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The Role of Algal Symbiont Community Dynamics in Reef Coral Responses to Global Climate Change

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UNIVERSITY OF MIAMI

THE ROLE OF ALGAL SYMBIONT COMMUNITY DYNAMICS IN REEF CORAL
RESPONSES TO GLOBAL CLIMATE CHANGE

By

Ross Cunning

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

December 2013

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THE ROLE OF ALGAL SYMBIONT COMMUNITY DYNAMICS IN REEF CORAL
RESPONSES TO GLOBAL CLIMATE CHANGE

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Reef Coral Responses to Global Climate Change.

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The continued growth and survival of reef-building corals is essential to sustain the goods and services provided by coral reefs, worth billions of dollars annually. However, warming oceans are causing more frequent and severe episodes of coral bleaching, the breakdown of symbiosis between corals and their algal symbionts (*Symbiodinium* spp.), which threatens corals' survival unless they can adapt or acclimatize. One way that corals may increase their thermal tolerance is by associating with different *Symbiodinium* types. Changes in partner abundance may also have functional consequences, but these symbiont dynamics are poorly understood. This dissertation aims to provide a clearer understanding of *Symbiodinium* community dynamics in corals by investigating changes in both partner identity and abundance over time, the factors driving these changes, and their functional consequences. First, I developed methods to analyze *Symbiodinium* community structure based on quantitative PCR, overcoming limitations of other techniques while providing a more physiologically relevant metric of symbiont density – the symbiont to host cell ratio. I then applied these techniques to simultaneously assay symbiont identity and abundance in corals under fluctuating environmental conditions. I show that naturally-variable symbiont densities converged under constant conditions to clade-specific equilibria and readjusted to new equilibria when conditions change. To explain these patterns, I used a mathematical

model to show that corals maintain an “optimal” symbiont abundance in a given environment that maximizes the net benefit of the symbiosis, suggesting that partner density regulation facilitates coral acclimatization in a dynamic environment. While symbiont abundance in the Pacific coral *Pocillopora damicornis* increased under warmer temperatures, corals with more symbionts were more susceptible to thermal bleaching, establishing a quantitative mechanistic link between symbiont abundance and the molecular basis for coral bleaching. Higher symbiont abundance also caused more severe bleaching in the Caribbean corals *Orbicella faveolata* and *Siderastraea siderea*, confirming the generality of this phenomenon and indicating that environmental conditions that increase symbiont densities, such as nutrient pollution, may exacerbate climate change-induced coral bleaching. However, corals may resist bleaching by increasing the relative proportion of thermally tolerant symbionts, which I show is more likely to occur when corals bleach more severely and recover at higher temperatures. These findings reconcile conflicting reports over whether corals change their symbionts in response to environmental stress by showing that the magnitude of symbiont shuffling is determined within an ecological framework by disturbance, niche differentiation, and competition among symbiont types. However, species-specific influences also determine symbiont community trajectories, evidenced by greater shifts to thermally tolerant communities in *S. siderea* compared to *O. faveolata*. To investigate potential tradeoffs associated with thermally tolerant symbionts, I measured growth rates of *P. damicornis* with different symbionts at three temperatures and found that while thermally tolerant symbionts reduced coral growth at cool temperatures, this disadvantage was ameliorated by warming, suggesting that in warmer oceans, these symbionts will benefit reefs by

enhancing coral survival at no cost to growth. Finally, a survey of thermally tolerant symbionts in different *Pocillopora* lineages revealed common associations across host taxa, indicating the widespread relevance of symbiont community variation and dynamics. In summary, this dissertation illustrates that coral performance and stress-tolerance are influenced by both symbiont identity and abundance, which are highly dynamic and change in accordance with principles of community ecology. These findings illustrate the importance of symbiotic dynamism in adaptation and acclimatization in diverse biological systems, while highlighting the major role that *Symbiodinium* community ecology may play in determining the future of coral reefs.

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Chapter 1

Introduction

Coral reefs are among the most biodiverse ecosystems on earth and provide goods and services estimated at \$375 billion annually (Costanza et al. 1997). They provide a food source and livelihood for hundreds of millions of people, especially in developing nations, and reef-based tourism supports economies around the globe. Coral reefs have captured the imaginations of people and cultures throughout history as an inspiration for art, exploration, and adventure. However, they have recently become the most prominent example of the decline of natural ecosystems due to human influence. In the Caribbean alone, coral cover has declined by an estimated 80% since the 1970s (Gardner et al. 2003).

Coral reefs are threatened by a variety of local and global stressors, including marine diseases, overfishing, pollution, eutrophication, and coastal development. However, the most significant threats facing coral reefs are rising sea temperatures and ocean acidification caused by anthropogenic CO₂ emissions (Pandolfi et al. 2003; Hoegh-Guldberg et al. 2007; Pandolfi et al. 2011). These local and global stressors have already contributed synergistically to the loss of 19% of the world's reefs (Wilkinson 2008), and some models predict that nearly all coral reefs will be unable to persist beyond the end of this century (van Hooidonk et al. 2013) unless the current course of climate change is dramatically reversed.

The proximate cause of much of the recent loss of coral reefs is coral bleaching, the breakdown of the symbiosis between reef-building corals and single-celled

dinoflagellate algae in the genus *Symbiodinium*. Elevated temperatures disrupt this symbiosis and algae are lost from coral tissues, thereby eliminating the autotrophic carbon source of algal photosynthesis that corals rely on for up to 90% of their nutrition (Muscatine and Porter 1977). Although corals can recover by re-establishing the symbiosis, severe or prolonged bleaching often leads to mass coral mortality (Baker et al. 2008).

