the symbiont to host cell ratio during Phase 1. Reference line denotes no change in symbiont density during Phase 1. ANCOVA reveals that the slope of the relationship is not significantly different between bleached (red) and control (blue) cores. Linear regressions show significantly negative slopes (p<0.0001), indicating that heat stressed cores with greater initial symbiont densities bleached more severely and that control cores with lower initial densities gained more symbionts.
Figure 3.5 More bleached coral gains proportionally more clade D symbionts. For those cores that changed their proportion of D1a during Phase 1, there is a significant relationship between the total symbiont density change and the change in the proportion of D1a (p=0.0368), with colonies that lose more symbionts becoming proportionately more dominated by D1a. There is no significant effect of bleaching treatment.
Figure 3.6 No effect of elevated $p$CO$_2$ on symbiont community dynamics. Mean total (panels A & B), clade C (C,D) and clade D (E,F) symbiont abundances over time in bleached (red) and control (blue) cores, at control (A,C,E) and elevated (B,D,F) $p$CO$_2$. Reference lines indicate the end of Phase 1 (heat stress) and the beginning of Phase 2 (elevated $p$CO$_2$). Letters indicate within-group significance over time, with asterisks denoting the effect of bleaching at each time point ($p=0.05 > \bullet > 0.01 > * > 0.001 > ** > 0.0001 > ***$). The effect of $p$CO$_2$ is not significant over time. Effect of bleaching is significant across time for the abundance of both clades C and D ($p<0.0001$). Control cores do not significantly change the abundance of D1a hosted over time ($p=0.5781$). Error bars are one standard error.
**Figure 3.7** Symbiont abundance correlates with symbiont gain during recovery. Relationship between total symbiont density at the start of Phase 2 and the change in symbiont to host cell ratio during Phase 2. Reference line denotes no change in symbiont density during Phase 2. ANCOVA reveals that the slope of the relationship is not significantly different between bleached (red) and control (blue) cores. Linear regressions show significantly negative slopes (bleached: p<0.0001; control: p=0.0184). There is no significant effect of pCO$_2$ level (p=0.3964).
Figure 3.8 Effect of bleaching and elevated $p$CO$_2$ on total calcification (g CaCO$_3$) during Phase 2. Both bleaching and $p$CO$_2$ have a significant effect on growth ($p<0.0001$), but there is no significant interaction between bleaching and elevated $p$CO$_2$ ($p=0.2$).
Figure 3.9 Effects of elevated $p$CO$_2$ on calcification rate and proliferation of clade D symbionts. Panels A and B show calcification rate (g CaCO3/day) from one time interval to the next at low and high $p$CO$_2$, respectively. Panels C and D show the proportion of D1a hosted by bleached (red) vs. control (blue) cores. The vertical reference line denotes the shift from Phase 1 to Phase 2 of the experiment, while the horizontal reference line in panels A and B indicates no net calcification. Letters indicate within-group significance over time, with asterisks denoting the effect of bleaching at each time point ($p=0.05 > \bullet > 0.01 > * > 0.001 > ** > 0.0001 > ***$). High $p$CO$_2$ significantly depresses calcification rate in both bleached and control cores ($p<0.0001$). The proportion of clade D in bleached cores increases significantly during Phase 2 at both $p$CO$_2$ levels ($p<0.0001$), but there is no significant effect of elevated $p$CO$_2$. Error bars are one standard error.
Figure 3.S1  July 2015 PAR measurements explain elevated photochemical efficiency ($F_v/F_m$) measurement. Note the drop in light levels from July 16-17, which helps to explain the elevated I-PAM measurement on July 17 (Figure 3.2).
Chapter 4

Identifying thermal tolerance thresholds in threatened Acropora cervicornis corals hosting A3 symbionts

Summary

Coral restoration efforts in the Caribbean focus heavily on the threatened staghorn coral Acropora cervicornis, which is a relatively quickly growing, thin-tissued species that can be propagated easily in nurseries by fragmentation. However, outplanting nursery-grown fragments can result in wasted effort when thermal stress events in the field cause outplants to succumb to bleaching-induced morbidity or mortality, and it is therefore of critical importance for nursery managers to understand the thermal stress response of A. cervicornis. Controlled application of heat stress in the laboratory has been demonstrated to elevate the thermal tolerance of other Caribbean coral species, resulting in “stress hardened” corals by promoting thermally tolerant Symbiodinium types in the symbiont community. The goal of this study was to explore the bleaching response of A. cervicornis to help develop a stress hardening protocol for this sensitive species. If successful, this methodology could be used to increase the thermal resistance in nursery fragments prior to outplanting, potentially increasing the success of restoration efforts.

This experiment examined the response of A. cervicornis hosting Symbiodinium type A3 to increasing severity and duration of thermal stress (31° to 32°C) and subsequent ramping (2°C/day) to recovery temperatures at 27° or 29°C. Imaging pulse amplitude modulated fluorometry was used to assess photochemical efficiency (Fv/Fm), and highly sensitive actin-based qPCR assays were used calculate the symbiont to host cell ratio (S:H), a metric of symbiont abundance. Corals were ramped to bleaching
temperature over one week and removed from heat stress after 2, 4, 10, and 16 days at temperature. Coral fragments stressed at 31°C for 2 days and those at 32°C for 4 and 16 days showed slight, but significant, declines in S:H (p=0.0108, p=0.0130, and p<0.0001, respectively), while Fv/Fm declined by only 31.2% in corals stressed for 16 days. Recovery temperature played a significant role in the response of corals during early recovery, with S:H tending to increase following the cessation of the thermal challenge at 27°C.

Fv/Fm showed a significant correlation with S:H that was dependent on the bleaching status of the coral. Unstressed corals had Fv/Fm values that were poorly explained by S:H, while the most stressed corals showed a significant positive correlation, indicating that the corals with the lowest S:H were accruing the most photodamage. This positive association was maintained during early recovery at both temperatures, but recovery temperature had a significant effect over longer time scales (>1 month), with corals at 29°C maintaining the positive correlation and corals at 27°C showing no relationship.

These results suggest that a stress threshold was surpassed between 10 and 16 days of heat stress, with corals at 32°C for 16 days showing significant declines in S:H and Fv/Fm. There was also a significant positive correlation between these two metrics in the most stressed corals, which was absent in corals stressed for shorter durations. While the applied thermal stress was not sufficient to induce changes in the symbiont community composition, this experiment represents an important first step in understanding in detail the stress response of this critical ecosystem engineer and reveals
that very early recovery may represent a point of intervention to influence the structure of symbiont communities as S:H changes rapidly at lower temperatures.

**Background**

Florida has experienced major declines in coral reef ecosystem health as a result of coral bleaching, disease, and other stressors. These declines have resulted in catastrophic losses of populations of the staghorn coral, *Acropora cervicornis*, which was listed as threatened under the ESA in 2006. Efforts are underway to restore local staghorn populations by outplanting fragments that have been grown in coral nurseries (e.g., Lirman et al. 2010; Johnson et al. 2011; Young et al. 2012; Lohr et al. 2015). But, once the fragments have been returned to the reef, they are subject to the same environmental stressors that affect the reef as a whole, including elevated temperatures due to global climate change. These hard-to-predict stress events can decrease the success and efficiency of these restoration efforts as fragments succumb to temperature stress and bleaching. For example, a recent outplanting effort on the northern Florida Reef Tract resulted in approximately 89% fragment mortality due to bleaching stress (C. Drury, unpublished data). Consequently, identifying and outplanting fragments that are more resistant to thermal stress would be a valuable compliment to these restoration efforts. Moreover, if the thermal tolerance of corals can be increased while they are in the nursery, then outplantation success will be less sensitive to thermal anomalies due to global climate change.

Stress hardening has been proposed as a way to introduce intra-generational changes in corals to increase their tolerance to subsequent stress (van Oppen et al. 2015) and is an example of “hormesis,” in which a beneficial response in an organism is
induced by exposure to a low dose of a stressor that would be damaging at higher levels. One way in which an increase in thermal tolerance may be achieved is by hardening fragments through controlled exposure to heat stress, which can result in changing symbiont communities (e.g., Silverstein et al. 2015; Cunning et al. 2015). Algal symbiont communities are dynamic in response to environmental change (Cunning & Baker 2014), a dramatic example of which is the bleaching response to elevated temperature. Temperature anomalies as low as 1-2°C above the mean summertime maximum can result in the breakdown of the coral-algal symbiosis (Hoegh-Guldberg et al. 2007). If the bleaching stress is not too severe or prolonged, the symbiont communities can recover, sometimes favoring different algal types potentially more suited to new environmental conditions (Buddemeier & Fautin 1993; Baker 2004).

The genus *Symbiodinium* is highly diverse, with nine subgeneric clades (A-I, Pochon & Gates, 2010) currently described, and changes in the relative proportions of different symbiont types within the community can have profound effects on holobiont response to environmental conditions and future stressors (Berkelmans & van Oppen 2006; Jones et al. 2008; Silverstein et al. 2015). Different types of *Symbiodinium* can vary in their physiological tolerances and, therefore, in their environmental optima (e.g., Iglesias-Prieto et al. 2004). For example, certain members of clade D, such as D1a in the Caribbean, have been shown to be more thermally tolerant and to experience photoinhibition at higher light levels than other *Symbiodinium* (Rowan 2004).

Thermally tolerant *Symbiodinium* in clade D have been found to emerge on reefs following bleaching events (e.g., Baker 2004), including D1a in the Caribbean (e.g., Kemp et al. 2014). Emergence of thermally tolerant symbiont types in this clade has
been associated with reduced coral mortality (e.g., Baker 2004) and elevated bleaching thresholds (Berkelmans & van Oppen 2006; Jones et al. 2008; Silverstein et al. 2015), although over time corals often revert to hosting their original symbiont type, which may be more optimal under non-stressful conditions (e.g., Thornhill et al. 2005; LaJeunesse et al. 2009). Recently, it has been argued that D1a is native to the Indo-Pacific and is an invasive type of *Symbiodinium* in the Caribbean (Pettay et al. 2015). However, there is no doubt that these symbionts are common on Florida’s reefs, and regardless of their origin, it is worth understanding the factors that control their emergence and dominance post-bleaching.

Experimental bleaching can result in symbiont community change in favor of thermally tolerant *Symbiodinium* in clade D, which have conferred higher bleaching thresholds to their coral hosts (Silverstein et al. 2015). These community shifts are enhanced under elevated recovery temperatures (Cunning et al. 2015). However, *A. cervicornis* is particularly sensitive to perturbation, and determining the degree of stress that will achieve the desired hormetic response without incurring unnecessary damage requires careful experimentation. To date, there has been limited experimentation on bleaching and recovery processes in *A. cervicornis*, and the factors that contribute to this species’ ability to resist thermal stress and recover from bleaching remain poorly understood. *A. cervicornis* in the Florida Keys have been found to host thermally tolerant members of *Symbiodinium* clade D (Baums et al. 2010) and they have also been dominant in Miami-Dade county (Port of Miami, unpubl. data). However, the capacity of local *A. cervicornis* to recover from bleaching with clade D symbionts, whether elevated recovery temperatures accelerate the inclusion of these thermally tolerant algae in the
symbiont community, and if these algae increase the bleaching threshold in *A. cervicornis* as they do in other species (e.g., Berkelmans & van Oppen 2006; Silverstein et al. 2015), remains unknown.

