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The Importance of the Rare: The Role of Background Symbiodinium in the Response of Reef Corals to Environmental Change

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THE IMPORTANCE OF THE RARE: THE ROLE OF BACKGROUND *SYMBIODINIUM* IN THE RESPONSE OF REEF CORALS TO ENVIRONMENTAL CHANGE

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Rachel N. Silverstein

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Reef ecosystems throughout much of the tropics are predicted to decline in coral cover and diversity as a result of ongoing climate change (ocean acidification, temperature increases, sea level rise), disease, pollution, and overfishing. Corals may be able to respond to some of these stressors by associating with diverse algal symbionts (*Symbiodinium* spp.) which vary in their physiological traits and therefore expand corals' realized niche space. This dissertation used high-sensitivity molecular techniques to investigate the presence and functional role of 'background' or 'rare' *Symbiodinium*, which occur at low abundance, and therefore may not be detected using standard molecular methods.

First, in order to determine the prevalence of mixed-clade symbiont communities (including potentially low-abundance populations), I used a highly-sensitive, real-time PCR assay to analyze archived DNA from a collection of geographically and phylogenetically diverse corals. I found that mixed-clade *Symbiodinium* communities were common, and that clades C and D were present in all 39 coral species examined. These findings provide strong evidence that no coral species is restricted to hosting only a single symbiont type.

I then investigated the functional role of low-abundance symbionts through a series of bleaching and recovery experiments involving the Caribbean coral *Montastraea cavernosa*. I monitored changes in symbiont community structure using newly-designed quantitative PCR assays, and monitored symbiont community function using chlorophyll fluorometry. Corals hosted only clade C symbionts before bleaching (except for 2 of 139 cores which hosted trace amounts of clade D as well). All bleached colonies (both herbicide-bleached and heat-bleached) recovered with dominant communities of clade D symbionts at both 24^{o}C and 29^{o}C recovery temperatures. Therefore, low-abundance (or even undetectable) symbionts became dominant in corals after disturbance. Increased temperatures, without acute disturbance, underwent less-dramatic, slower symbiont community changes. Corals that bleached, but which were not exposed to heat either during bleaching or during recovery, recovered with fewer D1a symbionts than corals bleached by heat or acclimated to higher temperatures.

During a third experiment, I used these same corals to investigate how changes in symbiont clade, past thermal history, and host genotype, affect coral thermotolerance during a second heat stress exposure. I found that, during heat stress, previously-bleached corals hosting D1a symbionts lost fewer symbionts and exhibited less photochemical damage than corals hosting C3 symbionts. Prior heat exposure, either during bleaching or during recovery, did not increase coral thermotolerance, unless it was also associated with symbiont community shifts to D1a-dominance. This demonstrates that rare (or even undetectable symbionts) can become dominant, and can eventually play a critical role in coral bleaching response.

Finally, a two-part experiment investigated the effect of incremental warming and cooling on these corals. D1a symbionts in corals that were incrementally heated to 33^{o}C had higher photochemical efficiency than cores containing C3 symbionts, and experienced less symbiont loss. During cooling, however, the photochemical efficiency of D1a was either equal to, or lower than C3. Despite this, fewer D1a symbionts were still lost compared to C3. This suggests that photochemical efficiency and symbiont loss may be decoupled from one another during stress, and that D1a symbionts may be generally more resistant to expulsion, regardless of their performance *in hospite*. This study also shows that *M. cavernosa* corals hosting D1a can expand their realized thermal niches wider corals hosting C3 symbionts, reinforcing the importance of functional redundancy in dynamic environments.

Together, these studies show that mixed algal symbiont communities can increase both the resistance and resilience of corals to stress and disturbance. These findings have indicate that symbiont community shifts have the potential to allow reef corals to rapidly adapt or acclimatize to environmental change.

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Chapter 1: Introduction

Dissertation Summary

Models predict that reef ecosystems throughout much of the tropics will decrease in coral cover, diversity, and/or undergo phase shifts away from coral-dominated reefs in the coming decades (Hughes et al. 2003, Donner et al. 2005, 2007, Hoegh-Guldberg et al. 2007). Already, corals have declined an estimated 80% in the Caribbean since the 1970's (Gardener et al. 2003), and 1-2% annually in the Indo-Pacific over the past 30 years (Bruno and Selig 2007, De'ath et al. 2012). Coral decline has been due to many local and regional factors, including disease outbreaks (Lessios et al. 1984, Brandt et al. 2007), overfishing (Jackson et al. 2001, Pandolfi et al. 2003), hurricanes (Lirman 2003), pollution (Wooldridge and Done 2009), *Diadema* loss (Hoegh-Guldberg et al. 2007, Anthony et al. 2008), and climate change (Hoegh-Guldberg et al. 1999, Hughes et al. 2003).

Most incidences of mass coral mortality have been the result of bleaching events, or the paling of corals as a result of the loss of symbiotic algae (*Symbiodinum* spp.) and/or algal pigments. Bleaching can occur due to a range of environmental stressors, such as changes in temperature, salinity, ultraviolet radiation, sedimentation and pollutants (Brown et al. 1997). However, most incidences of mass bleaching events are related to high temperature anomalies (Glynn et al. 1993). One of the worst recorded mass bleaching events occurred due to the 1997-1998 El Niño event in the eastern Pacific (Glynn 2001). The severity and frequency of sea surface temperature anomalies has been increasing over recent decades due to anthropogenic climate change (Hughes et al. 2003, Hoegh-Guldberg et al. 2007).

Corals vary in their resistance and resilience to bleaching, and bleaching is typically patchy, affecting adjacent colonies of the same species (as well as different parts of the same colony) to different degrees (Hughes et al. 2003). Variation in bleaching severity is cause by both extrinsic and intrinsic factors. Extrinsic factors include environmental conditions that vary by latitude (Glynn et al. 1996), depth (Mumby et al. 2001a), upwelling (Reigl 2003, Chou 2000, Chollett et al. 2010), island current flow (Karnauskas et al. 2012), cloud cover (Mumby et al. 2001b, West and Salm 2003), hurricane prevalence that can lower sea surface temperatures (Manzello et al. 2007), and aerosolized particles which may provide shading (Gill et al. 2006). Areas with high temperature variability have also been shown to be more resistant to bleaching, perhaps due to increased reserves of heat shock proteins and antioxidants, both of which can mitigate the effects of oxidative stress (Oliver and Palumbi 2011, Castillo et al. 2012). For example, reefs in the western Indian Ocean that experience high environmental variability have been shown to be more resistant to bleaching compared to areas that are relatively constant (McClanahan et al. 2007, Baker et al. 2008, Carilli et al. 2012). Similarly, during experimental bleaching, corals from lagunal environments with high temperature variability survived better than corals from adjacent environments with the same mean temperature, but less temperature variability (Oliver and Palumbi 2011). Past bleaching history and/or environmental conditions are important predictors of coral response to future stressors; and corals that have recovered from bleaching events are thought to be more resistant to future stress (Baker 2002, Baker 2004, Carilli et al. 2012). It is not known, however, whether this is due to physiological acclimatization of the coral

host (or its symbionts) or to changes in the composition of the symbiont communities, or both.

Variation in bleaching severity can also be due to intrinsic factors that can differ both among and within corals. For example, corals may produce fluorescent pigments (Salih et al. 2000), micosporine-like amino acids (Banaszak et al. 2000), heat shock proteins (Fang et al. 1997) and antioxidants (Lesser et al. 1997) that prevent the formation of (or damage from) reactive oxygen species (ROS) during bleaching. These changes likely last from days to weeks (C. Downs, Pers. Comm.). Coral survival may by further influenced by intrinsic differences in tissue thickness, heterotrophic feeding ability (Grottoli et al. 2006), and the ability to host thermally-tolerant symbionts (Goulet 2006, 2007, Baker and Romanski 2007, Mieog et al. 2007, Correa et al. 2009b, see Chapter 2). Symbionts themselves also contribute to acclimatization to environmental changes, as different strains may have different capacities for xanthophyll cycling, Mehler reaction speeds, antennae mobility, rate of D1 protein repair, and the degree of saturation of chloroplast lipid membranes (Tchernov et al. 2004). The coral "holobiont" refers to the coral animal and its consortium of symbiotic partners and associates, including the coral animal, the dinoflagellate algae symbionts (Buddemeier and Fautin 1993), and the Bacteria (Reshef et. al. 2007), and viruses (Correa et al. 2012). Changes in symbiont community identity in response to stress, lasting may last from months to years (Baker et al. 2004, Thornhill et al. 2006a, LaJeunesse et al. 2009), can help corals acclimatize to new environmental conditions.

Coral-Algal Symbiosis

Reef corals are able to build productive ecosystems in oligotrophic waters largely due to their mutualistic symbiosis with single-celled dinoflagellate algae in the genus *Symbiodinium*. Although morphologically cryptic, *Symbiodinium* are genetically diverse, with nine sub-generic clades (named A-I) currently recognized (Pochon & Gates 2010). Reef-building scleractinian corals most commonly associate with *Symbiodinium* in clades A-D (Baker 2003), although members of clades F and G have also been reported (Rodriguez-Lanetty et al. 2004, LaJeunesse et al. 2010) in these hosts.

Within these *Symbiodinium* clades, over 400 distinct ITS-2 rDNA "types" have been identified based on sequence differences and DGGE profiles (e.g., LaJeunesse 2002, 2003, 2004a, b, 2005), but it is not yet clear how many of these types represent distinct species (Correa & Baker 2009, Stat et al. 2011). The distribution of some *Symbiodinium* variants has been correlated with different environmental conditions, usually temperature or light (Rowan et al. 1997, Glynn et al. 2001, Baker 2003, Rowan 2004, Goulet et al. 2005, Warner et al. 1996, Berkelmans & van Oppen 2006, Abrego et al. 2008, Sampayo et al. 2008, Jones et al. 2008, Thornhill et al. 2008). Physiological variability among *Symbiodinium* variants likely contributes to the ability of a coral host to thrive in a variety of environmental conditions, such as gradients in depth, latitude, irradiance and temperature (e.g., Rowan & Knowlton 1995, Rowan et al. 1997, Baker 2001, Rodriguez-Lanetty et al. 2001, MacDonald et al. 2008, Thornhill et al. 2008, Sampayo et al. 2008). In a few cases, physiological studies have supported these correlations (e.g., Little et al. 2004, Tchernov et al. 2004, Cantin et al. 2009, Frade et al. 2008).

Recently, molecular evidence from several markers (including microsatellite, mitochondrial, ribosomal and chloroplast sequences) has been used to define *Symbiodinium* sub-cladal types as distinct species (LaJeunesse et al. 2012). However, the vast majority of reported variants have yet to be characterized physiologically, ecologically or geographically, and species delineations are particularly difficult to define in light of the lack of morphological features (Correa and Baker 2009, but see Sampayo et al. 2007).

Symbiodinium clade D has attracted particular interest in this regard because it includes opportunistic variants (e.g., Internal Transcribed Spacer-2, or ITS-2, types D1 and D1a) that can associate with hosts under environmental conditions, such as temperature extremes, that are typically sub-optimal for many members of other clades (reviewed by Baker 2003 and Stat and Gates 2011). Temperature has been shown to be the key determining factor in limiting species abundances and ranges (Hofmann and Todgham 2010), and therefore the ability to expand thermal tolerances by associating with thermally tolerant *Symbiodinium* has clear benefits for the coral holobiont. Members of clade D have been shown to increase the thermal tolerance of corals by 1-1.5^{o}C compared to other *Symbiodinium* types (Rowan 2004, Berkelmans and van Oppen 2006, Jones et al. 2008), and this may be a result of differences in the degree of lipid saturation of the *Symbiodinium* thylakoid membranes (Tchernov et al. 2004). Clade D symbiont have also been found to produce less reactive oxygen species, which my trigger bleaching, than other *Symbiodinium* types (McGinty et al. 2012). Clade D is also commonly found on reefs immediately following a bleaching event, either due to differential mortality of colonies that do not contain members of clade D, or because

clade D acts as a "pioneer" symbiont in the open niche space left by other symbionts in bleached coral tissue (Jones et al. 2008, Berkelmans and van Oppen 2006, van Oppen et al. 2005).

While clade D may allow corals to survive during high temperature events, there are likely to be trade-offs for coral fitness. This has provoked some researchers to suggest that the costs of hosting clade D symbionts may outweigh the benefits (Ortiz et al. 2012, Grottoli et al. 2012). Corals grow more slowly when hosting clade D, relative to clade C (Little et al 2004, Jones and Berkelmans 2010, Cunning et al. *in prep,* Ortiz et al. 2012, but see also Smith et al. 2010). Clade D is less efficient at translocating sugars to coral hosts (Cantin et al. 2009) which likely explains why corals harboring clade D may grow more slowly (Little et al. 2004, Cunning et al. *in prep*), and why it may not be the typical symbiont of any corals in their normal ranges. However, differences in growth rates and other potential tradeoffs have not been compared at the higher temperatures favored by clade D, so it is not clear if these tradeoffs are universally applicable to all environments. For example, Cunning et al. (*in prep*) suggests that, in the coral *Montastraea faveolata*, photochemical efficiency at 24^{o}C may be lower in clade D symbionts than in clade B sybmionts. However, during heat stress, clade D symbionts become more efficient than clade B symbionts. Similarly, Gillette (2012) presents data from *Pocillopora damicornis* suggesting that growth differences between corals hosting clade C and D symbionts disappear when temperatures are raised from 24^{o}C to 30^{o}C. Together, these studies suggest that clade D symbionts may be suboptimal at lower temperatures, but may be equally or more efficient than other symbionts at elevated temperatures.

Hosting clade D symbionts may allow corals to survive in more stressful environments, but also may mean entering into a less-fit symbiotic relationship, with unknown consequences for coral growth, reproduction, and disease resistance. Therefore, one outcome of chronically high sea surface temperatures in an era of climate change may be the proliferation of a sub-optimal symbiotic relationship for corals (Baker et al. 2004). This dissertation explores the potential expansion of corals' thermal niche by hosting clade D symbionts, and its implications for coral survival during warming and cooling oceans (See Chapter 5).

Tradeoffs of coral-algal symbiosis

While symbiosis can yield important benefits for mutualist partners, it can also mean that neither is stronger than the weakest partner. Studies have shown that zooxanthellate corals suffered higher extinction rates during the Cretaceous-Paleogene boundary than azooxanthellate corals, likely due to their restricted habitat ranges (Barbeitos et al. 2010). Corals are "producers within consumers", and thus are particularly susceptible to oxidative stress derived from light capture by *Symbiodinium* during photosynthesis. Reactive Oxygen Species (ROS) are natural by-products of aerobic metabolism, and are typically generated in high-energy systems in the presence of oxygen. Cells can deal with some degree of oxidative stress during normal function by antioxidants (e.g., superoxide dismutases, perodixases, carotenes, heat shock proteins 60 and 70, and ascorbic acids), chaperone proteins and other measures (Brown et al. 2002, Downs 2000, Rougee et al. 2006, DeSalvo et al. 2008, Rodriguez-Lanetty et al. 2006, Fitt et al. 2009). However, when light and/or heat stress overwhelms these ROS avoidance

measures, various parts of the host (e.g., host mitochondria, Lesser et al. 2006, Weis et al. 2008) and/or symbiont (e.g., thylakoid membranes, Tchernov et al. 2004; the D1 protein in the Photosystem II reaction center, Warner et al. 1996) can be damaged, disrupting the flow of photons through the photosynthetic machinery. This causes even more ROS to be formed, and triggers a bleaching cascade.

Symbiodinium can therefore be considered the source of oxidative stress, which triggers bleaching and has a negative effect on host fitness (which partner initiates the bleaching response, however, is unknown). Corals with higher symbiont densities are shown to bleach more during thermal stress (Cunning and Baker 2012, Wiedenmann et al. 2012). Coral "tissue balls" degrade and die faster during thermal stress when they have higher densities of *Symbiodinium* (Nesa et al. 2009). Conversely, aposymbiotic coral oocytes can survive in higher temperatures for longer amounts of time than symbiotic oocytes (Yakovleva et al. 2009, but see Baird et al. 2006).

As cost-benefit ratios are altered by climate change, shifts from mutualistic (mutually-beneficial) to antagonistic (parasitic) relationships are predicted to become more common (West et al. 2007, Kiers et al. 2010). This has already been observed in several plant-fungi and plant-insect mutualisms (Kiers et al. 2010). In coral-algal symbiosis, there are a few reports of "parasitic" symbionts causing a disease-like state in corals (Toller et al. 2001). Stat et al. (2008) reported that clade A *Symbiodinium* were significantly correlated with corals with impaired health, but Correa et al. (2009a) did not identify any "parasitic" symbiont types in their survey of *Symbiodinium* associated with diseased corals.

Paradoxically, while members of *Symbiodinium* in clade D provide clear benefit to coral hosts during suboptimal conditions, clade D has also been referred to as a "selfish opportunist" that represent a potentially "ominous sign" for corals (Stat and Gates 2011). Compared with other *Symbiodinium*, clade D might be considered to be less mutualistic, and more antagonistic, since it translocates less energy to the host (Cantin et al. 2009) and results in slower host growth rates at lower temperatures (Little et al. 2004, Jones and Berkelmans 2010, Cunning et al. *in prep*). However, these costs should be weighed against the benefit of increased survivorship of the coral host under conditions where clade D is favored.

While *Symbiodinium* may be the "weak link" in the symbiosis with respect to production of oxidative stress and restrictions of habitat to the photic zone, symbiosis is likely also responsible for the ecological dominance of reef building corals on modern reefs (Baird et al. 2006). Aposymbiotic corals, for example, make up almost half of the order Scleractinia, although they are largely absent from shallow reefs (Barbeitos et al. 2010). Coral-algal symbiosis evolved an estimated 240 million years ago, likely at the origin of the order Scleractinia, and this relationship has remained remarkably stable over evolutionary time (Gilbert et al. 2010), despite evidence that corals have abandoned a symbiotic state (and their clonal state) at periods of their the evolutionary history (Barbeitos et al. 2010). The competitive dominance of zooxanthellate corals on modern reefs suggest that, evolutionarily, any risks derived from associating with *Symbiodinium* are likely to be outweighed by the increased fitness (i.e., energy) that reef corals garner from hosting *Symbiodinium.*

How the cost-benefit ratio of this relationship will change under predicted climate change conditions will be a major factor determining the fate of zooxanthellate, reef-building corals in a world of warmer and more acidic oceans, and should be the focus of future studies.

Mutualisms in corals and other systems during climate change

Almost every living species engages in some form of mutualistic partnership. Symbiosis may link multiple species to a shared fate, compounding detriments from climate change (Thyliankis et al. 2008, Bascompte and Stouffer 2009, Dunn et al. 2009, Kiers et al. 2010). How symbioses might respond to changing environments therefore has important implications for the survival of both the symbiont and the host. A variety of outcomes for mutualists are possible, reviewed by Kiers et al. (2010), including: (1) abandonment of symbiosis, (2) the rapid evolution of adaptive traits, and (3) switching of mutualist partners. In considering the potential contribution of mutualisms to holobiont fitness during stressful environmental conditions, Kiers et al. (2010) raised two important questions: (1) have mutualisms evolved to be sufficiently resilient and flexible to withstand disturbance? If not, (2) can mutualist partnerships change rapidly enough to preserve symbioses during periods of environmental change?

Abandonment of Symbiosis

Studies have examined the possibility that, when at least one partner is negatively affected by changing environmental conditions, the symbiosis in which it is engaged could be abandoned. A 50-year dataset demonstrated that plants are now flowering earlier

in the year, while their butterfly (*Prunus*) partners develop later, disrupting the synchrony of the pollination scheme (Doi et al. 2008, Kiers et al. 2010). In the absence of pollinators, some plants have switched to abiotic pollination methods, such as water and wind distribution, in order to buffer themselves from damage due to the loss of symbiotic partnerships (Kaiser-Bunbury et al. 2010). Some plants growing in artificially-fertilized soils have also abandoned their mycorrhizal symbionts, as the need for increased nutrient uptake by the mycorrhizae is obviated by fertilizers. Other plants have lost the ability to form these mutualisms entirely (Wang and Qui 2006), making them particularly susceptible to mortality in the event that artificial fertilization ceases. Furthermore, if mycorrhizal symbiosis were to be abandoned entirely, it has the potential to impact entire ecosystems, as fungal hyphal networks sequester carbon and help stabilize soil (Kiers et al. 2010).

Reef corals have abandoned their symbiotic state over the course of their evolutionary history (Barbeitos et al. 2010). However, corals do not permanently abandon their obligate microbial symbioses during routine stress events. Over ecological time scales (rather than geological ones), bleaching represents a temporary breakdown of the symbiotic relationship between corals and algae. If corals remain in a bleached state for a long period of time, they often fail to gain sufficient energy for survival, unless they are able to easily increase their heterotrophic feeding (Grottoli et al. 2006). It has been predicted, therefore, that coral species which are best able to obtain energy from heterotrophy, and which are therefore less-dependant on energy from *Symbiodinium,* may have the best outcomes under climate change conditions which promote more frequent or

severe bleaching (Grottoli et al. 2006). These coral species may therefore be the most likely species to abandon their symbiotic relationships during climate change.

Evolved Stress Tolerance

Microbes have short generation times, and therefore have the potential for rapid adaptive changes under selective pressure (Meyer et al. 2006, Ivarsson & Holm 2008). Bennett et al. (1990) showed that, in a matter of days, under a high temperature treatment, *Escherichia coli* clones from a single individual evolved distinct high temperature-tolerant populations. Organisms involved in mutualistic relationships with microbial partners might similarly benefit from the spread of stress-tolerant emergent traits, and therefore the holobiont may be better able to deal with human-induced environmental impacts over short time scales (Correa and Baker 2010). Correa & Baker (2010) investigated how coral bleaching, by 1) disturbing the established population of resident symbionts, 2) favoring the survival of stress-tolerant symbionts, and 3) leaving open niches for the proliferation of uncommon symbiont types, which might lead to the spread of symbiotic "disaster taxa" within coral hosts. Despite long generation times and infrequent sexual reproduction, corals, via their partners and associates, including algae (Buddemeier and Fautin 1993), Bacteria (Reshef et al. 2006), Archaea, fungi, (Knowlton and Rohwer 2003, Rosenberg 2007), and even viruses (Correa et al. 2012), are predicted to have a higher adaptive capacity than might be otherwise expected (Correa and Baker 2010).

However, evolution of new traits in response to selection pressure can be difficult to test within obligate symbioses (Mueller et al. 2011). Some studies have attempted this

using experimental methods, or by observing symbiosis across steep ecological clines. Mueller et al. (2011) provided evidence for evolved cold tolerance in the symbiosis between *Atta texana* leafcutter ants and *Attamyces* fungal cultivars, which produce food upon which leafcutter ants depend. This symbiosis evolved as a tropical mutualistic symbiosis, however, *A. texana* and *Attamyces* have since developed a wide range across steep gradients that include low temperatures. Mueller et al. (2011) showed that the extended range of *Attamyces* and *A. texana* can be attributed to low temperature selection pressure resulting in the emergence of cold-tolerance traits in temperate populations.

Recent work with corals is showing similar findings. Symbionts from high temperature reefs exhibit greater thermotolerance when compared with the same symbiont type from lower temperature reefs, even after 30 generations (Howells et al. 2012). Similarly, van Oppen (2012) reported rapid evolution of symbiont strains under selective pressure for high temperature tolerance over very short timescales (months). These studies demonstrate the potential for rapid genetic evolution of *Symbiodinium* strains under selection for high temperature tolerance.

Partner Switching and Symbiotic Redundancy

Evidence from other systems suggests that, during disturbance, the ability to form diverse symbioses with multiple partners, or to evolve rapidly under selection from stress, may be key to the survival of mutualists. However, as pointed out by Wernegreen and Wheeler (2009) discussing the symbiosis between ants and the bacteria *Blochmannia*, “it is often difficult to distinguish whether symbiont proliferation and decline represent adaptive responses that benefit the host, or alternatively, a breakdown in the normal

regulation of symbiont numbers." This difficulty applies equally to coral-algal symbioses. Putnam et al. (2012) challenges the idea that partner switching is beneficial, and identify a correlation between coral species commonly found to host diverse symbiont communities and those generally acknowledged to bleach readily. However, their study did not experimentally test bleaching susceptibility in relation to diverse background clades, nor did it establish whether diverse symbiont communities resulted in bleaching or whether bleaching resulted in diverse symbionts.

Examples of partner switching in other systems are widespread, and have clear parallels to coral-algal symbiosis, despite the fact that the relative importance of partner switching to coral survival has been challenged (Hughes et al. 2003, Goulet 2006, Hoegh-Guldberg et al. 2007). In the case of the mutualism between *Camponotini* ants and *Candidatus blochmannia* bacteria, densities of symbionts are ontogenetically-mediated, increasing during developmental phases which are more metabolically intensive. This supports the idea that changes in symbiont densities might be adaptive (Wernereen and Wheeler 2009). In some cases, the relationships are environmentally-mediated, for example, the mutualism between the bark beetle (*Dendroctonus ponderosae)*, and two species of fungi (*Grosmannia clavigera* and *Ophiostoma montium,* Six and Bentz 2007). These fungi are critical for the survival of *D. ponderosae* because they supplement their hosts' diet, and there is some evidence that each fungus has differential effects on beetle fitness (Six and Paine 1998). The relative dominance of the fungal species within *D. ponderosae* appears to be mediated by temperature, and since most fungi have relatively limited temperature ranges, hosting "cool" and "warm" species of fungi may be a mechanism that allows beetles to expand their environmental range (Six and Bentz 2007).

In another example, pea aphids (*Acyrthosiphon pisum*) typically associate with a primary bacterial symbiont that is essential to their reproductive success and survival. However, between 35 and 80% of *A. pisum* populations also host a secondary bacterial symbiont that, under normal conditions, can be detrimental to host fitness (Koga et al. 2003). But, if the primary (dominant) symbiont has been experimentally removed, a secondary, low abundance, bacterial symbiont can proliferate within the aphid and assume the general function of the primary symbiont (Koga et al. 2003). This secondary bacterial symbiont has additionally been shown to confer thermotolerance to aphids exposed to heat stress (Montllor 2002).

Fungal endophytes associate with plants and confer tolerance to heat, salt, drought, and disease to their hosts in a habitat-mediated manner (Rodriguez et al. 2008). Fungal endophytes are even thought to have allowed for plants' evolutionary transition from aquatic to terrestrial ecosystems (Pirozynski and Malloch 1975). Rodriguez et al. 2008 showed that geothermal endophytes conferred heat but not salt tolerance, while coastal endophytes conferred salt but not heat tolerance to their hosts. These traits were shown to be transferable to plants after transplantation of the endophyte (Rodriguez et al. 2008). In another example, a type of geothermal grass associates with a fungus that allows it to survive at temperatures up to 65^{o}C. However, thermotolerance was found also to be dependent upon a virus that associates with the endophyte, thereby requiring three partners in order to confer heat resistance (Màrquez et al. 2007). Rodriguez and Redman 2005 hypothesize that the endophyte could increase stress tolerance by scavenging reactive oxygen species from the host, thereby preventing it from oxidative stress. ROS are likely triggers for bleaching in corals, and it is possible that different

bleaching tolerances of symbiont types relate to ROS production levels or ROS quenching abilities.

As with coral-algal symbioses, these systems routinely associate with multiple symbiotic partners and undergo dominance shifts between microbes, with varying effects on host fitness depending on environmental conditions. Mixed communities of *Symbiodinium* may therefore provide functional redundancy to a coral host, which can increase the likelihood that one of several mutualistic associations will be preserved during periods of environmental stress (*sensu* Yachi and Loreau 1999). Even if the persisting association confers only the same type (but not the same degree) of benefit, the host may benefit overall from avoiding an aposymbiotic state (Six and Bentz 2007). These examples show that the prevalence of background, functionally redundant microbial symbionts, as well as their dynamics, their interactions with dominant symbionts and hosts, and responses to different stressors, are important factors in understanding the symbiosis ecology of these different systems, and strongly argue for further research applying these concepts to coral-algal symbiosis.

Summary and Conclusions

This dissertation examines the prevalence and functional role(s) of rare, "background" symbionts in reef-building corals. I initially demonstrate that most (perhaps all) coral species can host mixed algal symbiont communities that include background symbionts at low abundance. I then conducted a series of bleaching and recovery experiments that were designed to investigate the role that these background symbionts might play in coral resistance and resilience. These experiments showed that

low abundance symbionts (undetectable using conventional molecular methods) can readily become dominant within disturbed coral hosts, and can increase coral thermotolance over a period of a few months. Distrubance was found to encourage more rapid and dramatic symbiont turnover. Additionally, the type of stress and recovery conditions influenced the symbiont communities present in hosts even 6 months post-bleaching. Although these experiments did not address whether these symbionts were acquired from the environment or were already present as members of the background community, it is clear that the functional redundancy provided by diverse symbiont communities can help corals survive climate change-induced stressors. In summary, symbionts that are, at the very least, rare members of the community, can be critically important inexpanding corals' realized thermal niches both at low and high temperatures.

Chapter 2

Specificity is rarely absolute in coral-algal symbiosis: Implications for coral response to climate change[1]

Summary

Some reef-building corals have been shown to respond to environmental change by shifting the composition of their algal symbiont (genus *Symbiodinium*) communities. These shifts have been proposed as a potential mechanism by which corals might survive climate stressors, such as increased temperatures. Conventional molecular methods suggest this adaptive capacity is not widespread because few (~25%) coral species are able to associate with genetically diverse *Symbiodinium*. However, these methods can fail to detect low abundance symbionts (<10-20% of the total algal symbiont community). Here we applied a high-resolution, real-time PCR assay to examine diversity in the lineages of *Symbiodinium* most commonly found in reef-building corals (clades A-D). We assayed 321 coral samples in 39 species of phylogenetically and geographically diverse corals, including 26 coral species thought to be restricted to hosting a single *Symbiodinium* clade ("symbiotic specialists"). We detected at least two *Symbiodinium* clades (C and D) in at least one sample of all 39 coral species tested; all 4 *Symbiodinium* clades were detected in over half (54%) of the 26 symbiotic specialist

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coral species. Furthermore, on average, 68% of all sampled colonies within a given coral species hosted two or more symbiont clades. We conclude that the ability to associate with multiple symbiont clades is common in among scleractinian (stony) corals, and that, in coral-algal symbiosis, "specificity" and "flexibility" are relative terms: specificity is rarely absolute. The potential for reef corals to adapt or acclimatize to environmental change via symbiont community shifts is therefore more phylogenetically widespread than has previously been assumed.

Background

Models predict that reef ecosystems throughout much of the tropics will decrease in coral cover, diversity, and/or undergo phase shifts in the coming decades (Hughes et al. 2003, Donner et al. 2005, 2007, Hoegh-Guldberg et al. 2007). An assumption of these models is that corals will be unable to compensate for increasing temperatures over relatively short timescales. The ability to associate with diverse symbionts has been suggested as one mechanism by which corals might be able to respond rapidly to environmental change (Buddemeier and Fautin 1993, Baker 2001, Baker 2004, Fautin and Buddemeier 2004, Berkelmans and van Oppen 2006, Jones et al. 2008, Baskett et al. 2009, Correa and Baker 2011). However, it has been argued that symbiont-mediated acclimatization is not feasible for most coral species based on perceptions that: (1) many coral taxa are "symbiotic specialists" that are restricted to hosting a single symbiont taxon, and (2) most scleractinian corals do not associate with thermo- and/or stress-tolerant algae, such as some members of *Symbiodinium* clade D (Hoegh-Guldberg et al. 2002, 2007, Goulet and Coffroth 2003a, b, Hughes et al. 2003, LaJeunesse et al. 2003,

2005, Goulet 2006, 2007, but see Baker 2003, Baird et al. 2007, Baker and Romanski 2007, Mieog et al. 2007, Correa et al. 2009b). In fact, perceived symbiont specificity has even been used as a criterion for recognizing both symbiont "species" (LaJeunesse et al. 2010) and cryptic coral species (Pinzon and LaJeunesse 2011). However, these perceptions were based on studies utilizing conventional, gel-based, molecular techniques such as analysis of Restriction Fragment Length Polymorphisms (RFLPs), Single Stranded Conformational Polymorphisms (SSCPs), and Denaturing Gradient Gel Electrophoresis (DGGEs) of nuclear ribosomal DNA (Figure 2.1). These methods are typically effective at detecting only the dominant, or most abundant, taxa in a (potentially) diverse symbiont community. Thornhill et al. (2006a) estimated that symbiont taxa representing < 5-10% of the total symbiont community are not detected using DGGE, while LaJeunesse et al. (2008) found that detection limits depended on symbiont type and molecular marker. For example using ITS-2, detections of some *Symbiodinium* (e.g., C1b-c) occur even when these variants comprise just 0.3% of the total community, while detections of other *Symbiodinium* (e.g., D1) occur only when variants represent 10-20% of the total symbiont community. Using the Internal Transcribed Spacer-1 (ITS-1) region marker, however, *Symbiodinium* D1 was not detected until it comprised between 50-90% of the total community, while *Symbiodinium* C1b-c remained detectable at 0.3% (LaJeunesse et al. 2008). These studies indicate that *Symbiodinium* variants that are not dominant (i.e. most abundant) community members may not be detected using conventional molecular techniques (Baker and Romanski 2007), even though they may be numerically abundant. For example, in a typical scleractinian coral containing 1 – 2 million symbionts per cm^2 of coral tissue (Drew

1972), a *Symbiodinium* taxon representing just 1% of the community would still represent 10,000 – 20,000 cells per cm^2 of coral tissue, but would not necessarily be detected using DGGE (LaJeunesse et al. 2008).