However, there is great variation in the susceptibility of reef corals to bleaching. Many factors may contribute to this intrinsic variation, including host protective mechanisms (Baird et al. 2009), environmental history or acclimatory state (Brown et al. 2000; Bellantuono et al. 2012), heterotrophic feeding (Grottoli et al. 2006; Anthony et al. 2009), or the specific type of *Symbiodinium* hosted by the coral (Glynn et al. 2001; Berkelmans and van Oppen 2006; LaJeunesse et al. 2007; 2010). The algal symbiont type can have an especially dramatic effect, such that when neighboring corals of the same species on a reef host different symbiont types, one may bleach and die while the other appears unaffected (Glynn et al. 2001). This dissertation seeks to improve our understanding of these relationships among corals and different *Symbiodinium* types and their impacts on coral function and fitness in the face of global climate change.

Symbiodinium diversity

Symbiodinium is a diverse genus comprised of nine major clades (A-I), each containing a further diversity of types (LaJeunesse 2001; Pochon and Gates 2010), which can have distinct physiological traits (Iglesias-Prieto and Trench 1994; 1997; Warner et al. 1999; Savage et al. 2002; Rowan 2004; Goulet et al. 2005; Loram et al. 2007b;

McGinty et al. 2012; Baker et al. 2013b). Certain taxa, particularly members of clade D, are heat-tolerant symbionts (Rowan 2004), conferring increased resistance to thermal bleaching on their hosts (Rowan et al. 1997; Glynn et al. 2001; Berkelmans and van Oppen 2006; LaJeunesse et al. 2010; McGinley et al. 2012). However, other symbiont types may confer other advantages. For example, certain clade C types may provide corals with more fixed carbon (Cantin et al. 2009), which may enable faster growth (Little et al. 2004; Jones and Berkelmans 2010). Symbiont taxa also differ in their ability to acquire inorganic nutrients (Baker et al. 2013b) and combat oxidative stress (McGinty et al. 2012).

These functional differences among *Symbiodinium* types cause them to be differentially suited to varied environmental conditions. Based on these differences, they are predictably distributed along gradients of light and temperature across reefs (Iglesias-Prieto et al. 2004; Fabricius et al. 2004; Baker et al. 2013a) and even within single coral colonies (Rowan et al. 1997; Kemp et al. 2008). These abiotically-mediated spatial patterns may also manifest in time: as environmental conditions change, it may be advantageous for corals to change their symbiotic associations. In fact, bleaching may provide an opportunity for corals to rid themselves of sub-optimal symbionts and establish associations with better-performing symbionts under the prevailing conditions. This phenomenon, called the Adaptive Bleaching Hypothesis (ABH) (Buddemeier and Fautin 1993; Fautin and Buddemeier 2004), suggests that changing symbiont communities over time may be a mechanism by which corals can rapidly adapt to changing environmental conditions (Baker 2001). However, the ABH has been controversial in the coral literature in recent decades, in part due to a prevailing notion

that most corals maintained a specific association with a single *Symbiodinium* type and were thus unable to change symbiotic partners (Goulet 2006; 2007; LaJeunesse et al. 2008).

However, much of this view was based on symbiont detection using molecular techniques such as restriction fragment length polymorphism (RFLP), single stranded conformation polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE), which often fail to detect symbionts that are present at abundances below 5-20% (Fabricius et al. 2004; Thornhill et al. 2006; LaJeunesse et al. 2008). More recently, assays based on quantitative PCR (qPCR) have been developed with the ability to detect symbionts with 100- to 1000-fold greater sensitivity (Mieog et al. 2007). Through the application of these high-sensitivity techniques, it is becoming increasingly clear that most corals associate with multiple *Symbiodinium* taxa (Baker and Romanski 2007; Mieog et al. 2007; Loram et al. 2007a; Correa et al. 2009; Silverstein et al. 2012). In fact, in a qPCR-based survey of 39 coral species worldwide, Silverstein et al. (Silverstein et al. 2012) found that 100% of species associated with both clades C and D *Symbiodinium*, while 54% of species associated with clades A, B, C, and D. This high incidence of symbiotic flexibility suggests that the potential for corals to acclimatize to changes in the environment by changing their symbiotic partners is widespread. However, the factors that realize this potential and drive symbiont community change over time are still poorly understood.

Symbiont diversity may change over time through the acquisition of novel *Symbiodinium* from the environment (i.e., symbiont “switching”), or through changes in the relative abundances of different types within a mixed community (i.e., symbiont

“shuffling”; (Baker 2003)); both of these mechanisms have been documented in corals (Lewis and Coffroth 2004; Berkelmans and van Oppen 2006; Coffroth et al. 2010).

Diversity may change seasonally, as evidenced by fluctuations in the relative dominance of clade C and clade D in a population of corals in Taiwan (Chen et al. 2005). Diversity may also change over time during initial symbiont uptake (Fitt 1985), early establishment of symbiosis (Coffroth et al. 2001), or surrounding bleaching events (Rowan et al. 1997; Toller et al. 2001; Baker 2001; Berkelmans and van Oppen 2006; Thornhill et al. 2006; Jones et al. 2008; LaJeunesse et al. 2009). Symbiont changes following bleaching events have shown adaptive significance consistent with the ABH: corals with heat-sensitive clade C symbionts transplanted into warmer water bleached and recovered with heat-tolerant clade D symbionts (Berkelmans and van Oppen 2006). However, other studies have shown corals to bleach and recover with the same symbiont types (Sampayo et al. 2008; Thornhill et al. 2009; Stat et al. 2009; LaJeunesse et al. 2010; McGinley et al. 2012), raising questions about the prevalence of adaptive community shifts in corals.

To address this uncertainty, one of the major goals of this dissertation is to investigate various factors that may promote or restrict changes in symbiont community structure over time. Importantly, community structure is defined not only by the diversity of taxa present, but also their abundance (i.e., density), a factor that is conspicuously missing from many analyses of *Symbiodinium* communities.