Stress hardening has not yet been performed with *A. cervicornis*, due to difficulties bleaching these corals and recovering them in the laboratory without incurring significant mortality. In a pilot study, we subjected 3 fragments from 18 genotypes (N=54) of *A. cervicornis* from the RSMAS Lirman Lab’s North Nursery (off of Elliott Key) to heat stress, ramping from 27°C to 32°C over one week and maintaining them at 32°C for 8 days before returning to 27°C at the same rate. All fragments exhibited complete loss of pigment and total mortality within 10 days at the recovery temperature. Given the outcome of this pilot study, the current study was designed to be a conservative exploration of the thermal stress response in this sensitive, thin-tissued coral species.

**Methods**

*Collection and acclimation*

Fragments from 4 genotypes of *Acropora cervicornis* were collected from approximately 7.5m depth at the RSMAS Lirman Lab’s experimental North Nursery, offshore of Elliott Key in the Florida Keys, on 3/3/16 (N=51 per genotype, ~7-14 cm long). They were returned to indoor tanks under controlled temperature and light (24°C, 300µM quanta m² sec⁻¹) with incoming seawater from Biscayne Bay filtered to 10 µm, mounted upright using epoxy-filled slices of PVC pipe. While they recovered from collection, the fragments were ramped slowly to 27°C over approximately one month in the indoor tanks under LED lights on a 12 hour light, dark cycle.
Experimental design

Four control fragments from each genotype (n=16) remained at 27°C at the start of the experiment, while the remaining fragments experienced ramping temperatures at a rate of 4°C per week (Figure 4.1). When the temperatures reached 29°C, 6 fragments of each genotype (n=24) were haphazardly removed from ramping, with 12 fragments held at 29°C (n=3 per genotype) and 12 returning to 27°C (2°C per day). The remaining fragments continued ramping until 31°C was reached (after one week), and 24 fragments were haphazardly removed from heat stress (n=6 per genotype), ramping down in temperature at a rate of ~2°C per day, with 12 fragments stopping at 29°C for recovery (after one day) and the other 12 continuing to ramp down to 27°C. 24 fragments continued to be removed from the temperature stress in this manner after 2, 3, 4, 6, 10, and 16 days at temperature. After two days of heat stress at 31°C, temperatures were increased to 32°C (1°C per day), and the lights were increased to 800μM quanta m\(^{-2}\) sec\(^{-1}\) over two days in the heat stress treatment.

Data collection

Symbiont community structure and function

The initial symbiont community in each fragment was sampled using the corner of a clean, single-edged razor blade to remove a single polyp from near the base of the fragment. All fragments were then sampled (1) at the start of the experiment (after 1 month ramping and acclimation to 27°C), (2) when samples reached 29°C during ramping to heat stress, (3) daily once bleaching temperatures were achieved, and (4) periodically into recovery. At each sampling point, a single polyp was removed from the same location on each fragment to minimize the potential effect of varying symbiont
abundance along the branch length. The tissue samples were preserved as cell lysates by heating to 65°C for 60 minutes in 300µL of 1% SDS in DNA buffer (Rowan & Powers 1991).

Imaging pulse amplitude modulated (I-PAM) fluorometry, which provides a measure of photochemical efficiency (Fv/Fm), was used to track symbiont community function at each tissue sampling point: daily during heat stress and approximately weekly during temperature ramping and recovery. Measurements were made from tissue immediately adjacent to the sampled polyp at each time point following 30 minutes of dark adaptation at the end of the 12 hr light cycle.

**Symbiont cell counts**

One fragment from each genotype was sacrificed for symbiont cell quantification at the start of the experiment before heat stress (27°C), at the end of ramping to 31°C, after 8 days in heat stress (32°C), and after long-term maintenance at 27°C (>2 months). The top centimeter of each sacrificed fragment was removed to eliminate a potential source of variability in the symbiont depauperate apical tip, and the top three centimeters of the remaining fragment were cut with bone cutters. Tissue was blasted from the 3 cm segment using a Water Pik with DNA buffer. The blastate was homogenized using a tissue homogenizer (following Szmant & Gassman 1990), and a 1mL aliquot was taken and preserved using 100µL of Lugols solution. Fragment surface area was estimated as a cylinder, with the diameter calculated as the average between the top and bottom cross sections of the blasted length (e.g., Roth et al. 2010). Symbiont cells were quantified using two independent replicate counts on a haemocytometer (Hausser Scientific) using a compound microscope at 100× magnification and were normalized to surface area.
DNA analysis

DNA was extracted from the tissue samples for corals exposed to 0, 2, 4, 10 and 16 days of heat stress and were analyzed using an organic extraction protocol modified from Baker, et al. (1997). DNA sequencing of ITS-2 rDNA amplicons retrieved by Denaturing Gradient Gel Electrophoresis (DGGE) was used to determine sub-clade symbiont types (e.g., LaJeunesse & Trench 2000). TaqMan-MGB (Life Technologies) assays targeting the actin gene region of both the coral host and symbiont clades A, B, C, and D were used to analyze a subset of initial and final DNA samples in duplicate through quantitative PCR (qPCR). The *A. cervicornis* assay included 200nM Acerv_F (5’-TCTGTACGCCAACACTGTGCTT-3’), 200nM Acerv_R (5’-AGTGATGCCAAGATGGAGCCT-3’), and 500nM AcervAct (5’-FAM-AGGAAATCACTGCTCTTTG-MGB-3’). Clade A was assayed using the primers and reaction conditions described in Chapter 2. Clade B was analyzed following Cunning, et al. (2015), while clades C and D were multiplexed using the primers and reaction conditions described in Cunning & Baker (2013).

Only *Symbiodinium* clade A was detected, and the remainder of samples were analyzed for clade A only using the primers and reaction conditions described in Chapter 2. 10µL reaction volumes with 5µL TaqMan Genotyping MasterMix and 1µL DNA template were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The StepOnePlus software package calculated cycle threshold (*C*_T) values with a Δ*R*_n=0.01 threshold fluorescence. The target DNA was considered present when amplification occurred in both technical replicates with *C*_T values no more than 1.5 cycles apart and when no target was detected in negative control reactions. *C*_T values
were used to calculate symbiont to host cell ratios (S:H) using the formula

\[ 2^{C_{r}(\text{host}) - C_{r}(\text{symbiont})} \]

**Data analysis**

Student’s t-tests were used to analyze initial vs. final values for S:H and \( F_v/F_m \) for each stress duration. Generalized linear models were used to assess the effect of recovery temperature on \( F_v/F_m \) and the interacting effects among S:H, bleaching status, and stress duration on \( F_v/F_m \). ANCOVA was used to assess the effect of recovery temperature on the relationship between S:H and \( F_v/F_m \) over time and to examine the relationship between S:H and symbiont cell counts at different temperatures. The statistical package JMP v13.0 was used to run all analyses.

**Results**

The thermal stress applied in this study had a significant effect on the photochemical efficiency of corals exposed to heat stress for more than 2 days (4 days: \( p=0.0183 \) and 6, 10, and 16 days: \( p<0.0001 \)) (Figure 4.2). However, even the most stressed group saw only a 31.2% decrease in \( F_v/F_m \), while fragments exposed for shorter durations had symbiont communities largely unaffected by heat stress, exhibiting minimal depressions of \( F_v/F_m \) (-2.8%, -5.1%, 7.4%, and -13.8% for corals exposed for 2, 4, 6, and 10 days, respectively). Recovery temperature significantly affected recovery of \( F_v/F_m \) only in corals stressed for 16 days, with fragments recovering at 29°C showing delayed recovery compared to those at 27°C for ~1 month (\( p<0.0001 \); Figure 4.2).

Temperature had a significant effect on S:H. Control coral S:H diverged from initial values, with increasing S:H at 27°C and depressed S:H at 29°C. Significant
differences by temperature were maintained for approximately 3 weeks (p-values ranging from <0.0001 to 0.0112), but oscillations in S:H values (especially at 27°C) ended in re-convergence and no difference by temperature (Figure 4.3A). Coral fragments stressed at 31°C for 2 days and those at 32°C for 4 and 16 days showed significant declines in S:H (p=0.0108, p=0.0130, and p<0.0001, respectively) (Figure 4.3B, C, E). These fragments also tended to exhibit significant increases in S:H at 5 days of recovery in corals at 27°C, while S:H in corals recovering at 29°C remained depressed (p<0.0001) (Figure 4.3B, C, E). As in the control corals, these differences by temperature were not long-lived, and were eliminated in as little as 2 weeks (e.g., Figure 4.3E).

Fv/Fm showed a significant correlation with S:H that was dependent on the bleaching status of the coral. Unstressed corals had Fv/Fm values that were poorly explained by S:H (R²=0.015), and this lack of correlation was found for stressed corals at 2, 4, and 10 days of heat exposure (Figure 4.4). However, corals under heat stress for 16 days showed a significant positive correlation (p=0.0264), indicating that the corals with the lowest S:H at that time point were accruing the most photodamage. This positive relationship (p<0.0001) was maintained during early recovery with no effect of recovery temperature, though faster recovery of both S:H and Fv/Fm were evident at 27°C (Figure 4.5). However, over longer time scales (>1 month), recovery temperature had a significant effect on the relationship, with corals at 29°C maintaining the positive correlation (p=0.0094) and those at 27°C showing no relationship between S:H and Fv/Fm (p=0.9133) (Figure 4.5). Correlations at >1 month recovery for corals stressed for shorter durations were not significant.
Temperature also may affect the relationship between symbiont density (cells/cm\(^2\)) and S:H. Unstressed corals sampled after long-term maintenance at 27° showed a significantly negative relationship, with lower S:H correlating with higher cell counts per square cm (p=0.0009). Corals sampled after 8 days at 32°C showed a positive (though non-significant) trend, and the interaction between temperature and S:H is significant at p=0.05629 (Figure 4.6).

**Discussion**

The conservative design of the current study, with corals removed from heat stress after very short durations, was based on the catastrophic response to thermal stress observed in the pilot study performed 2 years earlier, in which nursery-collected fragments were exposed to 32°C for 8 days, resulting in complete visual loss of pigment and total mortality. However, *A. cervicornis* fragments proved highly resistant to the thermal challenge applied in this study. In the current experiment, even 16 days at 32°C under high light caused only paling and minimal losses in S:H and F\(_v\)/F\(_m\) with no mortality.