High-resolution techniques, such as real time PCR (rt-PCR), have detection thresholds that are at least 100 – 1,000 times more sensitive than conventional techniques (Mieog *et al.* 2007). These techniques have recently revealed the presence of *Symbiodinium* clades at low abundance, as well as their dynamics within select coral species (e.g., Ulstrup and van Oppen 2003, Mieog et al. 2007, 2009, Ulstrup et al. 2007, Correa et al. 2009b, LaJeunesse et al. 2009, Coffroth et al. 2010, Yamashita et al. 2011). These findings support the idea that stony corals may be more flexible in their associations ("symbiotic generalists") than has been indicated using conventional molecular methods. However, it has remained unclear whether the relatively few host taxa surveyed using high-sensitivity techniques are representative of stony coral-*Symbiodinium* associations in general (Mieog et al. 2007, Correa et al. 2009b).

In this study, "dominant" symbionts will refer to symbionts that have been routinely detected using conventional molecular techniques (which may include more than one clade for a given coral species), and "background" symbionts will refer to symbionts which are newly detected using high sensitivity techniques. As they have not been previously detected with conventional techniques, they are likely present in corals at lower abundances.

Here, we assayed multiple individuals (N=4-12) of 39 geographically widespread and phylogenetically diverse coral species for the presence of *Symbiodinium* in clades A, B, C, and D. This study provides: (1) the most comprehensive, real time PCR survey of

symbiont diversity in corals to date, and (2) baseline data for symbiont community ecology studies.

Materials and Methods

DNA extraction and rt-PCR assay

Real time PCR assays were used to determine the presence/absence of *Symbiodinium* in clades A, B, C, and D in a total of 321 samples from 39 reef coral species, representing 28 genera and 14 families. Based on previous studies using conventional molecular techniques to detect *Symbiodinium* in coral hosts (through November 2009), 26 of these coral species were designated "symbiotic specialists" (defined here as species previously reported to host only a single *Symbiodinium* clade), while 13 were "symbiotic generalists" (defined here as species previously documented to host multiple *Symbiodinium* clades). Samples were collected from various locations worldwide between 1995 and 2009 (Table 2.1). Previous *Symbiodinium* detections reported between September 2006 and November 2009 are listed in Table 2.1. Detections prior to 2006 are summarized in Goulet 2006, and are included in this analysis but not enumerated here. Papers published since November 2009, using conventional molecular techniques as well as rt-PCR, may have found multiple *Symbiodinium* clades in some of these species (e.g. LaJeunesse et. al 2010, Finney et al 2010), but were not included in this analysis.

DNA extraction and purification methods were based on Baker et al. (1997). The rt-PCR assays and analyses followed Correa et al. (2009b) and had similar specificities,

efficiencies, and sensitivities to the assays described therein. Correa et al. (2009b) designed clade-specific primers within conserved regions of either the ITS1-5.8S-ITS2 (clade A) or LSU domain 2 (clades B-D) nuclear ribosomal DNA. Real time PCR assays were then performed on an Applied Biosystems (Foster City, CA) StepOne Plus machine. Each rt-PCR reaction contained 4.25µl of ddH_20, 6.25µl of Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and 0.5 µl each of forward and reverse 10µM primers (clades A-C only, clade D primers are used at 1µM concentrations due to their high sensitivity), with each clade occupying a different well, and 1µl of genomic DNA at 100 ± 5 ng/µl, for 12.5µl volume reactions. Each rt-PCR cycle consisted of a denaturing step at 95°C for 10 minutes, followed by 40 cycles of: 95°C for 30 seconds, annealing at 60°C for 30 seconds, and 72°C for 30 seconds. We scored positive amplifications as those that exceeded a fluorescence threshold value of 0.1 in <40 cycles. Each plate included a set of duplicate "no template controls" (NTC) for each primer set (with ddH_2O substituted for DNA). To avoid false positives, we scored any sample in which amplification occurred within 3 cycles of the NTC as a negative amplification (i.e., sample amplification was indistinguishable from background fluorescence). Each sample was run in duplicate, and each plate also included a duplicate set of positive control samples (DNA extracted from *Symbiodinium* in long-term culture obtained from S.R. Santos at Auburn University: culture FLAp1 for clade A, 703 for clade B, Mp for clade C, and A008 for clade D).

Melt curves (41 cycles of 30s, with an increase of 1°C per cycle from 55°C to 95°C) were generated for each rt-PCR run to confirm that observed fluorescent signals were derived from target amplicons and not primer-dimers. Products with a melt temperature of 80°C

(±1°C) were scored as target amplifications, while products melting at 72–75°C were scored as primer-dimers (based on Correa et al. 2009b).

Contamination tests

Previous studies of symbiont diversity in scleractinian corals using rt-PCR have considered any *Symbiodinium* detected to be *in hospite* (i.e., residing in a symbiosome, Mieog et al. 2007, Ulstrup and van Oppen 2003, Loram et al. 2007, Correa et al. 2009b, LaJeunesse et al. 2010). However, it is also possible that rt-PCR can detect symbionts that are merely: (1) surface contaminants (e.g., *Symbiodinium* that are free-living or recently expelled from other hosts); or (2) gut contaminants (i.e., recently ingested food items). To assess the extent to which rt-PCR detections reflect potential false positives for *Symbiodinium in hospite*, we analyzed samples of the azooxanthellate coral *Tubastrea coccinea* (N=13 total), using the methods described above, as a "biological negative control". Any positive amplifications for *Symbiodinium* from azooxanthellate samples must therefore have been derived from recently ingested algal symbionts or *Symbiodinium* cells adhered to the surface of coral polyps. Symbiont detections from azooxanthellate coral can be considered estimates of the relative proportion of rt-PCR detections of background symbiont populations from zooxanthellate (symbiotic) corals that may actually be "contaminant" *Symbiodinium.* *T. coccinea* were used from several different reef environments (Panama, N=4; Galapagos, Ecuador, N=2; West Palm Beach, FL, USA, N=4; Key Largo, FL, USA, N=3). To ensure that negative rt-PCR amplifications from *T. coccinea* samples reflected a lack of *Symbiodinium* DNA rather

than poor quality genomic DNA, we also amplified each *T. coccinea* sample using coral-specific primers (Wirshing and Baker, in prep.) for β-tubulin sequences.

Given the high sensitivity of rt-PCR, we also assessed any potential contamination resulting from the laboratory protocol of Baker et al. (1997) in paired, randomly selected coral samples (N=9). This extraction protocol uses an airbrush to blast tissue from coral skeletons using an EDTA-based (DNAB) buffer solution into a plastic bag, after which the bag is rinsed vigorously in running fresh water between each sample. The bag is replaced after ~5-10 samples. Although no contamination has been detected for this protocol using conventional, less-sensitive techniques such as DGGE, we investigated whether the highly sensitive rt-PCR technique might detect residual *Symbiodinium* cells remaining in collection bags after rinsing between samples. In order to assess potential laboratory contamination of coral samples, a randomly selected sample was airbrushed using the Baker et al. (1997) method to generate a "positive extraction control". Blasted tissue was then transferred from the plastic bag to a 1.5mL tube and extracted, and the bag was thoroughly rinsed as described above. A blasted buffer solution was then sprayed into the rinsed bag, without a coral sample, in order to generate a "negative extraction control". This negative control blastate was collected, extracted, and amplified using the same protocol as the positive control. A new bag was used for each of these paired positive and negative controls (N=9 pairs). Despite the geographically and temporally widespread nature of this data set, these controls are an appropriate way of assessing potential contamination levels because all samples in this study (zooxanthellate and azooxanthellate) were collected in the field, preserved, and

transported to the lab for DNA extraction in identical ways (described in Baker et al 1997), and are therefore comparable to one another.

SYBR assay specificity

The SYBR Green rt-PCR assays used in this study were fully validated in Correa et al. 2009b. However, SYBR Green rt-PCR assays have been suggested to be less specific to their target sequences than other rt-PCR assays, for example, those conducted with Taqman probes. We therefore conducted an extra specificity test of the SYBR Green assays used in this study. To do this, we randomly selected 9 samples from the total dataset (N=1 of each *Acropora cervicornis* from Panama, *Pavona clavus* from the Galapagos, *Pavona varians* and *Galaxea astreata* from the Gulf of Aqaba, *Stylocoeniella guentheri* from Japan, *Pocillopora eydouxi* from the Maldives and *Pachyseris speciosa* from Palau, as well as two samples of *Cyphastrea microphthalama* from the Arabian Gulf) and applied the SYBR Green assay for *Symbiodinium* clades A through D. We then sequenced all positive amplifications (N=16) using BigDye on a 3130xl Genetic Analyzer (Applied Biosystems) and identified the resulting sequences via BLASTn searches of the NCBI nr database.

Symbiodinium *diversity analyses within host species*

Within a particular host species, the percentages of colonies containing one, two, three, or four symbiont clades were calculated (Table 2.3), as well as the average number of colonies (± standard deviation) across all species (Table 2.3). These data were then grouped as follows: (1) all symbiotic generalist coral species, (2) all symbiotic specialist coral species, and (3) all coral species and are presented in Table 2.3.

Total Symbiodinium *community diversity analyses*

For each coral species, we calculated a Shannon's diversity index for the total *Symbiodinium* community detected, based on the combined positive symbiont detections from all sampled colonies. Shannon's t-tests (Hutcheson 1970) were then used to make pairwise comparisons of the Shannon's diversity indices (total *Symbiodinium* diversity) between all 39 coral species. After the Bonferroni correction, the p-value for these multiple tests of significance was set at 3.37×10^{-5}. This approach was also used to compare the total *Symbiodinium* diversity identified from Caribbean versus Indo-Pacific corals, and from symbiotic generalists versus symbiotic specialists. The Bonferroni correction was not applicable to these latter two tests, and therefore a P-value of 0.05 was used. All Shannon's diversity indices and Shannon's t-tests were calculated using the Biodiversity Calculator (Danoff-Burg and Xu 2005).

ANOVAs of linear regressions (performed in Microsoft Excel) confirmed that Shannon's diversity index results were not biased by the number of geographic regions or the number of coral colonies analyzed for a given coral species. P-values > 0.5 indicated that an independent variable (e.g., number of geographic regions) was not significant in explaining the Shannon's diversity index obtained for a given coral species. This same approach was also used to confirm that the number of colonies analyzed for a given coral species did not bias comparisons of total *Symbiodinium* diversity among coral species.

Clade-level Symbiodinium *diversity ordination analyses*

We used nonmetric multidimensional scaling (NMDS) with Bray-Curtis Index dissimilarities to visually represent configurations of the relative abundances (i.e., the number of times a particular clade was detected, normalized to the host sample size) of *Symbiodinium* in clades A-D hosted by different coral species (R v. 2.9.1, R Core Development Team 2009, Vegan library). Standard error ellipses delineate the standard error with respect to the relative abundances of symbiont clades within: (1) symbiotic specialist and symbiotic generalist coral taxa: and (2) Indo-Pacific and Caribbean regions, such that the center of the ellipse corresponds to the mean relative abundance. Because NMDS is a visual analytical tool and does not analyze statistically significance between groups, analysis of similarity (ANOSIM) was also performed in order to determine whether symbiont communities were significantly different between (1) symbiotic specialist versus symbiotic generalist categories of coral species, and between (2) Indo-Pacific and Caribbean corals (R v. 2.9.1, R Core Development Team 2009, Vegan library).

Results

RT-PCR results

Of the coral species examined here, 38 of 39 were found to associate with a *Symbiodinium* clade that had not previously been reported using conventional techniques (Table 2.1). *Pocillopora damicornis*, which was already known to host clades A, C, and D, was the exception, as it was not also found to associate with clade B in this study. (In subsequent studies, this host species has also been found in association with clade B with

DGGE analysis, LaJeunesse et al. 2010, Cunning and Baker 2012). In total, 78 previously unreported, clade-level, coral-symbiont detections are reported here (Table 2.1). We found that all 39 coral taxa contained *Symbiodinium* in clades C and D, in at least one of the colonies analyzed (N= 4 to 12 colonies per host taxon, Table 2.1).

Of the 26 coral taxa thought to be symbiont specialists, 14 (54%) associated with all 4 symbiont clades (A+B+C+D) in one or more samples, while 21 (81%) hosted at least 3 clades (either A+C+D or B+C+D) in one or more samples. Of the 13 coral taxa considered to be symbiotic generalists, 8 (62%) hosted all 4 clades in one or more samples, and all (100%) associated with at least 3 clades in one or more samples. Based on these rt-PCR assays, none of the symbiotic generalist species associated with only two clades.

Here, clade A was detected for the first time in samples from Bermuda (*Madracis mirabilis*, N=6), and clade D was detected for the first time in the Galapagos (*Pavona gigantea*, N=1; *Pavona clavus*, N=4; *Pocillopora damicornis*, N=3, Table 2.1).

Within-host species Symbiodinium *diversity comparisons*

In any given coral species, most individual colonies (mean 68% of all colonies of a given coral species) hosted more than one *Symbiodinium* clade. In 23% (N=9 of 39) of the coral species examined, every colony analyzed hosted multiple clades. On average, for a given coral species, 40% of the colonies analyzed hosted 2 clades, while 24% hosted 3 clades.

Between host species diversity comparisons

The *Symbiodinium* community diversity of each coral species was calculated using the Shannon Diversity Index. Values ranged from 0.52 (*Hydnophora exesa*) to 1.33 (*Colpophyllia natans*), but low sample sizes precluded some calculations (Table 2.2). Pairwise two-tailed Shannon's t-tests for all coral taxa (Figure 2.3a) showed that the relative diversity of coral-*Symbiodinium* associations (1) differed among species and (2) followed a gradient of overall symbiont diversity.

Although some coral species contained *Symbiodinium* communities that were significantly more diverse than others (Figure 2.3a), most host species examined in this study were equivalent in terms of their total *Symbiodinium* diversity (Figure 2.3b). Pairwise comparisons of *Symbiodinium* revealed that all coral species contained symbiont community diversity equivalent to at least 50% of the other 38 coral species (Figure 2.3b), including taxa previously considered to be symbiotic generalists and symbiotic specialists. When considered as groups, there were no differences in overall diversity between symbiotic specialists and symbiotic generalists (Shannon's t-test, P=0.06).

In a similar way to the comparisons of Shannon's diversity indices, the NMDS biplot compares the composition of the *Symbiodnium* communities present within each host species. However, this NMDS analysis additionally accounts for symbiont clade identity, rather than only the number clades detected, as well as relative evenness of those clades, as with Shannon's values. Furthermore, the biplot (Figure 2.2) illustrates similarities between coral host species based on the relative abundance of each of the symbiont clades A-D, rather than determining statistical significance between species having more or less diverse communities, as with Shannon's t-tests. The relatively low

stress of the NMDS ordination (0.1) indicates that the distribution of coral species based on the composition of their symbiont communities is well represented by the two axes. The biplot reveals that coral species from the Indo-Pacific region are more closely associated with symbionts from clades C and D than the five Caribbean coral species included in this study, which are more closely associated with clades A and B (Figure 2.2). In agreement with the hierarchical geographic diversity analyses performed using Shannon diversity index values, ANOSIM confirmed that the *Symbiodinium* communities hosted by Indo-Pacific versus Caribbean coral species host are significantly different from one another ($P=0.001$, $R^2=0.49$, Figure 2.2). ANOSIM further indicated that symbiont communities are not significantly different between symbiotic specialist and symbiotic generalist coral groups ($P=0.73$, $R^2=0.009$, Figure 2.2).

SYBR assay specificity

All of the 16 rt-PCR amplifications from 9 randomly selected coral samples selected for direct sequencing were specific to their target *Symbiodinium* clade, based on BLASTn searches of the NCBI nr database [*Genbank* accession numbers*;* clade A: AF427466 (N=4), EU074862 (N=1); clade B: DQ200710 (N=2); clade C: EF372040 (N=2), EF372052 (N=1), FJ851421 (N=1), AY903353, (N=1), EF372067 (N=1); clade D: AB248879 (N=3)].

Contamination tests

Out of 52 assays in which contaminant *Symbiodinium* could have been detected from azooxanthellate corals (13 *T. coccinea* samples x 4 symbiont clade assays per

sample), only one of the azooxanthellate *T. coccinea* samples (1.9%) amplified for one clade (C) of *Symbiodinium*. Out of 9 paired "positive" and "negative" controls analysed for potential contamination during the extraction protocol, 14 positive *Symbiodinium* clade detections were generated from the positive extraction controls (out of 36 possible detections: 9 samples x 4 clades), but only one negative extraction control sample amplified for a single clade (C) of *Symbiodinium* (1 detection out of 14 possible detections = 7.1%). Overall, we detected the presence of novel clades within this dataset 249 times. If extraction and biological contamination accounted for 2% and 7% of these detections, respectively, then we can estimate the number of false positives as 22 of 249 (9% of total), indicating that the vast majority (91%) of the novel coral-*Symbiodinium* associations we report do indeed represent *Symbiodinium in hospite.*

Between host species diversity comparisons

The diversity of coral-algal associations fall along a gradient of diversity (Figure 2.3a), along which coral species can be loosely grouped based on those associating with: 1) the highest *Symbiodinium* diversity (i.e., total symbiont community diversity detected is significantly more diverse than that found in >10.5% of the other host species examined in this study: N=6 of 39, 15.4%); (2) moderately high *Symbiodinium* diversity (i.e., total symbiont community diversity detected is significantly more diverse than 3-10.5% of the other host species examined in this study: N=11 of 39, 28.2%); and 3) moderate to low *Symbiodinium* diversity (i.e., total symbiont community diversity detected is not significantly more diverse than any of the other host species in this study: N=22 of 39, 56.4%, Figure 2.3a, Tables 2.1 and 2.2). These designations are relative; even the coral species with the lowest diversity indices contained at least two

Symbiodinium clades in at least one colony analyzed from a given species (generally clades C and D).

No relationship was found between Shannon's diversity indices and the number of colonies sampled per species (ANOVA, P= 0.31, d.f.=1), indicating that sampling effort had no effect on symbiont diversity. Furthermore, no relationship was detected between the number of geographic locations sampled (defined broadly by country, see Table 2.1) and the total symbiont community diversity (ANOVA, P=0.06, d.f.=1). However, Caribbean coral species were significantly more diverse than Pacific coral species (Shannon's t-test, $P=1.19x10^{-5}$), in agreement with previously published works (e.g., Baker and Rowan 1997, LaJeunesse et al. 2003).

Discussion

Diversity in coral-algal symbiosis

Real time PCR analysis reveals that the majority of the 39 reef coral species examined associate with a higher diversity of *Symbiodinium* clades than has been previously recorded using conventional molecular techniques (Figure 2.1). Overall, our results are still a conservative estimation of the true diversity of symbionts in coral species because: (1) we focused on detecting cryptic *Symbiodinium* diversity in symbiotic specialists, and included fewer symbiotic generalists; (2) the rt-PCR assays used detect only the most commonly observed symbionts of scleractinian corals (*Symbiodinium* in clades A-D), yet clades F and G have also been reported in a few scleractinian coral species (Rodriguez-Lanetty et al. 2004, LaJeunesse et al. 2010), and might therefore be detected from these coral species if appropriate assays were developed; (3) many coral

species are known to associate with multiple *Symbiodinium* types within a single clade (e.g., Pochon et al. 2004, Sampayo et al. 2008), and it is likely that the development of rt-PCR assays specific to *Symbiodinium* sub-clades would likely detect additional within-clade diversity; (4) the samples analyzed in this study originate from few of the full range of habitats and regions in which each host species may be found. Analysis of additional samples for each coral species may reveal additional symbiotic associations from spatial or temporal niches not sampled in this study; and (5) individual coral colonies can contain spatially heterogeneous distributions of *Symbiodinium* clades (e.g., Rowan et al. 1997, Kemp et al. 2007), but we sampled each colony only once. This study identifies high *Symbiodinium* diversity within 39 coral species using a relatively conservative approach, and it is therefore likely that additional diversity would be found if rt-PCR were to be expanded to other *Symbiodinium* clades and applied to other hosts, across their full biogeographic and environmental ranges (Baker 2003, Baker & Romanski 2007). Therefore, although corals clearly exhibit selectivity in their associations (e.g., Weis et al. 2001, LaJeunesse et al. 2002, Goulet 2006, 2007), strict host specificity to a particular symbiont type or clade does not appear to be the case for any coral species assayed to date, even corals thought to be highly selective.

Based on rt-PCR, the 39 scleractinian coral species examined here fall along a continuum in terms of the frequency with which they host multiple *Symbiodinium* clades, with all species tested being flexible to some degree. The total *Symbiodinium* clade diversity detected using rt-PCR was similar for most coral species (i.e., for a given coral species, Shannon's diversity index was statistically indistinguishable from the diversity indices for over half of the other 38 coral species examined, Figure 2.3b). Furthermore,

the total *Symbiodinium* diversity among corals previously thought to be symbiotic generalists compared to symbiotic specialists (as respective groups) was not statistically different (Shannon's t-test: P= 0.06; ANOSIM of NMDS ordination: P=0.73, R^2=0.009). Therefore, classifying corals species as either "specific" or "flexible" in their associations with *Symbiodinium* is an artificial dichotomy. Instead, a gradient of specificity exists over which scleractinian coral species vary in the frequency with which they: (1) are dominated by a particular *Symbiodinium* clade/type; and (2) contain background populations of additional *Symbiodinium* clades/types (Figure 2.3a, b, Table 2.1).

Furthermore, the overall finding that corals host a diversity of symbiont clades was often representative of the majority (average: 68%) of individual colonies analysed for a given species. This indicates that the ability of coral colonies to host multiple clades is common within populations, and not limited to a few unusual individuals within a coral species. Therefore, within a particular species, many colonies have the potential to benefit from associating with multiple symbiotic partners.

Are background rtPCR detections reflecting Symbiodinium in hospite?

Loram et al. (2007) indirectly assessed the nature of rt-PCR amplifications of *Symbiodinium* in the anemone *Condylactis gigantea* by comparing generated by this technique and RFLP surveys to visualizations of algal symbionts in the gastrodermis using Fluorescent In Situ Hybridization (FISH). Loram et al. (2007) found that FISH results more closely agreed with rt-PCR detections than RFLP detections, suggesting that rt-PCR depicts cnidarian symbiosis *in situ* more closely than conventional molecular techniques, and is not confounded by surface or gut contaminants. While our dataset consists of genomic DNA only and therefore cannot be analyzed with FISH, we did

attempt to account for environmental contaminants by analyzing samples of *T. coccinea,* an azooxanthellate coral, for the presence of *Symbiodinium* clades A-D. The assumption of this biological negative control was that any symbiont detections from these samples would be surface or gut contaminants, and not algae in symbiosis. It is possible, however, that *T. coccinea*, although thought to be azooxanthellate, may actually host some symbionts at abundances that are undetectable using conventional methods. In a recent study, for example, Wagner et al. (2011) identified symbionts within black corals, a group previously thought to be azooxanthellate.

Results from our azooxanthellate biological negative controls suggest that environmental contamination was minimal (~2%). Furthermore, we suggest that if gut and surface contamination were widespread in our dataset, this would have tended to homogenize our results. Most conspecific samples assayed, however, did not score positive symbiont detections for the same background clades, even when samples were collected from the same region, or even the same reef (Table 2.1). These data, combined with the findings of Loram et al. (2007), indicate that false positives due to contamination by free-living *Symbiodinium* are minimal for this study. However, further rt-PCR based research into the distribution and detection limits of surface and gut contaminant *Symbiodinium*, as well as the spatial and temporal distributions of symbiotic algal communities, are critical for understanding the extent to which background symbiont populations vary in their distribution, dynamics, and role(s) in corals throughout their ontogeny.

Laboratory contamination was also low for the methods and assays applied here, with only one of 14 potential "false positives" amplifying (~7%). In future studies, this

level of laboratory contamination could potentially be decreased even further by using a new bag for each airbrushed sample, or by using bone cutters rinsed in bleached, rather than simply rinsing with water or ethanol flaming (Singha et al. 1987, Bonne et al. 2008). While these potential bag cross-contaminations may not have affected earlier surveys using RFLP- or DGGE-based methods, our results show that studies using rt-PCR to detect low-level background symbionts should take additional steps to control for all sources of contamination, whether they be biological, environmental, and methodological.

High symbiotic diversity realized using sensitive molecular tools in other systems

Many metazoan species harbour symbiotic microbes, and recent studies from a variety of systems have also reported that microbial symbiont community diversity is greater than previously thought. For example, using rt-PCR, Olson et al. (2009) detected and quantified a greater diversity of nitrogen-fixing bacteria in close association with the corals *Montipora capitata* and *Montipora flabellata* than had not been previously observed using conventional techniques. These findings may partly explain how corals are able to thrive in low-nutrient environments. Furthermore, there was a strong positive correlation between the density of *Symbiodinium* cells in a coral sample and the number of gene copies detected from nitrogen-fixing bacteria, suggesting a relationship between coral-associated microbes and *Symbiodinium* communities. Flexibility in associations between coral and its symbiotic bacterial communities has also been proposed as a potential mechanism by which corals could rapidly acclimate to environmental changes (Reshef et al. 2006). In other systems, symbiotic bacterial diversity associated with

newly discovered, whale bone-eating polychaete worms, *Osedax* spp., was analyzed using rt-PCR (Goffredi et al. 2007). This approach showed differences in bacterial communities harboured by distinct species of *Osedax*, suggesting that *Osedax* species may occupy different ecological niches. Using another high-resolution technique (454-pyrosequencing), fungus-growing (attine) ants have also been shown to associate with more than one strain of antibiotic-producing *Pseudonocardia* bacteria, contradicting previous assumptions that a single ant nest hosts a single strain of *Pseudonocardia* (Sen et al. 2009).

High sensitivity molecular techniques are revealing that mixed microbial symbiont communities are common in many host taxa. Additionally, studies have also shown that these symbiotic relationships can exhibit flexibility between multiple partners. Redundant partners may confer the same *type*, although not necessarily the same *degree*, of benefit, but may prevent the host from experiencing an entirely aposymbiotic state during environmental stress (Six and Bentz 2007).

Implications of mixed symbiont assemblages within hosts

Intraspecific diversity in coral-algal symbiosis may provide a mechanism by which reef corals can respond to rapid environmental changes. Changes in the composition of symbiont communities within corals might allow corals to optimize aspects of their physiology in response to environmental stressors. It has been suggested, however, that (1) such symbiont shifts can only occur in a minority of coral species, namely those from which symbiont diversity has already been detected (Knowlton and Rohwer 2003, Goulet 2006, 2007, Hoegh-Guldberg et al. 2007), or that (2) shifts may be maladaptive for corals

(Putnam et al. 2012). Although reef corals clearly preferentially associate with certain dominant symbiont types (e.g., LaJeunesse et al. 2002, 2003, 2004a,b, 2005), our findings show that most, if not all, scleractinian coral species are capable of associating with multiple *Symbiodinium* clades, including members of both clades C and D. While our detection of diverse symbiont communities in corals does not directly address changes in stress tolerance or symbiont shifts over time (see Chapters 3-5), these data do show that coral species are not biologically restricted to associating exclusively with particular symbiont clades and/or types. This suggests that corals' cellular mechanisms for recognizing particular strains of *Symbiodinium* (e.g. Weis 2001,Wood-Charlson 2006, Dunn and Weis 2009, Dunn 2009) may be less definitive than they appear, perhaps because they are themselves environmentally or ontogenetically mediated. Future work should address the dynamics of background symbiont types over time and during episodes of coral bleaching, or the paling of corals as a result of the loss of symbiotic algae and/or algal pigments. Coral bleaching has been suggested to accelerate alterations in symbiont communities by disturbing an otherwise-stable *Symbiodinium* community (Buddemeier and Fautin 1993, Fautin and Buddemeier 2004).

Members of *Symbiodinium* in clade D (particularly ITS-2 types D1 and D1a) can be relatively thermo-tolerant, and coral colonies associating with members of this clade have exhibited increased bleaching resistance (Glynn et al. 2001, Baker 2004, Berkelmans & van Oppen 2006, Jones et al. 2008, LaJeunesse et al. 2009, but see Abrego et al. 2008). However, since we cannot assume that thermotolerance is characteristic of all members of clade D, future work should investigate variability in symbiont physiology within clade D (Douglas 2003, Tchernov et al. 2004), as well as the potential contribution of

Symbiodinium in other clades to coral survival during acute stress. In particular, symbiont type D1a is often referred to as putatively thermotolerant, but has not been directly examined (see Chapters 3-5).

Symbiodinium in clade C are recognized as common members of reef communities worldwide (LaJeunesse 2005), and it is not surprising that our analysis found members of this clade in every species examined. However, the fact that we also found *Symbiodinium* in clade D present in at least one sample of every host species examined, and across the same broad phyletic diversity of hosts as clade C (14 scleractinian coral families) suggests that members of clade D are also ubiquitous in reef communities and have a cosmopolitan (albeit frequently low abundance) distribution in hosts (Table 2.1). *Symbiodinium* in clades A and B were also common in our dataset (found in 32 and 24 of the 39 species examined, respectively), although within Indo-Pacific corals they were not as widespread among colonies of a given host species as clades C or D (Figure 2.2).

ANOSIM analysis revealed that the distribution of *Symbiodinium* clades among coral species was not significantly different based on previously designated symbiotic specialist or generalist hosts. However, differences were detected in the clade-level *Symbiodinium* communities found in Caribbean versus Indo-Pacific geographic provinces, which can also be observed in the NMDS biplot (Figure 2.2). Shannon's t-tests also indicated that Caribbean symbiont communities have greater clade-level diversity than those hosted by Indo-Pacific corals, agreeing with previous studies conducted using conventional molecular techniques (e.g. LaJeunesse et al. 2003). *Symbiodinium* diversity at the clade level has been correlated with patterns of bleaching severity on reefs over regional scales (Baker 2004), with regions characterized by more

diverse *Symbiodinium*, such as the Caribbean, tending to experience more frequent, but less severe bleaching events (Baker 2004). In contrast, regions with relatively low *Symbiodinium* diversity, such the Indo-Pacific, tend to experience less frequent, but more severe bleaching events (Baker and Rowan 1997, Baker 2004). The application of high-sensitivity techniques, such as real time PCR, to understanding microbial community structure in corals may help reveal how low abundance, background symbiont diversity further contributes to patterns of bleaching and recovery. Future surveys of global *Symbiodinium* diversity using high sensitivity molecular techniques and covering wide geographic and temporal scales will help us to understand how changing environmental conditions influence host-symbiont dynamics.

While symbiont identity has been correlated with holobiont physiological tolerance (e.g., Rowan et al. 1997, Glynn et al. 2001, Baker 2003, Rowan 2004, Berkelmans & van Oppen 2006, Abrego et al. 2008, Jones et al. 2008, Thornhill et al. 2008), the contribution of low abundance symbionts to the overall physiology of the holobiont is still unknown. It is also not yet clear at what numerical abundance background symbionts become physiologically relevant or how background symbionts may affect corals when they do become abundant, for example, after a disturbance such as bleaching or transplantation (i.e. proliferation of clade D, typically a background symbiont, within a coral post-bleaching). Furthermore, few studies have tracked shifts in symbiont communities over time to understand whether shifts are long-lived or ephemeral, or if they occur at all (Thornhill et al. 2006a, LaJeunesse et al. 2009, McGinley et al. 2012, see Chapters 3-5). Despite these unknowns, background *Symbiodinium* may provide functional symbiont redundancy to corals by persisting

during stressful conditions when dominant symbionts decline and by providing photosynthates to the host during bleaching and recovery, thereby preventing mortality (Mieog et al. 2007, Correa and Baker 2011, but see Putnam et al. 2012).

Coral bleaching has become increasingly common on reefs in recent decades due to climate-related stressors such as increases in sea surface temperature (Glynn et al. 2001, Hughes et al. 2003, Hoegh-Guldberg et al. 2007). During bleaching events, corals can suffer high mortality if they do not recover their algal symbionts within weeks to months (Glynn 1996). Without a mechanism to cope with rapid environmental change, coral bleaching will likely continue to result in widespread declines in coral cover (Hughes et al. 2003, Donner et al. 2005, Hoegh-Guldberg et al. 2007). Symbiotic flexibility may provide one mechanism by which corals can respond to changing environments, and data shown here suggests that this mechanism may be more widespread among diverse coral species than previously assumed.

Figures

Figure 2.1 Frequency distributions of *Symbiodinium* diversity in scleractinian corals identified (in previous studies) using conventional (e.g., DGGE, RFLP, SSCP) versus high sensitivity (rt-PCR) molecular techniques (in this study). Host species were designated as "symbiotic specialists" or "symbiotic generalists" based on previous symbiont detections using conventional techniques (Table 2.1). Conventional molecular techniques greatly overestimate the number of specific host species.