Symbiodinium density

To gain a full understanding of how symbiont communities affect coral responses to environmental change, we must understand the role of both symbiont diversity and

abundance. Symbiont population density can directly influence the costs and benefits of symbiosis (Holland *et al.* 2002; 2004). In corals, benefits derived from photosynthesis may increase with symbiont density but then decline at high levels (Hoogenboom *et al.* 2010). Symbiont density may also influence nutrient cycling within the coral (Wooldridge 2009), and stress responses (Nesa and Hidaka 2009; Nesa *et al.* 2012). However, little is known about symbiont density variation among corals and its functional consequences.

Most of what is known about symbiont density is related to acute stress and bleaching, which drastically reduces the number of symbionts in coral tissues. However, symbiont densities also change seasonally (Brown *et al.* 1999; Fagoonee *et al.* 1999; Fitt *et al.* 2000; 2001; Warner *et al.* 2002), which may reflect continuous equilibration of symbiont community structure to fluctuations in light, temperature, or other abiotic factors. Moreover, significant symbiont density variation also occurs among coral colonies on the same reef (Jones and Yellowlees 1997; Moothien-Pillay *et al.* 2005), which may be due to fine-scale microhabitat differences. These patterns suggest that symbiont density is variable and dynamic in corals; the density of symbionts may be even more dynamic than their diversity. However, the factors underlying this variation and dynamism are poorly understood, as are their functional consequences. For example, does a coral with more symbionts receive more or less benefit from the symbiosis, or differ in its susceptibility to stress? Understanding the drivers and consequences of symbiont density variation is another major goal of this dissertation.

Quantifying Symbiodinium community dynamics

One reason that *Symbiodinium* community dynamics are poorly understood is that methods to accurately quantify them have not been sufficiently developed. A comprehensive picture of symbiont community dynamics requires simultaneous assessment of both the identities and abundances of symbiotic partners, which has not been possible using traditional molecular techniques. Symbionts are usually identified by gel-based methods (LaJeunesse and Trench 2000) and sequencing of various genetic markers (Pochon et al. 2012). While these techniques can identify which taxa are present (above a threshold of 5-20%), they are not quantitative, and cannot determine the abundance of different types. To quantify abundance, cells are usually counted on a haemocytometer to calculate total areal cell density. However, because *Symbiodinium* types cannot be distinguished morphologically, the density of different types in a mixed community cannot be quantified. Furthermore, cell counting requires sacrificing significant amounts of coral tissue, preventing longitudinal studies of symbiont dynamics within individual corals. Areal metrics also fail to capture any information about the host (e.g., tissue thickness), which potentially limits their physiological relevance. To overcome these methodological limitations that hinder the study of symbiont community dynamics, a major goal of this dissertation work is to develop qPCR techniques that can simultaneously identify and quantify each member of the symbiont community.

Most qPCR assays used thus far to study *Symbiodinium* have targeted large subunit nuclear ribosomal DNA (LSU nrDNA), a gene region that is present at up to several thousands of copies within a single genome, and is highly variable among different types of *Symbiodinium* (Mieog et al. 2007; Loram et al. 2007a; Correa et al.

2009; Silverstein et al. 2012). Consequently, there is significant error involved in converting number of copies of the target gene present in a sample to number of cells of *Symbiodinium*. This difficulty renders these assays capable of only “order of magnitude” accuracy in estimating symbiont abundances. Additionally, without a means of standardizing the results of these assays, they are only capable of estimating relative ratios of one symbiont type to another, with no way of measuring cell density or absolute cell numbers.

To overcome these problems, Mieog et al. (2009) developed qPCR assays targeting low copy-number actin gene loci in *Symbiodinium* as well as coral hosts. By quantifying specific actin loci in the host and each symbiont type, these assays are able to estimate densities in the form of symbiont to host cell ratios for multiple symbiont taxa in mixed communities. Mieog and colleagues used SYBR Green chemistry in simplex rtPCR reactions to quantify *Symbiodinium* clade C and clade D densities within the coral *Acropora millepora*, although further sequencing of actin genes revealed that these assays may only be applicable to this particular host-symbiont association (Pochon et al. 2012).

In order to capitalize on the conceptual advantages of the actin-targeted qPCR assays while increasing their accuracy and applicability, this dissertation work develops Taqman assays for clade-level quantification of mixed *Symbiodinium* assemblages in a wide range of coral hosts. These assays are also engineered for multiplex capability for quantification of multiple symbiont clades in a single qPCR reaction, enabling high-throughput analysis that saves time and costs. Finally, only small DNA samples are required for such analysis, enabling repeated sampling of the same coral fragments over time.

These techniques enable comprehensive study of symbiont community dynamics by simultaneously identifying and quantifying multiple *Symbiodinium* types within a single coral. These capabilities facilitate new types of questions and experimental approaches to study the dynamics of these symbioses, how they fluctuate in space and time, and how they respond to environmental stress. This dissertation work utilizes this new quantitative approach to investigate the role of symbiont community dynamics in healthy corals under natural environmental fluctuations, in corals bleaching in response to acute stress, and in corals recovering from bleaching.

Symbiont community dynamics in healthy corals

Due to the optimization of different symbiont types to different environmental conditions, the composition of mixed *Symbiodinium* communities within a single coral may shift over time as changing environmental variables alter their competitive dynamics. In addition to changing partner identity, environmental fluctuation may drive changes in partner density. This dissertation documents patterns of symbiont community change in response to environmental variation (Chapter 2, Chapter 5), and then attempts to explain the observed patterns in terms of the costs and benefits of symbiosis (Chapter 5). In particular, I test the hypothesis that population densities of symbionts are maintained at specific equilibria dependent on their genetic identity and the prevailing environment. To investigate whether changes in symbiont density can represent a strategy to sustain maximum interaction benefit, we modeled density-dependent costs and benefits of symbiosis (*sensu* Holland *et al.* 2002) under a range of biotic and abiotic contexts and fit this model to the observed data.