It is difficult to determine what caused these opposing outcomes. The genotypes used in the current study were included in the pilot, and all corals had only *Symbiodinium* type A3 detected. While the two intervening years between experiments saw back-to-back bleaching events in Florida, no bleaching was observed in the nursery (C. Drury, pers. communication). It is possible that the corals switched to hosting a different, more thermally tolerant strain of A3, but it is highly unlikely especially in the absence of bleaching, as complete strain stability in *A. cervicornis* corals from nurseries in Florida has been found using microsatellite markers (J. Parkinson, *pers. communication*).
However, warm summers may have contributed to the corals’ thermal tolerance, and it is more likely that the elevated thermal tolerance observed in the current study is a result of changes in the host, such as acquired stress tolerance through acclimation to high temperature (e.g., Bellantuono et al. 2012; Bay & Palumbi 2015) or through potential increases in lipid reserves (e.g., Anthony et al. 2007) as a result of growth in the protected nursery.

The lack of a strong bleaching response did not provide the opportunity for thermally tolerant symbionts to proliferate in these corals (e.g., Cunning et al. 2015). Corals that were stressed for 10 days did not decline significantly in S:H, though they did exhibit declining $F_v/F_m$. Because the thermal challenge in this study was clearly not particularly stressful, the lack of response in S:H in those corals may not be surprising. Nevertheless, the results of this experiment suggest that a threshold was crossed between 10 and 16 days of heat stress, with corals at 32°C for 16 days showing not only significant declines in S:H and $F_v/F_m$ but also a significant positive correlation between the two, which was absent in corals stressed for shorter durations.

This positive correlation between S:H and $F_v/F_m$ may be an informative diagnostic tool for determining whether corals have passed their bleaching threshold. Coral bleaching has been hypothesized to be an extreme endpoint of normal cellular processes (Wooldridge 2009; Wooldridge 2013). Coral symbiont communities have been shown to be dynamic in response to changing environmental conditions, both seasonally (e.g., Fitt et al. 2000) and during stress recovery (e.g., Silverstein et al. 2014; Kemp et al. 2014; Cunning et al. 2015), and this dynamism has been suggested to be a result of continuous adjustment toward a context-dependent optimal abundance (Cunning & Baker 2014).
Significantly declining $F_v/F_m$ can indicate that the rate of damage to Photosystem II has exceeded the rate of repair (Warner et al. 1999; Roth et al. 2012; Osinga et al. 2012). However, significant differences in $F_v/F_m$ have also been observed in corals as a normal consequence of photoinhibition on both diurnal (e.g., Jones & Hoegh-Guldberg 2001; Hill & Ralph 2005) and seasonal (e.g., Warner et al. 2002; Hill & Ralph 2005; Sampayo et al. 2008; Suwa et al. 2008; Ulstrup et al. 2008) scales. Thus, even significant declines in photochemical efficiency or symbiont abundance must be interpreted carefully, as they are not necessarily indicative of coral-algal symbiosis breakdown but could instead be a result of normal cellular processes.

$F_v/F_m$ is a metric of photochemical efficiency that is independent of symbiont density, and it is therefore unsurprising that unstressed corals show no relationship between $F_v/F_m$ and S:H, which is a cell density based measure of symbiont abundance (Figure 4). Importantly, corals that were stressed for 10 days or less also showed no relationship between photochemical efficiency and S:H (Figure 4), indicating that the natural variability present in S:H was not predictive of photodamage in these fragments. Therefore, I hypothesize that while these corals were exhibiting potential signs of stress (depressed S:H and/or declining $F_v/F_m$), they had not passed their thermal stress threshold and were not yet on the path to a runaway bleaching process.

While the mechanisms of coral bleaching are not fully understood, it is understood to occur as a result of the expulsion or loss of damaged algal cells (e.g., Weis 2008). The significant correlation between low S:H and low $F_v/F_m$ in corals heated for 16 days, indicating that corals with the lowest symbiont abundance also contained the most damaged symbionts, strongly suggests that a stress threshold had been surpassed in these
fragments and that bleaching was beginning to occur. Corals in this treatment were also the only fragments to show any visual signs of paling. Interestingly, recovery temperature played a significant role over time in the relationship between symbiont abundance and damage in these corals, with those recovering at 27°C returning to an unstressed, uncorrelated state in 1 month. In contrast, corals recovering at 29°C maintained the positive relationship, suggesting that elevated temperature prolonged the recovery process.

Temperature also had significant effects on S:H, both in unheated control corals and in corals recovering from heat stress. It is possible that the changes seen in the S:H over time are due in part to sampled polyps taken from different places on the fragment, since symbiont communities are not homogenous along the length of *A. cervicorns* fragments (e.g., Gladfelter et al. 1989; Pillay et al. 2005; Wooldridge 2013). However, at a given time point, polyps were sampled from the same location on each fragment, so that potential source of variation over time does not explain the significant differences in S:H observed between temperatures on a given day. Similarly, it is unlikely that variability within a sampling location explains these differences, given the size of the error bars on a given day in relation to the differences between temperatures. Further, long-term maintenance at a given temperature resulted in consistent S:H values over time in control corals (Figure 3), suggesting that the early variability is not due entirely to sampling location.

The dramatic increases observed in S:H during early recovery may not be due entirely to a bloom in the symbiont community. As mentioned above, some of the variation over time could come from the sampling scheme. However, it is important to
note that the S:H metric is a ratio that can change with changing host cells as well as changes to the symbiont community. This caveat may prove to be especially important when studying *A. cervicornis*, a thin-tissued coral species (e.g., Loya et al. 2001), because changes to the number of host cells in more thinly-tissued species will have a greater impact on the S:H metric than the same absolute change in more thickly tissued corals such as *Montastrea cavernosa*. Host cell losses in response to the 4-5°C temperature shift over two days seen by corals recovering at 27°C may have contributed to the observed pattern. In future work, it will be important to compare the S:H measured from a single polyp with that calculated from sampling a larger area of tissue. Sacrificing more tissue may help to reduce noise in the S:H signal in this thin-tissued species.

Conspecific acroporids growing in differing environments have been found to vary significantly in their tissue thickness (Anthony & Hoegh-Guldberg 2003), and rapid tissue sloughing in this genus in response to stress and disease has been observed for decades (e.g., Peters 1984). Roth et al. (2012) found that acute temperature decreases of 5°C were more deleterious to fragments of *Acropora yongei* than a 5°C increase, but that cold-stressed corals were able to acclimate over a period of 2-3 weeks. This acclimation period closely matches what we documented in the current study, in which elevated S:H in corals recovering at 27°C from 2-4 days of heat stress at 31-32°C returned to pre-stress levels in under 3 weeks. Therefore, given the fact that stressed acroporids are known to lose host tissue rapidly and that S:H values would change with changing numbers of host cells, it seems likely that rapidly decreasing temperatures initiated an acute stress response that resulted in changes to the coral tissue architecture. Corals are well documented to exhibit changes in tissue thickness seasonally (Brown et al. 1999; Fitt et
al. 2000; Thornhill et al. 2011) as well as host cell loss and tissue thinning in response to stress (e.g., Gates et al. 1992; Fitt et al. 1993; Quan-Young & Espinoza-Avalos 2006; Ainsworth et al. 2008). This cell loss in response to stress may involve areal loss of both symbiont and host cells, but a greater net loss of host tissue would result in increases to the S:H metric, a phenomenon observed in Hydra symbioses (Douglas & Smith 1984). Because both coral tissue and algal communities may be in a highly dynamic state during early recovery, thermally tolerant Symbiodinium, such as D1a/S. trenchii, may have an opportunity to invade the disturbed symbiont community (see Stat & Gates 2010). Early recovery may therefore represent a further point of intervention in attempting to shape symbiont communities for increased thermal tolerance.

Changes in coral host cells can be readily examined in future studies by comparing S:H against symbiont cell counts in response to acute stress, which will reveal to what degree changes in S:H are due to changes in host tissue and will also serve to ground-truth the preliminary findings in the current study regarding the relationship between the two metrics. In unstressed corals maintained at 27°C, there was a negative relationship between S:H and cell count, which suggests that under non-stressful, steady-state conditions, increasing the total symbiont abundance per cm² supports greater proportional increases in host tissue. There is no significant relationship found in corals assessed at 32°C (due to small sample size), but these data suggest that there is a negative relationship between S:H and cell count in thin-tissued A. cervicornis. However, this relationship breaks down under stress, potentially reversing (p=0.05629) as S:H and cell counts decline due to bleaching.
Conclusions and Significance

The goal of this study was to gain a more nuanced understanding of the thermal stress response in the threatened coral species *Acropora cervicornis*, with the aim of identifying thresholds to inform the development of a stress hardening methodology for this sensitive species. While the applied thermal stress was not sufficient to induce changes in the symbiont community composition, this experiment represents an important first step in understanding in greater detail the stress response of this critical ecosystem engineer and reveals that very early recovery may represent a point of intervention to influence the structure of symbiont communities as S:H changes rapidly at lower temperatures.
**Figure 4.1 Schematic of experimental design.** Red color indicates increasing temperatures and heat stress, while blue indicates decreasing temperatures and recovery. Grey indicates those fragments removed from the experiment and sacrificed for symbiont cell counts. Line thickness is not to scale but indicates the numbers of fragments at a given temperature on each day, noted in the boxes.
Figure 4.2 Thermal challenge causes declines in photochemical efficiency ($F_v/F_m$). Red boxes indicate from left to right: warming from 27°C to 31°C, maintenance at 31°C for two days, and subsequent bleaching at 32°C. Fragments in heat stress for more than 2 days experienced significant declines in $F_v/F_m$ over the duration of the thermal stress (4 days: $p=0.0183$ and 6, 10, and 16 days: $p<0.0001$). However, mean photochemical efficiency values indicate only mild heat stress, with even those fragments at 32°C for 16 days experiencing a decline of only ~32% in $F_v/F_m$. Recovery temperature significantly affected $F_v/F_m$ values only in the most stressed fragments, with significantly lower photochemical efficiency at 29°C for approximately 1 month ($p<0.0001$). Error bars are one standard error and points over time are fitted with a cubic spline (lambda = 0.3) for visualization.
Figure 4.3 Symbiont communities respond dynamically to recovery temperature. The thermal challenge applied in this study is indicated by the red boxes from left to right: warming from 24°C to 27°C over one month, warming from 27°C to 31°C over one week and maintenance at 31°C for 2 days, followed by warming and maintenance at 32°C at increasing duration. The horizontal reference line indicates the mean S:H at the start of the heat stress. Significant declines in S:H were observed after 2, 4, and 16 days at the bleaching temperature (p=0.0108, p=0.0130, and p<0.0001, respectively). While the thermal challenge did not cause severe bleaching, symbiont communities nevertheless responded dynamically to the relief from elevated temperature conditions. S:H tended to increase dramatically after 5 days of recovery at 27°C, while fragments recovering at 29°C maintained unchanged or depressed S:H values. This overshoot at lower recovery temperature was not maintained, however, and S:H converged rapidly during recovery. Error bars are one standard error and points over time are fitted with a cubic spline (lambda = 0.3) for visualization.
Figure 4.4 Correlation between symbiont abundance and photochemical efficiency ($F_v/F_m$). The relationship between S:H and $F_v/F_m$ depends on both the bleaching status of the coral (unstressed, stressed, or recovering) and the number of days of heat stress experienced. S:H poorly explains the variance associated with photochemical efficiency in unstressed corals ($R^2=0.015$). Corals experiencing the longest duration of thermal stress showed a shift to a significantly positive relationship between S:H and $F_v/F_m$ ($p<0.0001$), with lower S:H values corresponding to more photodamage, suggesting that a stress threshold was passed between 10 and 16 days of heat stress. The significantly positive associations between S:H and photochemical efficiency for all stress durations during recovery indicate that there may be a lag in the response of S:H to thermal stress in this species.
Figure 4.5 Effect of recovery temperature on the relationship between symbiont abundance and photochemical efficiency ($F_{\text{v}}/F_{\text{m}}$). Recovery temperature influences the relationship between S:H and $F_{\text{v}}/F_{\text{m}}$ over time, with the positive association between the two metrics observed during early recovery at 27°C ($p=0.0517$) eliminated during longer term recovery ($p=0.9133$). This lack of correlation later in recovery at the lower temperature indicates that corals with lower S:H no longer have significantly more photoinhibition than those with higher S:H values. However, the positive relationship between S:H and $F_{\text{v}}/F_{\text{m}}$ at 29°C seen in early recovery ($p=0.0398$) is maintained over time ($p=0.0094$), indicating that the higher recovery temperature may be delaying recovery of photochemical efficiency and S:H.
Figure 4.S1  Effect of recovery temperature on the relationship between areal symbiont density and symbiont to host cell ratio. Symbiont density (cells/cm²) correlated with the S:H of clade A depending on the temperature at which the corals were sampled. Unstressed corals sampled after long-term maintenance at 27° showed a significant negative relationship, with lower S:H correlating with higher cell counts (p=0.0009). The interaction between temperature and S:H may be significant (p=0.05629), but suffers from a small sample size in the 32°C treatment (right panel).
Chapter 5