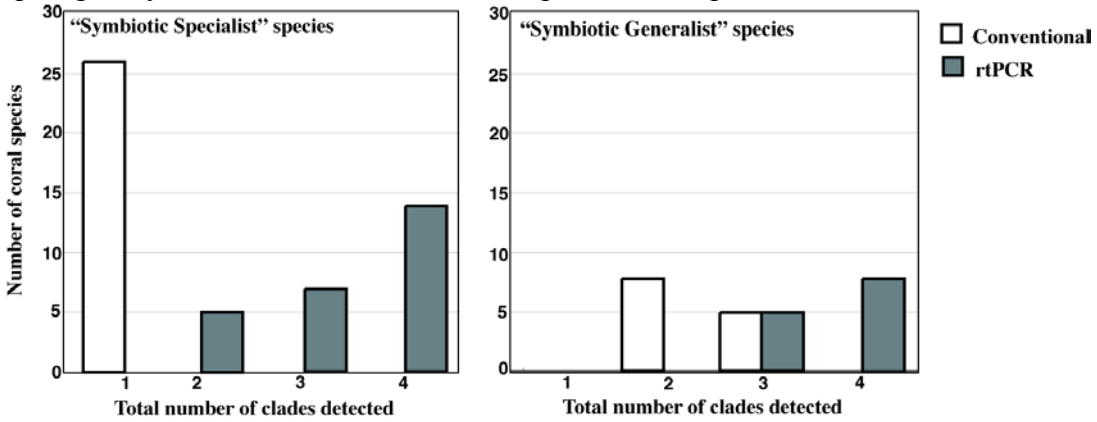


Figure 2.2 NMDS biplot showing configuration of the relative abundances of *Symbiodinium* clades within different coral host species. Boxed letters A, B, C, and D represent each clade's designated position within the biplot, around which the species scores of each coral were oriented. Most Indo-Pacific coral species cluster between clades C and D, while Caribbean coral species are more closely associated with *Symbiodinium* clades A and B. Open circles indicate Caribbean coral species, and full circles indicate Indo-Pacific coral species. Grey markers refer to symbiotic generalist species, while black markers refer to symbiotic specialist species. Solid ellipses in either grey or black indicate standard error of the symbiotic generalist (SG) and symbiotic specialist (SS) species groups, respectively, and long or short dashed-line ellipses indicate standard error (S.E.) of the Indo-Pacific (IP) and Caribbean (C) coral groups, respectively. Coral species are denoted by a four-letter species code where the first letter represents the genus and the remaining three letters represent the first three letters of the species name: *Acanthastrea echinata*=AECH, *Acropora cervicornis*=ACER, *Acropora humilis*=AHUM, *Acropora palmata*=APAL, *Acropora tenuis*=ATEN, *Coeloseris mayeri*=CMAY, *Colpophyllia natans*=CNAT, *Coscinaraea columna*=CCOL, *Cyphastrea micropthalma*=CMIC, *Dichocoenia stokesii*=DSTO, *Echinophyllia aspera*=EASP, *Favia stelligera*=FSTE, *Fungia fungites*=FFUN, *Fungia scutaria*=FSCU, *Galaxea astreata*=GAST, *Galaxea fascicularis*=GFAS, *Gardineroseris planulata*=GPLA, *Hydnophora exesa*=HEXE, *Leptoria phrygia*=LPHR, *Madracis mirabilis*=MMIR, *Montastraea curta*=MCUR, *Montastraea valenciennesi*=MVAL, *Montipora aequituberculata*=MAEQ, *Mycedium elephantotus*=MELE, *Pachyseris rugosa*=PRUG, *Pachyseris speciosa*=PSPE, *Pavona clavus*=PCLA, *Pavona gigantea*=PGIG, *Pavona varians*=PVAR, *Platygyra sinensis*=PSIN, *Plesiastrea versipora*=PVER, *Pocillopora damicornis*=PDAM, *Pocillopora eydouxi*=PEYD, *Pocillopora meandrina*=PMEA, *Porites panamensis*=PPAN, *Psammocora superficialis*=PSUP, *Stylocoeniella guentheri*=SGUE, *Stylophora pistillata*=SPIS, *Turbinaria reniformis*=TREN.

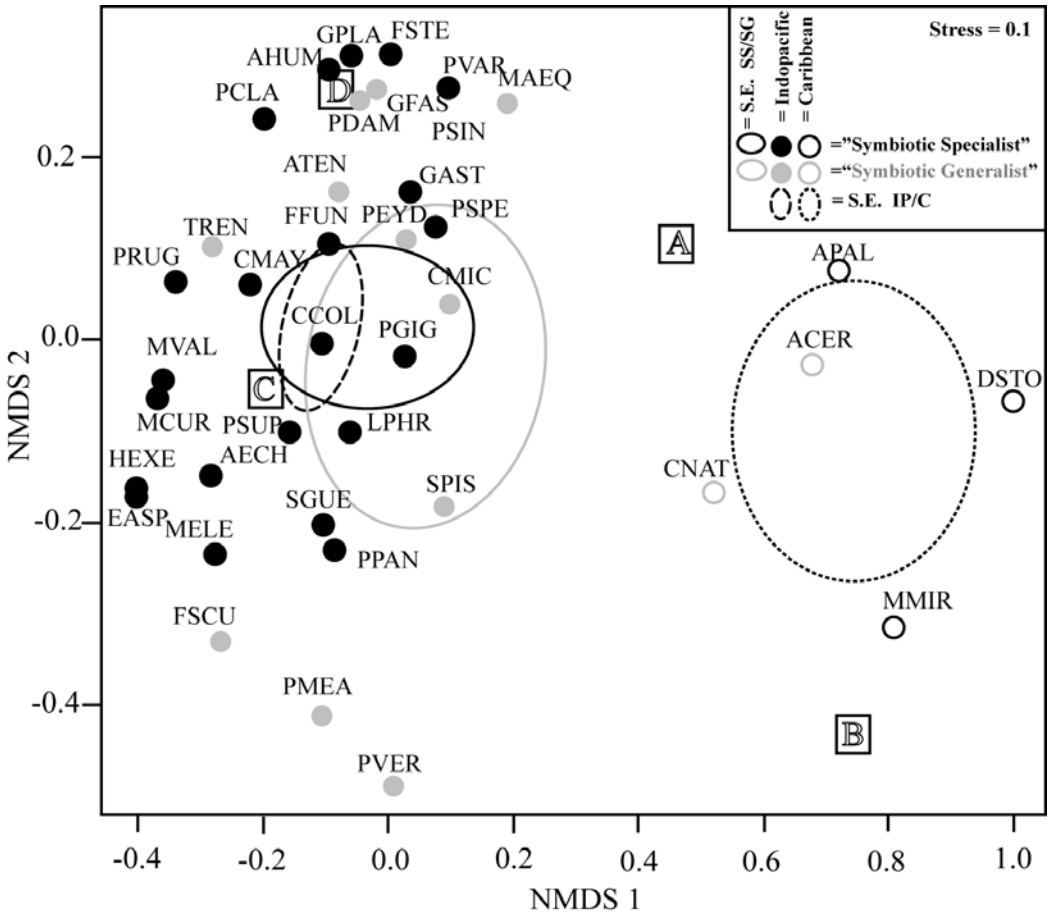
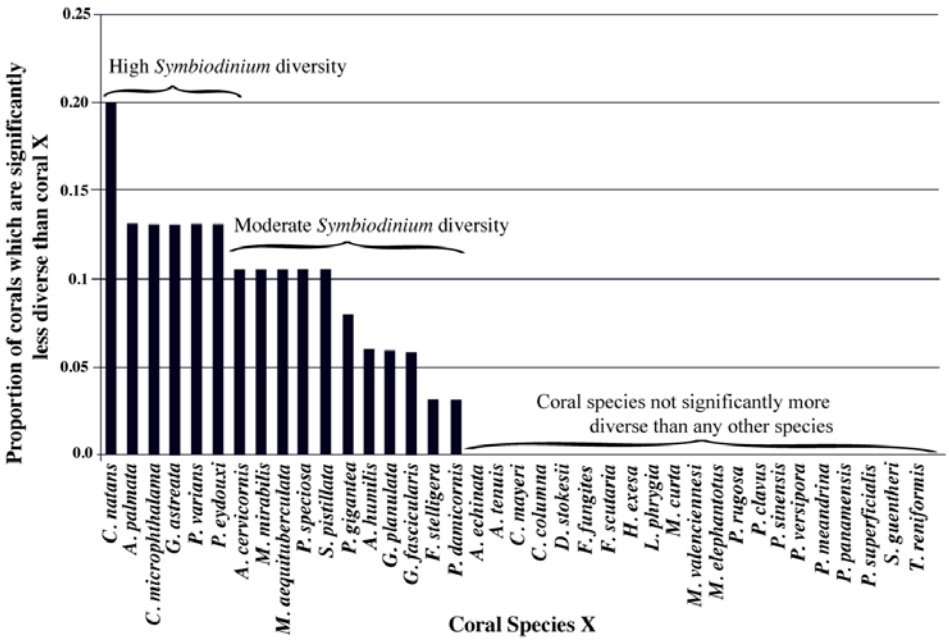

Stress = 0.1
= S.E. SS/SG
= Indopacific
= Caribbean
="Symbiotic Specialist"
="Symbiotic Generalist"
= S.E. IP/C
A
B
C
D
GPLA
FSTE
AHUM
PVAR
MAEQ
PCLA
GFAS
PDAM
PSIN
ATEN
GAST
FFUN
PEYD
PSPE
TREN
PRUG
CMAV
CMIC
CCOL
PGIG
MVAL
MCUR
PSUB
LPHR
HEXE
AECH
SPIS
SGUE
EASP
MELE
PPAN
FSCU
PMEA
PVER
APAL
ACER
DSTO
CNAT
MMIR
NMDS 1
NMDS 2
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

Figure 2.3 Comparisons of the relative *Symbiodinium* clade diversity hosted by 39 scleractinian coral species. Pairwise comparisons of the Shannon diversity indices for the total *Symbiodinium* community detected from each coral species were performed using the Shannon's t-test. **A. Proportion of 38 coral species (y-axis) that associate with a significantly higher *Symbiodinium* clade diversity than coral 'X' (x-axis).** Multiple symbiont clades were identified from all coral species, which vary along a gradient in the overall symbiont diversity they associate with. This gradient can be loosely characterized as hosting a: (1) high *Symbiodinium* diversity (N=6 of 39 coral species, 15.4%); (2) moderately high *Symbiodinium* diversity (N=11 of 39, 28.2%); or (3) moderate to low *Symbiodinium* diversity (N=22 of 39, 56.4%). **B. Proportion of 38 coral species (y-axis) that associate with a diversity of *Symbiodinium* clades similar to (i.e., not significantly different than) coral species 'X' (x-axis).** All coral species examined associate with *Symbiodinium* clade-level diversity that is statistically indistinguishable from at least half of the other 38 coral species analyzed.

2.3a



2.3b

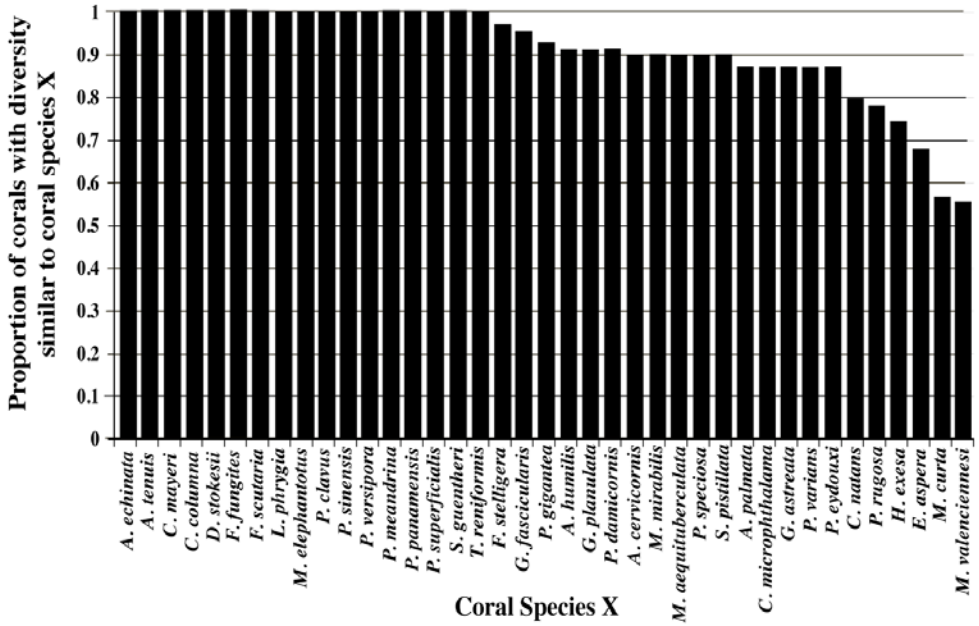

Proportion of corals with diversity similar to coral species X
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A. echinata
A. tenuis
C. mayeri
C. columna
D. stokesii
F. fungites
F. scutaria
L. phrygia
M. elephantotus
P. clavus
P. sinensis
P. versipora
P. meandrina
P. panamensis
P. superficialis
S. guentheri
T. reniformis
F. stelligera
G. fascicularis
P. gigantea
A. humilis
G. planulata
P. damicornis
A. cervicornis
M. mirabilis
M. aequituberculata
P. speciosa
S. pistillata
A. palmata
C. microphthalama
G. astreata
P. varians
P. eydouxi
C. natans
P. rugosa
H. exesa
E. aspera
M. curta
M. valenciennesi
Coral Species X

Table 2.1 Summary of the *Symbiodinium* diversity detected in this study using real time PCR and from previous studies using conventional molecular techniques.

Results of the real time PCR (rt-PCR) assay amplifying *Symbiodinium* clades A-D in 39 species of scleractinian corals, grouped by **Family**. **BP** indicates the biogeographic province from which the coral species are found: Indo-Pacific (IP) or Caribbean (C). **Previous studies using conventional molecular techniques**: The clades previously detected and the number of samples of each species analyzed **(N)** are shown. Based on these reports, each species is categorized as either "symbiotic specialist" (**S**: previously reported to host a single clade) or "symbiotic generalist" (**G**: previously known to host multiple clades). **This study, using high-sensitivity rt-PCR techniques:** Sample sizes (**N, number of coral colonies**), following by **Location,** which indicates regions from which the samples used in this study originated. **Cl** represents the number of clades identified in this study, followed by **Clade**, the number of each clade identified in each coral species. Species followed by asterisks have been found to host additional clades in previous rt-PCR studies (Correa et al. 2009b, Loram et al. 2007, Mieog et al. 2007). **Citations since 2006** reflect papers in which symbiont detections for each coral species were reported between September 2006 and November 2009. Prior to September 2006, citations can be found in Goulet 2006.

	Previous studies (conventional methods)	**This study (rt-PCR)**

Coral **Family**/*species*	**BP**	Clades previously detected **(N)**	**S/G**	**N**	**Location (N)**	**Cl**	Clade (# samples in which clade was detected)	Citations since 2006 (See Goulet 2006 for prior citations)
Acroporidae								
*Acropora cervicornis**	C	A, B, C *(360)*	G	8	Belize (4), Panama (4)	4	A(8) B(4) C(4) D(3)	Warner and Berry-Lowe 2006, Thornhill et al. 2006a, Smith et al. 2009
Acropora humilis	IP	C *(28)*	S	7	Madagascar (3), Gulf of Aqaba (4)	3	A(3) C(7) D(7)	Smith et al. 2009
*Acropora palmata**	C	A *(217)*	S	10	Bahamas (2), Belize (3) Panama (5)	4	A(10) B(4) C(4) D(4)	Thornhill et al. 2006a
*Acropora tenuis**	IP	B, C *(99)*	G	10	Gulf of Aqaba (3) Japan (5), Line Islands (3)	4	A(2) B(1) C(10) D(8)	Smith et al. 2009, Crabbe and Carlin 2009

Montipora aequituberculata	IP	C, D *(60)*	G	8	American Samoa (4), Madagascar (4)	4	A(4) B(2) C(6) D(7)	Dong et al. 2009, Smith et al. 2009, Ferrier-Pages et al. 2007
Agariciidae								
Coeloseris mayeri	IP	C (*10*)	S	5	Kenya (1), Lizard Island (4)	3	A(1) C(5) D(3)	(See Goulet 2006)
Gardineroseris planulata	IP	C (*5*)	S	8	Panama (8)	3	A(4) C(8) D(8)	(See Goulet 2006)
Pachyseris rugosa	IP	C (*7*)	S	10	Japan (4), Maldives (1), Lizard Island (4), Madagascar (1)	2	C(10) D(6)	Dong et al. 2008, Dong et al. 2009
Pachyseris speciosa	IP	C (*2*)	S		Maldives (1), Panama (3), Gulf of Aqaba (3), Lizard Island (1)	4	A(4) B(2) C(8) D(6)	Dong et al. 2008
Pavona clavus	IP	C (*17*)	S	8	Galapagos (4), Panama (4)	4	A (1) B(1) C(8) D(8)	LaJeunesse et al. 2008
Pavona gigantea	IP	C (*34*)	S	8	Galapagos (2), Panama (6)	4	A(3) B(2) C(8) D(4)	LaJeunesse et al. 2008

Pavona varians	IP	C (*22*)	S	10	Kenya (3), Madagascar (3), Panama (1), Gulf of Aqaba (3)	4	A(5) B(1) C(8) D(8)	Dong et al. 2009, Stat et al. 2009a
Astrocoeniidae								
*Madracis mirabilis**	C	B (*15*)	S	7	Bermuda (7)	4	A(6) B(7) C(4) D(1)	Frade et al. 2008, Loram et al. 2007
Stylocoeniella guentheri	IP	C (*1*)	S	11	Japan (11)	4	A(1) B(3) C(11) D(4)	(See Goulet 2006)
Dendrophylliidae								
*Turbinaria reniformis**	IP	C, D (224)	G	9	Japan (6), Line Islands (2), Australia (1)	3	B(1) C(9) D(6)	Ulstrup et al. 2006
Faviidae								
*Colpophyllia natans**	C	B, C (*31*)	G	8	Dominican Republic (1), Key Largo (4), Panama (3)	4	A(7) B(8) C(7) D(3)	Correa et al. 2009a
Cyphastrea microphthlama	IP	C, D *(6*)	G	9	Arabian Gulf (4), Gulf of Aqaba (1), Israel (1), Lizard Island (2)	4	A(5) B(2) C(9) D(5)	Mostafavi et al. 2007

Favia stelligera	IP	C (*10*)	S	4	Gulf of Aqaba (2), Kenya (1), Madagascar (1), Tanzania (1)	3	A(2) C(4) D(4)	(See Goulet 2006)
Leptoria phrygia	IP	C (*3*)	S	6	Japan (2), Kenya (1), Madagascar (1), Gulf of Aqaba (2)	4	A(2) B(1) C(6) D(2)	(See Goulet 2006)
Montastraea curta	IP	C (7)	S	12	Japan (9), Tanzania (3)	2	C(12) D(5)	(See Goulet 2006)
Montastraea valenciennesi	IP	C (*4*)	S	9	Japan (9)	2	C(9) D(4)	(See Goulet 2006)
Platygyra sinensis	IP	C (*3*)	S	6	Arabian Gulf (2), Kenya (1), Gulf of Aqaba (1), Lizard Island (2)	4	A(2) B(1) C(5) D(5)	Dong et al. 2008
Plesiastrea versipora	IP	B, C (*44*)	G	10	Arabian Gulf (1), Japan (3), Lizard Island (3), Australia (3)	4	A(1) B (4) C (9) D(1)	(See Goulet 2006)
Fungiidae								
Fungia fungites	IP	C (*3*)	S	5	American Samoa (2), Madagascar (3)	3	A(2) C(5) D(3)	Huang et al. 2006

Fungia scutaria	IP	A, C (*33*)	G	4	Hawaii (2), Line Island (1), Japan (1)	3	A(1) C(3) D(1)	Sebastian et al. 2009, Stat et al. 2009a
Meandrinidae								
Dichocoenia stokesii	C	B (*3*)	S	9	Bermuda (2), Key Largo (7)	4	A(5) B(9) C(1) D(4)	Correa et al. 2009a
Merulinidae								
Hydnophora exesa	IP	C (6)	S	11	Japan (10), Lizard Island (1)	2	C(11) D(3)	Dong et al. 2008
Mussidae								
Acanthastrea echinata	IP	C (2)	S	7	Israel (1), Japan (6)	3	A(1) C(7) D(2)	(See Goulet 2006)
Oculinidae								
Galaxea astreata	IP	C (*5*)	S	8	Kenya (4), Maldives (1), Gulf of Aqaba (1), Lizard Island (2)	4	A(3) B(2) C(8) D(7)	(See Goulet 2006)
Galaxea fascicularis	IP	C, D (*42*)	G	7	Israel, Japan (3), Kenya (4), Australia (4)	3	A(3) C(7) D(6)	Dong et al. 2008, 2009, Sebastian et al. 2009

Pectiniidae								
Echinophyllia aspera	IP	C (*2*)	S	7	Japan (7)	2	C(7) D(2)	(See Goulet 2006)
Mycedium elephantotus	IP	C (*3*)	S	10	Japan, Gulf of Aqaba	4	A(1) B(1) C(10) D(2)	(See Goulet 2006)
Pocilloporidae								
Pocillopora damicornis	IP	A, C, D (*784*)	G	10	American Samoa (1), Galapagos (3), Hawaii (6)	3	A(5) C(10) D(9)	Dong et al. 2008, 2009, Hill et al. 2009, LaJeunesse et al. 2007, 2008, Magalon et al. 2007, Sampayo et al. 2007, Smith et al. 2009, Stat et al. 2008, 2009a, b, Ulstrup et al. 2006, 2008
Pocillopora eydouxi	IP	A, C, D (*39*)	G	8	Hawaii (1), Japan (1), Line Islands (2), Maldives (1), Lizard Island (3)	4	A(3) B(2) C(8) D(6)	Magalon et al. 2007, Smith et al. 2008, Stat et al. 2009a
Pocillopora meandrina	IP	A, C, D (*79*)	G	10	Hawaii (6), Japan (4)	3	B(4) C(10) D(2)	Dong et al. 2009, LaJeunesse et al. 2007, 2008, Magalon et al. 2007, Stat et al.

								2009a
*Stylophora pistillata**	IP	A, C, D (*825*)	G	9	Gulf of Aqaba (1), Tanzania (2), Australia (5), Lizard Island (1)	4	A(1) B(5) C(9) D(5)	Barneah et al 2007, Dong et al. 2009, Ferrier-Pages et al 2007, Fitt et al. 2009, Hill et al. 2009, Lapert-Karako et al. 2008, Macdonald et al 2008, Sampayo et al. 2007, 2008, Sebastian et al. 2009, Smith et al. 2009, Stat et al. 2008, 2009b, Winters et al. 2009
Poritidae								
Porites panamensis	IP	C (55)	S	8	Panama (7)	4	A(1) B(2) C(7) D(2)	LaJeunesse et al. 2008
Siderastreidae								
Coscinaraea columna	IP	C (*7*)	S	5	Japan (2), Maldives (1), Lizard Island (2)	3	A(2) C(5) D(2)	(See Goulet 2006)
Psammocora superficialis	IP	C (*10*)	S	12	Arabian Gulf (4), Japan (5), Panama (2)	4	A(3) B(1) C(12) D(4)	LaJeunesse et al. 2008

Table 2.2 Summary of Shannon's diversity indices and pairwise two-tailed Shannon's t-tests of the total *Symbiodinium* community diversity detected within 39 scleractinian coral species. After Bonferroni correction, the p-value for the Shannon's t-tests was set at 3.37×10^{-5}. Blank squares indicate non-significant p-values derived from Shannon t-tests ($P \geq 3.37 \times 10^{-5}$), one asterisk (*****) indicates significant p-values ranging between $3.37 \times 10^{-5}$and 3.37×10^{-6}, two asterisks (******) indicate significant p-values ranging between $3.37 \times 10^{-6}$and 3.37×10^{-7}, and three asterisks (*******) indicate p-values smaller than 3.37×10^{-7}. **ND** indicates that there is no data because the t-test could not be performed. Raw Shannon Diversity Index= H'_1, Variance of Shannon Diversity Index= Var H'_1. **Coral species are indicated by numerals as follows:** *Acanthastrea echinata= (1)*, *Acropora cervicornis= (2)***,** *Acropora humilis*= (3), *Acropora palmata*= (4), *Acropora tenuis*=(5), *Coeleoseris mayeri*= (6), *Colpophyllia natans*= (7), *Coscinaraea columna*= (8), *Cyphastrea microphthalama*= (9), *Dicochoenia stokesii*= (10), *Echinophyllia aspera*= (11), *Favia stelligera*= (12), *Fungia fungites*= (13), *Fungia scutaria*= (14), *Galaxea astreata*= (15), *Galaxea fascicularis*= (16), *Gardinoceris planulata*= (17), *Hydnophora exesa*= (18), *Leptoria phrygia*= (19)**,** *Madracis mirabilis*= (20), *Montastraea curta*= (21), *Montastraea valenciennesi*= (22), *Montipora aequituberculata*= (23), *Mycedium elephantotus*= (24)*, Pachyseris rugosa*= (25), *Pachyseris speciosa*=(26)*, Pavona clavus*= (27), *Pavona gigantea*= (28), *Pavona varians*=(29), *Platygyra sinensis*= (30), *Plesiastrea versipora*=(31), *Pocillopora damicornis*= (32)**,** *Pocillopora eydouxi*= (33), *Pocillopora meandrina*= (34), *Porites panamensis*= (35), *Psammocora superficialis*= (36), *Stylocoeniella guentheri*= (37), *Stylophora pistillata*= (38), *Turbinaria reniformis*= (39).

SPECIES	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
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Table 2.3 Summary of the *Symbiodinium* clade-level diversity within individual based on rt-PCR. The average percentage of individual colonies harbouring one, two or more, two, three, or four symbiont clades within (1) symbiotic specialist species, (2) symbiotic generalist species and (3) all 39 coral species examined. The standard deviation (SD) for the average percentage in each category is also provided.

Average number of colonies per species ± SD which:	**Symbiotic Specialists**	**Symbiotic Generalists**	**All coral species**
Host 1 clade	36% ± 24.7	25% ± 24.9	32% ± 25.3
Host 2+ clades	64% ± 24.7	75% ± 24.9	68% ± 25.3
Host 2 clades	40% ± 17.1	40% ± 18.9	40% ± 17.7
Host 3 clades	20% ± 15.5	32% ± 20.3	24% ± 18.2
Host 4 clades	5% ± 6.2	4% ± 7.4	4% ± 6.7

Chapter 3:

Stress, temperature, and rare symbionts influence coral recovery from bleaching

Summary

Corals can lose their algal symbionts (*Symbiodinium* spp.), or "bleach", due to exposure to a variety of environmental stressors. To date, most episodes of mass bleaching on coral reefs have been the result of high temperature stress. In order to better understand the effect of heat stress and bleaching on algal symbiont community dynamics in corals, we exposed specimens of the Caribbean coral *Montastraea cavernosa* to either heat stress (32^{o}C) or to a herbicide (DCMU) for 10 days, and then allowed the corals to recover for 6 months at different temperatures (24 or 29^{o}C). We used quantitative PCR and chlorophyll fluorometry to assess the community diversity, abundance, and photochemical function of symbionts during bleaching and recovery. All corals initially hosted only clade C symbionts (*Symbiodinium* C3), but changed to hosting clade D-dominated (*Symbiodinium* D1a) communities after bleaching. Both bleaching stressor (heat or DCMU) and recovery temperature (24^{o}C vs. 29^{o}C) affected the relative abundance of different *Symbiodinium* in recovered corals. Corals entirely naïve to heat during both bleaching and recovery (bleached with DCMU, recovered at 24^{o}C) also recovered with D-dominated communities, but with significantly less clade D and more clade C compared to corals bleached with heat or recovered at 29^{o}C. These results show that: 1) bleaching promotes rapid change in symbiont communities, 2) symbionts that are undetectable (even using qPCR) prior to bleaching can readily become dominant after disturbance, and 3) both bleaching stressor and temperature regime can influence the

recovery trajectories of algal symbiont communities. These findings provide insight into the response of coral-algal symbioses to stress, and have implications for the resilience of reef corals, and the ecosystems they build, to climate change.

Background

The symbiosis between corals and their dinoflagellate algal symbionts (*Symbiodinium* spp.) is an essential prerequisite for the ecological dominance of corals on modern-day reefs, yet the symbiosis also increases their susceptibility to environmental stress. Breakdown in symbiosis, known as coral bleaching, in which algal symbionts and associated pigments are lost from the host coral, occurs in response to exposure to temperature, salinity, pollution, light or other environmental extremes (Baker et al. 2008). The tolerance of corals to these bleaching stressors is partly dependent on the identity of the symbiotic algae they host, and changes in *Symbiodinium* community composition is one mechanism by which corals may acclimatize to environmental change (Rowan et al. 1997, Glynn et al. 2001, Baker et al. 2003, Rowan et al. 2004, Goulet et al. 2005, Berkelmans and van Oppen et al. 2006, Warner et al. 2006, Abrego et al. 2008, Jones et al. 2008, Sampayo et al. 2008, Thornhill et al. 2008). Widespread, rapid changes in symbiont identity typically occur after episodes of coral bleaching (Baker 2001, 2004, Berkelmans and van Oppen 2006, Jones et al. 2008, LaJeunesse et al. 2009). Stress-tolerant symbionts may populate recently-bleached corals, uptaken either from the external environment (symbiont switching) or from background, residual populations present before stress (symbiont shuffling, Baker 2003, Stat et al. 2006).

Often, symbiont community change in response to bleaching involves the proliferation of symbionts in clade D (Glynn et al. 2001, Berkelmans and van Oppen 2006, Jones et al. 2008, LaJeunesse et al. 2010). Certain types of symbionts within clade D, such as D1a, have been linked to thermotolerance (LaJeunesse et al. 2009, see Stat and Gates 2011 for review), and corals that experienced community shifts in favor of this clade have been shown to increase their thermal tolerance by 1-1.5^{o}C compared to corals that have not undergone shifts, and therefore still contained thermally sensitive symbionts in clade C (e.g., Berkelmans and van Oppen 2006, Jones et al. 2008). Symbionts in clade D are more common in hosts on high-temperature reefs, in sub-optimal environments, and after bleaching events, likely due to the preferential survival of corals dominated by clade D and/or the proliferation of clade D in recovering corals (Glynn et al. 2001, Baker et al. 2004, Thornhill et al. 2006a, Jones et al. 2008, LaJeunesse et al. 2009). Jones and colleagues (2008) found that both differential mortality and symbiont community shifts resulted in a higher clade D prevalence in *Acropora millepora* colonies after a bleaching event. Clade D's prevalence post-bleaching may be transient, and corals may eventually revert back to their pre-bleaching symbionts. However, this process may take months to years (Toller at al. 2001, Thornhill et al. 2006a, Jones et al. 2008, LaJeunesse et al. 2009). Symbiont community changes in response to stress may have adaptive value in increasing survivorship of corals following acute disturbance (i.e., bleaching, Buddemeier and Fautin 1993, Baker 2001, Fautin and Buddemeier 2004, Buddemeier et al. 2004). However, symbiont community change also may occur without acute disturbance (i.e., visual bleaching). Some studies have found changes in the dominant symbiont type without a bleaching event (Chen et al. 2005). In contrast, several studies report stable

symbiont communities over long time periods (LaJeunesse et al. 2005, Thornhill et al. 2006b, McGinley et al. 2012). To address the impact of disturbance on symbiont communities, we compared the rate and degree of symbiont community change in corals maintained at 29^{o}C (from an initial acclimation temperature of 24^{o}C), either with intact symbiont communities or immediately post-bleaching.

Clade D symbionts are often associated with heat stress specifically. Here, we compared the abundances of clade D in corals after heat- and herbicide-induced bleaching. In this way, we can distinguish which responses are specific to thermal stress, and which are related to bleaching generally. The herbicide, DCMU or Diuron™ (*N*'-(3,4-dichlorophenyl-*N,N*-dimethylurea), inhibits photosynthetic function by blocking electron transport from the reaction center of photosystem II (Bowyer et al. 1991). This blocks the flow of light energy, and leads to the formation of highly damaging reactive oxygen species (ROS) (Jones et al. 2004), triggering bleaching (Weis 2008). Because bleaching by both DCMU and heat are light-dependent (Jones et al. 2004), heat and DCMU may result in similar intracellular physiological responses. The viability of the resident and/or expelled symbionts after treatment is not known, and may differ between thermal and chemical exposure.

Recent advances in genetic techniques, mainly related to the quantification of symbionts within corals using quantitative or real-time PCR (Mieog et al. 2007, 2009, Cunning and Baker 2012), have increased our ability to study fine-scale changes in the density of different *Symbiodinium* taxa within the host. The density of symbiont cells can now be quantified from small, rapidly healing tissue biopsies that do not require sacrificing the entire core, unlike traditional cell counts which require sacrificing large

amounts of tissue. Recent surveys using qPCR have revealed that background symbiont clades are common in scleractinian corals (Ulstrup and van Oppen 2003, Mieog et al. 2007, Correa et al. 2009b, LaJeunesse et al. 2010, Chapter 2), with 'background' (i.e., low abundance) levels of clade D symbionts now having been detected in all species for which they have been surveyed broadly using qPCR (Ulstrup and van Oppen 2003, Mieog et al. 2007, Correa et al. 2009b, Chapter 2). The potential function of these background symbiont clades has not yet been fully explored. It has been hypothesized, however, that even at low abundance, cryptic symbionts may increase functional redundancy during stress (Mieog et al. 2007, Correa et al. 2009b, Chapter 2, but see McGinley et al. 2012). By analyzing Single Stranded Conformation Polymorphisms (SSCP) in the ITS1 region of ribosomal DNA, Jones and colleagues (2008) detected background levels of clade D in *A. millepora*, and found that these levels increased post-bleaching. LaJeunesse and colleagues (2010) found, using qPCR, that 7 of 8 Caribbean coral species harbored background clade D symbionts, which also increased in abundance after bleaching. The only coral that did not harbor background clade D pre-bleaching, *Montrastrea cavernosa,* was also found to host clade D after recovering from bleaching.

Here, we monitored the *Symbiodinium* community structure and function within the Caribbean coral *Montastraea cavernosa* during bleaching in response to two stressors-- heat (32^{o}C) and the herbicide Diuron ® (DCMU at 24^{o}C)-- as well as during recovery post-bleaching at two different temperatures (24^{o}C and 29^{o}C).

Methods

Coral Collection and Preparation

Nine colonies of *Montastraea cavernosa* were collected at 20 m depth from Breakers reef (N26 42.18', W80 01.01' W) in February 2011 (Florida Fish and Wildlife Conservation Commission Special Activity License #SAL-11-1182-SRP). Per the terms of the collection permit, all colonies were 22.9 cm (9") in diameter or less.

After three weeks in shaded, outdoor holding tanks at the University of Miami's Experimental Coral Hatchery, replicate 2.54 cm (1") diameter cores were taken using a drill press (153 cores total, from 9 colonies, N=12 to 20 per colony). The skeleton was trimmed to just below the polyp depth (~1 cm thickness) and then affixed to a ceramic "reef plug" (Boston Aqua Farms) using "coraffix", a cyanoacrylate adhesive gel (Two Little Fishes, Florida, USA). These corals were placed in experimental aquaria (described below), and were allowed to recover for two months. Corals were fed throughout the experiment twice weekly with Reef Chili (Bulk Reef Supply). Corals showed feeding behavior, lateral extension of tissue over the exposed skeleton, and extratentacular budding of new polyps during this period.