Symbiont community dynamics during bleaching

The process of coral bleaching involves loss of *Symbiodinium* cells from coral tissues, and occurs in response to a number of stressors including high light, temperature extremes, hyposalinity, and pollution. Several studies have shown a relationship between the dominant symbiont type and bleaching (Rowan et al. 1997; Glynn et al. 2001; Berkelmans and van Oppen 2006; Sampayo et al. 2008; LaJeunesse et al. 2009). This dissertation aims to create a more complete picture of how symbiont taxa influence the bleaching process by quantifying dynamics of the entire symbiont community during bleaching (Chapters 2, 3, 4).

If symbiont loss is indeed dependent on symbiont genetic identity, then the dynamics of bleaching in corals hosting multiple symbiont taxa will give great insight into this relationship. Rates and patterns of symbiont loss may differ depending on the abundances of certain taxa or thresholds of community diversity. In addition, symbiont density may significantly influence bleaching susceptibility. Answering these questions will give us a better understanding of the process of coral bleaching and how it is dependent on *Symbiodinium* community structure (Chapters 2, 3, 4).

Symbiont community dynamics during recovery

As the symbiont community recovers from bleaching, it may undergo successional changes as it moves toward a stable symbiosis (Toller et al. 2001). The dynamics of recovering symbiont communities are likely complex, and may be shaped by interactions among diverse algal types, host regulatory control, and external environmental forcing. Berkelmans and van Oppen (2006) showed that corals

transplanted to a warmer environment bleached and recovered with a different symbiont type that was better suited to the prevailing environmental conditions. Conversely, other studies have shown no changes in the dominant symbiont type in corals that experienced a natural thermal bleaching event followed by a return to normal environmental conditions in the field (Sampayo et al. 2008; Stat et al. 2009). Together, these results suggest that the recovery environment may have a significant effect on recovering symbiont community composition. In other words, a sustained environmental change may cause a community shift, whereas a constant environment may favor the re-establishment of the same symbiotic community (Toller et al. 2001). There is also evidence that the densities and taxa of remnant symbionts in bleached tissue may determine the trajectory of recovery (Toller et al. 2001). To address these issues, this dissertation will test the effects of bleaching severity and recovery temperature on symbiont community change following bleaching (Chapters 3 and 4).

Consequences of symbiont community changes

If symbiont communities change over time, it is important to understand how these changes may affect coral health and performance. To address this issue, I measure growth rates of corals with either clade C or clade D *Symbiodinium* at three different temperatures. This will determine whether corals switching or shuffling to clade D-dominated communities will experience significant reductions in growth, and whether this tradeoff will change as temperatures rises (Chapter 6).

Lastly, I investigate how widely applicable changes in symbiont communities may be in *Pocillopora* corals, as some evidence suggests that only a subset of these corals

are capable of hosting clade D (Pinzon and LaJeunesse 2011). Through a survey of eastern Pacific *Pocillopora*, I investigate symbiosis specificity, and thus the extent to which symbiont community dynamics may play a role in their response to climate change (Chapter 7).

Summary

An overarching goal of this dissertation is to understand the dynamics of *Symbiodinium* as an ecological community within a single coral. As the first quantitative study of *Symbiodinium* community dynamics, this dissertation will elucidate (1) drivers of *Symbiodinium* community change, (2) the dynamics of *Symbiodinium* communities in healthy, stressed and recovering corals, and (3) the role of *Symbiodinium* community dynamics in coral acclimatization to environmental change. In addressing these questions, this study considers both symbiont identity and abundance as important factors in determining these outcomes. The focus in this dissertation on symbiont abundance represents a significant contribution to the field as many previous studies have only investigated the effects of symbiont identity on the function of coral-algal symbioses. In Chapter 8, I summarize the importance of symbiont abundance in reef corals and its implications for understanding coral responses to environmental change.

Chapter 2

Excess algal symbionts increase the susceptibility of reef corals to bleaching¹

Summary

Rising ocean temperatures associated with global climate change are causing mass coral bleaching and mortality worldwide (Baker et al. 2008). Understanding the genetic and environmental factors that mitigate coral bleaching susceptibility may aid local management efforts to help coral reefs survive climate change. While bleaching susceptibility depends partly on the genetic identity of a coral's algal symbionts (Glynn et al. 2001), the effect of symbiont density, and the factors controlling it, remain poorly understood. By applying a novel metric of symbiont density (Mieog et al. 2009) to study the coral *Pocillopora damicornis* during seasonal warming and acute bleaching, we show that symbiont cell ratio density is a function of both symbiont type and environmental conditions, and that corals with high densities are more susceptible to bleaching. Higher vulnerability of corals with more symbionts establishes a quantitative mechanistic link between symbiont density and the molecular basis for coral bleaching, and indicates that high densities do not buffer corals from thermal stress, as has been previously suggested (Stimson et al. 2002). These results indicate that environmental conditions that increase symbiont densities, such as nutrient pollution (Falkowski et al. 1993; Fabricius 2005), will exacerbate climate change-induced coral bleaching, providing the first mechanistic explanation for why local management to reduce these stressors will help coral reefs survive future warming.

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Background

Coral reef ecosystems have high biodiversity and economic value, and are facing numerous threats from local stressors and global climate change (Hoegh-Guldberg et al. 2007). Elevated seawater temperatures disrupt the mutualistic symbiosis between corals and their dinoflagellate algae (genus *Symbiodinium*), resulting in coral “bleaching” (stress-induced loss of symbionts), which has led to episodes of mass coral mortality on reefs worldwide (Baker et al. 2008). The frequency and intensity of bleaching are projected to increase with anthropogenic climate change (Hoegh-Guldberg et al. 2007).