Repeated thermal stress interacts with gradual warming to promote thermotolerant algal symbionts and elevate bleaching thresholds of reef corals

Summary

Coral reefs are increasingly threatened by the effects of global climate change, which are predicted to result in rapidly declining reef health due to increasingly frequent and severe bleaching events. However, predictive models forecasting the occurrence and impacts of annual mass bleaching by mid-century generally have not included the potential for corals to elevate their bleaching resistance through adaptive mechanisms, such as changes in symbiont community composition. Furthermore, the degree to which warming baseline temperatures might interact with repeated bleaching events to elevate thermal stress tolerance has never been investigated experimentally and consequently has not been included in predictive modeling efforts.

The goal of the current study is to elucidate the roles of repeated, acute thermal stress (4 days at 32°C) and gradually warming temperatures (28-30°C, increasing by 1°C every 4-5 weeks) on symbiont community structure and function, and their eventual impacts on coral holobiont stress response. Replicate cores of the Caribbean coral Montastraea cavernosa hosting only Symbiodinium C3 symbionts were assigned to one of four treatments: (1) gradual warming only, (2) periodic acute thermal stress only, (3) gradual warming plus periodic acute thermal stress, or (4) no gradual warming or periodic thermal stress (i.e., control: maintenance under constant thermal conditions). At the end of the experiment, cores from all treatments were subjected to a final thermal
challenge at 32°C for 8 days to assess how treatment conditions had affected the bleaching response. Quantitative PCR (qPCR) assays targeting the actin gene region of the coral host and algal symbionts were used to calculate the symbiont to host cell ratio (S:H), a metric of symbiont abundance, and chlorophyll fluorometry (I-PAM) was used to track photochemical efficiency (Fv/FM) of the symbiont communities.

In corals exposed only to periodic acute thermal stress, both mean Fv/Fm and S:H ratio declined after each thermal stress event, but recovered significantly by the end of each recovery period. In contrast, corals exposed to periodic acute thermal stress in combination with gradual warming showed no declines in S:H ratio in response to heat after the first thermal stress event and no decline in Fv/Fm during the third event. Both the gradual warming treatment and the repeated thermal stress treatment significantly increased the proportion of thermotolerant Symbiodinium D1a/S. trenchii over time. However, none of these symbionts was detected in bleached cores until after the second thermal stress event, and unbleached cores under gradually warming conditions did not have detectable proportions of these symbionts until warming reached 30°C (~9% D). Exposure to gradual warming punctuated by iterative stress interacted to cause significant stepwise increases in the mean proportion of clade D (to ~70% D at the end of the 3rd recovery period). However, these symbiont community changes were accompanied by reductions in Fv/Fm, such that communities dominated by clade D performed with ~25% less efficiency than C-dominated communities when not exposed to acute thermal stress.

The final thermal challenge revealed significant differences in bleaching response based on cores’ thermal history, with exposure to gradual warming plus periodic thermal stress having no effect on mean S:H or Fv/Fm. Moreover, during thermal stress, corals
whose symbiont communities comprised only D1a performed with ~25% greater efficiency compared to those with only C3. Cores that experienced only gradual warming or only periodic thermal stress exhibited declines in Fv/Fm, as well as significant S:H losses that were no different from control colonies that had not been gradually warmed or periodically stressed.

This study is the first to experimentally examine the combined effects of repetitive thermal stress events and warming temperatures on bleaching response and symbiont community structure and function. Exposure to either gradual warming or repeated thermal stress alone was insufficient to elevate thermal tolerance as measured here. Instead, the combination of warming and repeated acute stress exposure most strongly promoted clade D and significantly altered the response of the holobiont to the applied stress, enabling corals to host thermally tolerant symbionts and avoid symbiont loss. These results strongly suggest that repeated, sub-lethal bleaching on short time scales can effect symbiont community changes that are amplified by gradual warming between successive stress events, leading to elevated bleaching thresholds, and demonstrate the importance of including in predictive models this mechanism of thermal acclimation.

Background

Coral reefs are threatened by the effects of global climate change (e.g., Hoegh-Guldberg et al. 2007), and increasingly frequent and severe thermal anomalies are of particular concern. Prolonged average sea surface temperatures as little as 1-2°C above the local average summertime maximum can result in the phenomenon known as coral bleaching (Glynn 1991). Bleaching is the stress-mediated breakdown of the obligate
relationship between corals and their communities of endosymbiotic dinoflagellate algae in the genus *Symbiodinium*. Bleaching can result in significant coral mortality if the bleaching stressor is too severe or prolonged (e.g., Glynn 1993; Glynn et al. 2001; Baird & Marshall 2002). Sub-lethal bleaching can result in a suite of deleterious effects on the coral host, including depressed lipid reserves (Porter et al. 1989; Fitt et al. 1993; Rodrigues & Grottoli 2007; Grottoli et al. 2015), reduced growth rates (e.g., Goreau & Macfarlane 1990; Baird & Marshall 2002), and lowered fecundity (Szmant & Gassman 1990; Jones & Berkelmans 2011).

Recent climate models predict rapidly declining reef health due to the increasing frequency and severity of coral bleaching events (e.g., Frieler et al. 2012; van Hooidonk et al. 2013; van Hooidonk et al. 2014), with severe annual bleaching predicted to occur by 2055 at 90% of all reef locations (van Hooidonk et al. 2014). Some models address the possibility of thermal adaptation in the coral host (Frieler et al. 2012) and the influence of symbiont types with differing thermal tolerances on reef responses to warming (Baskett et al. 2009; Ortiz et al. 2013a; Ortiz et al. 2013b; Fabina et al. 2013). Importantly, inclusion of genetic and community level variation in symbiont thermal tolerances has been shown to significantly improve modeled reef responses to climate change (Baskett et al. 2009; Fabina et al. 2013).

*Symbiodinium* is a highly diverse genus, with nine currently described clades (A-I, Pochon & Gates 2010) and numerous sub-clade types (e.g., LaJeunesse 2001; Rodriguez-Lanetey 2003; Van Oppen et al. 2005; Coffroth & Santos 2005). Different *Symbiodinium* types have been found to vary in their physiological tolerances (e.g., Tchernov et al. 2004; McGinty et al. 2012; Wang et al. 2012), and, therefore, in their
environmental optima (e.g., Iglesias-Prieto et al. 2004). For example, some members of clade D have been shown to be more thermally tolerant than other types, experiencing photoinhibition at higher light (Rowan 2004) and temperature (Wang et al. 2012) and exhibiting no increase in reactive oxygen species production with elevated temperature (McGinty et al. 2012).

Increases in the relative abundance of *Symbiodinium* in clade D have been observed in the field following natural bleaching events (Baker et al. 2004), including D1a in the Caribbean (LaJeunesse et al. 2009; Kemp et al. 2014). Corals that recover from bleaching with symbionts in clade D exhibit increases in bleaching thresholds of at least 1.5°C in response to future stress (Berkelmans & van Oppen 2006; Silverstein et al. 2015). However, it has been argued that post-bleaching recovery with symbionts in clade D may not be beneficial to the coral host due to reduced translocation of photosynthate (Cantin et al. 2009) and lower growth rates in corals hosting these symbionts (Little et al. 2004). Clade D *Symbiodinium* have been described as weedy opportunists with parasitic characteristics (Stat & Gates 2010; Lesser et al. 2013), whose post-bleaching rise to dominance is often assumed to be the result of out-competition of other symbiont types (e.g., McGinley et al. 2012; but see Baker et al. 2013). Under these assumptions, some models have shown rapid declines in Caribbean coral cover as a result of the spread and dominance of symbionts in clade D, suggesting that reefs ultimately will persist longer if corals maintained associations with their “business as usual” symbionts that are assumed to confer greater direct physiological benefits, such as faster growth rates (Ortiz et al. 2013a; Ortiz et al. 2013b).
However, it has been demonstrated that such tradeoffs to hosting thermally tolerant D symbionts can be context dependent, with declines in growth associated with hosting D eliminated at higher temperatures (Cunning et al. 2014). Even incremental changes in community composition can result in improved community function under stress (Cunning et al. 2015), and the amount of clade D with which a stressed community recovers has been shown to depend on both the duration of bleaching stress and the recovery temperature (Cunning et al. 2015). However, the subsequent response of these modified symbiont communities to thermal stress was not tested, and we lack an understanding of how coral symbiont communities will recover from repeated, short-term thermal stress events such as those predicted to occur as climate change proceeds and which are already starting to occur on today’s reefs. Furthermore, it is critical to reef management that we assess the degree, if any, to which repeated bleaching influences future thermal tolerance.

The goal of this experiment was to examine the effects of repeated, acute thermal stress and gradual warming on the symbiont community structure and function in an important reef-building Caribbean coral, Montastraea cavernosa, and to assess the impact of exposure to these conditions on thermal tolerance. We define increasing thermal tolerance as improved performance in the measured physiological parameters in response to repeated stress compared to the stress response of previously unstressed corals (e.g., Grottoli et al. 2014). We hypothesize that (1) repeated, short-term temperature stress on corals will result in stepwise increases in the proportion of thermally tolerant Symbiodinium D1a; (2) increases in these thermotolerant symbionts will occur more rapidly in corals also experiencing gradually warming recovery
temperatures: (3) beyond some thermal threshold, warming temperatures will also promote the proliferation of D1a, even in the absence of acute stress, and (4) increases in the abundance of D1a improves the thermal tolerance of coral hosts under stress.