Cores were randomly allocated among treatment groups (6 groups, N=24-25 per group), such that at least two cores from each coral colony (genotype) were present in each treatment group.

Experimental Setup

Recovery and Acclimation

Corals were housed in an indoor facility in flow-through 100-gallon epoxy tanks. Tanks were supplied with sand- and UV-filtered seawater, which was pumped from Biscayne Bay, and was held at 24^{o}C with a TR20 SeaChill chiller in each of four identical tanks. Seawater flowed into the tank at a rate of 10 mL s^{-1} (water turnover rate ~10.5 h). Two pumps also circulated water within each tank.

Light (190-280 μmol quanta m^{-2} s^{-1}) was provided using 400 W metal halide ballast and pendant lights (IceCap Inc., USA) on a 12-hour light-dark cycle. Light and temperature levels were recorded every 15 minutes using HOBO temperature and light pendant data loggers (Onset Corp UA-002-64). Corals were fed with Reef Chili coral food (added directly to tanks) 2-3 times per week.

Bleaching conditions

Corals (N=24-25) were exposed to either: (1) heat stress, 32 ^{o}C for 10 days (2) herbicide (450 μg L^{-1} DCMU for 10 days at 24^{o}C), (3) 'cool control' conditions (24^{o}C for 10 days), or (4) 'warm control' conditions (29^{o}C for 10 days). See Figure 3.1. Control temperatures (24^{o}C and 29^{o}C) were maintained using the chillers, and the heat stress temperature (32^{o}C) achieved by adding 300 W aquarium heaters (Jaeger and ViaAqua).

For the herbicide bleaching treatment, DCMU was added to a 20 L closed system tank with a circulating pump and an airstone. Water temperature in these tanks was maintained at 24^{o}C by immersing the tanks within larger epoxy 100-gallon tanks, whose temperature was controlled using a TR20 SeaChill heater/chiller. Light and temperature

levels were recorded every 15 minutes using HOBO temperature and light pendant data loggers (Onset Corp UA-002-64). Water was changed every 48 hours, with fresh aliquots of DCMU added to each water change.

Recovery conditions

Immediately after the 10-day bleaching exposure (heat or herbicide), corals were placed in one of two recovery treatments (24^{o}C and 29^{o}C), with two replicate tanks for each treatment. Unbleached corals remained at the same temperature in their respective tanks during the recovery phase, while heat- and DCMU-bleached cores were allocated to one of the two replicate recovery tanks at each temperature. A system of heaters and chillers were used to maintain tank temperature within 1^{o}C of the target temperature (Sea Chill, Jaeger and ViaAqua 300W). At least one core from each coral colony (genotype) from each treatment was represented in each replicate tank (N=24 per treatment, N=12 per treatment per recovery tank). Corals remained in these recovery conditions for 89 days. After 89 days, half of the cores from each treatment group were used in a subsequent bleaching experiment (see Chapter 4), but the other half remained at recovery conditions for a further three months and were monitored regularly using chlorophyll fluorometry and qPCR (see Figure 3.1 for schematic of experimental design).

Chlorophyll Fluorometry

Measurements of *Symbiodinium* photosynthetic function in each sample were taken before DCMU or heat exposure, three days after initiation of stress exposure, and approximately every week to 10 days thereafter, for a period of 13 weeks. Measurements

of maximum quantum yield of photosystem II (Fv/Fm) were made using an Imaging Pulse Amplitude Modulated (I-PAM) fluorometer (Walz, Effeltrich, Germany) in the early morning (08:00h), before lights were turned on (there was no ambient sunlight in these experiments). Photographs also were taken under the same conditions using a light box, after I-PAM measurements were completed.

Tissue Sampling

Small coral tissue (and embedded *Symbiodinium)* samples were taken using sterile razor blades immediately before and after the 10-day stress exposure period, and then after 3 months and 6 months of recovery. Tissue samples were immediately transferred from the razor blade to a 1% solution of sodium dodecyl sulfate (SDS) and cooked for 1-1.5 hours at 65^{o}C to produce a cell lysate for archival purposes. DNA was then extracted from this archive using established organic extraction protocols (Baker et al. 1997).

Symbiont typing

The internal transcribed spacer-2 (ITS-2) region of ribosomal DNA (rDNA) was amplified and *Symbiodinium* types isolated using denaturing gradient gel electrophoresis (DGGE), per established methods (LaJeunesse et al. 2001, revised in Silverstein et al. 2011). Excised bands were amplified with BigDye 75.6.8.2, sequenced using a 47000XL Genetic Analyzer at the University of Miami's Core facility, edited with Geneious Pro (v. 4.6.4) and compared to known sequences in Genbank.

Quantitative PCR assay

Quantitative ("real-time") PCR (qPCR) assays were used to quantify the number of copies of specific actin loci in clades C and D *Symbiodinium*, as well as in the coral host in each sample, in order to calculate a ratio of the number of symbiont cells to the number of host cells. *Montastraea cavernosa* primers and probes were designed for this study from cloned actin gene sequences (n=11) from 5 *M. cavernosa* colonies using Allele ID 7.7 (Premier Biosoft) and Primer Express (Applied Biosystems). *Montastraea cavernosa* primers and probes were designed from cloned actin gene sequences (n=11) from 5 *M. cavernosa* colonies using Allele ID 7.7 (Premier Biosoft) and Primer Express (Applied Biosystems). Unlabelled primers were purchased from IDT and fluorescently-labeled MGB Taqman probes from Applied Biosystems (F: 5'-CGT TGA CAT CCG TAA GGA TCT CTAT-3', R: 5'-CAA TGA TCT TAA TCT TCA TGG TTG GT- 3', Probe: NED- CCA ACA CTG TCC TCT CT-MGB). Primer and probe efficiency were calculated from standard curves run in triplicate, as described in Cunning and Baker (2012). Standard curve template DNA was produced from purified PCR products of clone isolates (diluted to a range of 10^1 - 10^5 copies μL^{-1} for the symbiont assays, and 10^2 - 10^6 copies μL^{-1} for the coral assays). Clades C and D assays were designed by Cunning and Baker (2012).

All qPCR reactions were run on an Applied Biosystems (Carlsbad, California, USA) Step One Plus, 96-well machine. The thermal cycling program consisted of an incubation step at 50^{o}C for 2 minutes, followed by 95^{o}C for 10 minutes, and then a series of 40 cycles of: denaturing at 95^{o}C for 15 seconds, and annealing/extension at 60^{o}C for 60 seconds. We scored positive amplifications as those that exceeded a fluorescence

threshold value of 0.01 in <40 cycles in both replicate wells. Samples in which replicate wells did not agree within 2-3 Ct's were re-run. If replicates were still not similar, the sample was excluded from the analysis. Between 7 and 16% of samples were excluded at each time point because they either did not amplify (even after re-extraction), duplicates did not agree (even after re-extraction and/or repeated assays), or were found to be statistical outliers (defined as more than two standard deviations away from the mean). Each plate included a set of duplicate "no template controls" (NTC) for each primer set (with ddH_2O substituted for DNA). Any sample in which amplification occurred within 3 cycles of the NTC was scored as a negative amplification to avoid inclusion of false positives (i.e., sample amplification indistinguishable from background fluorescence). Each plate also included a duplicate set of positive controls for each assay that were derived from clone isolates which exactly matched primer and probe sequences. Each sample was run in random order in duplicate.

Fluorescence adjustments

Fluorescence intensity differed among the Taqman MGB probes due to their particular sequences and reporter dyes (clade C probe: VIC, clade D probe: FAM, *M. cavernosa* probe: NED), therefore an adjustment value was calculated to normalize the fluorescence intensity among probes. Two standard curves with a known number of actin gene copies (ranging from 10^1 to 10^6, see methods below) from two different clone isolates per target were used for standardization (N= 4 standard curves per assay from two different plate runs). All assays were normalized to the clade D assay, which had the lowest Ct values, in order to account for inherent differences in the strength of the fluorescence between different probes. Least square mean values determined that *M.*

cavernosa amplified a mean of 3.43±0.17 cycles later than clade D, while clade C amplifies 1.67 ± 0.18 cycles later than clade D. These values were subtracted from the raw Ct values for the *M. cavernosa* and clade C assays prior to analysis.

Actin loci copy number

In order to calculate the mean number of copies of the actin gene per cell, tissue from corals containing either only clade C or clade D symbionts (determined by qPCR analysis), was removed using filtered seawater blasted through an airbrush. Symbiont cells were isolated by centrifugation and counted using a haemocytometer. Each cell slurry was counted in 4 replicate aliquots, and each aliquot was divided into 5 count areas. Approximately 1,500 cells for each sample were counted. Known quantities of cells were assayed against standard curves of known numbers of copies (10^1-10^5) derived from purified PCR-amplified clone isolates which were nanodropped and quantified. As described above, at least two standard curves from known quantities of different purified PCR products were prepared on each plate for each assay. Three colonies of clade C-only (between 3 and 4 individual extractions per colony), and 2 colonies (1 and 3 individual extractions per colony) of clade D-only were assayed. Copy numbers were estimated from an average of a normally-distributed subset these amplifications, such that clade C copy number was estimated from 33 separate amplifications, and clade D from 22 amplifications. These amplifications were adjusted for an estimated 95% extraction efficiency (Cunning and Baker 2012).

Because known numbers of coral cells could not be obtained, host copy number could not be determined. For simplicity, we therefore assumed a value of 1 for all genotypes in this analysis, and did not adjust for ploidy. Consequently, symbiont to host

cell ratios (see below) should be considered relative measures of the differential abundance of clades C and D, and not the mean abundance of symbiont cells in each host cell.

Statistical analyses

Statistical tests were performed in JMP (version 10.0.0, SAS). Raw Ct values were adjusted for fluorescence and copy number before analysis. Results are reported as symbiont to host (S:H) cell ratios, which express the relative number of *Symbiodinium* cells to coral host cells. S:H ratios are log-distributed and data was log-transformed. Because over 90% of colonies were >90% dominated by either clade C or clade D symbionts (3. 5), corals were designated as either clade C- or clade D-dominated, and changes in total *Symbiodinium* density (C+D) were used for most analyses.

Values for photochemical efficiency (Fv/Fm) were arcsin transformed for statistical analysis. Fv/Fm values also were compared using JMP models and were normally distributed. Relative proportions of clade C or D in each treatment group were compared using one-way ANOVAs with a Bonferroni-corrected alpha-value of 0.003 (Table 3.1), and a Tukey's HSD test where appropriate.

Results

Photosynthetic Function

Bleaching phase

Fv/Fm values in both DCMU and heat treatments decreased significantly ($P<0.0001$) after 3 days of stress exposure (Figure 3.2), and these declines continued until the end of stress exposure on day 10. DCMU-bleached corals had greater declines in

Fv/Fm (-97.2%) than heat-bleached (-55%) corals (Figure 3.2). Photochemical efficiency in unbleached corals maintained at 24°C and 29°C showed no change during the 10-day period (Figure 3.2).

Recovery phase

Once stress was removed, both DCMU- and heat-treated corals recovered photochemical efficiency, with DCMU-treated corals recovering more quickly than heat-treated corals. Five days after the cessation of DCMU treatment, these corals showed Fv/Fm values that were no different from the heat-bleached corals, despite the fact that DCMU-treated corals had considerably lower Fv/Fm values by the end of the stress exposure (Figure 3.2).

After 12 days in recovery conditions, recovery temperature (24°C vs. 29°C) did not have a significant effect on photochemical efficiency ($P=0.48$), but bleaching stressor (DCMU vs. heat) was highly significant ($P<0.001$). However, 7 days later (t=19 days) recovery temperature had a significant effect on Fv/Fm ($P=0.002$), but bleaching stressor was no longer significant ($P=0.47$). After 19 days at recovery conditions, photochemical efficiency of corals at 29°C recovered faster than those at 24°C, regardless of bleaching stressor (Figure 3.2), recovering to unbleached control values after 35 days of recovery. Fv/Fm values of corals at 24°C took 2-3 times longer (74 days to 3 months) to recover to control levels (Figure 3.2).

Visual observations during recovery

Corals regained pigmentation in striped patterns that radiated around the mouth, parallel to the mesenteries (Figure 3.6c, d). These stripes first appeared as pigmented

"flecks" (Figure 3.6c). I-PAM analysis suggests these stripes and flecks represent proliferation from low-abudance populations of symbionts, either residual or acquired from the environment, since they were photosynthetically active (Figure 3.6e). Corals typically regained full pigmentation 3-4 weeks following the appearance of stripes. The rate of recovery of photosynthetic function agreed with the rate of appearance of "flecks" and "stripes", appearing first in corals held at 29^{o}C. Within a treatment group, the rate of re-pigmentation also seemed to vary consistently by coral genotype, which had been fully crossed among treatments.

Genetic Analyses

ITS-2 genotyping

Sequencing of dominant bands excised from DGGE profiles revealed that all initial samples were dominated by *Symbiodinium* C3, while recovering corals tended to be dominated by *Symbiodinium* D1a, a putatively thermotolerant symbiont (LaJeunesse et al. 2010). No other types were detected by DGGE.

Coral genotype analysis

Analysis of the nine *M. cavernosa* colonies with 8 microsatellite markers (Table 3.2, Serrano et al. *in prep*.) showed the colonies were genetically distinct genotypes (no shared alleles across all 8 loci).

Actin copy numbers in Symbiodinium

Symbiodinium C3 was estimated to have approximately 50 (50.5±4.2) copies of the actin gene per symbiont cell, while clade D, ITS-2 type D1a, was estimated to have a copy number of 3 (3.3±0.2). Symbiont to host (S:H) ratios were adjusted by these values prior to analysis.

Symbiodinium *community dynamics*

All corals began the experiment with similar densities of *Symbiodinium* C3 (Figure 3.1, 3.4, 3.5); two colonies had slightly higher densities and one colony had slightly lower densities. Coral genotype was not a significant factor in experimental results, however. Two cores (N=2 of 139) were found to have trace amounts (<1%) of clade D *Symbiodinium* prior to the start of the experiment. Although cores had been randomly assigned to treatments, by chance, both of these corals had been allocated to the heat-bleached treatment with a recovery temperature of 24^{o}C (Figure 3.5).

At the end of the 10-day stress exposure period, only clade C *Symbiodinium* was detected in bleached cores (Figure 3.1, 3.4, 3.5) presumably *Symbiodinium* C3, although this was not verified by DGGE). Four cores (of 140) also had detectable levels of clade D (two of which had D before the stress treatment). Although there was no visual bleaching, and no observed decline in Fv/Fm, control corals at 29^{o}C lost more symbionts over the 10-day period, compared to control corals at 24^{o}C (P=0.0059, Figure 3.3, 3.4).

Corals treated with DCMU and heat both experienced similar reductions in symbionts (>95%, P=0.67, see Figure 3.3, 3.4). Corals with higher initial symbiont

densities lost a greater proportion of their symbiont communities during stress ($P<0.0001$, Figure 3.3, see Cunning & Baker 2012). Coral genotype had no effect on symbiont loss during bleaching ($P=0.1$).

After 3-6 months of recovery at 24°C and 29°C, corals had mixed communities of *Symbiodinium* in both clades C and D, but were dominated by clade D symbionts (Figure 3.1, 3.5). Clade D symbionts are hosted at approximately 2-3 times higher densities than clade C symbionts (Figure 3.4).

Unbleached control corals at 24°C did not acquire clade D symbionts after 3 or 6 months (Figure 3.1, 3.4, 3.5). Unbleached control corals at 29°C, however, hosted trace levels (<1%) of clade D in 3 cores (of 20) after 3 months (all other cores had no detectable clade D). Control corals at 29°C experienced rapid increases in the abundance of clade D between three and six months, from <1% in only 3 cores to 41.9% (±7.7%) clade D across all cores (Figure 3.1, 3.4, 3.5).

Recovery temperature and bleaching stressor post-bleaching symbiont community

Symbiont community recovery was affected by both bleaching stressor and recovery temperature, with bleaching due to heat stress and recovery at higher temperatures promoting recovery with more clade D symbionts. Among cores bleached with the same stressor, those which recovered at 24°C had significantly more clade C ($P<0.02$) compared to those which recovered at 29°C, both at three and six months post-bleaching. For example, DCMU-bleached corals which recovered at 24°C (i.e., corals not exposed to heat either during bleaching or during recovery) hosted 34% clade D symbionts after 6 months, whereas DCMU-bleached corals which recovered at 29°C (that

hosted the same type and amount of symbionts immediately after stress) hosted >99% clade D symbionts after 6 months. Exposure to heat, either during bleaching (32^{o}C) or recovery (29^{o}C), resulted in corals with equal amounts of clade D symbionts (>99%) at the end of the recovery period (P>0.07, Table 3.1, Figure 3.5)**.**

Discussion

Previously undetectable symbionts become dominant after disturbance

Changes in algal symbiont communities after disturbance may be the result of the acquisition of symbionts from environmental sources ("switching") or the proliferation of residual symbiont populations inside coral hosts ("shuffling") (Baker 2003). Despite being undetectable in >98% of experimental cores prior to the experiment, *Symbiodinium* in clade D (ITS-2 type D1a) became the dominant symbiont in almost all recovered corals. Only corals that had not experienced heat during either bleaching or recovery ("heat-naïve" corals) were exceptions to this finding. Similarly, LaJeunesse et al. (2009), using qPCR, did not detect clade D in *M. cavernosa* prior to a natural bleaching event in Barbados in 2005, but found that it was the dominant symbiont in this coral species 4-8 months post-bleaching. These joint findings suggest that *M. cavernosa* either routinely harbors undetectable populations of clade D (perhaps in spatially-isolated pockets of tissue that were not sampled by either study), or is readily capable of acquiring clade D from the environment.

It is possible that our experimental corals may have acquired clade D symbionts during the experiment, since these corals were potentially exposed to *Symbiodinium* in the incoming water supply and/or via other corals present in the experimental tanks that were hosting clade D (*S. siderea* and *M. faveolata*, Cunning et al. *in prep*). Previous work has shown that symbionts expelled from corals can be viable (Ralph et al. 2001) and that adult corals can acquire symbionts from the environment (Coffroth et al. 2010). However, we cannot exclude the possibility that clade D symbionts may also have been present in the corals (either at extreme low abundance that was undetectable by qPCR or in pockets of unsampled tissue); consequently, the origin of the clade D in recovered corals cannot be determined by this experimental design.

Regardless of the source of the clade D symbionts, these data demonstrate that symbionts that are undetectable, even when using high-resolution molecular methods (qPCR), can be critical components of coral recovery after disturbance, and may be important factors in coral resilience. The dominance of previously-undetectable symbionts in corals recovering from bleaching highlights the fact that "snapshot" sampling studies of *Symbiodinium* diversity within healthy corals may not capture the full breadth of coral-*Symbiodinium* associations (Baker and Romanski 2007), and illustrates the value of monitoring studies over time (e.g., Thornhill et al. 2006a, LaJeunesse et al. 2009). Methods of visualizing the location of particular symbionts within coral tissues, such as fluorescent labeling of individual *Symbiodinium* (Soffer et al. 2010) or Fluoresence *In Situ* Hybridization (FISH), would help identify the origins of novel *Symbiodinum* during community changes.

Symbiont communities are dominated by clade D symbionts post-bleaching

The expulsion of symbionts during bleaching promoted rapid symbiont community change during recovery (Baker 2001). Corals recovered with more *Symbiodinium* in clade D, and at a faster rate, when they were bleached (either by 32^{o}C heat stress or DCMU), compared to corals that were acclimated to warmer (29^{o}C) temperatures without acute bleaching (Figure 3.1). However, corals that did not bleach also increased their abundance of clade D after 6 months to levels approaching those in the corals that bleached (Figure 3.5, Table 3.1). Whether this would occur in the field, however, is uncertain, as corals may not experience sustained, elevated temperatures for several months at a time, as they did here. Therefore, bleaching and subsequent recovery may provide the most rapid potential acquisition of thermotolerant symbionts.

Changes in symbiont dominance may be proportional to symbiont loss during bleaching (Toller et al. 2001, Baker 2001, Cunning et al. *in prep*), enabling a non-resident symbiont to become established. Recent studies have quantified the amount of disturbance and correlated it to symbiont turnover (Cunning et al. *in prep*). However, sub-visual bleaching symbiont density changes can also occur (Stimson 1997, Fagoonee et al. 1999, Fitt et al. 2000) although as many as half of the symbiont community may have been lost before becoming visually apparent (Fitt et al. 2000). *Symbiodinium* compositions can shift between dominant communities of clades C and D in *Acropora palifera* on a seasonal basis also without apparent bleaching (Chen et al. 2005). LaJeunesse et al. (2010) showed that both bleached and unbleached colonies increased the amount of clade D that they harbored after a warming event, concluding that temperature increases that did nto cause visual bleaching may also promote symbiont

community change. Cunning and Baker (2012) similarly found increases in clade D *Symbiodinium* density in D-dominated *Pocillopora damicornis* in response to warming temperatures that did not cause bleaching. Future work should establish how the low levels of clade D acquired by corals under chronic warm conditions might contribute to increased thermotolerance and/or higher survivorship following bleaching, and how many of these symbionts are required to mitigate bleaching and mortality.

Higher temperatures post-bleaching promote faster recovery with more clade D symbionts

Corals regained their photochemical efficiency and pigmentation faster at higher recovery temperatures (Figure 3.2), perhaps as a result of increased replication (Strychar et al. 2004), or higher rates of photoprotection and enzyme activity at higher recovery temperatures (Iglesias-Prieto et al. 1992, Rowan 2004). Photosynthesis and respiration in corals is elevated at temperatures between 29^{o}C and 32^{o}C (Castillo and Helmuth 2005). Additionally, symbiont division rates (mitotic index, MI) have been shown to be elevated at higher temperatures (Strychar et al. 2004, Wooldridge 2012), which may have resulted in the more rapid repopulation of symbiont communities observed.

Therefore, recovery may have been slower at lower temperatures due to slower symbiont reproduction rates, lower rates of photoprotection and/or less enzyme activity. Therefore, bleaching due to non-heat stressors, such as pollutant exposure (Jones et al. 2004, Jones and Heyward 2003), low salinity (van Woesik et al. 1995), or winter cold snaps (Kemp et al. 2011, Lirman et al. 2011), might incur higher mortality as a result of

slower recovery times as they could also co-occur with low temperatures. For example, Lirman et al. (2011) reported an 11.5% mortality rate during a cold snap in Florida in early 2010, compared to a 0.5% mortality rate during the previous five summers, including the summer of 2005 during which warm-water bleaching was reported.

Corals bleached with heat recover with more clade D symbionts than corals bleached with herbicide

Corals bleached with heat stress had more clade D *Symbiodinium* after 3-6 months of recovery, compared to those bleached with DCMU. All bleached corals (with heat or DCMU) recovered with communities that were dominated by clade D symbionts, but DCMU-bleached corals that recovered at 24^{o}C (and which were therefore naïve to heat bleaching and during recovery), had the most symbionts in clade C (~36% of the symbiont community). This suggests that, while the stress itself might be transient, the type of stress can alter community recovery trajectories for months post-exposure. This indicates the influence of some mechanism beyond stochastic repopulation of a vacant niche; the effect of heat exposure has enduring physiological effects which long-lasting effects on the recovery of bleached tissues. This could either be due to: (1) the enhanced ability of clade D symbionts to invade heat-bleached tissues, (2) the decreased ability of clade C symbionts to thrive in heat-bleached tissues, or (3) a decrease in host specificity mechanisms after heat stress, which allowed opportunistic (clade D) symbionts to proliferate.

Additionally, Wooldridge (2012) proposes a model of symbiont shuffling based on symbiont growth rates (MI), which are positively correlated with temperature

(Strychar et al. 2004). Evidence suggests that corals may favor slow growing (low MI, such as clade D) symbionts during higher temperatures, possibly as a way to regulate algal density (Baghadasarian and Muscatine 2000). Therefore, fast-growing symbionts (high MI) appear to be selected against during periods of increased temperature (McCloskey et al. 1996, Baghadasarian and Muscatine 2000). Selection by the host for slower-growing symbionts after heat exposure, either during bleaching or during recovery, could result in the observed high abundances of clade D symbionts in corals exposed to heat. The selection against fast-growing symbionts may be less severe without heat exposure specifically, for example, in the DCMU-bleached corals at 24^{o}C, which allowed for a greater amount of clade C symbionts to populate after bleaching.

***Rapid colonization vs. competitive displacement in different* Symbiodinium**

Coral bleaching results in vacant niches within the host that can be repopulated by *Symbiodinium*. In some coral species this appears to follow a predictable pattern of succession (such as clade C monoculture → clade D-dominated mixed community → reversion to initial clade C monoculture, e.g., Thornhill et al. 2006a, LaJeunesse et al. 2009). Recent research has focused on developing models of microbial community succession that parallel well-known studies of plant community succession (Fierer et al. 2010). Here, disturbance (*sensu* Connell 1978) increased diversity within *Symbiodinium* communities, with clade C monocultures transitioning to mixtures of both clades C and D after recovery. However, increased diversity post-disturbance also implies that species differ in their relative competitive and colonization abilities (Tilman et al. 1994). Clade D symbionts have been suggested to possess distinct, "opportunistic" traits and exhibit

distribution patterns that are different from other symbiont clades (Stat and Gates 2010). Clade D symbionts are more likely to be present in bleached tissues than other clades (Glynn et al. 2001, Berkelmans and van Oppen 2006, LaJeunesse et al. 2009, Jones et al. 2008, this study, but see also Sampayo et al. 2008, Oliver and Palumbi 2009, Stat et al. 2009). However, their ability to be good colonizers in bleached tissues during stress (i.e. hardiness, opportunist) also may make them poor competitors after stress (i.e. sensitivity to bleached tissue conditions). After pre-bleaching conditions have been restored both *ex situ* and *in situ*, weaker comptetitive ability by clade D symbionts may lead to a reversion back to initial symbiont communities (Thornhill et al. 2006a, LaJeunesse et al. 2009).

With increasingly frequent disturbance, the poorest colonizers are predicted to go extinct first (Tilman et al. 1994). In corals, this might include the most productive symbionts with the highest MI that are the best competitors, such as members of clade C *Symbiodinium*. In this experiment, corals exposed to heat, either during bleaching or during recovery, experienced a nearly complete local (i.e. within-colony) clade C extirpation, hosting <1% clade C *Symbiodinium,* even after 6 months of recovery. On a global scale, clade C symbionts have declined on reefs that have undergone warming (Baker et al. 2004). The negative selection against fast-growing (high MI) symbionts during stress has similarly favored an increase in dominance in slow-growing (low MI) symbionts (Baker 2004, Oliver and Palumbi 2009).

Future work should seek to understand: 1) why some symbionts are inherently more susceptible to bleaching than others (e.g., C3 vs. D1a); 2) how heat stress specifically prevents some symbionts from recovering (e.g., C3) or promotes the recovery of others (e.g., D1a), 3) how long symbionts remain vulnerable to eviction by the host's

immune system post-stress, 3) why some symbionts (e.g., D1a) appear to be able to colonize bleached coral tissue better than others (e.g., C3).

Summary and conclusions

This study demonstrates that *Symbiodinium* types that are undetectable in corals prior to bleaching can be essential components of coral recovery after bleaching, and therefore important elements of coral resilience. It also shows that, although symbiont communities can change slowly in the absence of bleaching (provided corals are exposed to prolonged, chronic warming conditions), bleaching promotes more rapid and dramatic changes in symbiont communities. This study supports previous findings that the abundance of clade D (in this case D1a) increases following heat exposure. It also shows that bleaching as a result of heat stress, compared to low temperature herbicide exposure, increases the amount of clade D symbionts present, even after 6 months of recovery, suggesting that it is not just the creation of vacant niche space in the coral host that determines the recovery trajectories of symbiont communities, but also longer-term physiological or community changes that take place as a result of stress exposure. Potentially, succession and recovery with clade D symbionts post-disturbance within a coral colony could lead to increased thermotolerance for corals and enhanced survival during climate change.

Figure 3.1 Schematic showing experimental design. Light blue backgrounds represent corals at 24°C, while dark blue boxes represent corals at 29°C. Unbleached control corals remained at 24°C for the duration of the experiment (3-6 months), or were placed at 29°C without acute bleaching. Orange boxes represent heat stressed corals, while green boxes represent DCMU-bleached corals. These corals were then allowed to recover at either 24°C or 29°C for a period of three-six months. Orange and green shading during recovery periods indicate the type of bleaching stress (i.e. green for DCMU-bleaching, orange for heat-bleaching).

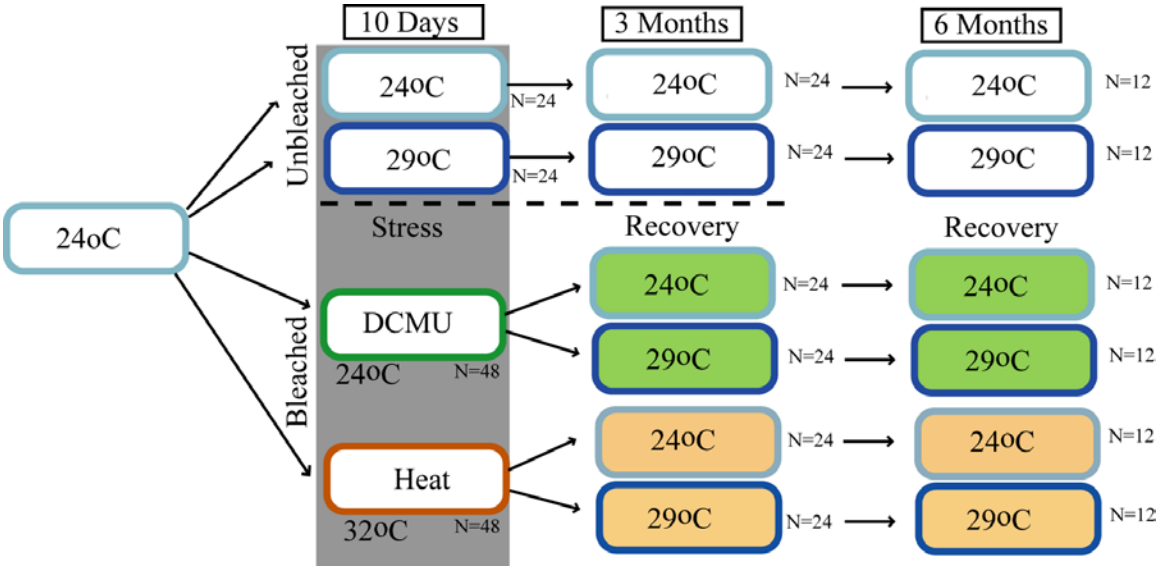


Figure 3.2 Maximum potential quantum yield (Fv/Fm) of symbionts within corals exposed to, and recovering from, bleaching due to heat and herbicide exposure. Corals treated with DCMU (light and dark green lines) experienced greater declines in photosynthetic efficiency (Fv/Fm), but recovered quickly after the removal of DCMU. After 5 days, DCMU-bleached corals had equivalent Fv/Fm values to heat-bleached corals. After 19 days, Fv/Fm values were more closely associated with recovery temperatures than bleaching stressor. Fv/Fm of recovering corals at held at 29^{o}C (darker shades) recovered to levels similar to those of the control (unbleached) corals more rapidly than those held at 24^{o}C (lighter shades). Orange shading indicates the 10-day period of stress exposure, white areas indicate the 6-month recovery period. Bars indicate standard error.