The susceptibility of corals to bleaching varies greatly (Baker et al. 2008), and an important management objective is to identify which reef areas and coral species are most resilient to the effects of global climate change. Variation in bleaching susceptibility may depend on a combination of host genetic effects (Baird et al. 2009), environmental history and acclimatization (Brown et al. 2000), and the symbiotic algal community (Glynn et al. 2001; LaJeunesse et al. 2010). Genetic variation among algal symbionts underlies physiological differences (Iglesias-Prieto and Trench 1994; Rowan 2004) that affect the performance of the symbiosis, with certain thermally tolerant *Symbiodinium* in clade D conferring increased resistance to bleaching on their coral hosts (Glynn et al. 2001; Berkelmans and van Oppen 2006). We hypothesize that symbiotic function, and thus bleaching susceptibility, is also dependent on the density of algal symbionts. However, the factors influencing symbiont density, its dynamics, and its effects on the coral host are poorly understood, possibly because the traditional technique of measuring density per unit area may be unable to reveal these relationships.

To investigate the connections between environmental change, quantitative symbiont community dynamics, and coral response, we used quantitative PCR (qPCR) to track changes in the “cell ratio density” of different symbionts over time in the coral *P. damicornis*. This metric of symbiont density - a ratio of the total number of symbionts to the total number of host cells (Mieog et al. 2009) - is distinct from both areal symbiont density (symbionts per cm²) and “cell-specific density” (CSD; Muscatine et al. 1998; Shick et al. 1999), a microscopic metric of the average number of symbionts contained within the symbiosomes of individual endodermal cells.

Materials and Methods

Coral collection and maintenance

Colonies of *Pocillopora damicornis* (n=53) were collected from 3-6 m depth at Taboga, Panama (8°48'0 N, 79°32'60 W) on 11 February 2009 and held in running seawater at the Smithsonian Tropical Research Institute's Naos Marine Laboratory. The following day, corals were transported by air to the University of Miami Experimental Hatchery where they were maintained in a shaded outdoor tank (receiving approximately 30% of ambient sunlight, reaching peak midday irradiance of ~500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ during summer months) supplied with seawater from Biscayne Bay (tank effluent treated to prevent discharge of propagules to the environment). Colonies were maintained during a 6-month period of natural seasonal warming that culminated in a bleaching event. Temperatures experienced by the corals were recorded prior to collection by a HOBO data logger near the collection site, and during the study by a HOBO data logger in the corals' holding tank (except 8/14-8/18 recorded by a NOAA buoy near the seawater

intake for the holding tank). Small branch tips (<1 cm³) were clipped from each colony three times during the warming period (2/12/09, 4/11/09, 6/14/09), and from a subset of colonies (n=28) twice during bleaching (8/13/09 and 8/18/09). Tissue samples were preserved in 1% SDS in DNAB, and DNA was extracted using a modified organic extraction protocol.

Symbiont community analysis

Symbiodinium types hosted by the corals were identified by denaturing gradient gel electrophoresis (DGGE) and sequencing of the second internal transcribed spacer-2 region of ribosomal DNA (ITS2 rDNA). This gene region was amplified using the primers “ITSintfor2” and “ITS2clamp” (LaJeunesse and Trench 2000), and amplification products were separated by DGGE (35-75% gradient) using a CBS scientific system. Dominant bands on the gel were excised, re-amplified, and sequenced using BigDye Terminator v3.1 cycle sequencing kit and an Applied Biosystems 3130xl Genetic Analyzer (Foster City, CA, USA).

Symbiont cell ratio densities (symbiont to host cell ratios (Mieog et al. 2009)) were quantified from each DNA sample using qPCR. Primers and probes for qPCR assays targeting specific actin loci in *P. damicornis*, *Symbiodinium* clade C, and clade D (JN546581-JN546583) were developed based on an alignment of 68 coral and *Symbiodinium* actin sequences (see *Actin gene sequencing*, below). Each assay was validated for target specificity and amplification efficiency, and clade C and D assays were further optimized to be carried out in multiplex (see *qPCR assay development*, below). All qPCR reactions were performed using a StepOnePlus™ Real-Time PCR

System (Applied Biosystems, Foster City, CA). Reaction volumes were 20 μ L, with 10 μ L Taqman® Genotyping Master Mix and 1 μ L genomic DNA template. The *P. damicornis* assay included 100 nM forward primer (PdActF, 5'-GAGAAGCTCTGCTATGTTGCCA-3'), 200 nM reverse primer (PdActR, 5'-TCCACGGAATCGCTCGTT-3'), and 100 nM Taqman probe (PdActProbe, 5'-NED-AGACTGCTGCCTCAAC-MGB-3'). The multiplexed *Symbiodinium* clades C and D assay included 50 nM clade C forward primer (CActF, 5'-CCAGGTGCGATGTCGATATTC-3'), 75 nM clade C reverse primer (CActR, 5'-TGGTCATTCGCTCACCAATG-3'), 100 nM clade C probe (CActProbe, 5'-VIC-AGGATCTCTATGCCAACG-MGB-3'), 50 nM clade D forward primer (DActF, 5'-GGCATGGGGTAAGCACTTCTT-3'), 75 nM clade D reverse primer (DActR, 5'-GATCCTTGAAGTAGCCTTGAAAC-3'), and 100 nM clade D probe (DActProbe, 5'-6FAM-CAAGAACGATACCGCC-MGB-3'). Thermal cycling conditions consisted of initial incubation at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 1 minute. Cycle threshold (C_T) values were calculated by the StepOnePlus™ software package using automatic baseline calculations and a set fluorescence threshold of $\Delta R_n=0.01$.

Positive amplifications were counted only when both technical replicates produced C_T values <40 and there was no amplification in no-template control reactions. C_T values were adjusted for differences in fluorescence intensity among the three different reporter dyes associated with the Taqman MGB probes. These differences were calculated from average C_T values produced by standard curves of copy number standards ranging from 10^8 to 10^2 , which revealed that the *P. damicornis* assay (NED

dye) and clade C assay (VIC dye) produced C_T values 4.48 ± 0.12 and 2.68 ± 0.14 (SE) cycles higher than the the clade D assay (FAM dye), respectively. Symbiont to host cell ratios were then calculated from adjusted C_T values by using the formula: $2^{(C_{T(\text{host})} - C_{T(\text{symbiont})})}$, and then dividing by the symbiont to host ploidy ratio (1/2), DNA extraction efficiency ratio (0.828), and target locus copy number ratio (3 for clade C, 1/3 for clade D; see *Estimation of actin loci copy numbers*, below).