**Methods**

Three parent colonies of the Caribbean coral *Montastraea cavernosa* were collected from approximately 17m depth off of West Palm Beach, Florida, and were returned to the lab at the University of Miami. Replicate cores (5cm$^2$) were generated from each colony (n=88, 83, and 80; total N=251) using a drill press and mounted on plaster Reef Plugs (Boston Aquafarms) after a tile saw was used to trim the bases to standard height parallel to the tissue surface to minimize variation in the light field. Cores were allowed to recover from coring for two months in indoor flow-through tanks maintained at 27°C under custom LED lights (Build-my-LED) at 200μmol quanta m$^{-2}$ sec$^{-1}$ on a 12 hour light:dark cycle.

Once cores were fully recovered, with tissue growth observed on the edges and tentacles regularly extended for feeding, they were assigned haphazardly to one of four treatments: (1) gradual warming only, (2) periodic acute thermal stress only, (3) gradual warming plus periodic acute thermal stress, or (4) no gradual warming or periodic thermal stress (i.e., control: maintenance under constant thermal conditions). Each treatment was replicated in five independent 10 gallon aquaria (n=12-13 cores per aquarium), with acute thermal stress at 32°C occurring in a separate tank to ensure cores from different treatments experienced consistent stress conditions. Because the goal of the experiment was to contrast the effects of baseline warming with intense perturbation, cores in the periodic acute stress treatments were not ramped to target temperature
(32°C), but rather were placed immediately into pre-heated tanks for 4 days. Temperatures in the aquaria in warming treatments were ramped by 1°C after every punctuated thermal stress event, such that gradually warming cores exposed to acute stress experienced stepwise increases in temperature for each recovery period (Figure 1), corresponding to a warming rate of ~0.2-0.25°C per week.

Tissue biopsies were taken from each core with the corner of a new, single-edged razor blade from approximately 1/3 of the circumference of a single calyx wall. Samples were preserved as tissue lysates in 1% SDS in DNA buffer (Rowan & Powers 1991). All cores were sampled at the start and end of each 4-day thermal challenge, as well as midway through the first recovery period, with care taken not to biopsy previously sampled tissue at each time point. DNA was extracted from the tissue samples using an organic extraction protocol modified from Baker et al. (1997). TaqMan-MGB (Life Technologies) assays targeting the actin gene region of both the coral host and symbiont clades A, B, C, and D were used to analyze a subset of initial and final DNA samples in duplicate through quantitative PCR (qPCR). 10µL reaction volumes with 5µL TaqMan Genotyping MasterMix and 1µL DNA template were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Clade B was analyzed using the primers and conditions described in Cunning, et al. 2015, while M. cavernosa was assayed as described in Silverstein, et al. 2015. Clade A was assayed using the primers and conditions described in Chapter 2. Clade C and D assays were multiplexed, using the primers and reaction conditions described in Cunning & Baker (2012). The StepOnePlus software package calculated cycle threshold (Ct) values with a ΔRn=0.01 threshold fluorescence.
The target DNA was considered present when amplification occurred in both technical replicates with $CT$ values no more than 1.5 cycles apart and when no target was detected in negative control reactions. $CT$ values for *Symbiodinium* clade C were reduced by 3.798 cycles to correct for differences in fluorescent signal intensity between the TaqMan-MGB fluorophores used for clades C and D. This fluorescence correction factor was calculated based on standard curves generated following Cunning and Baker (2013). Adjusted $CT$ values were then used to calculate symbiont to host cell ratios using the formula $2^{CT_{\text{host}}-CT_{\text{symbiont}}}$. Denaturing Gradient Gel Electrophoresis (DGGE) was used to confirm sub-clade types (e.g., LaJeunesse & Trench 2000).

Photochemical efficiency ($Fv/Fm$) was measured using an Imaging Pulse Amplitude Modulated fluorometer (I-PAM, Walz, Germany) following 30 minutes of dark adaptation at the end of the 12 h light cycle. I-PAM measurements were taken at each tissue sampling point, midway through each recovery period, and every other day during the final thermal challenge.

**Statistical analyses**

Generalized linear models were used to assess the effects of baseline temperature and acute heat stress over time on Fv/Fm and on total, clade C, and clade D S:H values. ANOVA was used to test for differences in mean relative Fv/Fm among treatments, and Student’s t-tests were employed to test for differences between initial and final relative S:H for bleached corals in different baseline temperature treatments at each acute stress. The statistical program JMP v13.0 (SAS) was used to run all tests.
Results

First three acute thermal stress events

Symbiont community function

Cores subjected to repeated acute thermal stress that always recovered at 27°C showed significant declines in Fv/Fm during each acute thermal stress (p<0.0001) but recovered by the end of each recovery period (Figure 2). In contrast, repeatedly bleached cores recovering at increasing temperatures (28°C, 29°C, and finally 30°C) showed significant declines in Fv/Fm (p<0.0001) for the first two bleaching events, and did not fully recover Fv/Fm following the second bleaching event (recovery at 29°C; p=0.3016; Figure 2). However, these cores did not suffer any further reduction in photochemical efficiency during the next (third) stress event (p=0.1645; Figure 2). Control cores that were never subjected to bleaching stress and were simply maintained at 27°C also showed increasing Fv/Fm values during each recovery period, suggesting that Fv/Fm may be depressed by sampling, and then recover. Unbleached cores under gradually warming conditions exhibited declining Fv/Fm when temperatures reached 30°C (p=0.0277).

In heat stressed cores, there was a significant interaction between stress event (1st, 2nd, or 3rd) and recovery temperature (27°C vs. warming) on the mean relative Fv/Fm maintained during heat stress (p=0.0002; Figure 3). Stressed cores maintained significantly higher photochemical efficiency at the second bleaching event compared to the first (p<0.0001). However, only cores recovering at warming temperatures showed a significant gain in thermal tolerance at the 3rd thermal stress (after recovery at 29°C), retaining 92.7% of Fv/Fm on average (p<0.0001).
Symbiont community structure

Initial communities contained only clade C symbionts [Symbiodinium C3, Silverstein et al. 2015]. Cores recovering at 27°C showed significant declines in total S:H relative to controls during the first three acute stress events (p=0.0131, p=0.0757, and p=0.0385, respectively; Figure 4). In contrast, corals experiencing stepwise increases in recovery temperature showed no significant decline in total S:H relative to unbleached controls in response to the second and third thermal challenges (p=0.3261 and p=0.9466; Figure 4).

Clade D was first detected in bleached samples at the end of the second recovery period, with higher recovery temperatures significantly increasing the proportion of clade D hosted (p<0.0001; Figure 5). Clade D was not detected in unbleached cores experiencing gradually warming temperatures until after they had been maintained at 30°C, achieving a mean proportion of ~9% in the community. Repeated bleaching plus gradually warming recovery temperatures significantly interacted to promote clade D symbionts (p<0.0001; Figure 5).

Final bleaching challenge

Symbiont community function

During the final bleaching challenge, there was a significant interaction between bleaching history and time, both for cores at the control temperature (27°C; p=0.0023) and for those recovering under gradual warming (p<0.0001; Figure 6). Repeatedly bleached cores that recovered at 27°C had significantly lower initial Fv/Fm than those that had not yet been bleached, but maintained more photochemical efficiency at the end of the 8-day thermal challenge. Cores that had experienced gradual warming showed a
similar interaction (p<0.0001). However, repeatedly bleached cores recovering under warming conditions exhibited no significant declines in Fv/Fm during the final thermal challenge (p=0.1531), while gradually warmed cores experiencing acute heat stress for the first time showed declines in Fv/Fm (p<0.0001; Figure 6).

**Symbiont community composition**

Total S:H during the final thermal challenge was significantly affected by maintenance temperature (control vs. warming), bleaching history (repeat vs. first), and time (p<0.0001; Figure 8). All interactions (but maintenance temperature*time: p=0.0552) were highly significant (p-values ranging from 0.0008 to <0.0001; Figure 8). Clade C abundance was significantly affected by maintenance temperature, bleaching history, and time, with a significant interaction between maintenance temperature and bleaching history (p-values range from 0.0064 to <0.0001; Figure 8). Finally, clade D S:H showed a significant interaction between maintenance temperature and bleaching history (p<0.0001), but did not change significantly over time (p=0.2952; Figure 8).

**Symbiont community structure and function**

The proportion of clade D in all gradually warmed corals at the start of the final bleaching challenge correlated negatively with the symbiont community’s photochemical efficiency (b = -0.092, p<0.0001; Figure 9), such that corals hosting only D had a 25% reduction in photochemical efficiency compared to those hosting only C. In contrast, the relationship showed the opposite pattern at the end of the thermal stress, with higher proportions of clade D correlating positively with community function (b = 0.059,
p=0.0020; Figure 9), such that corals with only D performed 25% more efficiently after 8 days at 32°C (Cunning et al. 2015).

**Discussion**

This study is the first to examine the effect of gradually warming temperatures punctuated by repeated short-term heat stress on coral tolerance to subsequent thermal challenge. To date, other studies that have examined the effects of repeated bleaching on corals have performed only two stress events, *i.e.*, only one episode of ‘repeat’ bleaching (Grottoli et al. 2014, 2015; Silverstein et al. 2015), and only Silverstein et al. (2015) stressed corals that had recovered from the first bleaching at different temperatures. Although the timescales used in this study do not correspond to real-world warming and bleaching forecasts (projected warming of 1°C over decadal timescales and bleaching projected to occur annually (e.g., van Hooidonk et al. 2013)), this study nevertheless demonstrates that stepwise symbiont community change can occur through repeated, short-term perturbation without wholesale, potentially catastrophic symbiont community loss.

*Stepwise increases in clade D*

A number of previous studies on coral-algal symbiosis have concluded that the capacity of corals to flexibly associate with novel symbionts, or of background symbionts to become dominant in the community, ia highly limited (e.g., Goulet & Coffroth 2003; Goulet 2006; Thornhill et al. 2009; Coffroth et al. 2010; LaJeunesse et al. 2010; Lee et al. 2016; but see Baird et al. 2007; Baker & Romansky 2007; Cunning et al. 2015; Silverstein et al. 2015). However, very few coral bleaching experiments have examined
coral response to repeated thermal stress (but see Grottoli et al. 2014, 2015; Silverstein et al. 2015). Although this work does not elucidate the origin of these symbionts, which could have arisen from existing symbionts present at very low, ‘background’ levels (e.g., McGinley et al. 2012; Silverstein et al. 2012) and/or been acquired anew from the water column (e.g., Coffroth et al. 2010; Jones et al. in prep), clade D proliferated in corals that contained no detectable D at the start of the experiment (detection limit of multiplexed D and C assays determined to be $10^5$ C:10D, Cunning & Baker 2013). These results illustrate the need for caution when using single-stress experiments to extrapolate the consequences of more frequent bleaching on the coral-algal symbiosis; in the current experiment, very different conclusions might have been drawn if we had terminated the experiment after a single bleaching event.