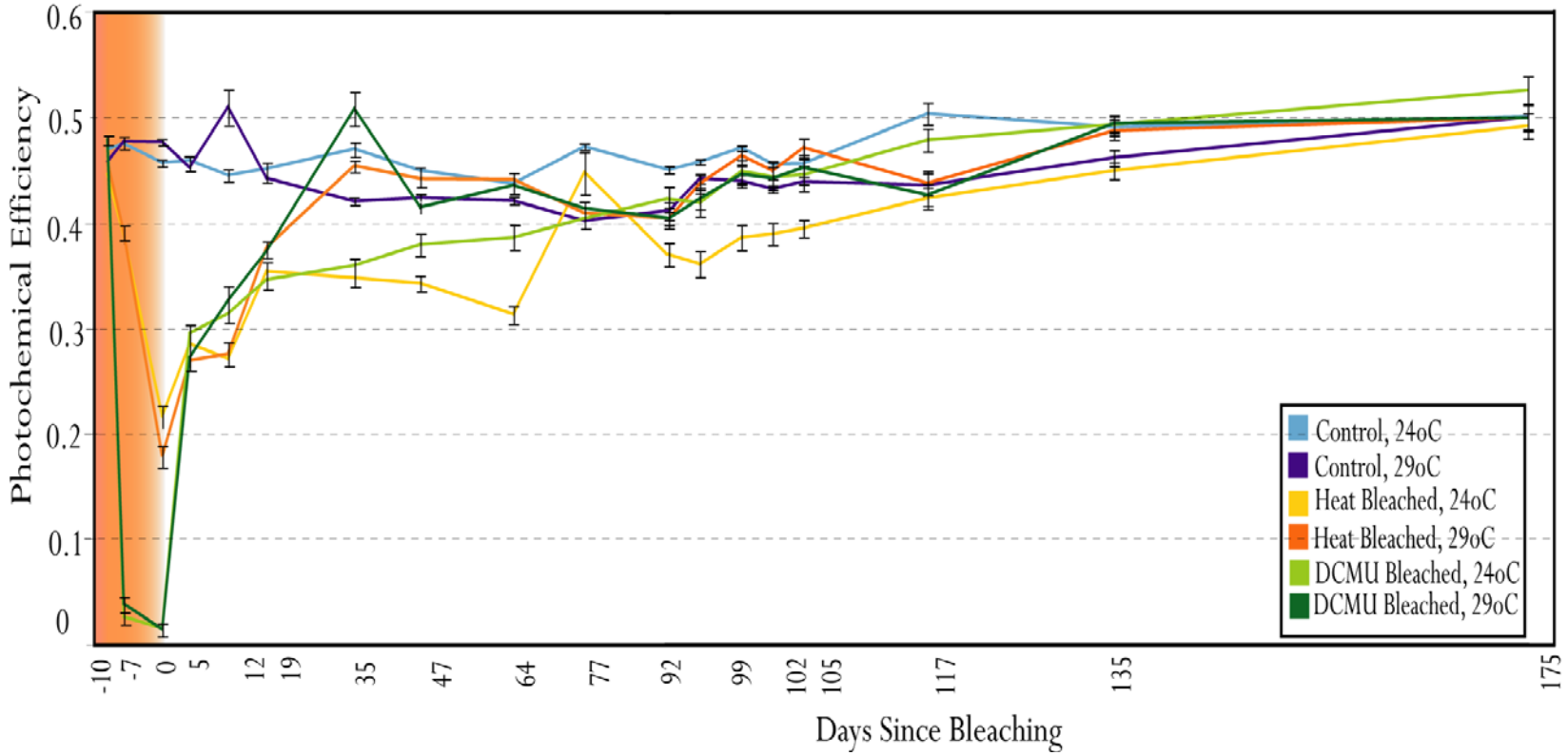

Photochemical Efficiency
0.6
0.5
0.4
0.3
0.2
0.1
0
-10
-7
0
5
12
19
35
47
64
77
92
99
102
105
117
135
175
Days Since Bleaching
Control, 24oC
Control, 29oC
Heat Bleached, 24oC
Heat Bleached, 29oC
DCMU Bleached, 24oC
DCMU Bleached, 29oC

Figure 3.3 Change in symbiont density during 10-day stress exposure of: 1) unbleached corals at 24°C, 2) unbleached corals at 29°C, 3) heat-bleached corals and 4) DCMU-bleached corals as a function of initial symbiont density. Corals bleached with DCMU and heat (32°C) lost similar numbers of symbionts during the 10-day period (P=0.24). Corals exposed to 29°C lost most symbionts during the 10-day period, compared to those exposed to 24°C, but did not bleach visually or lose photochemical efficiency (P=0.04,see Figure 2).

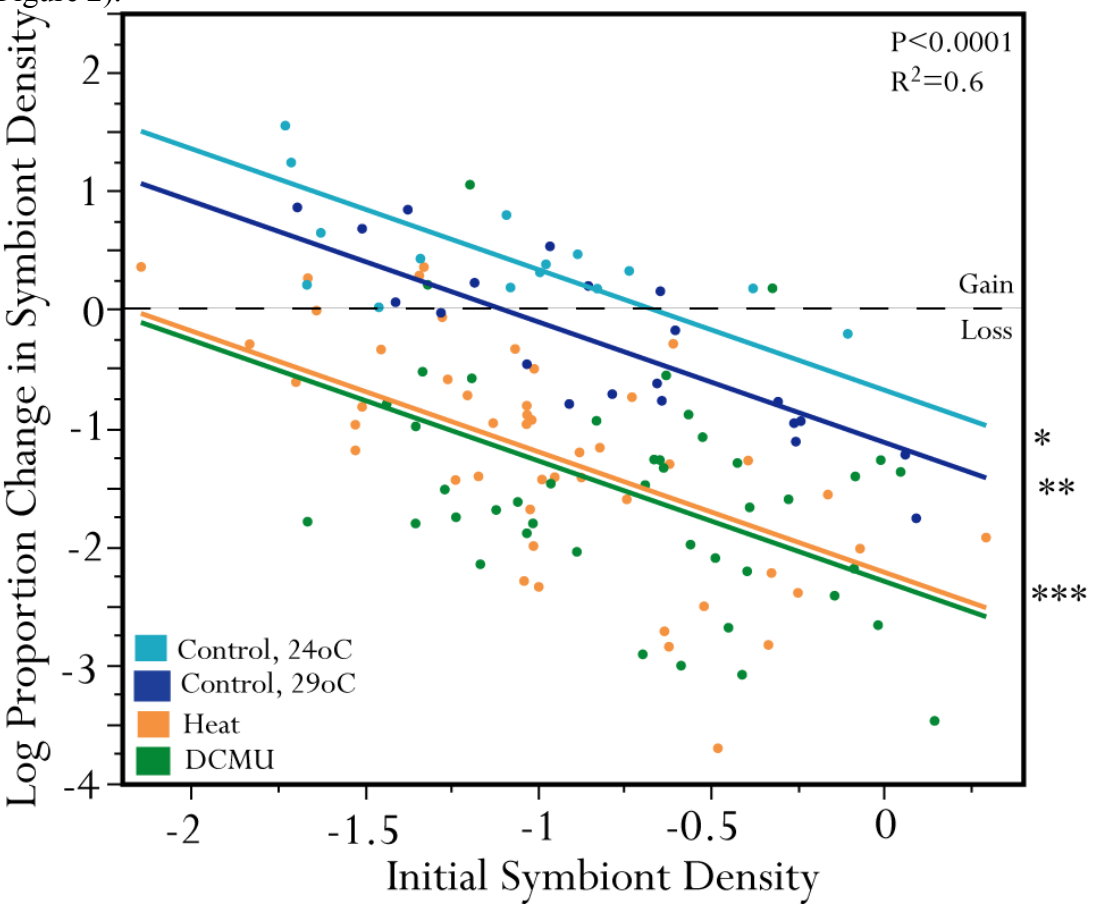


Figure 3.4 The total symbiont community density (height of bar, S:H) and proportion of clade C (blue) and D (red) in unbleached, heat-bleached, and DCMU bleached corals at (A) 24°C and (B) 29°C recovery temperature, and (C) unbleached corals acclimated to either 24oC or 29°C. Samples represent symbiont communities before experimental manipulations, after (1) 10 days at 32°C/DCMU/or 24°C/29°C, (2) three months after stress, and (3) six months after stress. Unbleached corals at 29°C acquired ~41% clade D after 6 months, while unbleached corals at 24°C did not acquire any clade D symbionts after 6 months, suggesting increased invasibility of clade D at higher temperatures. Corals that had bleached recovered with >99% clade D at both 24°C and 29°C, however, corals at 24°C had slightly higher amounts of clade C symbionts. Corals dominated by clade D also have a higher overall symbiont density ($P<0.025$). Error bars represent standard error for the total symbiont community (clades C+D).

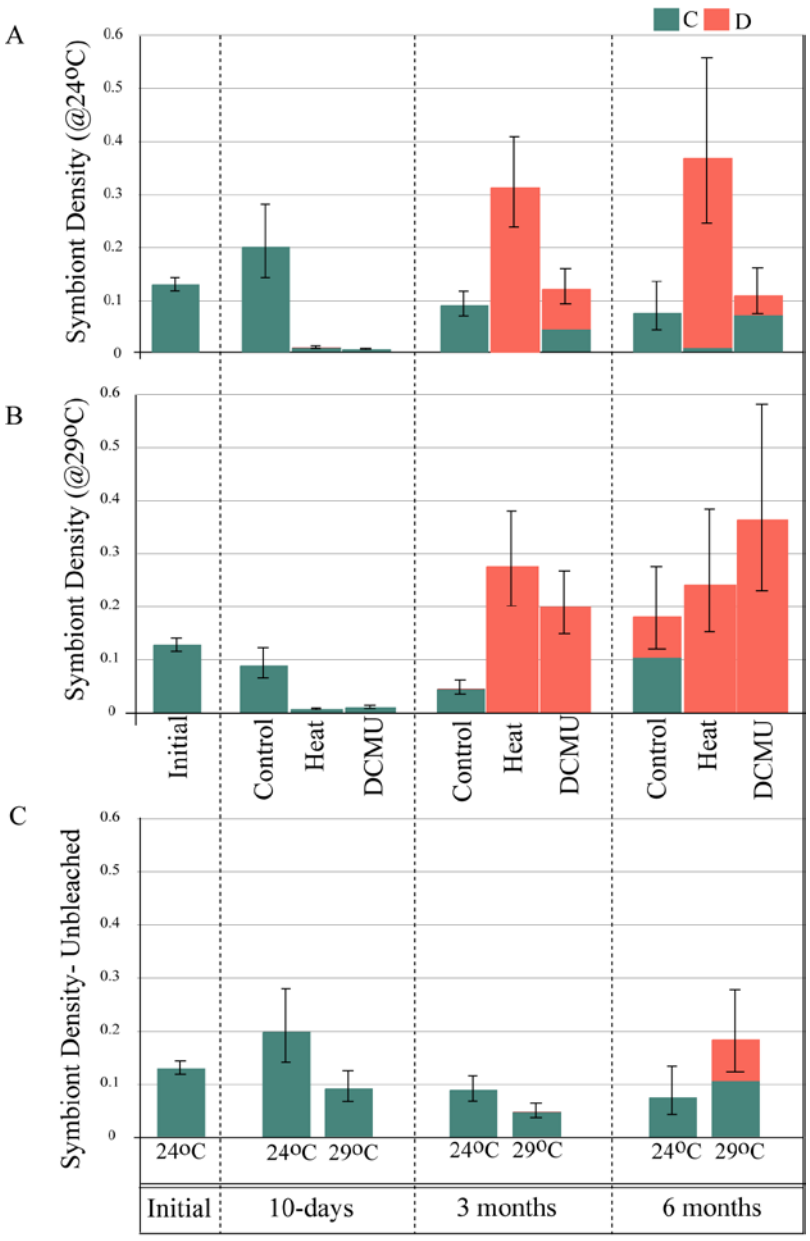

A
B
C
C
D
Symbiont Density (@24ºC)
Symbiont Density (@29ºC)
Symbiont Density- Unbleached
0.6
0.5
0.4
0.3
0.2
0.1
0
Initial
Control
Heat
DCMU
Control
Heat
DCMU
Control
Heat
DCMU
24ºC
24ºC
29ºC
24ºC
29ºC
24ºC
29ºC
Initial
10-days
3 months
6 months

Figure 3.5 Cell ratio densities of *Symbiodinium* in clades C and D. All corals were monocultures of clade C symbionts (circles), both prior to bleaching and during bleaching, except for a few corals (n=2 and 4, respectively) which contained low levels of clade D (triangles). After three months of recovery, bleached corals recovered with communities dominated by clade D symbionts (diamond), while unbleached corals (light and dark blue) remain dominated by clade C symbionts. DCMU-bleached corals at 24°C (light green) most commonly hosted mixed communities of clades C and D (open triangles). Heat-bleached corals recovering at 24°C (yellow) and 29°C (orange), and DCMU-bleached corals recovering at 29°C (dark green) hosted equivalently-high levels of clade D symbionts. Heat-bleached corals recovering at 29°C hosted the most corals with no detectable clade C symbionts. After 6 months of recovery, unbleached corals at 29°C hosted greater amounts of clade D symbionts, in approximately equal amounts to clade C symbionts. The dotted diagonal line represents equal amounts of clades C and D in a sample.

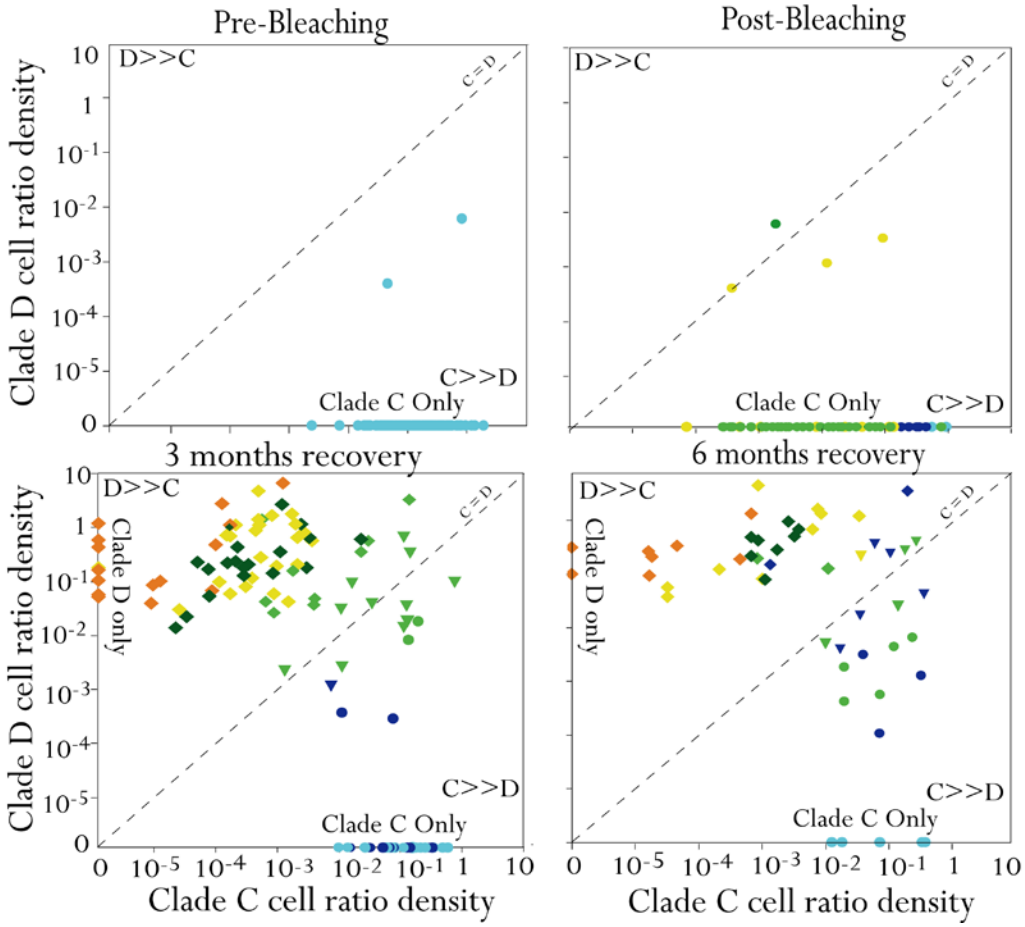


Figure 3.6 Images of cores of *Montastraea cavernosa* in various conditions: A) healthy, B) bleached, C) recovering with stripes, D) close-up of polyp with stripes, E) background fluorescence in I-PAM image.

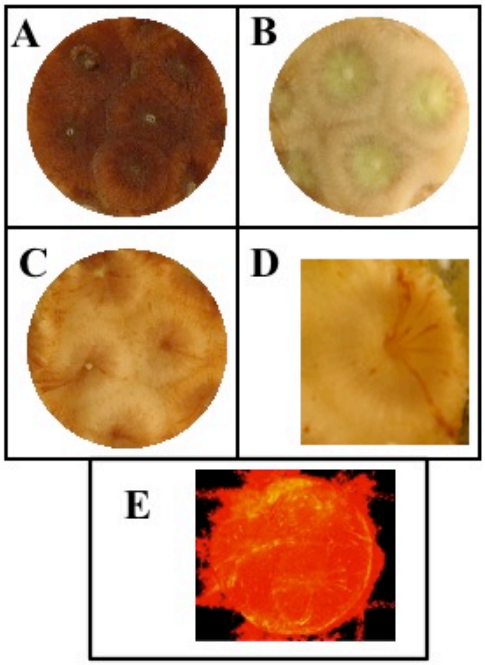


Table 3.1 Summary of statistically significant differences (determined by one-way ANOVA) in the amount of clade C and clade D symbionts in samples of each treatment after 6 months of recovery from stress. Statistically significant relationships are based on a Bonferroni-corrected alpha value of 0.003.

	Control-24°C	**Control-29°C**	**Heat-24°C**	**Heat-29°C**	**DCMU-24°C**	**DCMU-29°C**
Control-24°C	XXX	$C_{24}=C_{29}$, $D_{24}<D_{29}$	$C_{Control}=C_{Heat}$, $D_{Control}<D_{Heat}$	$C_{Control}>C_{heat}$, $D_{Control}<D_{heat}$	$C_{Control}=C_{DCMU}$, $D_{Control}<D_{DCMU}$	$C_{Control}>C_{DCMU}$, $D_{Control}<D_{DCMU}$
Control-29°C		XXX	$C_{Control}>C_{Heat}$, $D_{Control}=D_{Heat}$ (P=0.03)	$C_{Control}>C_{heat}$, $D_{Control}=D_{heat}$	$C_{Control}=C_{heat}$, $D_{Control}=D_{heat}$	$C_{Control}>C_{DCMU}$, $D_{Control}=D_{DCMU}$ (P=0.02)
Heat-24°C			XXX	$C_{24}=C_{29}$ (P=0.02), $D_{24}=D_{29}$	$C_{DCMU}>C_{heat}$, $D_{DCMU}<D_{heat}$	$C_{DCMU}=C_{heat}$, $D_{DCMU}=D_{heat}$
Heat-29°C				XXX	$C_{DCMU}>C_{heat}$, $D_{DCMU}=D_{heat}$ (P=0.006)	$C_{DCMU}>C_{heat}$, $D_{DCMU}=D_{heat}$
DCMU-24°C					XXXX	$C_{24}=C_{29}$ (P=0.01), $D_{24}<D_{29}$
DCMU 29oC						XXXX

Table 3.2 Microsatellite markers (N=8) used to identify *Montrastrea cavernosa* colonies used in this study as non-clones (Serrano et al. *in prep.*).

Genet ID	Allele 4_1	Allele 4_2	Allele 18_1	Allele 18_2	Allele 29_1	Allele 29_2	Allele 41_1	Allele 41_2	Allele 49_1	Allele 49_2	Allele 65_1	Allele 65_2	Allele 46_1	Allele 46_2	Allele 97_1	Allele 97_2
1	147	147	227	227	171	175	396	408	255	255	154	154	133	139	171	175
2	147	147	224	230	171	175	396	408	246	330	154	154	133	139	163	171
3	144	144	230	233	175	175	408	412	330	333	154	154	133	139	167	175
4	144	156	230	242	0	0	0	0	330	330	154	154	0	0	175	175
5	144	165	233	236	0	0	0	0	330	333	154	160	133	133	175	175
6	153	165	230	233	0	0	0	0	330	333	154	154	133	139	171	175
7	147	165	236	251	0	0	0	0	330	330	142	160	133	133	175	175
8	177	203	233	233	0	0	0	0	330	330	154	154	133	139	171	175
9	147	179	233	236	0	0	0	0	324	330	154	160	133	133	175	175

Chapter 4:

Changes in algal symbiont community, not exposure to heat, explains increased coral thermotolerance following bleaching

Summary

Prior exposure to stress can increase coral thermotolerance, but whether this is due to physiological acclimatization or to changes symbiont community composition, has not been directly investigated. To differentiate between these mechanisms, we used quantitative PCR (qPCR) and chlorophyll fluorometry to assess the community structure and function of *Symbiodinium* in the Caribbean coral *Montastraea cavernosa* in a repeated (two-phase) stress experiment. *M. cavernosa* containing only *Symbiodinium* in clade C (C3) were exposed for 10 days to either high temperature (32^{o}C) or to herbicide (450µg/L DCMU) at low temperature (24^{o}C). Both treatments produced bleached corals, but the DCMU-treated corals were naïve to direct heat stress. Corals were then allowed to recover at either 24^{o}C or 29^{o}C for three months. All corals recovered with *Symbiodinium* communities dominated by clade D (D1a), which has been associated with stress-tolerance. Corals were then stressed again by exposure to 32^{o}C for 10 days. Corals that had previously bleached and recovered with clade D symbionts exhibited less photodamage than corals dominated by clade C symbionts. Corals initially bleached by DCMU or heat showed the same thermotolerance, indicating that the change in symbiont type and not prior heat exposure was responsible for this acquired thermotolerance. Additionally, corals that had been acclimated to 29^{o}C for three months demonstrated more photodamage and symbiont loss than those at 24^{o}C, possibly due to exhaustion of

physiological acclimatization mechanisms during long-term exposure to 29^{o}C, or due to an inability by corals to regulate symbiont proliferation at higher temperatures. These results suggest that the increased thermotolerance arose due to changes in algal symbiont community composition, and not due to physiological acclimatization due to past high temperature exposure, either acute or chronic. Additionally, there may be a cost to long-term acclimatization to constant warm temperatures, even if the temperatures appear sub-lethal and do not cause bleaching.

Background

Coral reefs are threatened by elevated sea surface temperatures, chronic ocean acidification, and other anthropogenic stressors. Coral cover has declined by ~80% in the Caribbean since the 1970s (Gardner et al. 2003) and ~2% annually in the Pacific (Bruno and Selig 2007) from a combined group of stressors. One major source of coral die off has been episodes of mass coral bleaching, which have been linked to high sea surface temperatures (see Baker et al. 2008 for review). In order to avoid further bleaching and mortality as a result of increased temperatures, it has been estimated that coral thermotolerance will need to increase by 0.2-1.0^{o}C per decade in order to keep pace with current warming ocean conditions (Donner et al. 2005).

Corals' ability to adapt to changing environments is evidenced by their persistence over geological time (Veron 1995). However, the regional disappearance of corals during periods of climatic change also suggests they can be vulnerable: coral reefs ceased to exist in the geological record of the eastern Pacific for a ~2,500 year period, corresponding with episodes of climate variability (Toth et al. 2012). This raises

concerns over the ability of coral reefs to acclimatize and/or adapt rapidly enough to keep pace with current climatic shifts (Hoegh-Guldberg et al. 2007). Acclimatization refers to compensatory changes in an organism's metabolic function in response to natural environmental change (Gates and Edmunds 1999). Corals may adapt or acclimatize to environmental changes via: (1) physiological or cellular mechanisms, such as antioxidants and heat-shock proteins (Brown et al. 2002, Middlebrook et al. 2008), (2) symbiont community changes (Buddemeier and Fautin 1993, Rowan et al. 1997, Baker 2001, Baker 2003, Berkelmans and van Oppen 2006, Jones et al. 2008), or (3) genetic adaptation of either the coral host (Hughes et al. 2003) or its symbionts (e.g., Howells et al. 2012). Physiological changes in either the host or the symbiont have been shown to occur in response to stress (Brown et al. 2002, Middlebrook et al. 2008). These cellular alterations occur over rapid time scales (hours or days) and can last for days to weeks (Middlebrook et al. 2008). Corals exposed to elevated temperatures 1 or 2 weeks prior to thermal stress, for example, experienced less photochemical impairment, and had increased non-photochemical quenching and xanthophyll cycling during thermal stress (Middlebrook et al. 2008). Similar preconditioning via chronically high levels of irradiance led to host upregulation of antioxidants, fluorescent photoprotective pigments, and heat-shock proteins, and resulted in increased thermotolerance (Brown et al. 2002). Similarly, preconditioning corals at 28^{o}C for 10 days prior to high temperature stress (31^{o}C) increased bleaching resistance compared to controls, without any change in algal symbiont communities (Bellantuono et al. 2011).

Coral bleaching itself also has been suggested to be an extreme outcome of an acclimatization mechanism involving changes in symbiont densities and pigments (Gates

and Edmunds 1999). Large-scale *Symbiodinium* community changes occur most frequently after recovery from an acute disturbance, such as a bleaching event (Baker 2001, Glynn et al. 2001, Toller et al. 2001, Baker et al. 2004, Berkelmans and van Oppen 2006, Jones et al. 2008, LaJeunesse et al. 2009, see also Chapter 3). *Symbiodinium* community recovery post-bleaching typically occurs over several months (see Chapter 3), and can be maintained from weeks to years (Toller et al. 2001, Thornhill et al. 2006a, LaJeunesse et al. 2009). Symbiont community changes in the absence of acute disturbance likely requires sustained environmental change, and may take even longer to occur than changes following recovery from bleaching (Chen et al. 2005, see also Chapter 3). Post-bleaching symbiont communities are often dominated by *Symbiodinium* in clade D (such as ITS-2 types D1 and D1a), which have been associated with an increase in thermal tolerance of 1-1.5^{o}C (Berkelmans and van Oppen 2006).

Jones et al. (2008) showed that coral colonies hosting clade D *Symbiodinium* survived heat stress better than those hosting clade C symbionts, and that corals changed from hosting clade C to clade D symbionts during recovery from a bleaching event. In a transplantation experiment, bleached corals that recovered with clade D symbionts survived subsequent heat stress better than those that bleached and recovered with clade C symbionts (or those that hosted clade C symbionts and did not bleach), regardless of whether they were acclimated to higher temperatures or not (Berkelmans and van Oppen 2006). Therefore, coral bleaching may provide a means of rapid, non-Darwinian adaptation to high temperatures by promoting partner switching (Buddemeier and Fautin 1993, Baker 2001, Baker 2004, Fautin and Buddemeier 2004, Berkelmans and van Oppen 2006, Jones et al. 2008). However, symbiont communities do not always change

following bleaching events (LaJeunesse et al. 2005, Thornhill et al. 2006, McGinley et al. 2012), suggesting the capacity for change is likely related to the degree of symbiont loss, the types of residual symbiont remaining, and the coral species involved.

Corals have been thought to have a limited adaptive capacity due to their long generation times, low genetic diversity, and asexual reproductive strategies (Hoegh-Guldberg et al. 2007). However, *Symbiodinum* and other microbial symbionts may have higher adaptive potential than their coral hosts, due to short generation times and genetic isolation (Correa and Baker 2011). When isolated from the host, symbionts have been shown to increase their thermotolerance in a matter of several hours, by increasing the thermostability of their thylakoid membranes (Hill et al. 2009). *Symbiodinium* of the same genetic identity can acquire heritable, thermotolerant traits via genetic adaptation over a time scale of several months (Howells et al. 2012, van Oppen ICRS 2012).

In this study, a series of experiments were designed to distinguish the relative contributions of (1) prior bleaching, (2) prior acute thermal stress, (3) acclimation to chronically-high temperatures, (4) dominant symbiont clade, and (5) host genotype, to changes in thermotolerance over a three-month period.

Methods

Initial bleaching and recovery conditions

Experimental conditions (tanks, lights, water flow) are described in Chapter 3. *M. cavernosa* cores were either: (1) not bleached, (2) bleached with DCMU at 24^{o}C, or (3) bleached with heat (32^{o}C), according to the conditions described in Chapter 3. All cores were then acclimatized for three months to either 24^{o}C or 29^{o}C. After three months,

bleached corals recovered with dominant (>99%) communities of *Symbiodinium* clade D, although the heat-naïve treatment group (DCMU-bleached, 24°C recovery temperature) had significantly less clade D (~63%) than the other bleached groups (see Chapter 3). The treatments (N=24 cores/group), followed by the proportion of clade D in parentheses, were as follows: 1) unbleached controls recovered at 24°C (no detectable clade D), 2) unbleached controls recovered at 29°C (0.9±3.3% clade D in 3 of 20 cores, no detectable clade D in all others) 3) heat-bleached corals recovered at 24°C (99.6±3.1% clade D) heat-bleached corals recovered at 29°C (99.9±3.7% clade D) 5) DCMU-bleached corals recovered at 24°C (63.5±3.3% clade D) and 6) DCMU-bleached corals recovered at 29°C (99.6±3.4% clade D).

Repetitive thermal stress experiment

Half of the cores (N=12) from each of the six treatment groups remained at their respective recovery temperatures (24°C or 29°C) for a further 3 months (total of 6 months "recovery" time from initial stress exposure). The remaining cores (N=12 per treatment group) were exposed to a 10-day thermal stress event (32°C). After this 10-day stress period, corals recovered for the remainder of the three-month period at their original recovery temperature. The experimental design of both bleaching phases is illustrated in Figure 4.1.

Treatment groups were designated as follows: "Initial Stress Exposure-Recovery Temperature-Second Stress Exposure." For example, the group of corals that was initially bleached with DCMU, then recovered at 24°C, and was then exposed to 32°C was "DCMU-24°C-Heat". The half of the DCMU-bleached corals that recovered at 24°C, but

remained at control conditions during the second phase, was "DCMU-24^{o}C-Control." Corals that were not previously bleached were referred to as "Unbleached Controls."

Symbiodinium *Community Function*

Imaging-Pulse Amplitude Modulated Fluorometry (I-PAM), described in Chapter 3, was used to take measurements of dark-adapted photochemical efficiency (maximum quantum yield, Fv/Fm) on days 0, 3, 7, and 10 during the thermal stress event, as well as every 1 to 2 weeks during the three-month recovery period. After each I-PAM measurement, a photograph of each core was taken under standardized irradiance conditions.

Genetic analyses

Tissue samples for qPCR analysis of algal symbiont communities were collected: 1) before the start of thermal stress, 2) after 10 days of thermal stress, and 3) after three months of recovery. DNA from these samples were analyzed using a clade-specific, actin-based assay (Mieog et al. 2009) optimized for *Symbiodinium* clades C and D (Cunning and Baker 2012), and the coral *Montastraea cavernosa* (see Chapter 3). Copy number was estimated (see Chapter 3) to be approximately 50 (50.5±4.2) for clade C symbionts (type C3) and 3 (3.3±.2) for clade D symbionts (type D1a).

Statistical analyses

After recovery from the initial bleaching (i.e., at the start of this experiment), most corals (91.1%) were >90% dominated by either clade C or clade D symbionts

(Figure 4.3). Only 11 samples of 72 which had any clade D had less than 90% clade D. Corals were therefore categorized as either "C-dominated" (>90% clade C) or "D-dominated" (>90% clade D) corals, and were analyzed by these categories. A few cores (N=11 of 124) were not >90% dominated by one clade and were categorized as "C+D" corals; these corals were excluded from the analysis because they were too few to form a statistically rigorous third group. A sensitivity analysis was conducted in order to ensure the appropriate thresholds for defining "dominant" clade were used (i.e., a threshold below which background symbionts did not affect overall community function).

Between 19 and 33% of samples at each time point were not included in analysis because they were statistical outliers (i.e., were more than two standard deviations away from the mean of each treatment group), did not amplify during qPCR (even after re-extraction), or had technical replicates which did not agree (even after re-extraction and re-amplification).

Symbiont communities were expressed as a ratio of the number of copies of the symbiont actin gene to the number of copies of the host actin gene, adjusted by the mean copy number of the symbiont actin genes (S:H actin ratios, see Chapter 3). S:H actin ratios were adjusted for fluorescence disparities and log-transformed prior to analysis (see Chapter 3). Maximum quantum yield data (Fv/Fm) was arcsine-transformed prior to analysis. Models were fitted using JMP (9.0.2, SAS) and one-way ANOVAs or ANCOVAs followed by Tukey's HSD test. An alpha value of 0.05 was used for all tests.

Results

Control corals maintained at 24°C or 29°C

Fv/Fm values of corals maintained at 24°C or 29°C remained relatively constant (i.e., did not drop below 0.4) during the course of the experiment, with the exception of the heat-24°C-Control corals, which began with Fv/Fm levels of 0.38±0.4. These corals, however, ended with mean Fv/Fm values of 0.49±0.2 (Figure 4.2a). Control (unbleached) cores at 24°C did not contain any clade D symbionts, even after 6 months (including initial bleaching and recovery phase). Control cores at 29°C had low (but still detectable) levels of clade D in 3 cores of 20 after 3 months (mean of cores containing clade D: 6.1±4.2%, mean of all unbleached cores at 29°C: 0.86±0.69%). After 6 months, all unbleached cores at 29°C (N=10) had acquired significant numbers of clade D *Symbiodinium* (41.9±7.7% of the total symbiont community, see Figure 4.1).

Corals exposed to 32°C for 10 days

During heat stress, previously-unbleached corals (that were still dominated by clade C) from both 24°C and 29°C experienced equivalent declines in Fv/Fm (-48.2± 0.1%, P=0.66). This decline in photochemical efficiency was similar to the decline observed during the first heat bleaching treatment (see Chapter 3). Previously unbleached corals lost ~75% (24°C) and 93.5% (29°C) of their symbionts (least square means adjusted for initial symbiont density) during heat stress. Among previously bleached (and D-dominated) corals, heat-bleached corals which recovered at 24°C and 29°C had equivalent Fv/Fm levels (P=0.50); heat-bleached corals recovered at 29°C were

equivalent to DCMU-bleached corals recovered at 29°C (P=0.44), which in turn were equivalent to DCMU-bleached corals recovered at 24°C (P=0.10).

Overall, corals dominated by clade C lost more symbionts (P<0.001, Figure 4.3) and showed greater reductions in photochemical efficiency (P<0.0001, Figure 4.2) than clade D-dominated corals when exposed to 32°C. Clade D-dominated corals retained 63.9 (standard error: +0.5 and -0.3%), while clade C-dominated corals retained only 11.7 (standard error: +0.23 and -0.17%) of their initial symbiont communities (least square means adjusted for density). Higher proportions of clade D resulted in less symbiont loss (P=0.0004, R^2=0.58, Figure 4). These findings were unchanged by altering the threshold of corals considered "D-dominated" from 90% to 95%, 96%, 98%, 99%, and even 99.7% of the total community (only one sample hosted between 90 and 95% clade D, and no samples hosted between 95 and 99% clade D, 5 samples hosted between 99% and 99.7% clade D, P<0.005 for all tests). C-dominated corals with background D were rare (only 5 of 49 cores, ranging from 0.4% to 9.1% clade D), and therefore this analysis was not conducted on clade C-dominated corals.

Clade D-dominated corals that had been previously exposed to heat stress lost the same amount of symbionts as those previously bleached with DCMU (P=0.06, Figure 4.4); however, heat-bleached corals showed smaller reductions in photochemical efficiency than DCMU-bleached corals (P<0.0001, Figure 4.2). Initially DCMU-bleached, D-dominated corals hosted the same amount of background clade C symbionts (P=0.23), but significantly lower amounts of clade D symbionts compared to initially heat-bleached corals (P=0.007). Changing the thresholds for clade D-dominance (from 90% to 95%, 96%, 98%, 99%, and 99.7%) did not alter these results for photochemical

efficiency or symbiont loss comparisons, however. DCMU-bleached and heat-bleached corals did not lose significantly different amounts of symbionts (P=0.06) at the 90% dominance threshold, and became more similar as the threshold was raised to 95% (P=0.14) and up to 99.7% (P=0.38), reinforcing the finding that past heat exposure did not influence symbiont loss during this second heat stress event. Changes in photochemical efficiency also were unaffected by D-dominance thresholds (P<0.0001 at all levels).