Actin gene sequencing

Actin sequences were obtained from samples of *P. damicornis* and its particular clade C and D symbionts, as well as from *Symbiodinium* in clades A, B, C and D. Actin genes were amplified using universal actin forward primer 2 (5'-CGGCTACTCCTTYACCACMA-3') and universal actin reverse primer (5'-TCRCCCTTGGAGATCCACAT-3'; Mieog et al. 2009). PCR was carried out in 25 μ L reactions consisting of 0.5 μ M forward and reverse primers, 0.2 μ M each deoxynucleotide (Bioline, Boston, MA, USA), 2.0 μ M $MgCl_2$, 1x Green GoTaq® Flexi Buffer, and 1 U GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA). Reaction conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 74°C for 1 min. PCR products were cloned using the pGEM®-T Easy Vector System (Promega, Madison, WI, USA), and inserts were amplified using M13 primers and sequenced using BigDye Terminator v3.1 cycle sequencing kit and an Applied Biosystems 3130xl Genetic Analyzer (Foster City, CA, USA).

qPCR assay development

68 coral and *Symbiodinium* loci were sequenced and manually aligned using Geneious Pro v4.7.6 (BioMatters), incorporating other available sequences from GenBank. Using this alignment, primers and probes for taxon-specific qPCR assays targeting *P. damicornis*, *Symbiodinium* clade C, and *Symbiodinium* clade D were developed. Melting temperatures were 58-60°C for selected primers, and 68-70°C for Taqman® MGB probes (as determined by PrimerExpress (Applied Biosystems, Foster City, CA)). Amplicons produced by each primer set were between 100-150bp in length. Selected primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and Taqman® MGB probes by Applied Biosystems.

Each set of primers and probes was tested for specificity to its intended target by assaying DNA extracted from 21 cultures of *Symbiodinium* in clades A, B, C, and D. Primer-probe sets were considered specific by the positive amplification of their intended target and the lack of amplification of all other targets. Amplification efficiency of each primer-probe set was evaluated by assaying serial dilutions of copy number standards. Copy number standards were created for each target by amplifying each target sequence from a plasmid, determining DNA concentration of purified PCR products, calculating into copy numbers, and diluting appropriately. Amplification efficiency was calculated from the slope of the standard curve resulting from assaying copy number standards in a log dilution series from 10^5 to 10^1 copies in quadruplicate (Table S1). All primer-probe sets showed equally high efficiency (*P. damicornis* assay=95.7%; clade C assay=94.9%; clade D assay=95.3%), allowing comparison among assay results. Standard curves for all three assays are presented in Table 2.1.

Clade C and clade D assays were optimized to be carried out in multiplex.

Limiting primer concentrations were first determined for simplex assays by independently diluting forward and reverse primers in increments from 900 nM to 50 nM and selecting the lowest combination of primer concentrations that reduced ΔR_n without significantly reducing the C_T . Probe concentrations were similarly minimized by assaying a dilution series from 250 nM to 50 nM. To validate multiplex capability, two dilution series were assayed in both simplex and multiplex in which either the clade C or clade D target DNA was held at 10^5 copies per reaction, while the other target was serially diluted 1:10 down to 10 copies per reaction. This was designed to test the lower limit of detection of a low-abundance target in the presence of the maximum possible amount of high abundance target (the scenario in which the inhibitory effects of competitive PCR are most significant). *P. damicornis* actin was also included at 10^5 copies per reaction to ensure no inhibitory effects of non-target template. Resulting standard curves from simplex and multiplex reactions were compared by ANCOVA, and slopes were found to be identical for both targets in simplex and multiplex (clade C: $F=2.54$, $p=0.12$; clade D: $F=1.21$, $p=0.28$), indicating equal amplification efficiency in simplex and multiplex for both targets. Intercepts were also identical in simplex and multiplex (clade C: $F=0.99$, $p=0.33$; clade D: $F=4.32$, $p=0.04$), indicating multiplex reactions were not experiencing any inhibition. Multiplexed clade C and D assays thus accurately quantify mixed samples of clade C and D targets in proportions ranging from 10^5 C:10 D to 10 C: 10^5 D.

Estimation of DNA extraction efficiency

Extraction efficiency was estimated for the extraction of host and symbiont DNA from coral samples and the extraction of symbiont DNA from isolated cells. DNA was extracted from eight fragments of *P. damicornis* and six different *Symbiodinium* cultures (Y109, FLAp1, FLAp2, PurPflex, A008, and A013). The chloroform phase that is normally discarded during organic extraction was retained and pelleted, and DNA was re-extracted. qPCR assays were then used to estimate the ratio of DNA in the second extraction to the first extraction, and this ratio was used to calculate a theoretical total amount of DNA that would be recovered after three subsequent re-extractions, assuming the same proportion remaining in the chloroform phase each time. The ratio of the amount of DNA recovered in the first extraction to the theoretical total amount of DNA was then calculated as the extraction efficiency. Extraction efficiencies were $95.5 \pm 1.4\%$ for symbiont DNA extracted from isolated cells, and $81.3 \pm 2.0\%$ and $98.2 \pm 0.43\%$, respectively, for symbiont and host DNA extracted from coral samples. Thus, a symbiont to host extraction efficiency ratio of 0.828 ($81.3/98.2=0.828$) was used in calculating symbiont cell ratio densities.