If stress-tolerant symbionts, such as *Symbiodinium* D1a/S. trenchii, can readily increase in abundance following disturbance and are relatively more difficult to lose once acquired (Silverstein et al. 2017), a ratchet-like rise to dominance can be expected, the speed of which would be driven by the frequency of perturbation to the system and the baseline conditions between disturbances. Members of *Symbiodinium* clade D are often found in disturbed corals (e.g., Baker 2004; Stat & Gates 2010; Lesser et al. 2013; Silverstein et al. 2015; Cunning et al. 2015), and they can be tenacious in the face of attempts to dislodge them (Silverstein et al. 2017). In the current study, clade D exhibited such stepwise, ratchet-like increases in abundance, with gradually warming recovery temperatures between thermal stress events further driving its proliferation. These results expand upon previous work that showed that more severe bleaching and elevated recovery temperatures promoted bleached *Orbicella faveolata* recovery with
clade D (Cunning et al. 2015) and demonstrate that a change in dominant symbiont type can result from repeated acute stress in combination with increasing baseline temperature.

In the current study, clade D symbionts were not detected in acutely stressed corals until after recovery from the second thermal stress, and corals experiencing only warming conditions gained detectable levels of D only once warmed to 30°C. These results support the hypothesis that there appears to be a thermal threshold beyond which clade D is promoted even in the absence of acute perturbation and corroborate suggestions that a threshold temperature exists beyond which clade D is favored (Baker et al. 2004; Baker et al. 2013), although this threshold may vary between regions (Oliver & Palumbi 2009). In this experiment, this threshold appears to be ~29.5°C.

Consequences for the host

Acquisition of thermal tolerance

Corals that hosted elevated levels of clade D symbionts as a result of gradual warming plus repeated thermal stress exhibited higher thermal tolerance, in that they did not experience declines in S:H or Fv/Fm by the third episode of acute stress. Elevated thermotolerance was maintained during the final thermal challenge, when they again showed no declines in mean total S:H or mean Fv/Fm and were visibly more pigmented than cores in the other treatments. In contrast, corals that consistently recovered from bleaching at 27°C showed significant declines in both S:H and Fv/Fm after each thermal stress event. Total S:H at the start of the second bleaching stress was significantly elevated compared to controls, suggesting that stressed corals were still recovering from the first bleaching event and S:H had not yet stabilized, which may explain the higher variability in response to the second bleaching stress. This overcorrection is in keeping
with other work that has found that symbiont communities recovering from bleaching can overshoot unstressed levels (Kemp et al. 2014; Silverstein et al. 2015; see Chapter 4).

Cores that experienced only warming or only acute periodic stress exhibited the same mean S:H loss during the final stress as corals in the control treatment which saw neither warming nor episodic stress, indicating that neither gradual warming nor prior experience of repeated thermal stress alone was sufficient to cause an increase in thermal tolerance. Without punctuated, acute perturbations, the symbiont communities in corals exposed only to gradual warming achieved a mean proportion of only ~9% D, which did not prove sufficiently protective during the final stress event. Indeed, cores experiencing gradual warming showed declining Fv/Fm during the experiment, which could indicate accumulating stress as temperatures gradually warmed, increasing photoinhibition in the Symbiodinium C3 symbionts that dominated these corals (e.g., through damage to the D1 protein (Warner et al. 1999) or impaired repair mechanisms (Takahashi et al. 2004)).

Corals which gradually warmed experienced increasingly smaller temperature increases to reach the episodic heat stress target of 32°C. This could have resulted in a weaker response to perturbation, and a less severe bleaching response. It is also possible that the higher thermotolerance of corals that experienced both acute stress and gradual warming could have resulted solely from host acclimatory mechanisms in response to the combined exposure to those conditions (e.g., Bellantuono et al. 2012; Guest et al. 2012; Bay & Palumbi 2015). However, members of clade D have been shown to have enhanced heat tolerance (e.g., McGinty et al. 2012; Wang et al. 2012) and to confer elevated bleaching thresholds to their coral host (Glynn et al. 2001; Berkelmans & van Oppen 2006; Jones et al. 2008; Silverstein et al. 2015). In addition, Silverstein et al.
(2015) found that hosting clade D, and not prior exposure to heat stress, caused *M. cavernosa* to be more thermally tolerant. Therefore, it is likely that in light of the congruence between the S:H data and the Fv/Fm data, the increased abundance of *Symbiodinium* D1a/*S. trenchii* in corals that were repeatedly bleached and gradually warmed contributed significantly to their increased resistance to thermal stress.

**Potential costs and benefits**

Certain members of clade D have often been characterized as weedy, selfish opportunists (e.g., Stat & Gates 2010), whose mutualistic benefits may be reduced compared to other types. These costs have been most notably demonstrated in a reduction of translocated photosynthate to the host (Cantin et al. 2009) leading to slower host growth rates (e.g., Little et al. 2004). These reductions presumably allow for higher rates of photosystem repair (Tchernov et al. 2004), which may be an important target of heat damage (Takahashi et al. 2004), enabling these symbionts to maintain elevated performance under thermal stress compared to other, more sensitive types (e.g., McGinty et al. 2012; Wang et al. 2012). However, these tradeoffs are themselves likely to be environmentally mediated (Cunning et al. 2014).

High performance under stress in clade D symbionts was illustrated after the final thermal challenge, in which D-dominated communities in gradually warmed corals performed with ~25% greater efficiency than those dominated by C. Thus, the benefits of increased clade D in these gradually warmed, previously bleached corals were twofold: first, these corals maintained S:H values indicating symbionts were not being expelled in response to stress, and secondly, these corals maintained photophysiological function, resulting in significantly higher photochemical efficiency compared to
communities still dominated by clade C. This pattern could not be tested in corals that had not been gradually warmed because they hosted only minimal levels of clade D symbionts. However, in these corals that had been maintained at the control baseline temperature, there was a significant interaction of bleaching history over time on Fv/Fm, such that cores that had experienced acute stress (and hosted ~4.6% clade D on average) showed less decline in Fv/Fm and maintained greater mean Fv/Fm during the final thermal stress than control cores that had not bleached (and hosted 0.2% clade D on average). These findings support previous work in which *Orbicella faveolata* corals hosting clade D symbionts performed less efficiently than those hosting clade B under normal conditions, but outperformed B dominated communities following heat stress (Cunning et al. 2015).

Given this context-dependent performance, broad categorization of a given symbiont type as “sub-optimal” may be inappropriate, because the relative costs and benefits associated with hosting a given symbiont should always be considered within its environmental context. For example, recent work has demonstrated that the negative growth trade-off often associated with hosting D was lost when corals were grown at higher temperatures (Cunning et al. 2014). In the current study, the response of symbiont communities to the final thermal challenge further supports the context-dependence of tradeoffs associated with different symbiont types. Mean clade D S:H did not change significantly in any treatment, but mean clade C S:H declined across all treatments, indicating a preferential loss of clade C from mixed communities. This preferential loss suggests that, in the context of thermal stress, the more damaged clade C symbionts were less beneficial than were the clade D symbionts, which were able to maintain
photochemical efficiency.

Organisms engaged in mutualisms must balance the costs and benefits associated with potential partners (e.g., West et al. 2002), with the optimal partner maximizing the net benefit to the host under a given set of conditions (Holland et al. 2002; Cunning & Baker 2014). However, the environment is not static: as conditions change, the costs and benefits associated with a given partner must be integrated over time. Therefore, it follows that a highly beneficial partner under normal conditions may be selected against under less favorable conditions if its benefits to the host are sharply reduced. “Sub-optimal” partners, such as D1a, may exhibit greater stability in their net benefit to the coral host across a broad range of conditions. This cost/benefit balance may underpin the success of clade D in associating with hosts in highly variable environments (e.g., Oliver & Palumbi 2009; Baker et al. 2013), in stressful temperature regimes (Fabricius et al. 2004), or on reefs recovering from bleaching (e.g., Baker 2004; Kemp et al. 2014), helping to explain the mechanism behind the ratchet-like increases in abundance.