Among clade C-dominated corals, acclimation temperature did not affect photochemical efficiency (P=0.92, Figure 4.2) or symbiont loss (P=0.07, Figure 4.5a). Among clade D-dominated corals, those at 24^{o}C lost fewer symbionts (P=0.04, Figure 4.5b) and also experienced smaller declines in Fv/Fm (P<0.0001, Figure 4.2) compared to corals acclimated to 29^{o}C. The significance of these results was unaffected by the threshold value set for D-dominance.

Initial (pre-stress) symbiont density did not have a significant effect on the degree of photodamage observed (P=0.44), however initial symbiont density of both clades was a highly significant factor in symbiont loss during stress (P<0.0001), with corals containing more symbionts showing greater symbiont loss (See Chapter 3). Corals that began with higher symbiont densities lost a greater proportion of their symbiont communities overall (P<0.0001). Therefore, comparisons of symbiont density changes include initial symbiont densities as a factor in the analysis (ANCOVAs, Figure 4.3, 4.4).

Colony genotype was not a significant factor in either photosynthetic efficiency or symbiont loss (P=0.49).

Recovery of corals from heat stress

After 15 days at recovery conditions, corals that were maintained at 29°C regained photochemical efficiency faster than those maintained at 24°C (P=0.0003). Clade C-dominated corals at 29°C recovered to initial Fv/Fm values after 15 days, while those at 24°C did not reach control Fv/Fm levels during the entire 3-month recovery period. All previously-bleached corals (i.e., clade D-dominated) reached and/or exceeded their initial Fv/Fm levels after just 15 days of recovery. Corals that were dominated by clade C prior to bleaching recovered from bleaching with >99% clade D symbionts after three months, as a similarly stressed and recovery corals had shown in response to the first bleaching event (See Chapter 3).

Discussion

Symbiont community change post-disturbance and clade-dependent responses

Corals that had bleached previously (and had consequently switched from hosting communities dominated by clade C to clade D) avoided both major photodamage (Figure 4.2) and symbiont loss during the second stress exposure (Figure 4.3). Corals that had not previously bleached were still dominated by clade C symbionts, and therefore underwent major declines in photochemical efficiency and symbiont density during the second stress exposure as well.

These results indicate that bleaching promotes rapid turnover of algal symbiont communities, and can drive changes to clade D-dominance, resulting in increases in coral thermotolerance. However, bleached corals also are at a higher risk of mortality (Glynn et al. 1996), highlighting a potential trade-off between rapidly-acquired thermotolerance

and mortality risk. Recently, researchers have suggested that net costs to hosting clade D *Symbiodinium* outweigh the overall benefits (Grottoli et al. 2012, Ortiz et al. 2012). Clade D symbionts appear to be more resistant to stress and produce less ROS than other symbiont types (McGinty et al. 2012), but are also associated with less-efficient energy transfer, decreased calcification, lower metabolism, and slower growth of the coral host (Little et al. 2004, Cantin et al. 2009, Grottoli et al. 2012). However, higher thermotolerance of clade D will result in avoided symbiont loss in corals that host these symbionts, and will contribute to higher bleaching resistance. Similarly, if residual clade D symbionts accelerate recovery of bleaching coral tissue, then they may contribute to increased coral resilience in response to bleaching. Together these factors will increase coral survivorship and hence improve overall coral fitness. Therefore, although corals hosting clade D symbionts may experience lower growth rates when not exposed to warmer conditions, higher rates of survivorship during stressful conditions might easily compensate for this disadvantage, and should be incorporated into net cost-benefit analyses. Studies designed to evaluate coral survivorship post-bleaching in the absence of clade D symbionts could assess the degree to which clade D is either necessary for recovery, or an opportunist that prevents other symbiont types from proliferating. Studies also are needed that compare the growth rates and metabolism of corals hosting clade D and other symbionts under different environmental conditions, including warmer temperatures at which clade D-dominated corals might grow faster than clade C-dominated corals. Studies that compare the performance of coral hosts containing different symbionts at high and low temperatures would better predict the contribution and role of clade D to coral survival in warmer oceans. Finally, coral growth and/or

metabolism likely decreases after a bleaching event, regardless of the composition of the recovered symbiont communities. For example, corals have been shown to suspend reproduction for two years following a bleaching event (Ward et al. 2000). Therefore, metrics of coral health are likely to confound the effects of recovery with clade D from the after-effects of stress generally, unless careful controls are designed. Studies that distinguish between these factors would better elucidate the robustness of clade D-dominated corals.

Higher symbiont densities result in more bleaching

High *Symbiodinium* density at the start of high temperature stress increased the proportion of symbionts lost during bleaching. This has been observed in *Pocillopora damicornis* corals during a natural warming and bleaching event (Cunning and Baker 2012), and *Montastraea faveolata* during experimental bleaching (Cunning et al. *in prep)*. These observations have some precedent: Coles and Jokiel (1978) related pigmentation to symbiont density, and reported that bleaching at 32.5^{o}C was most severe in corals that were initially darkly pigmented. Similarly, corals that had paled (due to low temperature preconditioning) prior to heat exposure, survived heat stress better than darkly-pigmented corals, perhaps as a result of additional evictions of algae during pre-conditioning (Coles and Jokiel 1978). *Acropora millepora* with higher initial symbiont densities lost a greater proportion of their symbionts in a repeated thermal stress experiment (Middlebrook et al. 2012). Cunning and Baker (2012) also show this trend in *Pocillopora damicornis*, and suggest that increased bleaching at high symbiont densities may be due to the cumulative effect of reactive oxygen species (ROS) produced by each *Symbiodinium* cell. Nesa et al.

(2012) also show that coral "tissue balls" degraded and died faster when hosting higher symbiont densities. Conversely, it has been suggested that high densities of symbionts may reduce the severity of bleaching by decreasing light stress in corals via self-shading (Enriquez et al. 2005, Teràn et al. 2010). Possibly, light fields, and thus irradiance stress, are higher when symbiont densities are decreased (Enriquez et al. 2005, Teràn et al. 2010), but ROS production (which may trigger bleaching) is also reduced, therefore bleaching is also less severe. These differing mechanisms also are potentially explained by differences in areal versus S:H ratio symbiont density metrics.

Because symbiont density is a determinant of symbiont loss during stress, environmental conditions that result in increased symbiont densities (such as nutrient pollution) may make corals more susceptible to bleaching (Wiedenmann et al. 2012, Cunning and Baker 2012). Corals in areas of upwelling or nutrient runoff do have decreased bleaching thresholds (D'Croz et al. 2001, Wooldridge and Done 2009); the highest bleaching thresholds (>33^{o}C) are found in corals living in oligotrophic waters (Wooldridge 2012). Local efforts to improve water quality and to control nutrients may therefore elevate coral bleaching thresholds and avoid severe coral bleaching (Wooldridge and Done 2009, Kelly et al. 2011).

Chronic acclimation to high, but sub-bleaching, temperatures does not impart thermotolerance

Chronic acclimation to higher temperatures (with and without bleaching) did not prevent photodamage (Figure 4.2) or symbiont loss during heat exposure (Figure 4.5). In fact, clade D-dominated corals that were pre-acclimated to higher temperatures (29^{o}C vs. 24^{o}C) were more susceptible to heat stress (32^{o}C), despite experiencing a less severe

temperature change during stress (3°C vs. 8°C). This trend was not observed in clade C-dominated corals perhaps due to either 1) low sample sizes, or 2) the large number of symbionts lost from corals that had been acclimated to both temperatures obscured detection of this trend (Cunning and Baker 2012).

Corals in some areas are routinely exposed to temperatures that far exceed typical bleaching thresholds elsewhere. For example, corals in the Arabian Gulf commonly experience temperatures >34°C without bleaching (Coles et al. 1988). This indicates that chronic exposure to higher temperature, can result in acclimatization to higher temperatures. However, several studies have found contrasting effects of preconditioning on coral thermotolerance, with some studies finding that preconditioning did not increase stress tolerance, and in some cases even lowered it, as we documented here. For example, corals that were pre-exposed to cold stress (15°C) were less fit during future cold stress than corals which were not pre-exposed, possibly due to decreased metabolic reserves or sub-lethally damaged cells and proteins (Saxby 2001). Similarly, *Acropora millepora* preconditioned to heat for three months did not lose fewer symbionts when exposed to thermal stress, although preconditioned corals did show higher non-photochemical quenching ability (Middlebrook et al. 2012). Moreover, no additional thermotolerance was acquired by *A. millepora* transplanted to a chronically high temperature site 6 months prior to acute thermal stress, unless they also experienced changes in *Symbiodinium* communities to favor *Symbiodinium* in clade D (Berkelmans and van Oppen 2006).

In contrast, other studies have shown that corals already living in chronically high-temperature inner lagoon reefs responded to heat stress better than corals living in

lower temperature, outer lagoon areas (Castillo and Helmuth 2005). Similarly, corals living in tropical conditions had a higher thermal threshold than sub-tropical corals (Coles et al. 1976). However, in neither of these studies was *Symbiodinium* identity evaluated, and therefore corals in chronically higher temperatures may have hosted high temperature-specialist symbionts in addition to undergoing potential local genetic adaptation (Jokiel and Coles 1990). Local genetic adaption within the same symbiont type also could have occurred in response to chronic high temperature (Howells et al. 2012), and symbionts can also undergo relatively rapid (months) adaptation under selective pressure for thermotolerant traits in the laboratory (van Oppen 2012). However, the length of time required for Darwinian adaptation to result in higher thermal thresholds in corals or symbionts *in situ* is not known (Coles and Brown 2003).

Some studies have shown that corals pre-conditioned to high (but sub-bleaching) temperature and light levels can avoid bleaching via upregulation of physiological mechanisms to deal with stress (Brown et al. 2002, Middlebrook et al. 2008, Bellantuono et al. 2011). These studies mainly examined acute or short periods (days to weeks) rather than chronic (>3 weeks) acclimation over long time scales (Bellantuono et al. 2011, Middlebrook et al. 2008), as examined in this study. Middlebrook et al. (2008) found a bleaching-avoidance response in corals which had been pre-exposed to heat for two weeks prior to heat stress. Bellantuono et al. 2011 found similar results. However, Middlebrook et al. (2012) examined preconditioning over a longer period (3 months), and found that it did not prevent symbiont loss during future thermal stress, although non-photochemical quenching was enhanced in preconditioned corals. This implies that a 3

month interval may be sufficiently long to negate any potential benefit of thermal preconditioning.

In the current study, acclimation to 29^{o}C was chronic and constant, rather than acute or variable. Earlier long-term studies found increased thermotolerance in corals acclimated to high (28^{o}C) temperatures for a similar amount of time (56 days) prior to heat stress being applied (Coles and Jokiel 1978). This suggests the effect may be species-specific, or that 29^{o}C was more stressful over longer periods of acclimation than 28^{o}C. The difference in the results might also be a result of the prior thermal history of the corals involved, or the genetic identity of the symbionts concerned, which was not examined by Coles and Jokiel (1978). Alternatively, chronic high temperatures may not be as effective as temperature variability in promoting acclimatory responses (Oliver and Palumbi 2011). Thermal variability may allow corals' acclimatory responses to remain active, while avoiding exhausting resources. Studies in American Samoa, for example, found that, corals in more thermally-variable pools had higher photochemical function when exposed to acute heat stress compared to corals from less thermally variable pools, even though the mean temperature was the same (Oliver and Palumbi 2011). Reefs in the western Indian Ocean with variable thermal regimes have also been shown to also have lower bleaching incidence relative to areas with relatively constant temperatures (McClanahan et al. 2007, Baker et al. 2008, Carilli et al. 2012). In this study, the lack of variability and the constantly elevated temperatures for several months may have limited corals' ability to deal with heat stress.

In addition to possible chronic thermal stress, preconditioning could also lead to lower bleaching thresholds if the symbiont mitotic index (MI), or the proportion of

symbionts undergoing cytokinesis within the symbiosome, increased at higher temperatures. Increased temperature has been shown to increase symbiont MI (Bhagooli and Hidaka 2002, Strychar et al. 2004), and the faster repopulation and recovery of symbionts at 29°C compared to those at 24°C (See Chapter 3) suggests a higher proliferation rate of symbionts at 29°C. Higher MIs have been correlated with lower bleaching thresholds in corals (Wooldridge 2012). It is therefore possible that conditioning to higher temperatures increased *Symbiodinium* MI and resulted in a more severe bleaching response than corals acclimated to lower temperatures, even when accounting for differences in total symbiont density.

The decreased performance of corals acclimated to 29°C, when exposed to stress, compared to those at 24°C, highlights the importance of time scales involved in coral stress response. Physiological mechanisms occur faster (minutes to days, Yakovleva et al. 2004) than algal symbiont community changes (weeks to months, see Chapter 3), and therefore may help corals survive during stress events. However, physiological changes, once activated, may also be shorter-lived (days to weeks, C. Downs, pers. comm.) than symbiont community changes, which typically revert back to pre-stress symbiont communities after months to years (Toller et al. 2001, Berkelmans and van Oppen 2006, Thornhill et al. 2006a, LaJeunesse et al. 2009, see Chapter 3). Therefore symbiont community changes might provide durable thermotolerance over longer periods of time. Finally, genetic adaptation is generally slow (years to decades, although it may be faster for the symbiont than the host), by may provide more permanent thermotolerant traits. When considering conservation for corals in warmer oceans, opportunities for intervention and enhancement of thermotolerance exist at each time scale: helping corals

to survive during bleaching, enhancing or promoting symbiont community changes, and protecting or transplanting therotolerant genotypes.

This study demonstrates that corals can acquire thermotolerance over a period of three months by changing symbiont communities following recovery from a bleaching event. Prior exposure to acute heat stress for 10 days, or chronic high temperature exposure for three months appears to provide minimal, if any, increase in bleaching resistance, if not also associated with *Symbiodinium* community change. Increased thermotolerance was primarily a consequence of alterations in the algal symbiont community as a result of disturbance, and not a result of physiological acclimatization due to past high temperature exposure, either acute or chronic.

Although additional work is needed to understand the implications, both positive and negative, for corals hosting clade D, corals dominated by these symbionts do allow corals to avoid symbiont loss during high temperature exposure, and disturbance during bleaching does appear to promote relatively rapid, acquired thermotolerance via symbiont community changes. There may also be an additional cost to chronic exposure to warm temperatures, even if bleaching is not observed. Therefore, corals living in consistently warmer oceans, as is predicted to occur under climate change conditions, may be more susceptible to stress.

Figures.

Figure 4.1. Schematic showing experimental design. All corals were acclimated to 24°C for two months prior to the start of the experiment. Corals were then either not bleached and remained at (1) 24°C or (2) were placed at 29°C, (3) bleached with DCMU at 24°C, or (4) bleached with heat at 32°C for 10 days. Corals were then allowed to recover at either 24°C or 29°C for three months. After recovery, half of the corals from each treatment group were exposed to a second heat stress event (32°C for 10 days), identical to the first one. Corals were again allowed to recover at either 24°C or 29°C for three months. Outer boxes represent the tank temperature: light blue backgrounds represent corals at 24°C, while dark blue boxes represent corals at 29°C. Orange boxes represent periods of heat exposure for 10 days (32°C), the green box represented DCMU-bleaching. Stress exposure periods are shown in grey boxes.

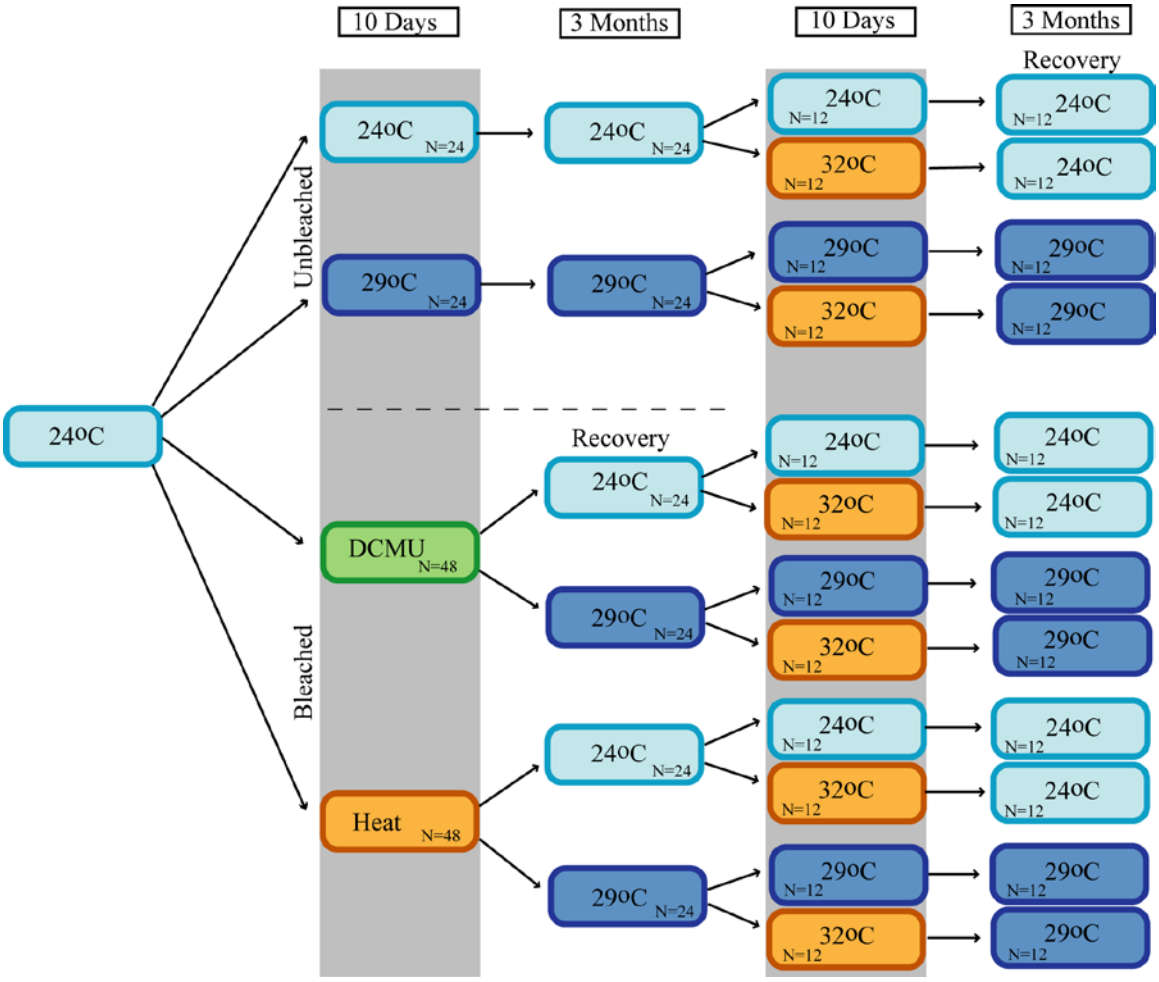


Figure 4.2 Maximum potential quantum yield (Fv/Fm) of *Symbiodinium* in experimental corals. Declines in Fv/Fm are typically indicative of photodamage. Orange shading indicates the 10-day period of stress exposure, white areas indicate the subsequent 6-month recovery period. Unbleached corals are shown in blue (dark blue: 29°C, light blue: 24°C); heat-bleached corals are shown in orange (29°C) and yellow (24°C); DCMU-bleached corals are shown in green (dark green: 29°C, light green 24°C). Error bars represent standard error. 2a) Mean Fv/Fm values depicting all treatments during the both the first and the second bleaching events. Corals dominated by clade C experienced declines in Fv/Fm values after 10 days at 32°C during both thermal stress events, similar to results from the initial heat stress period. 2b) Enlargement of Fv/Fm values for the second stress exposure period. 2c) Change in Fv/Fm values during the second 10-day stress exposure, standardized to initial values (i.e., all starting values are zero).

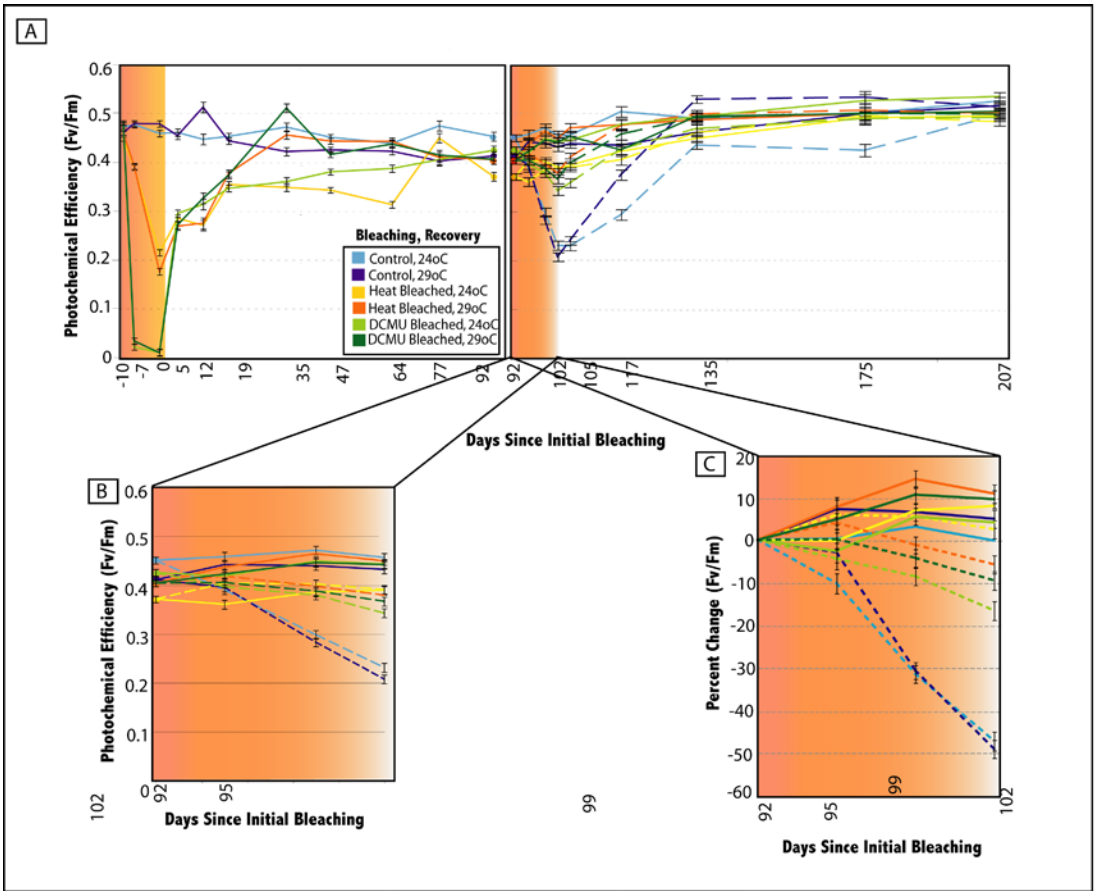
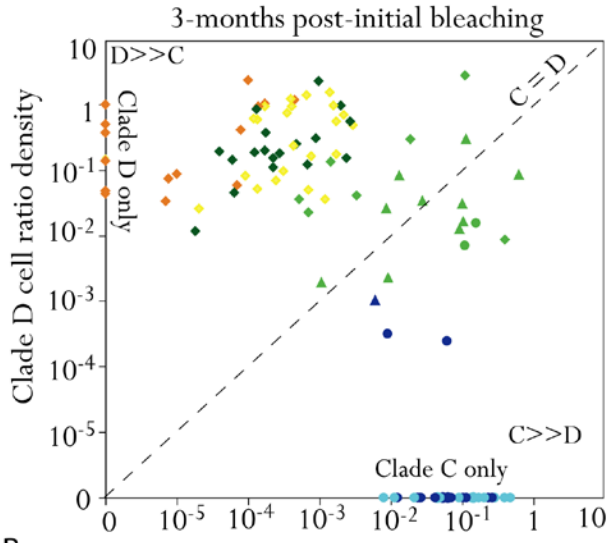
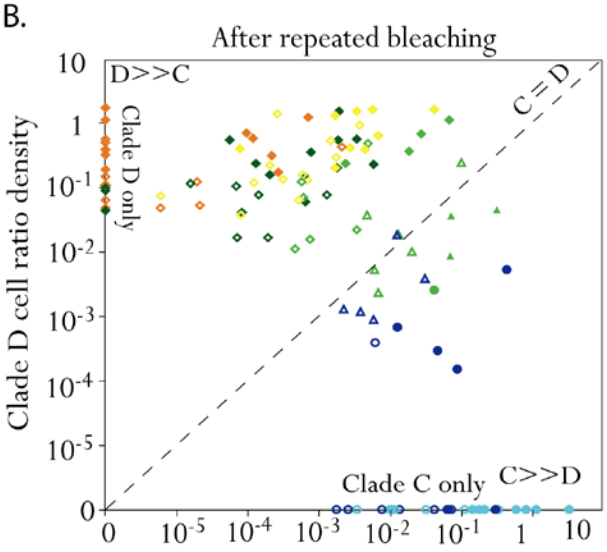


Figure 4.3 Cell ratio densities of clades C and D symbionts. Clade C-dominated corals are shown by circles, clade D-dominated corals by diamond markers, and corals with mixed-clade communities by triangles. Open markers were exposed to 32^{o}C, while solid markers remained at control temperatures. Unbleached corals are shown in blue (dark blue: 29^{o}C, light blue: 24^{o}C); heat-bleached corals are shown in orange (29^{o}C) and yellow (24^{o}C); DCMU-bleached corals are shown in green (dark green: 29^{o}C, light green 24^{o}C). A. Symbiont communities three-months after the initial bleaching (at the start of this experiment). B. Symbiont community compositions after 10-days exposed to 32^{o}C (open markers), or 10-days at recovery temperatures (solid markers). C. Symbiont community compositions three months after the second stress exposure. The dotted line represents equal amounts of clades C and D in a sample.

A.


3-months post-initial bleaching
D>>C
C = D
Clade D only
C>>D
Clade C only
Clade D cell ratio density
10
1
10-1
10-2
10-3
10-4
10-5
0
0
10-5
10-4
10-3
10-2
10-1
1
10

B.


After repeated bleaching
D>>C
C = D
Clade D only
Clade C only
C>>D
Clade D cell ratio density
10
1
10-1
10-2
10-3
10-4
10-5
0
0
10-5
10-4
10-3
10-2
10-1
1
10

C.

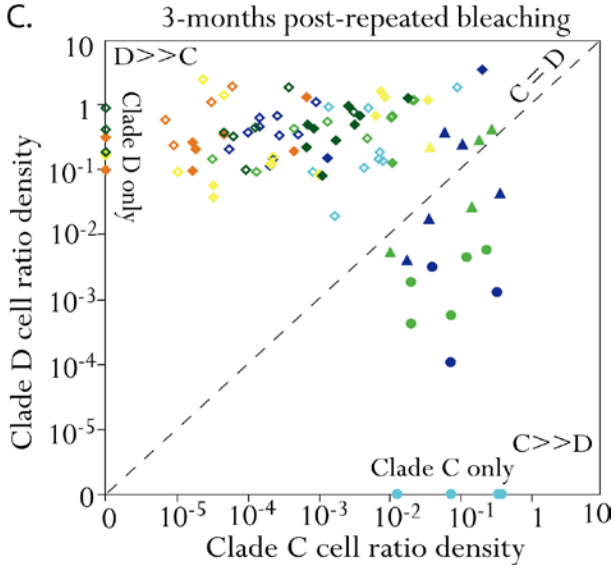

3-months post-repeated bleaching
D>>C
C = D
Clade D only
C>>D
Clade C only
Clade D cell ratio density
Clade C cell ratio density
10
1
10-1
10-2
10-3
10-4
10-5
0
0
10-5
10-4
10-3
10-2
10-1
1
10

Figure 4.4 ANCOVA showing the symbiont density changes during 10-day thermal stress between clade D (red) and clade C (green) dominated corals. Corals hosting clade D symbionts lost fewer symbionts than corals hosting clade C symbionts (P=0.001, R^2=0.52). Corals also lost more symbionts when total symbiont density was higher, regardless of symbiont type (P<0.0001).

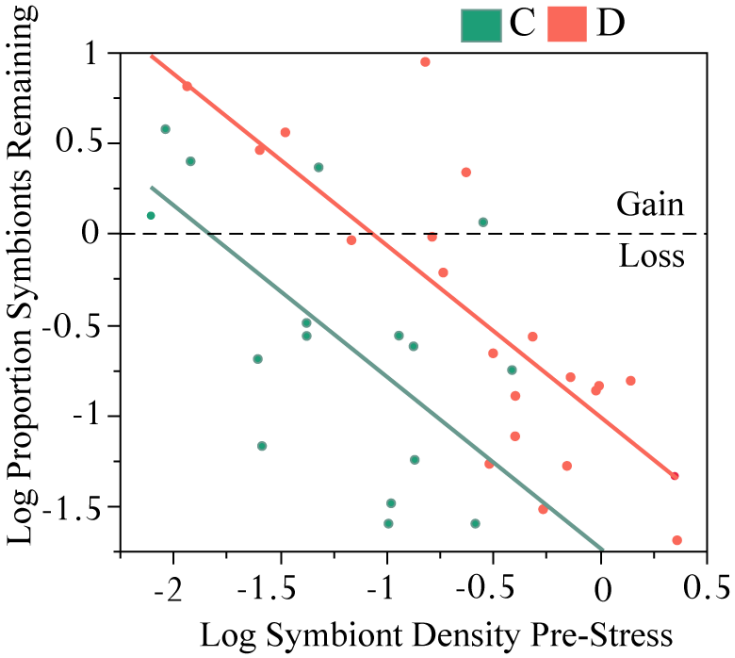


Figure 4.5 The proportion of symbionts lost during repeated heat stress. Corals shown here were all dominated by clade D, and were initially bleached with heat (orange) or DCMU (green). The loss of symbionts between these two groups was not significantly different (P=0.06), and both groups lost more symbionts when initial symbiont densities were higher (P<0.0001). Previous exposure to heat, as opposed to bleaching at low temperatures, therefore did not help corals to avoid symbiont loss during future heat stress.

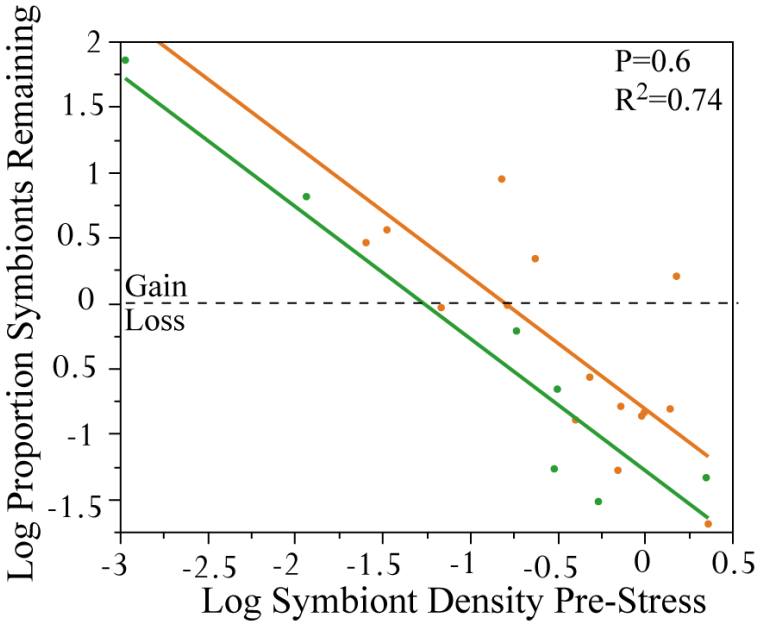
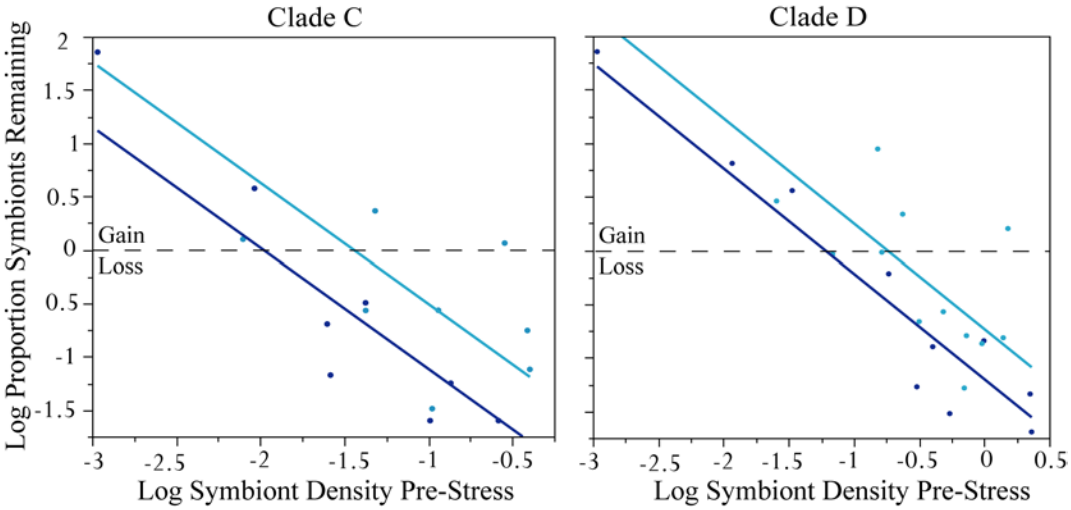


Figure 4.6 The change in total symbiont community density during thermal stress between corals acclimated to either 24°C (light blue) or 29°C (dark blue). For corals dominated by clade C, there was no significant effect of acclimation temperature (P=0.07). There was a significant interaction (0.02). For clade D-dominated corals, corals acclimated to 29°C lost more symbionts than those at 24°C (P=0.04). Corals with higher initial symbiont densities lost more symbionts during stress (P<0.0001). There was no significant interaction term (P=0.32).