Estimation of actin loci copy numbers

In order to accurately quantify cell numbers, the number of copies of the targeted actin locus for *P. damicornis* and *Symbiodinium* clades C and D per genome must be estimated. For *P. damicornis*, copy number was estimated by comparing the amplification of the actin assay to the amplification of a partial SRP54 intron, a purportedly single-copy locus (Concepcion et al. 2008). New primers for *P. damicornis*

SRP54 were developed: PdamSRP54_F (5'-TTAAAGGCAATGATCTACTGGTCATAG-3') and PdamSRP54_R (5'-TTAGGCCACATGAATTGCCA-3'). A standard curve was produced using these primers that indicated 97.7% amplification efficiency, allowing comparison between the SRP54 and actin assays. DNA from six different *P. damicornis* individuals was subsequently amplified with both primer sets, and ΔC_T analysis revealed an average actin to SRP54 ratio of 3.03 ± 0.43 (standard error). Copy number for *Symbiodinium* in clades C and D hosted by *P. damicornis* was estimated by assaying known numbers of algal cells freshly isolated from colonies in which only one clade was detected. Cells were isolated separately from 2 colonies hosting only clade C and 2 colonies hosting only clade D, counted with a haemocytometer, and divided into 100,000 cell aliquots. DNA was extracted from 3 separate aliquots of each sample, and quantified by qPCR using a standard curve of copy number standards and assuming 95.5% DNA extraction efficiency from isolated cells (see above). The targeted actin locus copy number was estimated at 8.95 ± 0.20 in clade C cells and 1.09 ± 0.05 in clade D cells. Therefore, the symbiont to host copy number ratios used in cell ratio density calculations were 9/3 (=3) for clade C, and 1/3 for clade D. A symbiont to host ploidy ratio of 1/2 (Santos and Coffroth 2003) was also used in calculating symbiont cell ratio densities.

Comparison of symbiont cell size

Symbiodinium cell sizes were measured by isolating symbionts from corals identified as hosting only clade C or D, and photographing cells at 600x magnification on a light microscope. Image analysis software was used to measure the maximum diameter

of intact, non-dividing clade C cells (n=115) and D cells (n=129). Clade C symbionts were larger than clade D symbionts (11.0 vs. 8.6 μm diameter, $t=-16.8$, $p<0.0001$). Under a model of space-limitation, smaller clade D cells may have a higher theoretical maximum density, but it is unlikely that the values reported here represent space-limited maxima, because some colonies with high symbiont densities actually decreased over time (Fig. 2.3a). It is more likely that the lesser increase in clade C densities compared to clade D arose from differential effects of the changing environment on the balance of growth and expulsion for the two symbiont types.

Investigation of other density-dependent factors

Although our data support the increased bleaching susceptibility of higher standing stocks during the earlier stages of bleaching, this relationship disappeared by the end of the bleaching event, as symbiont densities in bleached colonies approached zero. We suggest that high densities will initially bleach more severely due to elevated ROS generation, but during prolonged or extreme bleaching events, densities may fall below a threshold where reduced self-shading creates an environment of increased light stress, initiating a positive feedback that drives symbiont communities to minimum densities (Enríquez et al. 2005). This resulted in the disappearance of a statistical relationship between initial density and bleaching severity by 18 August.

Statistical analyses

Total symbiont cell ratio densities (sum of clade C and clade D cell ratio densities in each colony) were log-transformed for statistical analyses. Relative change over time

during the warming period was analyzed by ANCOVA with dominant clade as a fixed factor and initial density (February) as the continuous covariate. To confirm that model effects were not due to added correlation resulting from the predictor ($\log(\text{Feb. density})$) being used in the calculation of the response ($\log(\text{June density}/\text{Feb. density})$), ANCOVA was also used to analyze $\log(\text{June density})$ by clade with $\log(\text{Feb. density})$ as the continuous covariate. This independent analysis revealed the same significant effects of clade and initial density on change over time, and thus we chose to present the model involving relative change as the response variable due to ease of interpretation. Relative change over time during bleaching (From June to August 13) was also analyzed by ANCOVA with clade as a fixed factor and initial density (June) as the continuous covariate. Model effects were again verified by an independent ANCOVA, as above, using final density (August 13) as the response variable, and the same results were obtained. All analyses were conducted using R v2.9.1.

Results and Discussion

We found that colonies were dominated either by *Symbiodinium* in clade C (type C1b-c) or D (type D1) (Fig. 2.1). Warming increased cell ratio symbiont densities (Fig. 2.2), with greater increases occurring in clade D-dominated colonies, and colonies with lower initial densities (Fig. 2.3a). When acute bleaching occurred, densities decreased (Fig. 2.2), with greater decreases in clade C-dominated colonies, and colonies with higher initial densities (Fig. 2.3b). These trends reveal a new perspective on how symbiont densities are shaped by their environment, and how high densities of symbionts become a liability for their host in times of abiotic stress.

During warming, mean symbiont cell ratio density increased (Fig. 2.1), in contrast to the typical decreases observed using areal density metrics (Fitt et al. 2000). This difference is likely a consequence of standardizing symbionts to host cell numbers, which may change over time, rather than surface area, which is constant. We hypothesize that coral host cells are lost during seasonal warming due to increased metabolic demands that expend energy reserves and decrease coral tissue biomass (Fitt et al. 2000; Thornhill et al. 2011). Additionally, decreased heterotrophy during summer months (Ferrier-Pagès et al. 2011) may promote reductions in prey-capturing cnidocyte and mucocyte cells that do not contain symbionts. As host cells are lost, symbionts are expelled as a regulatory mechanism to maintain cell ratio densities below a critical threshold. Thus, although both host and symbiont cell numbers decrease during seasonal warming, a greater net loss of host cells results in an increased symbiont to host cell ratio, which we suggest is a physiologically more meaningful metric of symbiont density.

The discovery that symbiont cell ratio density increases during seasonal warming but decreases during thermal stress (Fig. 2.2) suggests that the cellular and molecular underpinnings of seasonal areal symbiont loss may be different from acute thermal bleaching. Although it has been suggested that the two phenomena are differing endpoints of the same stress-induced signaling cascade (Warner et al. 2002; Wooldridge 2009), our results suggest that seasonal loss may be primarily due to reductions in host cell numbers that drive compensatory symbiont expulsion to avoid excessive symbiont load. This perspective suggests that seasonal loss of symbionts may not represent “bleaching” as a result of acute photoinhibition (Fitt et al. 2000), and is instead a regulatory mechanism that reduces future bleaching susceptibility.