Conclusions and Significance

Repeated acute thermal stress interacted significantly with gradual warming to promote stepwise increases in clade D in the symbiont community. Clade D symbionts were also promoted in corals that were gradually warmed to 30°C in the absence of acute stress. Dominance by these thermally tolerant algae helped to elevate the corals’ bleaching threshold, maintaining symbiont abundance and photochemical efficiency in response to heat stress, even when it lasted longer than previous episodes which had previously caused bleaching. As climate change continues to affect reefs, stress tolerant *Symbiodinium*, such as those in clade D, may prove to be a lifeline for corals
experiencing repeated exposure to elevated temperatures. Ratchet-like increases in clade D abundance in response to repeat bleaching events and warming baseline temperatures may be an important mechanism by which clade D achieves local dominance on reefs. These results have important implications for coral bleaching response and survivorship under climate change scenarios and suggest new directions for predictive modeling approaches seeking to integrate biological response into forecasts describing reef futures.
Figure 5.1 Schematic of temperature regime by treatment. Note that warming treatments increase by 1°C at the end of each thermal stress event. All cores were challenged with thermal stress for 8 days at the end of the experiment.
**Figure 5.2 Acute thermal stress results in significant declines in photochemical efficiency (F.v/F.m) in repeatedly bleached cores.** Bleached cores recovering at 27°C show significant increases in F.v/F.m following each thermal stress event (p<0.0001), while stressed cores recovering at gradually warming temperatures do not recover F.v/F.m following the second and third thermal stress events. Unbleached cores under gradually warming conditions exhibit significant declines in F.v/F.m when temperatures reached 30°C (p=0.0277).
Figure 5.3 Effect of repeated thermal stress and gradual warming on proportion of photochemical efficiency ($F_v/F_m$) retained. Final relative to initial $F_v/F_m$ in cores experiencing acute heat stress reveals a significant interaction between stress event (1$^{st}$, 2$^{nd}$, or 3$^{rd}$) and recovery temperature (27°C or warming) ($p=0.0002$). Cores retain significantly more photochemical efficiency at the second bleaching event compared to the first ($p<0.0001$). Only cores recovering at warming temperatures showed a significant gain in thermal tolerance at the 3$^{rd}$ thermal stress, retaining 92.7% of $F_v/F_m$ on average ($p<0.0001$).
Figure 5.4 Effects of repeated thermal stress and gradual warming on relative symbiont abundance. Total S:H of bleached relative to unbleached cores for the first three bleaching events, with the reference line at 1 indicating no difference in S:H between bleached cores and unbleached controls. Cores recovering at 27°C show significant declines in total S:H relative to controls during the first three acute stress events (p=0.0131, p=0.0757, and p=0.0385, respectively). In contrast, corals experiencing stepwise increases in recovery temperature show no significant declines in total S:H during the second and third thermal challenges (p=0.3261 and p=0.9466).
Figure 5.5 Interaction of repeated thermal stress and gradual warming to promote clade D symbionts. Thermal stress events are indicated on the x-axis in red. Clade D was not significantly hosted by bleached corals until after recovery from the second bleaching event (control: $p=0.0042$; warming: $p<0.0001$). Unstressed cores under gradually warming conditions did not host detectable clade D until maintenance at 30°C ($p<0.0001$). Repeated bleaching and gradually warming recovery temperatures interact to promote clade D symbionts ($p<0.0001$).
Figure 5.6 Effect of bleaching history and time on photochemical efficiency (Fv/Fm) during final thermal challenge. Examination of photochemical efficiency during the final bleaching challenge reveals significant interactions between bleaching history and time both for cores at the control temperature (27°C; p=0.0023) and for those recovering at gradually warming temperatures (28-30°C; p<0.0001). Repeatedly bleached cores that recovered at 27°C had significantly lower initial Fv/Fm than those that had not yet been bleached, but maintained more photochemical efficiency at the end of the 8 day challenge. Cores that had experienced gradual warming showed a similar pattern, with repeatedly bleached cores exhibiting no significant declines in Fv/Fm (p=0.1531), while cores seeing acute heat stress for the first time lost Fv/Fm (p<0.0001; logistic fit, AICc - 486.5277). Bar graphs show the proportion of Fv/Fm lost during the final thermal challenge, with letters indicating significant differences at α = 0.05.
Figure 5.7 Effect of thermal history on proportion of photochemical efficiency ($F_v/F_m$) retained during final thermal challenge. Final relative to initial $F_v/F_m$ in cores at the final thermal challenge reveals a significant effect of maintenance temperature, with cores under gradually warming conditions retaining higher proportions of $F_v/F_m$ during heat stress than those maintained at 27°C ($p<0.0001$). Similarly, bleaching history had a significant effect on proportion of $F_v/F_m$ retained, with repeatedly bleached cores at both maintenance temperatures losing less photochemical efficiency than cores experiencing thermal stress for the first time ($p<0.0001$). There was no significant interaction between bleaching history and maintenance temperature.
Figure 5.8 Effect of bleaching history on symbiont community dynamics during final thermal challenge. Initial to final total S:H (solid), clade C S:H (dotted), and clade D S:H (dashed) during the final, 8 day long bleaching challenge. For total S:H, maintenance temperature (control vs. warming), bleaching history (repeat vs. first), time, and all interactions are significant (p-values range from 0.0008 to <0.0001; but maintenance temperature*time p=0.0552). For clade C, maintenance temperature, bleaching history, Time, and maintenance temp*bleaching history are significant (p-values range from 0.0064 to <0.0001). For clade D, maintenance temperature, bleaching history, and the interaction between the two are significant (p<0.0001).
Figure 5.9 Correlation of proportion D with photochemical efficiency ($F_v/F_m$) before and after final challenge. Proportion of clade D hosted in gradually warmed corals (displayed as arcsin square root transformed values for visualization) showed a significant correlation with $F_v/F_m$. Model parameters ($b = -0.092$, $p < 0.0001$) indicate that before stress, corals hosting only D had a 25% reduction in photochemical efficiency compared to those hosting only C, while the positive association in corals after heat stress ($b = 0.059$, $p = 0.0020$) indicates that corals with only D performed 25% more efficiently.
Chapter 6

Conclusions

This dissertation focused on examining environmental factors that influence the reassembly of bleached symbiont communities in Caribbean corals. The studies reported in Chapters 2-5 represent a significant contribution to our understanding of symbiont community dynamics during bleaching recovery under different conditions and of the factors that contribute to the proliferation of thermally tolerant symbionts in clade D. While these studies included three Caribbean coral species and examined symbiont reassembly in responses to different perturbations under different recovery conditions, some common findings suggest areas for future research. Furthermore, this research can be directly applied to real-world conservation efforts, potentially increasing the thermal tolerance of nursery corals.

Overcorrections and oscillations in S:H following short-term perturbation

A common thread throughout this work was the overcorrection in S:H in response to mild or short-term perturbation. Elevations in symbiont abundance have been previously observed in corals recovering from bleaching (Kemp et al 2014; Silverstein et al. 2015). Kemp et al. (2014) saw a three-fold increase in areal cell densities during post-bleaching re-proliferation, resulting in a great than expected cell density with respect to normal seasonal oscillations. Similarly, Silverstein et al. (2015) found that S:H values overshot initial levels during bleaching recovery. However, both of these studies observed recovery dynamics on the scale of months.
Examining the S:H metric on a relatively fine time scale revealed dramatic short-term fluctuations in symbiont abundance, on the scale of days (Ch. 4) to weeks (Ch. 3, 5) following perturbation. In *Acropora cervicornis* recovering for 5 days at 27°C after 4 days at 32°C, S:H increased ~4-fold over the S:H of fragments recovering at 29°C (Ch. 4). In *Montastraea cavernosa* recovering for ~3 weeks at 28°C after 4 days of thermal stress, S:H similarly was elevated by ~4-fold compared to unstressed controls, while cores recovering at 27°C from the same stress saw ~2.5-fold S:H increases over controls (Ch. 5). And, *M. cavernosa* saw ~3-fold increases in S:H after exposure to high natural light (Ch. 3).

The calculation of S:H using qPCR can be associated with high variability due to the logarithmic error inherent to the technique (Cunning & Baker 2014), necessitating methodological care and consistency in DNA quality, for example. This variability may be more apparent when samples are taken on a fine time scale and when the perturbation to the system is not strong enough to result in a clear signal of symbiont cell loss. However, the patterns of overcorrection observed in this dissertation are unlikely to be errors, because they occurred consistently following perturbation, rather than randomly, and thus probably represent a real phenomenon.

These swings in S:H may not be due entirely to changes in symbiont abundance. Changes to host tissues resulting in host cell losses would also act to increase the measured S:H. More rapid changes in host tissue than symbiont cells have been demonstrated in the thermal stress response leading to a bleaching cascade (Ainsworth et al. 2008) and may also be occurring during early recovery from a short-term perturbation. Indeed, short-term perturbation has been shown to stress the symbiosis without losses in
symbiont cell abundance, causing instead symbiont pigment losses and declines in animal protein concentration (e.g., Dove et al. 2006). Cunning & Baker (2014) reviewed the utility of different metrics of symbiont abundance and argued that normalizing symbiont abundance to a host biological parameter, such as number of host cells, was particularly useful when addressing functional and physiological variation in the coral-algal symbiosis. The results of this dissertation demonstrate that the interpretation of these large changes in S:H on short time scales would benefit from the inclusion of areal cell counts.

If areal density and S:H change at different rates, those differences could be attributed to more rapid changes in host tissue than in algal cells (e.g., Ainsworth et al. 2008). For example, the negative relationship between areal symbiont cell density and S:H under normal conditions suggests that colonies with more symbiont cells have disproportionately more host cells, causing S:H to decline (Ch. 4). This relationship may be a result of additional symbionts supporting disproportionately more host biomass, perhaps as a result of increased reproductive output or energy storage, both of which involve host cell types that might not be expected to contain symbionts. However, this relationship breaks down (or even reverses) in corals experiencing thermal stress, resulting in pathogenic coral bleaching, indicating that the relationship may be context-dependent and thus potentially diagnostic of host physiological status. Examining the coral-algal symbiosis using both metrics simultaneously thus could help interpret changes in S:H over time and elucidate the nature of the overcorrections and oscillations observed throughout this dissertation.
Cunning & Baker (2014) suggest that in complex, highly variable reef environments, corals may be continuously adjusting S:H to approach an ever-changing optimum. This hypothesis is supported by the convergence of S:H values in corals held under steady-state experimental conditions (Cunning et al. 2015a). In this dissertation, corals did not approach equilibrial S:H smoothly following short-term or mild perturbation, instead exhibiting pendulum-like oscillations following initial overcorrections (Ch. 3, 4, 5). This phenomenon was especially apparent in *A. cervicornis* due to the frequency of sampling (Ch. 4), and stabilization of S:H in *A. cervicornis* appeared to oscillate with decreasing amplitude, converging on a presumed optimum S:H (Cunning & Baker 2014). This pendulum-like oscillation before equilibration was also observed in *Montastraea cavernosa* in response to high light (Ch. 3), where control corals saw significant increases in S:H before declining and re-stabilizing at a lower value. After short-term thermal stress, *M. cavernosa* showed a similar pattern, overshooting initial values before beginning to re-equlibrate and doing so at different rates based on recovery temperature (Ch. 5).

Exploring these overcorrections and oscillations in S:H following perturbation is an important area for future research. Perturbations that push the system past some threshold exceed the resilience of this system (preventing the return swing of the pendulum) and result in a bleaching cascade and the eventual breakdown of the symbiosis. Thus, examining the degree of perturbation and the overcorrection that occurs in response may provide valuable insights to the resilience of the coral-algal symbiosis and could allow for the more precise pinpointing of stress thresholds.
Recovery with Symbiodinium in clade D

Climate change is threatening reefs on a global scale, creating the potential for stressors, such as increasing $p\text{CO}_2$ and increasingly frequent and severe thermal anomalies, to act synergistically to influence the reassembly of coral symbiont communities. By itself, elevated $p\text{CO}_2$ did not affect symbiont community reassembly or recovery with thermally tolerant symbionts (Ch. 3). High $p\text{CO}_2$ has been shown to increase the severity of bleaching in corals under high light (Anthony et al. 2008), and bleaching severity impacts the structure of recovering symbiont communities (Cunning et al. 2015b). Therefore, it is possible that differential reassembly of the bleached communities would have been observed at different $p\text{CO}_2$ levels if the cores had been heat stressed under high light and varying $p\text{CO}_2$. However, it would not have been possible to distinguish between the effect of bleaching severity and the impact of elevated $p\text{CO}_2$ itself on the bleaching recovery process.

While other studies have shown no change in coral-associated microbe or symbiont communities along natural pH gradients (e.g., Meron et al. 2012), Chapter 3 was the first to demonstrate no significant effect of elevated $p\text{CO}_2$ on bleached coral recovery or symbiont abundance as normalized to a host biological metric (i.e., S:H). The results of Chapter 3 suggest that ocean acidification levels projected for end of century (~900ppm) may not play a strong structuring role in the ability of corals to recover with clade D symbionts. Furthermore, elevated $p\text{CO}_2$ did not interact synergistically with thermal stress to affect calcification rates in this study. This finding is in keeping with Ban et al. (2013), who found in a meta-analysis that most interacting stressors work additively on corals, rather than synergistically.
In contrast, acute thermal stress and gradually warming baseline temperatures interacted with strong synergism to promote clade D in the symbiont community (Ch. 5). Chapter 5 is the first experiment to include more than two thermal stress events in conjunction with warming baseline recovery temperatures. The combination of these environmental variables drove the stepwise proliferation of clade D. The speed of this ratchet-like rise to dominance is likely determined by the severity of the perturbation to the symbiont community, the frequency of perturbation, and the baseline conditions between each perturbation. While the timeframe of the thermal challenges employed in this experiment is accelerated in comparison to the real-world stressors that reefs are experiencing under climate change, this study nevertheless is a practical demonstration of the ratchet-like trajectory of D1a in repeatedly perturbed symbiont communities under warming baseline conditions.