Chapter 5:
The effect of incremental temperature change on algal symbiont community structure and function in corals: A comparison of upper and lower thermal limits

Summary

Climate change is predicted to expose corals to chronic, as well as acute, temperature stress. Here, the effect of gradual increases and decreases in temperature on the symbiosis between the Caribbean coral *Montastraea cavernosa* and different algal symbionts (*Symbiodinium* C3 and D1a) was studied experimentally. We used corals of the same genotype which were either dominated by C3 or D1a and acclimated them for 6 months to either 24°C or 29°C. Corals acclimated to 24°C were then cooled (-1°C/week) to 15°C over a period of 9 weeks, while corals acclimated to 29°C were then warmed (+1°C/week) to 35°C over a period of 6 weeks. Corals' symbiont community structure and function were monitored using quantitative PCR (qPCR) and Imaging Pulse Amplitude Modulated (I-PAM) fluorometry, respectively. More D1a symbionts remained in symbiosis than C3 symbionts at both temperature extremes. During cooling, > 90% of C3 symbionts were lost after exposure to 15°C for 1 week, while clade D symbionts were not lost. However, despite fewer symbionts lost, D1a symbionts had equivalent or lower photochemical efficiency than clade C3 symbionts at all temperatures below 24°C. During heating from 29°C to 33°C, corals dominated by C3 lost >90% of their symbionts, and remaining symbionts were photochemically impaired. In contrast, corals dominated by D1a did not experience symbiont loss or photochemical impairment until temperatures exceeded 33°C. However, at temperatures >34°C, both symbiont types experienced high symbiont losses and photodamage. These data suggest that, when

controlling for coral genotype, the upper thermal threshold of D1a in *M. cavernosa* is at least 1-2^{o}C greater than C3, and the lower thermal threshold is at least 2^{o}C below C3. As corals continue to face thermal extremes during climate change conditions, hosting D1a symbionts may help corals increase their thermotolerance, but limits to compensation still exist.

Background

While corals have survived changing environments over geological time (Veron 1995), current climate change-induced alterations in ocean temperature and chemistry may be too rapid for coral acclimatization (Hoegh-Guldberg et al. 2008). In addition to other perturbations, climate change is predicted to produce both acute as well as chronic alterations to sea surface temperature, and to potentially produce extreme high and possibly low temperature conditions. Corals have been shown to experience bleaching, or the breakdown of coral-algal symbiosis, from both warm (Hoegh-Guldberg et al. 1999) and cold (Saxby et al. 2003) thermal anomalies. Corals in some areas could face bleaching stresses induced by both high summer temperatures and by low winter temperatures (Hoegh-Guldberg and Fine 2004, Lirman et al. 2010). In 2010, corals in Florida experienced both one of the coldest winters and the hottest summers on record (Roth et al. 2012). Corals must therefore be able to survive both high and low temperature extremes, in particular on high latitude reefs.

As oceans continue to warm and high temperature-induced bleaching becomes more frequent, corals globally may have begun to shift to hosting more symbionts within clade D (Baker et al. 2004), which is known to include thermotolerant members (e.g.,

ITS-2 type D1a). However, while these symbionts may be tolerant of high temperatures (see Chapter 3, 4 and LaJeunesse et al. 2009), their tolerance to low temperature conditions has not been directly studied (but see LaJeunesse et al. 2008, 2010, and McGinley et al. 2012 for a study of ITS-2 type D1 in *Pocillopora damicornis* during cold stress). If D1a symbionts are less able to tolerate cold temperatures, corals that host symbiont communities dominated by D1a may be more vulnerable to periods of cold stress. In this study, we examine chronic temperature stress at both the upper and lower thermal limits for corals, over a period of several weeks, focusing on how past thermal history and symbiont community identities might expand or contract corals' potential temperature ranges.

High temperature stress has been implicated in most mass-bleaching events to date (Glynn et al. 1996). Many tropical corals already live near their upper thermal thresholds, and increases of 1-2^{o}C above the local mean thermal maximum can induce widespread coral bleaching and mortality if prolonged (Mayer 1914, Coles and Brown 2003). Mean global sea temperatures are projected to increase by 1.1-6.4^{o}C over the current century (IPCC, 2007), and El Niño conditions are predicted to become more severe (Urban et al. 2000). Therefore, to avoid frequent episodes of mass bleaching, corals will require an increase in thermal tolerance of 0.2-1^{o}C per decade (Donner et al. 2005).

Corals vary in their thermal thresholds depending on local temperature regime, suggesting possible mechanisms for increasing temperature limits (Hughes et al. 2003, Berkelmans and van Oppen 2006). These include physiological acclimatization (e.g., Brown et al. 2002), changes in bacterial symbiont communities (e.g., Reshef 2007,

Gilbert et al. 2012), and algal symbiont community shifts (e.g., Baker et al. 2004, Berkelmans and van Oppen 2006). For example, Pacific acroporid corals that changed to hosting clade D *Symbiodinium* following transplantation to a warmer environment increased their thermal tolerance by 1-1.5^{o}C, compared to transplanted conspecifics that still contained members of clade C (Berkelmans and van Oppen 2006). Similarly, the Caribbean coral *Montastraea cavernosa* reduced its bleaching response during a 10-day exposure to 32^{o}C after changing from hosting C3 symbionts to being dominated by D1a symbionts (see Chapters 3 and 4).

Coral bleaching due to low temperatures has also been shown to occur (Steen and Muscatine 1987, Saxby et al. 2003, Hoegh-Guldberg 2005, LaJeunesse et al. 2008, 2010), and its relevance has recently increased due to the high coral mortality during the 2010 Florida cold temperature event, and due to predictions about the increasing frequency and severity of climate events such as La Niña (which bring cooler conditions, Urban et al. 2000). During the January 2010 cold snap in the Florida Keys, temperatures reached 12^{o}C, resulting in widespread coral bleaching and eventually 11.5% coral mortality (compared to 0.5% mortality recorded even in the summer of 2005, when warm-water bleaching was widespread, Lirman et al. 2011). Previous reports of cold-induced coral mortality in the Florida Keys were reported in the winters of 1969-1970, 1976-1977, and 1980-1981 (Kemp et al. 2011). These reports demonstrate that cold exposure on the Florida Reef tract recurs, but may be sufficiently infrequent to make acclimatization difficult for corals.

Cold bleaching appears physiologically similar to heat stress because it triggers reduced Photosystem II quenching **(**Hoegh-Guldberg et al. 2004), decreased Fv/Fm

values (Kemp et al. 2011), and symbiont loss (Saxby et al. 2003, LaJeunesse et al. 2008, 2010). Photosynthetic impairment during cold stress was found to be light-dependent, similar to stress observed during high temperature exposure (Saxby et al. 2003). Corals exposed to both heat stress and cold stress experienced decreased growth and symbiont densities (Roth et al. 2012). Several studies have also reported observations of cold stress causing host cell detachment, the formation of pellets of loose host cells, and eventual expulsion propelled by cilia and muscle contraction (Hoegh-Guldberg et al. 1989, Gates et al. 1992).

Several studies have examined the responses of different clades of *Symbiodinium* to high temperature stress, and have found that certain members of clade D are more thermotolerant than other symbiont types (Rowan et al. 2004, Berkelmans and van Oppen 2006, Jones et al. 2008, but see Abrego et al. 2008). However, relatively few studies have examined the responses of different symbiont types to low temperature events. ITS2 type B2 symbionts in culture were found to be most resilient to cold stress (Thornhill et al. 2008), and in *Pocillopora damicornis,* colonies dominated by D1 survived cold stress better than those dominated by clade C1b-c symbionts (LaJeunesse et al. 2010). However, this is the first study in which corals of the same genotype, but with different symbiont communities, are compared during decreasing, as well as increasing, temperature stress. From this experimental design, we hope to control for host genotypic effects and determine thermal tolerance limits for *M. cavernosa* hosting either *Symbiodinium* C3 or D1a.

Incremental temperature change may also produce different physiological responses than acute changes (see Chapters 3 and 4). Because incremental changes are

usually slower than acute changes, corals may have a chance to acclimatize, or undergo compensatory changes in metabolic function in response to environmental variation (Gates and Edmunds 1999). For example, corals can activate or upregulate some cellular processes or pathways to deal with temperature change (such as the production of antioxidants and heat shock proteins, or photoacclimation involving non-photochemical quenching) when preconditioned to sub-bleaching temperature stress (Brown et al. 2002, Middlebrook et al. 2008, Bellantuono et al. 2011). However, coral bleaching due to acute, extreme thermal "shock" (both hot and cold) likely occurs because corals' acclimatization mechanisms are overwhelmed, resulting in damage to host cells, often leading host cell detachment (Gates et al. 1992). This mechanism may lead to colony mortality rather than recovery (Gates et al. 1992). Jokiel and Coles (1977), for example, reported high mortality when corals were exposed to temperatures 4-5^{o}C over their typical summer maxima for 1-2 days. Corals also bleached at lower temperatures when exposure was prolonged, but this type of stress resulted in less mortality (Coles and Jokiel 1978). Here, we studied the response of coral-algal symbioses during a period of increasing or decreasing temperatures (+/- 1^{o}C/week) to test the effect of gradual temperature change on corals and their diverse symbiotic communities. By including corals of the same genotype with different thermal histories and dominant symbiont clades, the effect of coral genotype can be controlled for, and the influence of past thermal history and symbiont community composition can be examined. The goal of the current study was to identify upper and lower bleaching thresholds for C3 and D1a, and to compare associated photochemical impairment between these types, while controlling for host genotypic effects.

Methods

Production of coral clones containing different symbionts

To produce coral cores of the same genotype with different stress histories and algal symbiont community composition, six months prior to the start of this experiment, corals were either: 1) bleached with heat (32°C for 10 days, N=24), 2) bleached with DCMU (450µg/L for 10 days at 24°C, N=24) or 3) not bleached (control, N=24). Corals were then allowed to recover for three months at either 24°C or 29°C. Corals that had bleached from both heat and DCMU recovered with *Symbiodinium* communities dominated by D1a, while corals which were not bleached remained dominated by C3 (see Chapter 3). After recovery, half of the corals from each treatment group (N=12) were exposed to a second bleaching event (32°C for 10 days), while the other half remained in recovery conditions (24°C or 29°C). After 10 days, all corals were allowed to recover at their initial recovery temperatures for a further three months (see Chapters 3, 4). Unbleached corals acclimated to 29°C for six months also increased their abundance of clade D symbionts, although not to same levels as corals recovered from bleaching (see Chapter 3). These treatments resulted in corals of the same genotype that had different thermal and bleaching histories, and algal symbiont communities that were dominated by different *Symbiodinium*.

Experimental set-up

Corals were maintained under conditions described in Chapter 3. Corals (including heat-bleached, DCMU-bleached, and unbleached controls) that had been

maintained at 29°C for the previous six months were warmed to 35°C in two replicate tanks at a rate of 1°C per week (i.e., a 6-week warming period with corals spending the final week at 35°C). In addition to these corals (most of which were dominated by D1a), unbleached corals (that had been previously maintained at 24°C for 8 months, and which contained only C3 symbionts) were also added to the warming treatment (N=11).

Corals that had been maintained at 24°C for the previous 3 months were cooled to 15°C in two replicate tanks at a rate of 1°C/week (i.e., a 9-week cooling period with corals spending the final week at 15°C).

Imaging Pulse Amplitude Modulated (I-PAM) fluorometry was used to measure the maximum quantum yield (dark adapted Fv/Fm, photochemical efficiency) of *Symbiodinium* in the experimental treatments after 1 week at each temperature. Measurements were taken in the morning before lights were turned on. After I-PAM measurements were completed, photographs were taken under standardized conditions (see Chapter 3). Temperatures were then increased or decreased the following morning after I-PAM readings, before lights were turned on to begin the next day.

In warming treatments, tissue samples were collected for quantitative PCR (qPCR) analysis (see Chapter 3) at the start of the experiment (29°C), after 4 weeks (33°C) and after 6 weeks (35°C). In cooling treatments, tissue samples were collected at the start of the experiment (24°C), after 4 weeks (20°C), after 6 weeks (18°C), and after 9 weeks (15°C). Corals were not sampled at every 1°C temperature increment to avoid excessive damage from repeated sampling.

Analysis

Symbiodinium community structure was determined using an actin-based qPCR assay (Mieog et al. 2009) of *Symbiodinium* clades C and D (Cunning and Baker 2012) and the coral host (see Chapter 3). Symbiont densities for each clade were calculated as the ratio of the number of cells of each *Symbiodinium* clade to the number of cells of the coral host, and were expressed as a symbiont to host cell ratio (S:H, Mieog et al. 2009, Cunning and Baker 2012). The relative abundance of each clade was obtained by correcting for the number of copies of the targeted actin locus in each *Symbiodinium* cell (50 for C3 and 3 for D1a, see Chapter 3). S:H ratios were log-transformed, and maximum quantum yield (Fv/Fm) data were arcsine-transformed prior to analysis. Models were fitted using JMP (9.0.2, SAS), one-way ANOVAs, MANOVAs and ANCOVAs, followed by Tukey's HSD tests. Matching pairs t-tests were used to compare within-clade abundance between time points. An alpha value of 0.05 was used for all tests. Between 7.0 and 16.2% of samples from each warming time point, and between 4.6 and 37.5% of samples from each cooling time point were not included in analysis either because (1) they were statistical outliers (i.e., were more than two standard deviations away from the mean), (2) the replicate wells did not agree within 2-3 Ct's (even after re-amplification), or (3) the samples did not amplify (even after re-extraction).

As a result of prior bleaching and recovery, experimental corals had different thermal histories, despite being the same genotype (see Chapters 3 and 4). At the start of this experiment, most corals (87.5% in cooling treatment and 93.1% in warming treatment) were >90% dominated by either clade C or clade D symbionts (Figure 5.3). The effect of past thermal history was evaluated using ANOVA and MANOVA in both

heating and cooling experiments. Sensitivity analysis was conducted in order to determine an appropriate threshold levels for low-abundance (<10%) symbionts in corals considered "dominated" by a single clade (i.e., a threshold below which background symbionts did no affect overall community function). Corals were then pooled either by dominant clade ("C" or "D"), or were designated as mixed communities (not dominated by either clade, "C+D") for analysis.

Results

Incremental Cooling

Effect of previous stress exposure and background clades during cooling

Previous stress exposure (see Chapters 3 and 4) did not significantly influence changes in symbiont densities (P-values at all time points for all clades were >0.2 for symbiont loss). However, because previous stress exposure resulted in dominance by clade D, the effect of past thermal history could only be tested in clade D-dominated corals (i.e., there were no clade C-dominated colonies that had been previously stressed). The number of C+D samples were too low (N=8 of 44) to be robustly analysed. Corals initially bleached with heat had the least amount of background clade C (unbleached: 3.6±0.9%, DCMU: 3.1±1.6%, heat: 0.4±0.2% clade C; P=0.0085), but the same amount of clade D symbionts (P=0.91). Stress exposure 3-months prior to this experiment did not alter the amount of C or D present in D-dominated corals (P>0.67). There was no interaction between the stress exposures 3- and 6-months prior to this experiment (P>0.6). Similarly, there was no difference in photochemical efficiency between corals that had bleached once, and those that had bleached twice (P=0.30).

However, the photochemical efficiency of clade D-dominated corals during cooling was significantly higher in the corals that had not bleached during the first exposure (6-months prior), compared to those which had bleached either with DCMU or heat ($P<0.0001$, Figure 5.1c). This effect was dependent on the threshold set to define background symbionts. When the threshold for background clade C symbionts was ≤4%, these groups (unbleached, DCMU-bleached, and heat-bleached) were no longer significantly different from one another ($P=0.19$, Figure 5.1b). This suggests the effect of past thermal history may be an artefact of high background levels of clade C symbionts in D-dominated corals, rather than an effect of past bleaching history (Figure 5.1b & 5.1c). Because clade C symbionts had higher photochemical efficiency than clade D symbionts during cooling, these background clade C symbionts may have increased the overall Fv/Fm values in initially-unbleached corals (Figure 5.1b). Since background clade C symbionts explained the effect of first stress exposure of photochemical efficiency, we pooled our analysis by dominant clade (>90%) rather than by past thermal history. C-dominated corals with background D were rare (only 4 of 8 colonies, ranging from 0.8% to 3.6% clade D). Therefore this analysis was not conducted on clade C-dominated corals. Coral genotype was not a significant factor in any tests.

Symbiont community function during cooling

All symbionts showed declining Fv/Fm values during cooling, and at 23^{o}C and 21^{o}C, symbionts of both clades had equivalent Fv/Fm values (Figure 5.1). Figure 5.1 represents Fv/Fm change over time based on designations of "C", "D" or "C+D" corals at the initial time point, and corals did not switch categories over time, even if their symbiont communities changed. At temperatures below 21^{o}C, however, corals dominated

by clade C symbionts had equal ($P>0.08$), or significantly less, photodamage ($P<0.04$) than D-dominated corals. Symbionts in mixed communities (C+D) had intermediate Fv/Fm values that were in between those of corals dominated by either C and D symbionts.

Symbiont community structure during cooling

Symbiont loss in response to cooling was most severe in corals with initially higher densities of symbionts ($P<0.0001$, Figure 5.5a). Symbiont loss between clades was therefore analyzed using ANCOVA, with pre-cooling symbiont density as the continuous covariate (Figures 5.5a, 5.6a, 5.7a). Clade C-dominated corals lost ~60% of their original population when cooled to 18^{o}C (6 weeks), and >98% at 15^{o}C (9 weeks, Figure 3a, 6a, 7a). *Symbiodinium* C3 were lost in higher proportions than D1a at 21^{o}C and 15^{o}C (Figure 5.3a, 5.5a, 5.6a, 5.7a), despite clade C showing higher photochemical efficiency at low temperatures (Figure 5.1). Clade D-dominated corals retained >90% of their symbiont population at 21^{o}C and 18^{o}C, and actually increased in density at 15^{o}C, although this increase was not significant (Figure 5.6a, 5.7a). C+D corals had symbiont densities that were intermediate between C- and D-dominated corals, and which steadily declined at cooler temperatures. No mortality was observed in any colonies.

Incremental Warming

Effect of previous stress exposure during heating

Previous stress exposure (see Chapters 3 and 4) did not affect either the decline in symbiont density or photochemical efficiency at any time point (P-values at all time points for all clades were >0.2 for symbiont loss and >0.05 for photochemical efficiency,

ANOVA and Tukey's HSD). However, because previous stress exposure always led to dominance by clade D, this could only be tested in clade D-dominated corals (i.e., there were no clade C-dominated colonies that had been previously stressed). Sample sizes for C+D corals were too low (N=5 of 59) to be robustly analysed. Therefore, we excluded prior stress exposure as a factor in our analysis and pooled all cores by dominant clade, regardless of the stress treatment that resulted in D-dominance.

Effect of background symbiont thresholds during heating

The past thermal history of D-dominated corals resulted in the same amount of clade D symbionts (P=0.91, 6-months prior bleaching; P=0.68, 3-months prior bleaching), but different levels of background clade C symbionts (P=0.001, 6-months prior bleaching; P=0.003, 3-months prior bleaching). There was no interaction between the first and second stress exposures on the amount of clade C present (P=0.24), however there was an interaction between first and second stress exposure on the amount of clade D present (P=0.02). During heating, the initial amount of (background) clade C in D-dominated corals did not affect the relative change in symbiont density (symbiont density change was the same at all threshold levels of background clade C between 0% and 10%, P<0.0001) or the decline in photochemical efficiency (relative change in Fv/Fm was the same at all threshold levels of background clade C between 10% and 0%, P=0.02). We therefore used a 10% background clade threshold for defining D-dominated or C-dominated corals.

However, any effect of background clades on the total community function during heating may be masked, because corals that were dominated by clade D contained very few clade C symbionts (53 of 54 coral cores contained <2% clade C, and just one

core had 5.4% clade C symbionts), which may have obscured any signal related to background clade C symbionts. Similarly, C-dominated corals with background D were also rare: 13 samples (of 72 total) were clade C-dominated and only 4 of these contained background clade D symbionts (ranging from 0.2% - 7.4%). Therefore, low sample sizes hindered robust statistical analysis of the function or contribution of background clade D symbionts to overall coral performance in C-dominated corals.

Coral genotype was not a significant factor in community structure, except for the symbiont density change between 29^{o}C and 33^{o}C, where two genotypes (of nine) lost fewer clade D symbionts than one other genotype. The remaining 6 genotypes were not significantly different from either group.

Symbiont community function during heating

While clade C symbionts showed a reduction in Fv/Fm values at all temperatures >29^{o}C, clade D-dominated corals actually showed increased Fv/Fm values at 30^{o}C ($P<0.0001$). However, after 1 week at 31^{o}C, clade D symbionts began a slight decline, and were significantly lower than initial Fv/Fm values by 1% ($P=0.007$, Figure 5.2). Figure 5.2 shows Fv/Fm change over time based on designations of "C", "D" or "C+D" corals at the initial time point, and corals remained pooled by these initial categories during the experiment, regardless of any subsequent change in symbionts (i.e., if C+D corals lost clade C symbionts and became D-dominated during heating, they remained in the C+D category for the purposes of analysis). The Fv/Fm values for all symbionts' declined steadily with continued heating. Clade D symbionts showed higher Fv/Fm values than clade C-dominated corals at all temperatures up to 33^{o}C ($P<0.0001$, Figure 5.2). At temperatures >33^{o}C, both clade C- and D-dominated corals had the same

relative change in Fv/Fm values (P>0.1, Figure 5.2). After 1-week exposure to 35°C, 54% of corals of both symbiont types had no detectable photochemical activity (i.e., Fv/Fm=0; 60% of C-dominated corals, and 51.7% of D-dominated corals). After 1 week at 35°C, several corals (N=11), all of which had not been previously bleached, also had no detectable *Symbiodinium* by qPCR analysis.

C+D corals had Fv/Fm values that were intermediate between clade C- and D-dominated corals. Fv/Fm values of C+D corals were equivalent to those of both C- and D-dominated corals at all temperatures, except 30°C and 32°C, when Fv/Fm values of C+D corals were equivalent to clade D-dominated corals, but higher than those of clade C-dominated corals.

Symbiont community structure during heating

Corals with greater proportions of clade D symbionts lost fewer symbionts overall (Figure 5.4b). Symbiont loss was dependent on initial symbiont density, with corals containing higher densities of symbionts prior to warming losing more symbionts in response to warming (P<0.0001, Figure 5.5b). Clade D-dominated corals lost a greater proportion of their symbiont communities (P<0.0001), but this same trend was not observed in clade C-dominated corals, likely because of the severe symbiont loss observed in all corals (P=0.12). Therefore, symbiont loss within each clade was analysed over a density gradient (Figure 5.5b).

Clade C symbionts were lost in greater proportion than clade D symbionts at all elevated temperatures (30-35°C, P<0.0004, 5.5b, 5.6b, 5.7b). Accounting for the effect of symbiont density, clade C dominated corals lost >99.9% of their initial symbionts after one week at 33°C, and >99.99% of their initial symbionts after one week at 35°C (Figure

5.6b, 5.7b). In contrast, clade D dominated corals lost only 9.5% of their symbionts after 1 week at 33°C, and >88% of after 1 week at 35°C (Figure 5.6b, 5.7b). Together, these data indicate that *Symbiodinium* D1a has higher photochemical efficiency between 29°C and 33°C compared to C3, but this effect may be lost at temperatures >33°C. However, at D1a symbionts are lost less frequiently than C3 symbionts during both heating and cooling.

All corals of one genotype ("Genet 4", N=6) died after one week at 33°C, regardless of the individual core's prior thermal history or *Symbiodinium* community composition. Four cores of a second genotype ("Genet 5") and one core of "Genet 6" died after 1 week at 34°C (See Chapter 3, Table 3.2, for list of genotypes, Serrano et al. *in prep*). No other mortality was observed.

Discussion

The effects of cooling on *Symbiodinium* community structure and function

Corals dominated by *Symbiodinium* C3 had equal or greater photochemical efficiency at all temperatures <24°C, compared to those dominated by D1a (Figure 5.1). This higher photochemical efficiency during cooling was a feature not only of clade C-dominated corals, but was also observed in clade D1a-dominated communities that contained ≥4% background C3 symbionts. These data suggest that, even at relatively low-abundance, symbionts (in this case C3) can contribute to overall photochemical function during stress.

C3 and D1a symbionts may differ in response to cooling as a result of differences in enzyme efficiency or membrane fluidity. Decreased enzymatic activity leads to an

over-reduction of the light reactions, damage to PSII, increased ROS production (Saxby et al. 2003) and eventual coral bleaching (Lesser et al. 1996, Weis 2008). *Symbiodinium* D1a symbionts produced less ROS during thermal stress (McGinty et al. 2012, type D1), but may be less efficient than other symbiont types, translocating less carbon to their coral hosts (Cantin et al. 2009), and leading to slower coral growth (Little et al. 2004, Cunning et al. *in prep*.). Possibly, these symbionts are particularly susceptible to decreased enzyme activity, although other factors could also contribute to a lack of photosynthate transfer as well. Membrane fluidity has been shown to be a critical determinant of heat tolerance in symbionts (Tchernov et al. 2004) is also thought be important in cold tolerance (Thornhill et al. 2008). Differences in the lipid composition of chloroplast thylakoid membranes appear to determine membrane stability to thermal stress, with thermotolerant symbionts (including some members of clade D) having been shown to have higher levels of lipid saturation (Tchernov et al. 2004). Future work should compare thylakoid membranes from different symbiont types under low temperature stress to determine physiological mechanisms for cold tolerance.

Despite having equal or greater Fv/Fm values at all temperatures $<24^{o}$C, C3 symbionts were still lost at a higher rate than D1a symbionts (Figure 5.5a, 5.6a, 5.7a). This suggests that symbiont expulsion may be decoupled from photochemical impairment. Clade D symbionts may resist eviction or may avoid detection by host immune systems, even when impaired (Weis 2008), possibly due to less ROS production (McGinty et al. 2012). Moreover, over seasonal timescales, clade D-dominated, high latitude *Pavona* corals outperformed clade C-dominated corals in both cool and warm temperatures (Suwa et al. 2008). Similarly, *Pocillopora damicornis* dominated by D1

symbionts had higher survivorship than conspecifics dominated by C1b-c during a cold bleaching event (LaJeunesse et al. 2010).

In this study, no mortality was observed after 1 week at 15°C, although temperatures below 15°C were not tested. The lack of mortality at 15°C, and lack of symbiont loss in clade D-dominated corals, suggests that *M. cavernosa* may be able to survive at even lower temperatures than 15°C. While chronic heating reached a temperature at which photosynthesis largely ceased (between 34°C and 35°C), the thermal minima for these symbionts is likely below 15°C. However, there is likely species-level variation in thermal tolerance (Muscatine et al. 1991, Kemp et al. 2011). Muscatine and colleagues (1991) observed no symbiont eviction from *M. cavernosa* even at extreme low temperatures (12°C), unlike in *Seriatopora* and *Acropora*, which both lost half of their symbionts after 4 hours at 14°C and 12°C. Similarly, short-term exposure to 12°C resulted in partial mortality in some species such as *Montastraea faveolata* and *Porites astreoides*, while other species, such as *Siderastrea siderea,* recovered readily (Kemp et al. 2011).

The effect of warming on *Symbiodinium* community structure and function

Corals that were dominated by D1a symbionts had a higher thermal tolerance than corals that were dominated by C3 symbionts (Figure 5.2, 5.4b, 5.5b, 5.6b, 5.7b). Severe photodamage and symbiont loss was observed in C3 symbionts at temperatures >29°C, but in D1a symbionts this was only observed at temperatures >33°C (Figure 5.2). D1a has often been referred to as "putatively" thermotolerant (e.g., Correa et al. 2009b), but few studies, if any, have systematically tested its thermotolerance. Berkelmans and van

Oppen (2006) showed that corals hosting clade D symbionts had higher thermal tolerance than corals hosting clade C symbionts. Higher thermotolerance of D1a symbionts has also been observed in response to repetitive bleaching at 32°C (see Chapter 4).

However, following exposure to temperatures >33°C (34°C and 35°C) the density of both symbiont types declined precipitously, and many corals (54%) registered no photochemical efficiency (Figure 5.4b, 5.5b, 5.6b, 5.7b), indicating a near-complete loss of photochemical function. Therefore, temperatures >33°C appear to exceed the thermal limit for photochemical function for both of the *Symbiodinium* types investigated here. These findings support previous studies of cultured *Symbiodinium*, which indicated collapse of photosynthesis at temperatures of 34°C-36°C (Iglesias-Prieto et al. 1992).

Because experimental cores were not sampled at every 1°C temperature increment (to avoid excessive damage to cores due to repeated sampling), the exact thermal thresholds for symbiont loss (based on qPCR) cannot be determined. Fluorometric data indicates that D1a symbionts have a thermal limit between 1 and 2°C higher than C3 symbionts (Figure 5.2), until 33°C. Considering the qPCR data jointly with the fluorometric data (i.e., using combined metrics of symbiont community structure and function), the thermal threshold for D1a can be estimated to be >33°C and <35°C, while for C3 it is >29°C and <33°C (Figure 5.4b, 5.6b, 5.7b). However, this experiment investigated chronic heating (rather than a discrete thermal anomaly), and this could have resulted in either cumulatively greater total stress for corals, or (if temperature change was sufficiently gradual for acclimatization to occur), less stress overall. Therefore, our threshold estimates could differ from studies that employed discrete thermal challenges to study thermotolerance (Berkelmans and van Oppen 2006, Chapter 4). Regardless, our

results are remarkably similar to the only other estimate of the thermal limits of clade D compared to clade C symbionts, which indicated a 1-1.5^{o}C elevation in the bleaching threshold when corals host clade D (Berkelmans and van Oppen 2006). Future studies should assess thermal thresholds under both acute thermal stress and chronic incremental temperature change, with frequent tissue sampling at each temperature.

This study was unique in its use of the same host genotype with different symbiont communities to examine thermal threshold limits. Although we found no effect of host genotype on symbiont loss or photochemical efficiency, there was a genotypic effect on coral mortality. This highlights the importance of host genotype in predicting coral mortality and resilience, as well as the recognition that differences between individuals, as well as species, will likely play a significant role in determining thermal tolerance. Possibly, some host tissues are more susceptible to ROS stress and damage, or are more prone to host cell detachment responses, which can lead to higher mortality during bleaching. Studies that examine more genotypes of diverse coral species, and which incorporate the role of bacterial symbionts in thermotolerance (Reshef et al. 2007) will help broaden our understanding of thermal limits for coral holobionts.

Tradeoffs between *Symbiodinium* C3 and D1a

The abundance of clade D symbionts on reefs increases following bleaching events and may become more common on reefs as sea surface temperatures continue to warm (Baker et al. 2004, Jones et al. 2008, LaJeunesse et al. 2009). This study shows that D1a symbionts appear to be more resistant to eviction by the host under both heating and cooling treatments. Consequently, rather than viewing D1a as high temperature specialists, it may be more accurate to view C3 symbionts as high efficiency symbionts,

but only within a very narrow temperature range. A tradeoff for corals may therefore exist between symbiont retention during stress and a highly efficient symbiotic partnership during non-stressful conditions.

Recent studies have suggested that coral species with diverse symbioses are "ecological losers" because these corals are more likely to bleach, therefore they conclude that diverse symbioses may be maladaptive (Putnam et al. 2012). However, causality is not established by these data (i.e., did diverse symbioses cause bleaching susceptibility, or did bleaching result in more diverse symbioses?), and this argument only holds if higher rates of bleaching universally lead to higher rates of mortality. Corals with diverse symbionts might bleach more readily because they have greater capacity to recover with more different symbionts, i.e., they have greater functional redundancy in their associations (Baker 2007). In the present study, bleaching and recovery with different dominant symbionts increases the potential range of temperatures for corals, making corals less, rather than more, susceptible to bleaching due to either warm or cool thermal exposure. Future studies should compare survivorship of corals during stress that bleach readily and host multiple symbiont types to those that rarely bleach and infrequently change their dominant symbiont type.

Studies have shown that reef ecosystems are highly susceptible to declines in coral growth rates due to competition with algae and predation that is dependent on size class (Mumby et al. 2007). Therefore slower growth of corals containing clade D symbionts has been suggested as having a net-negative effect on reefs as a whole (Ortiz et al. 2012). However, the growth rate of corals hosting clade D has not been compared at higher temperatures, where clade D is potentially a better-suited symbiont. The

possibility exists that, at higher temperatures, the growth rates of corals hosting clade D will be equivalent, or higher, than corals hosting clade C symbionts (e.g., Smith et al. 2008). Because D1a typically increases in corals after stress, further studies are also needed to distinguish the confounding effects of hosting D1a versus the general metabolic declines of hosts recovering from bleaching (Grottoli et al. 2012).

The potential cost of hosting symbionts with lower efficiency but a wider range of temperature tolerances should also be evaluated in terms of growth, metabolism, disease resistance, and potential reproduction, over a range of temperatures (Stat and Gates 2011). Mixed symbiont communities are typically considered to have higher functional redundancy (Yachi and Loreau 1999), which may help corals maintain critical symbioses during a wider range of environmental conditions (see Introduction). Ultimately, data from this study suggest that coral survivorship may be highest when hosting a diverse and flexible symbiotic community in order to benefit from high efficiency carbon transfer during periods of low environmental stress, as well as increased survivorship during periods of thermal extremes.

Figures.