This laboratory experiment may provide a mechanistic explanation for the dominance of clade D observed in the field at Horseshoe Reef, which had experienced slightly warmer mean temperatures, as well as greater and more frequent positive thermal anomalies, compared to the less variable Emerald Reef (Ch. 2). The observational nature of Chapter 2 precludes the definitive statement that the differing thermal histories at the two sites caused the observed symbiont community variation. Nevertheless, the findings are consistent with observations that warmer and more variable environments promote clade D symbionts (e.g., Oliver et al. 2009; Baker et al. 2013; Stat et al. 2013). While it is certainly possible that other, unmeasured factors contribute to the local abundance of D1a at Horseshoe Reef, the site’s thermal history and the ratchet mechanism of D proliferation likely play an important role.
Consequences of recovery with D1a

Clade D has been shown to translocate less photosynthate (Cantin et al. 2009) and cause coral hosts to grow more slowly (Little et al. 2004) under normal baseline conditions. Predictive models using such parameters have suggested that bleached coral recovery with and dominance by D1a in the Caribbean would have net negative consequences for reefs compared to recovery with the “native” symbiont type (Ortiz et al. 2013a; 2013b). However, this modeled outcome has been called into question with the demonstration of the context-dependence of coral growth with clade D symbionts, such that the growth tradeoff disappeared when corals were grown at higher temperatures (Cunning et al. 2014).

This dissertation further supports the contention that the coral-algal relationship should always be considered within its environmental context (Cunning & Baker 2014), an assertion that is especially important to consider when building predictive models that may inform management and policy decisions. Corals under gradually warming conditions dominated by clade D performed with ~25% greater efficiency under stress compared to corals that only hosted clade C (Ch. 5; see also Cunning et al. 2015b). The effects of differing thermal histories (the experience of acute thermal stress in addition to gradual warming or not) and of the resulting symbiont community structure (dominance by clade D or not) on this pattern of photochemical efficiency under stress are difficult to distinguish. Because symbiont community structure results in part from the thermal history of the holobiont (e.g., Cunning et al. 2015b), the two variables are closely related in general. However, Silverstein et al. (2015) showed that elevated thermal tolerance was conferred by hosting clade D, rather than the corals’ thermal history, suggesting that the
improved photochemical efficiency under stress of communities dominated by clade D compared to those dominated by C in Chapter 5 is indeed a result of the different symbiont communities themselves and not the thermal histories that led to dominance by different types. However, analysis of changes to host gene expression in response to repeated acute stress and gradual warming might also elucidate what role host transcriptional acclimatization might have played in elevating thermal tolerance in these corals.

Bleaching recovery with clade D not only affected the performance under stress of the symbiont community itself (Ch. 5), but also proved to be of neutral (Ch. 3) or positive (Ch. 2, 5) benefit to the coral host. Recovery with clade D did not hinder the recovery of calcification rates in previously bleached corals at either elevated or ambient $pCO_2$ (Ch. 3). Furthermore, corals that had high levels of clade D on average as a result of repeated acute thermal stress and gradually warming baseline temperatures exhibited elevated thermal tolerance, such that by the third stress event, those cores were not showing additional declines in photochemical efficiency or S:H (Ch. 5). During the final thermal challenge, cores that had experienced only acute stress or only gradual warming showed the same stress response as control cores, indicating that the only cores to have elevated their bleaching threshold were those that were dominated by clade D as a result of their thermal history.

Thus, hosting thermally tolerant clade D in the context of thermal stress not only can be beneficial to symbiont community photochemical efficiency (Ch. 5), but also can directly benefit host physiology by elevating bleaching thresholds and increasing stress tolerance (Ch. 5). These experimental findings were supported in the field at Horseshoe
Reef, where colonies of *Orbicella faveolata* that hosted higher proportions of D1a showed evidence of greater resource allocation to reproduction, spawning from a greater proportion of the colony surface area. While it has been suggested that clade D is detrimental to coral reproduction (Jones & Berkelmans 2011), this dissertation again demonstrates the context-dependence of the coral-algal symbiosis. At Horseshoe, colonies hosting stress tolerant symbionts appear to have had an advantage because spawning was occurring in the environmental context of thermal stress, with a greater proportion of polyps participating in reproduction, thereby increasing the colony’s fitness.

*Application to conservation*

This dissertation can directly inform real-world, ongoing conservation efforts. Coral nurseries are a widely used conservation tool in the Caribbean, used to propagate tens of thousands of coral fragments in Florida alone (Lirman et al. 2010; Lirman & Schopmeyer 2016). The threatened species *Acropora cervicornis* is commonly grown in these coral gardens, because its relatively rapid growth and habit of reproduction by fragmentation make it an ideal candidate species for this conservation method. Managers plant fragments back to the reef with the goal of repopulating habitats with this ecologically valuable species. A recent restoration guide outlines outplanting site selection criteria, discussing stressors such as wave exposure, predator abundance, and human activities (Johnson et al. 2011), but it does not address the need to mitigate for the looming threat of increasingly frequent and severe bleaching events due to climate change. Unfortunately, outplants are vulnerable to bleaching-induced mortality, as was exhibited during the back-to-back bleaching events in Florida in 2014 and 2015 (Lirman
& Schopmeyer 2016). Rearing and outplanting fragments requires considerable resources (Johnson et al. 2011), so restoration efforts would benefit greatly by improving the thermal tolerance of coral fragments before they leave the nursery.

As models are now predicting annual bleaching on most coral reefs by the middle of this century (van Hooidonk et al. 2013; 2015), reef managers increasingly are considering more active manipulation of corals for conservation, including assisted migration and assisted evolution (van Oppen et al. 2015). Ethical concerns have arisen in response to the possibility of managers’ taking more manipulative actions, including worries about outbreeding depression, introduced species, and other unintended impacts (Baker et al. in prep). While such concerns should be carefully considered, the development of methodologies to actively improve reef response to climate change also should not be delayed, so that by the time their use has been approved, they are ready for safe and effective deployment.

Stress hardening has been suggested as a way to manipulate the thermal tolerance of coral colonies with the goal of improving outplant survivorship (van Oppen et al. 2015). For example, the ratchet mechanism explored in Chapter 5 could be used to develop a prescription of stress and recovery conditions that promote clade D with minimum stress to the coral host. The development of this stress hardening methodology for use with A. cervicornis could provide a scalable tool for the generation of stress tolerant fragments for outplanting (Figure 6, 1a-3a). The response of A. cervicornis fragments in Chapter 4 to the applied stress was an important first step in the development of this methodology, not least because it was a successful application of heat stress in the lab with no associated mortality (Ch. 4). Furthermore, if high light can
be used as a stressor to perturb the symbiosis, fragments could be stress hardened *in situ* at nurseries by exposing them to high irradiance stress for short periods during the summer months, so that the combined photoinhibition results in bleaching. This could be achieved using floating subsurface rafts, removing the need to heat stress in the laboratory and greatly improving cost effectiveness and scalability. Stress hardening techniques could also be used with more slowly growing massive colonies, by re-implanting manipulated cores into the parent colony as tissue plugs now hosting thermally tolerant D1a (Figure 6, 1b-3b), which could result in the spread of clade D into the parent tissue (Baker et al. *in prep*), improving the thermal tolerance of large, valuable colonies.

Because even incremental changes in the proportions of thermally tolerant symbionts have been shown to improve function during stress (Cunning et al. 2015a; Ch. 5), these manipulations would not necessarily have to result in wholesale switches in clade dominance for there to still be improvements to the holobiont stress response. Recent work has demonstrated that even under continuously cooling temperatures, D1a is not readily lost from host tissue, suggesting that not only are these symbionts stress tolerant, they may also be adept at avoiding expulsion even when they are not performing well (Silverstein et al. 2017). However, even if the change in symbiont communities reverts to the original composition over time (e.g., Thornhill et al. 2006; Coffroth et al. 2010), and clade D is slowly lost from host tissue, even 1% of the symbiont population represents >10,000 cells per cm$^2$ (e.g., Fitt et al. 2000), potentially providing a lifeline for more rapid recovery in outplanted fragments.
Chapter 5 showed clade D proliferating in corals that had no detectable D symbionts at the start of the experiment, indicating they were either acquired from the environment, invaded from elsewhere in the colony where they were of relatively high abundance, and/or proliferated from pre-existing, undetectable background levels. Any of these options demonstrate that, given the appropriate environmental context, extremely low abundance symbionts can become vital, dominant members of a coral’s symbiont community that affect future stress tolerance (Berkelmans & van Oppen 2006; Silverstein et al. 2015; Ch. 5).

Summary

This dissertation explored the effects of thermal history, stress duration, and recovery conditions on symbiont community reassembly following perturbation. Using three important Caribbean coral species, these studies revealed common patterns in the response of perturbed symbiont communities to stress. The relationship between thermal history and the local dominance of thermally tolerant symbionts in the field was explored mechanistically in the lab. Importantly, the relative costs and benefits of hosting different symbionts was shown to be context-dependent, with corals hosting clade D benefitting from the relationship under stress, but suffering an apparent penalty otherwise. Managers may be able to take actions to promote these thermally tolerant symbionts if the frequency and/or severity of stress is expected to increase, potentially improving the efficacy of existing restoration activities. Moving forward, research should further examine the relationship between cell counts and S:H and explore oscillations in the symbiosis to better understand when corals have passed stress thresholds. This may help inform the development of a methodology to stress harden
corals by harnessing the ratchet-like proliferation of clade D. As reefs continue to experience the intensifying effects of climate change, these thermally tolerant symbionts may become critical lifelines for corals, elevating their bleaching thresholds and improving coral survivorship in a warming ocean.
Figure 6.1 Illustration of possible stress-hardening methodologies. 1a-3a show fragments of *Acropora cervicornis* brought into the lab from the nursery and returned to the field after controlled application of heat stress. Similarly, 1b-3b indicate the potential use of tissue plugs as vectors for inoculation of large parent colonies by thermally tolerant in the field.
Works Cited


LaJeunesse, T.C. et al., 2010. Long-standing environmental conditions, geographic isolation and host-symbiont specificity influence the relative ecological dominance and genetic diversification of coral endosymbionts in the genus Symbiodinium. Journal of Biogeography, 37(5), pp.785–800.


Lee, M.J. et al., 2016. Most low-abundance “background” Symbiodinium spp. are transitory and have minimal functional significance for symbiotic corals. *Microbial Ecology*.


McGinley, M. et al., 2012. Symbiodinium spp. in colonies of eastern Pacific Pocillopora spp. are highly stable despite the prevalence of low-abundance background populations. *Marine Ecology Progress Series*, 462, pp.1–7.