Figure 5.1 ***Relative percent change in photochemical efficiency (Fv/Fm) of symbiont communities dominated by C3, D1a and C3+D1a during experimental cooling.*** **5.1a:** Lines indicate relative percent change in Fv/Fm values. Red lines indicate corals that were >90% dominated by D1a symbionts, green lines indicate corals >90% dominated by clade C3 symbionts, and purple lines indicate corals that were not >90 % dominated by either C3 or D1a symbionts at the start of heating or cooling. Asterisks indicate a significant difference ($P<0.05$) between C3- and D1a-dominated corals. All corals exhibited decreased efficiency as temperatures decreased, regardless of symbiont community compositon, although C3-dominated corals always showed equal or higher Fv/Fm values compared to D1a-dominated corals, or corals with mixed communities. **5.1b**: Photochemical efficiency of clade D-dominated corals with up to 10% background clade C symbionts (solid dark red line), between 10% and 4% clade C symbionts (solid light red line), and between <4% background clade C symbionts (dashed light red line). **5.1c:** Photochemical efficiency of all D-dominated symbionts (up to 10% clade C symbionts, solid red line) between corals initially bleachied (either with heat or DCMU, orange dashed line) or not initially bleached (orange solid line) 6 months prior to this experiment. Figures 5.1b and 5.1c demonstrate the similarity in Fv/Fm values between background clade C symbionts and past thermal history, where the disparity in Fv/Fm values based on past thermal history is likely an artefact of background clade C symbionts).

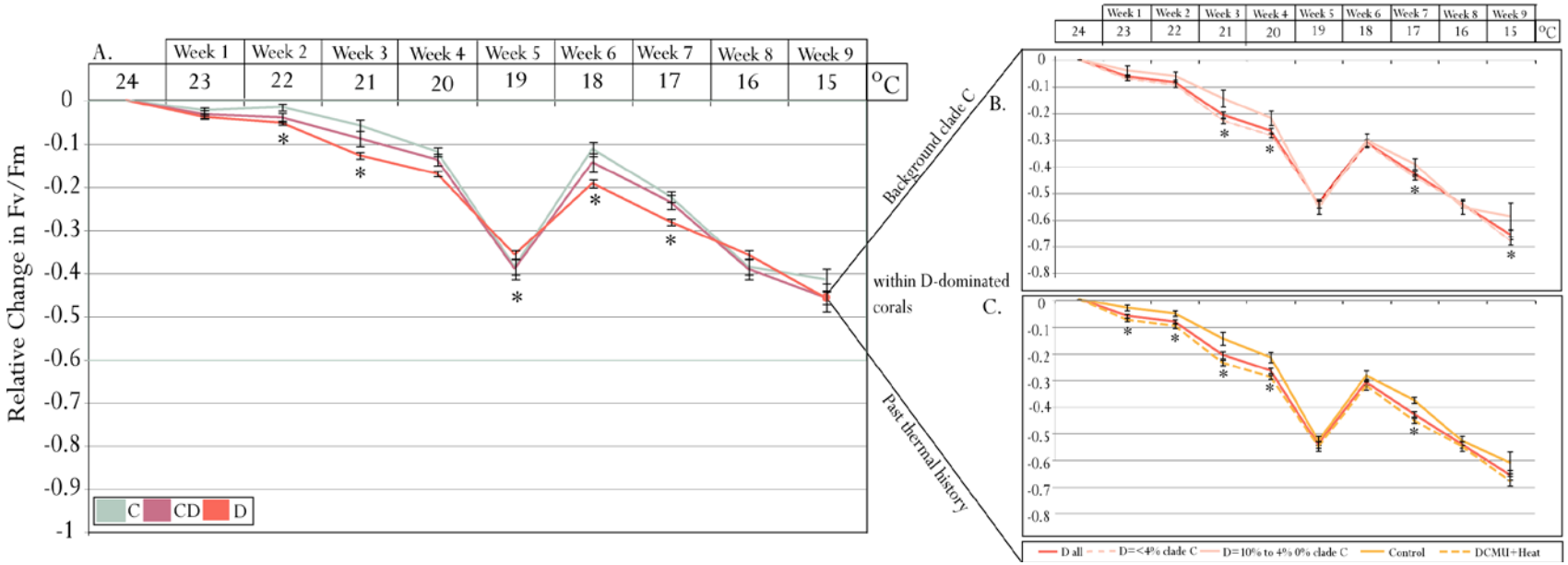

A.
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Week 7
Week 8
Week 9
24
23
22
21
20
19
18
17
16
15
°C
Relative Change in Fv/Fm
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1
C
CD
D
Background clade C
within D-dominated corals
Past thermal history
B.
C.
D all
D=<4% clade C
D=10% to 4% 0% clade C
Control
DCMU+Heat

Figure 5.2 ***Relative change in photochemical efficiency (Fv/Fm) of symbiont communities dominated by C3, D1a and C3+D1a during the experimental heating.*** C3 symbionts experienced immediate declines in Fv/Fm, while D1a symbionts showed equal or higher Fv/Fm values than C3-dominated corals until temperatures exceeded 33°C.

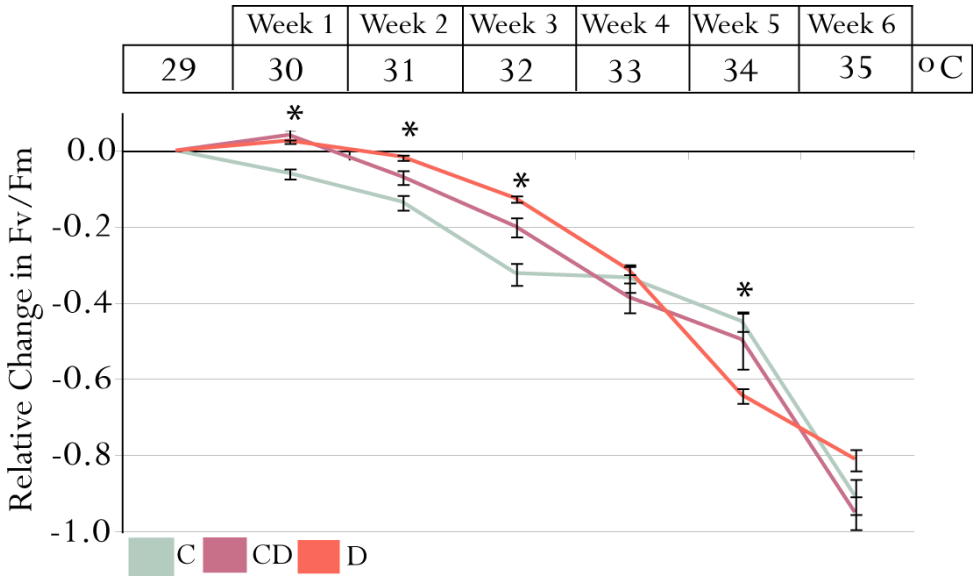
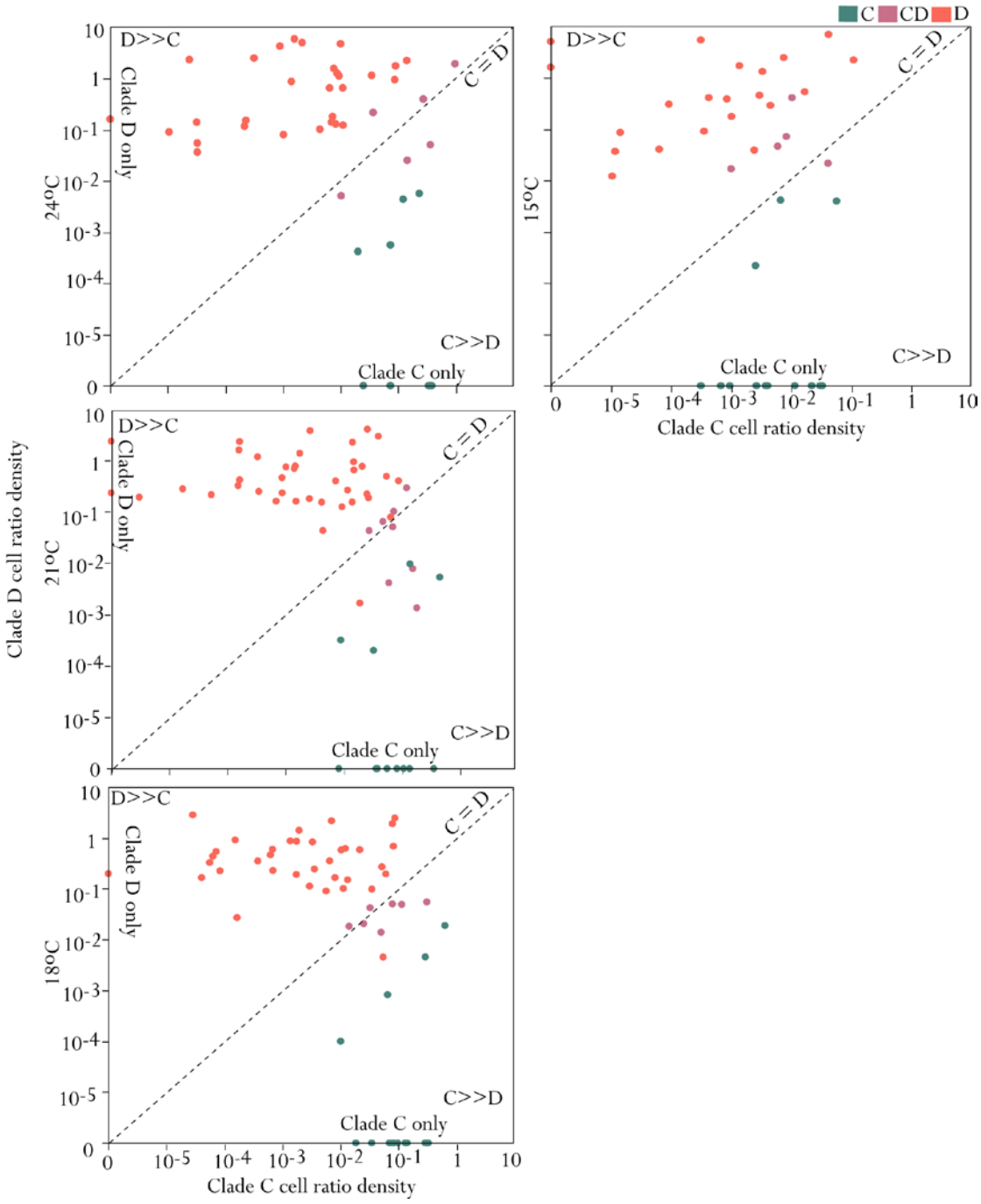


Figure 5.3. Cell ratio densities (log scale) of C3 and D1a symbionts. Clade C-dominated corals are shown by green markers, clade D-dominated corals are red markers, and corals with mixed-clade communities are purple markers. The dotted line represents equal amounts of clades C and D in a sample. **5.3a: Symbiont densities during incremental cooling**. The panels represent symbiont communities at the start of this experiment, after 1 week at 21^{o}C, then after 1 week at 18^{o}C, and finally after 1 week at 15^{o}C. **5.3b**: **Symbiont densities during incremental heating**. The panels represent symbiont communities at the start of this experiment, after 1 week at 33^{o}C, after 1 week at 35^{o}C.

5.3a


C
CD
D
D>>C
C=D
C>>D
Clade D only
Clade C only
24°C
21°C
18°C
15°C
Clade D cell ratio density
Clade C cell ratio density
10
1
10-1
10-2
10-3
10-4
10-5
0

5.3b

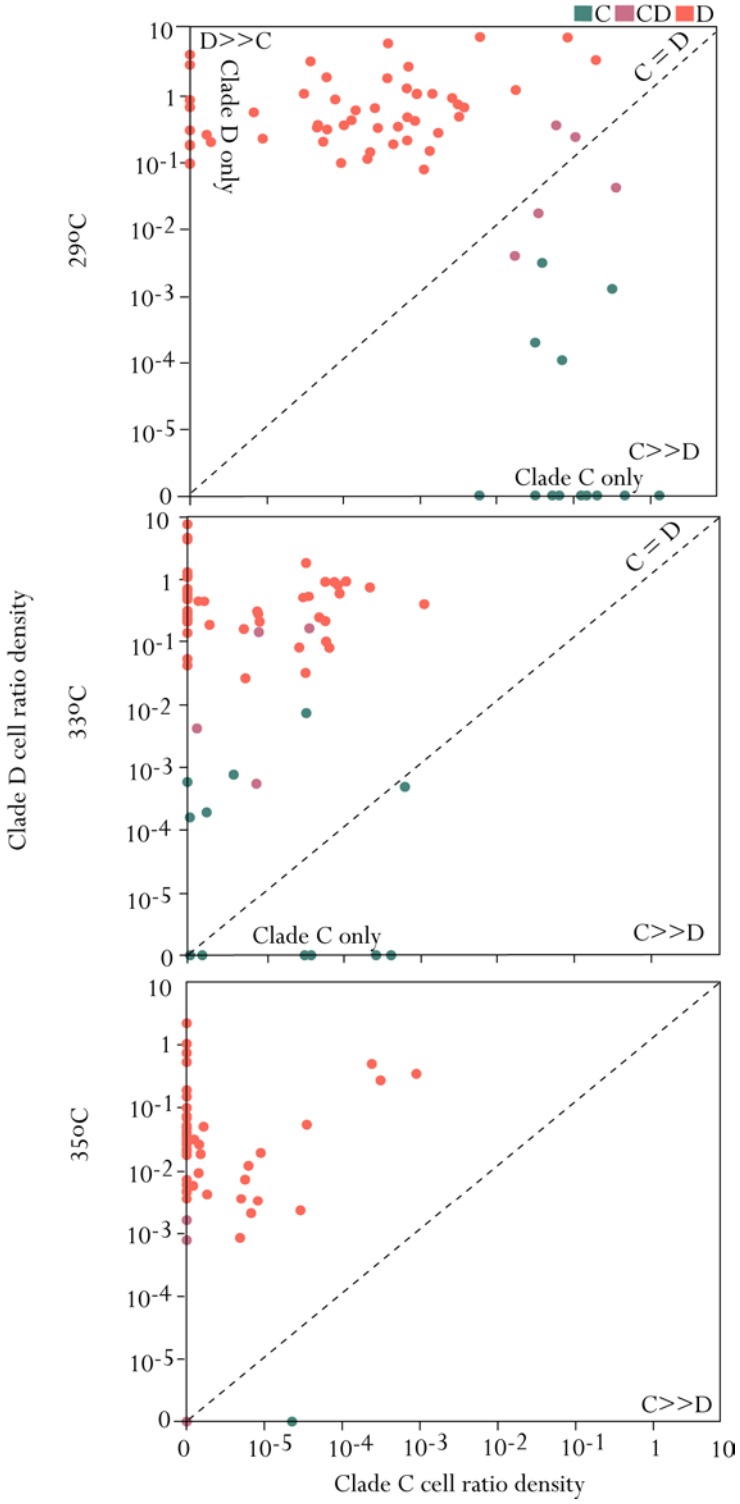

C CD D
D>>C
Clade D only
C = D
C>>D
Clade C only
29ºC
33ºC
35ºC
Clade D cell ratio density
Clade C cell ratio density
10
1
10-1
10-2
10-3
10-4
10-5
0

Figure 5.4 Symbiont density changes during cooling (5.4a) and heating (5.4b). Red lines indicate best fit line; dashed line indicates no symbiont density change. **5.4a Relative change in symbiont density at the start of the experiment (24°C), to 21°C, 18°C and 15°C**. Linear regressions revealed no relationship between clade D symbionts and symbiont loss between 24°C and 21°C (P=0.8) or 18°C (P=0.7). However, between 24°C and 15°C, linear regressions had positive slopes, indicating that colonies with more clade D lost fewer symbionts (P=0.005). **5.4b Relative change in symbiont density at the start of the experiment (29°C), 33°C and 35°C**. Linear regressions reveal a positive slope (P<0.0001) at each thermal interval (between 29°C to 33°C and 35°C), indicating that corals with a greater proportion of clade D symbionts lost fewer symbionts during heating. Between 29°C and 33°C, corals with high amounts of clade D did not lose symbionts; however, between 33°C and 35°C, even corals with high amounts of clade D lost symbionts. These data suggest that over 33°C there is little added benefit for coral, in terms of avoided symbiont loss, in hosting clade D.

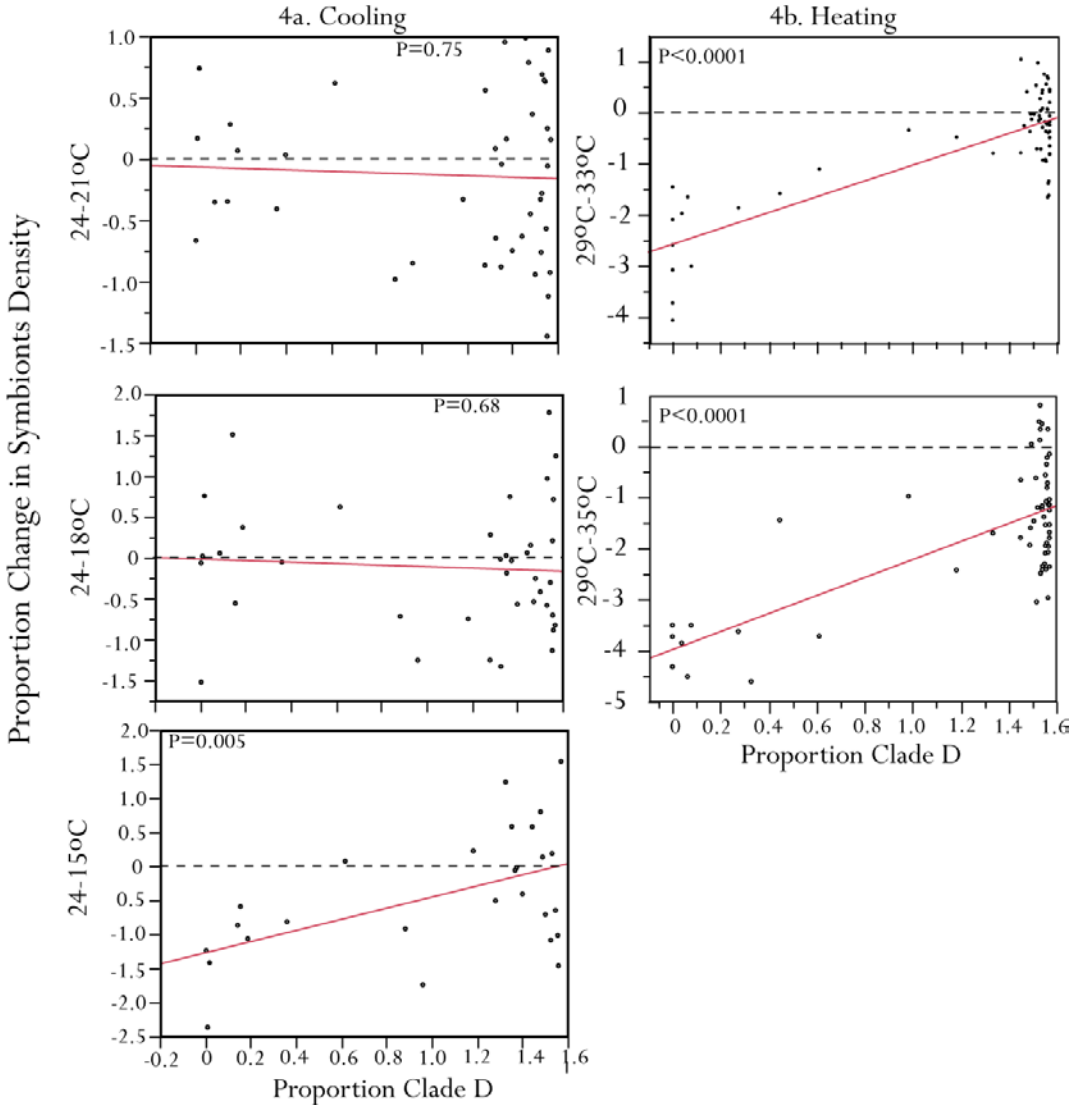


Figure 5.5 Clade- and density-dependent changes in symbiont communities during cooling (5a) and warming (5b). **5.5a**: Differences in relative symbiont loss in corals dominated by clade C, D, or C+D across a gradient of initial symbiont density between 24°C and 21°C, 18°C and 15°C. The effect of clade becomes a significant factor in determining symbiont loss only after one week exposure to 15°C, as C3 symbionts are lost in greater proportion than D1a. **5.5b**: Differences in relative symbiont loss between C, D, and C+D dominated corals across a gradient of initial symbiont density. D1a symbionts lose fewer symbionts at both 33°C and 35°C.

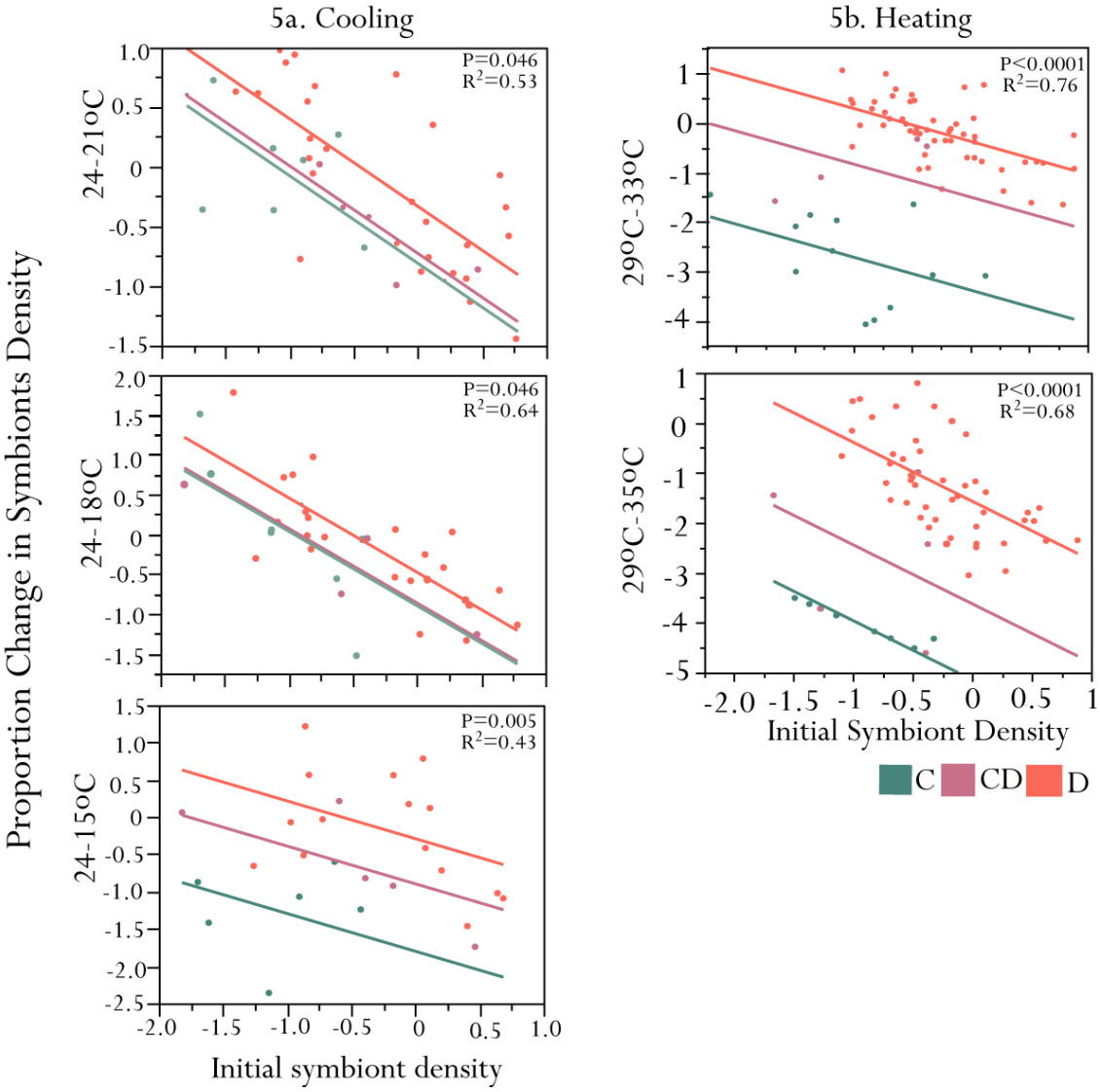
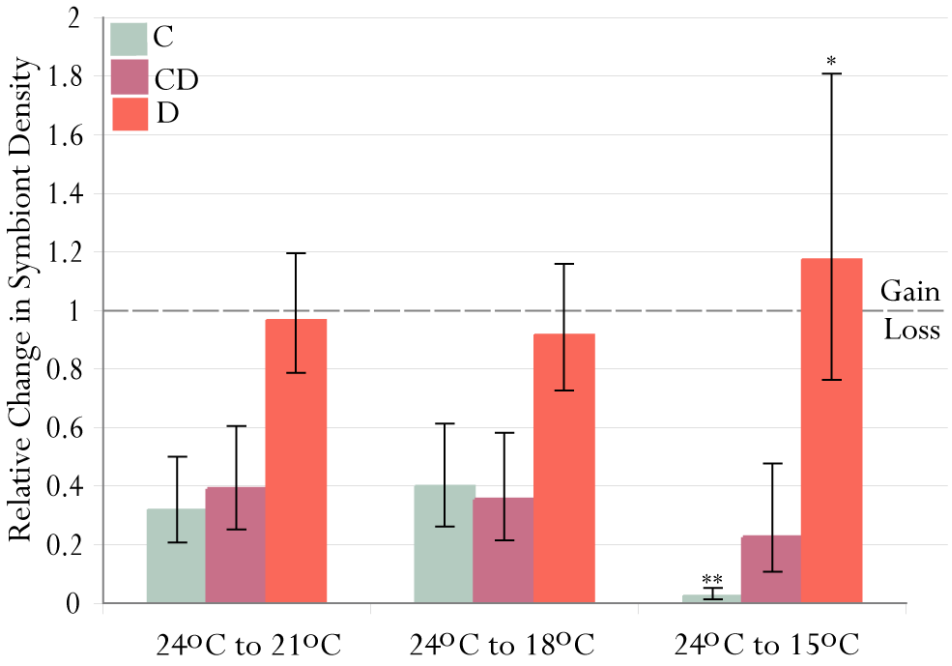


Figure 5.6 ***Log ratio relative change in*** **Symbiodinium** ***density compared to initial samples***. One asterisk represents a significant (P<0.05) difference between the relative changes in C3 and D1a density. Two asterisks represent a significant change (P<0.05) in within-clade comparisons to the initial time point. **5.6a: Adjusted mean relative change clade C and D symbionts remaining between initial (24°C) and 21°C, 18°C, and 15°C.** Clade D symbiont abundance is unchanged during cooling (P=0.78), while clade C symbionts experience decline of between 60 and 70% between 18°C and 21°C, followed by a significant loss of over 95% of its symbionts after 1 week at 15°C. **5.6b**: **Adjusted mean relative change of symbionts of C3 and D1a remaining from 29°C to 33°C, and then from 33°C to 35°C.** During the first time interval, more clade D *Symbiodinium* is retained than clade C *Symbiodinium* (P<0.0001). From 33°C to 35°C, both symbiont clades experience high losses, although clade C still has significantly higher declines (P<0.0001).

5.6a



5.6b

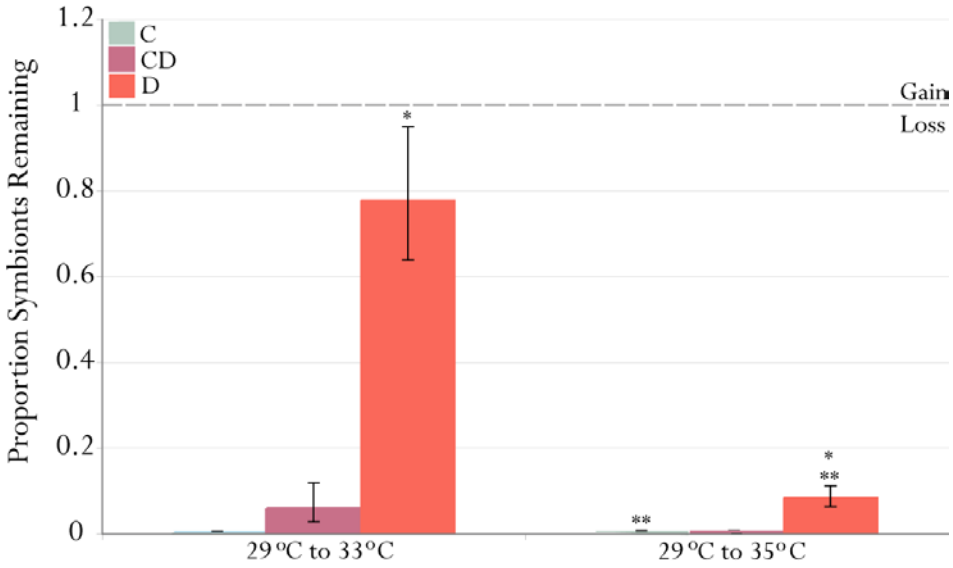
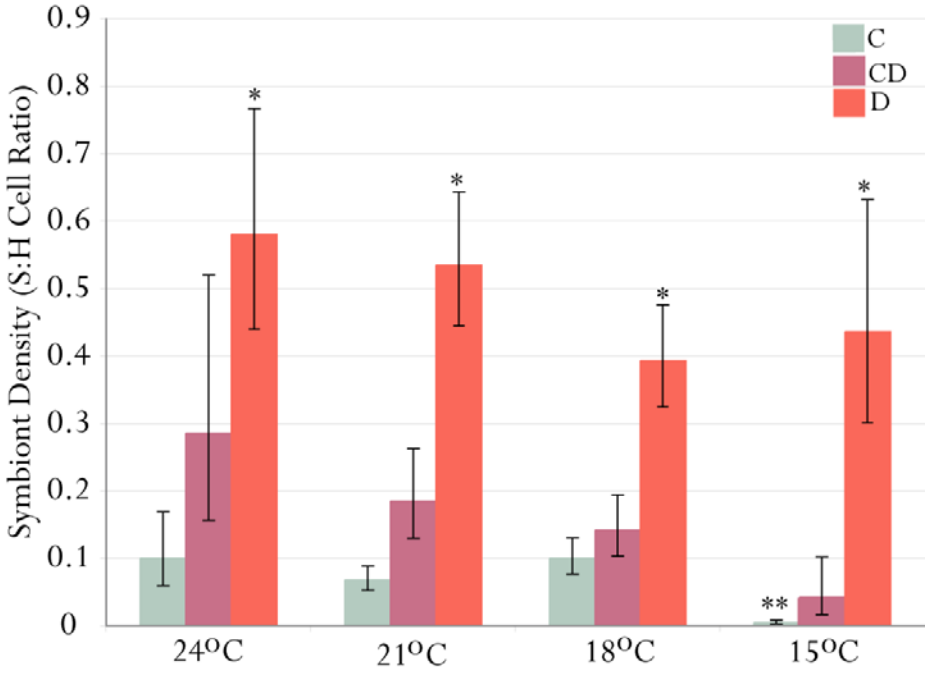
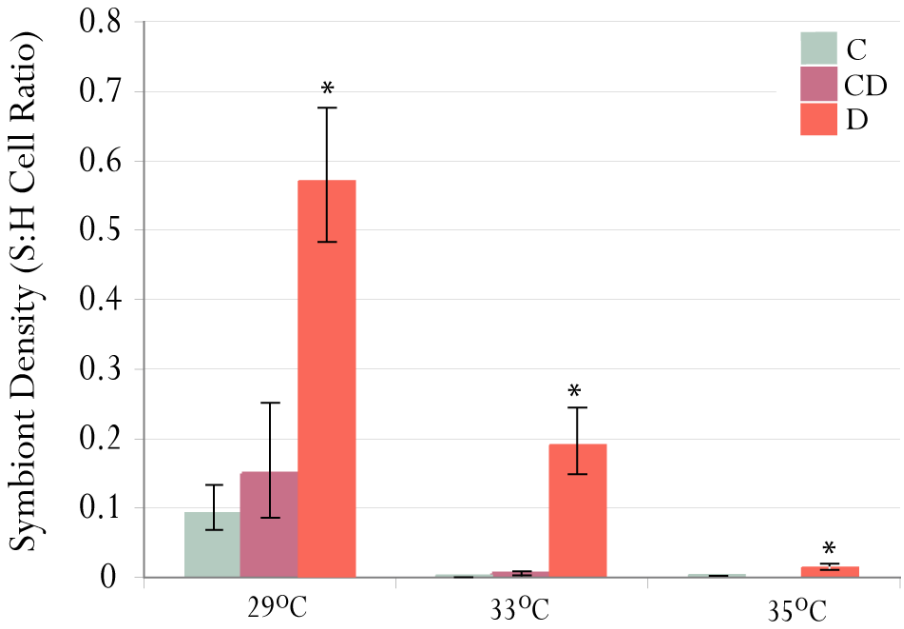

1.2
1
0.8
0.6
0.4
0.2
0
Proportion Symbionts Remaining
C
CD
D
Gain
Loss
*
**
*
**
29 °C to 33 °C
29 °C to 35 °C

Figure 5.7 ***Symbiont community composition (S:H ratio) in corals dominated by different symbiont types at each time point.*** One asterisk indicates time points where density of C3 was significantly different from D1a density. **5.7a: Symbiont community density in corals during chronic cooling.** Clade D-dominated and C+D corals are unchanged at all time points (P>0.23), whereas clade C-dominated corals are significantly lower than at other time points at 15°C, as indicated by two asterisks (P<0.004). **5.7b: Total symbiont community density in corals during chronic heating.** Symbiont density in clade D-dominated corals declines steadily across all temperatures, whereas clade C symbiont density is almost entirely diminished after 1-week exposure to 33°C. Within-clade, all clades have significantly lower densities than at the previous time point (P<0.01).

5.7a



5.7b


Symbiont Density (S:H Cell Ratio)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C
CD
D
*
*
*
29°C
33°C
35°C

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