The seasonal increase in symbiont cell ratio density within a colony depended on both the identity of the symbionts and their initial density. Although no visual differences were apparent, densities increased more in colonies dominated by clade D than clade C (161% vs. 35% increase), and in colonies with lower initial densities. Colonies with the highest initial densities actually decreased during seasonal warming (Fig. 2.3a). We hypothesize the intermediate cell ratio densities toward which colonies converged (0.289 for clade D, 0.077 for clade C; Fig. 2.3a) represent an optimal symbiont density that is dependent on both symbiont type and environment. Dynamically changing environments will drive compensatory changes in the identity and density of symbionts, resulting in the continuous adjustment of symbiont communities. The higher optimal density of clade D compared to clade C is consistent with previous observations (LaJeunesse et al. 2010), and may reflect differences in cell size (see *Comparison of symbiont cell size*, above), or differences in their rates of proliferation and expulsion. Warming may induce greater expulsion of clade C symbionts relative to clade D by increasing their naturally faster growth rates, which reduces the carbon they transfer to the host (Lesser 1996; Wooldridge 2009).

At the peak of seasonal warming (Aug.), elevated temperatures caused acute coral bleaching, and symbiont cell ratio densities decreased in nearly all colonies. Clade C-dominated colonies paled severely, associated with an 88.4% decrease in cell ratio density. Clade D colonies, although not visually bleached, nevertheless also experienced a 16.7% decrease in cell ratio density. This difference in bleaching severity is likely due to clade C symbionts being more susceptible to photodamage during heat/light stress (Rowan 2004), and therefore producing more reactive oxygen species (ROS) that trigger

bleaching (Lesser 1996; Downs et al. 2002; Tchernov et al. 2011). While the greater bleaching susceptibility of clade C colonies has been well documented (Glynn et al. 2001; LaJeunesse et al. 2010), we show for the first time a significant additional effect of symbiont density: colonies with high symbiont cell ratio densities bleached more severely, regardless of symbiont type (Fig. 2.3b). We attribute this effect to higher total ROS production in corals with more symbionts (Nesa and Hidaka 2009). Even relatively thermotolerant clade D symbionts that produce low levels of ROS on a per-cell basis may cumulatively produce high total levels when maintained at high densities, explaining why even some D-dominated colonies experienced large reductions in symbiont cell ratio density (up to 76.6%). Moreover, symbionts at high density may generate more ROS per cell as CO₂-limitation compromises algal photosynthesis (Wooldridge 2009), further exacerbating ROS-mediated bleaching.

The simultaneous effects of identity and density establish a quantitative link between symbiont communities and the molecular basis of coral bleaching by showing that a ROS-mediated bleaching cascade can be activated by either a few highly-stressed symbionts, or many slightly-stressed symbionts. This mechanistic framework predicts an environment- and identity-dependent symbiont cell ratio density threshold above which corals bleach, which likely reflects a maximum amount of ROS that can be detoxified before bleaching is triggered (Downs et al. 2002; Tchernov et al. 2011). Our data empirically support the existence of this threshold (where no symbiont loss is predicted during thermal stress (Wooldridge 2012)), and show that excess symbionts elicit a proportionally more severe bleaching response (Fig. 2.3b). We therefore hypothesize that coral bleaching severity is determined by the total amount of ROS generated by algal

symbionts relative to the coral's detoxification capacity, and suggest that the symbiont cell ratio density is an accurate indicator of this relationship.

Because these thresholds reflect the changing physiological relationship between a coral and its symbionts, they may only be detectable by symbiont cell ratio densities, which quantify both symbiotic partners. Measuring symbionts per unit area is unlikely to reveal these relationships, because these metrics cannot detect cellular changes in the coral host which drive symbiont regulatory processes and determine the coral's capacity to tolerate stress. Future studies utilizing cell ratio density may reveal further interactions between symbiont identity and density and coral responses.

These findings suggest that corals with high symbiont cell ratio densities are more vulnerable to climate change-induced bleaching events, and that corals are at elevated bleaching risk when they reach their seasonal density peak, which we show coincides with the warmest temperatures. Additionally, corals in environments favoring elevated symbiont populations (e.g., low light (Titlyanov et al. 2001) or high nutrient environments (Falkowski et al. 1993; Fabricius 2005)), may be more susceptible to bleaching stressors (Wooldridge and Done 2009), regardless of symbiont type. Most importantly, our results indicate that coral reef bleaching severity may be influenced by any interacting stressor that affects symbiont densities, including ocean acidification as a result of increasing $p\text{CO}_2$ (Rodolfo-Metalpa et al. 2010) and poor water quality (Fabricius 2005). In particular, this framework explains how terrestrial runoff and eutrophication, by increasing symbiont densities, may increase the susceptibility of reef corals to bleaching (Wooldridge and Done 2009; Wiedenmann et al. 2012). Local management to reduce these stressors may therefore significantly help corals survive climate change.

Figure 2.1 Cell ratio densities of clades C and D symbionts. Densities of each clade in each sample prior to bleaching (n=159; 53 colonies, each sampled 12 Feb., 11 Apr., 14 June) show a high incidence of mixed communities (64.1% of colonies) that were heavily dominated by one clade with background populations of the other. Open triangles represent colonies categorized as C-dominated (99.6-100% clade C), and closed triangles are D-dominated (87.6-100% clade D). All colonies maintained C- or D-dominance throughout the study, except for a single colony (gray triangle) that changed from C- to D-dominance during seasonal warming, which was omitted from further analyses between C and D colonies. The dotted line represents equal amounts of clades C and D in a sample.

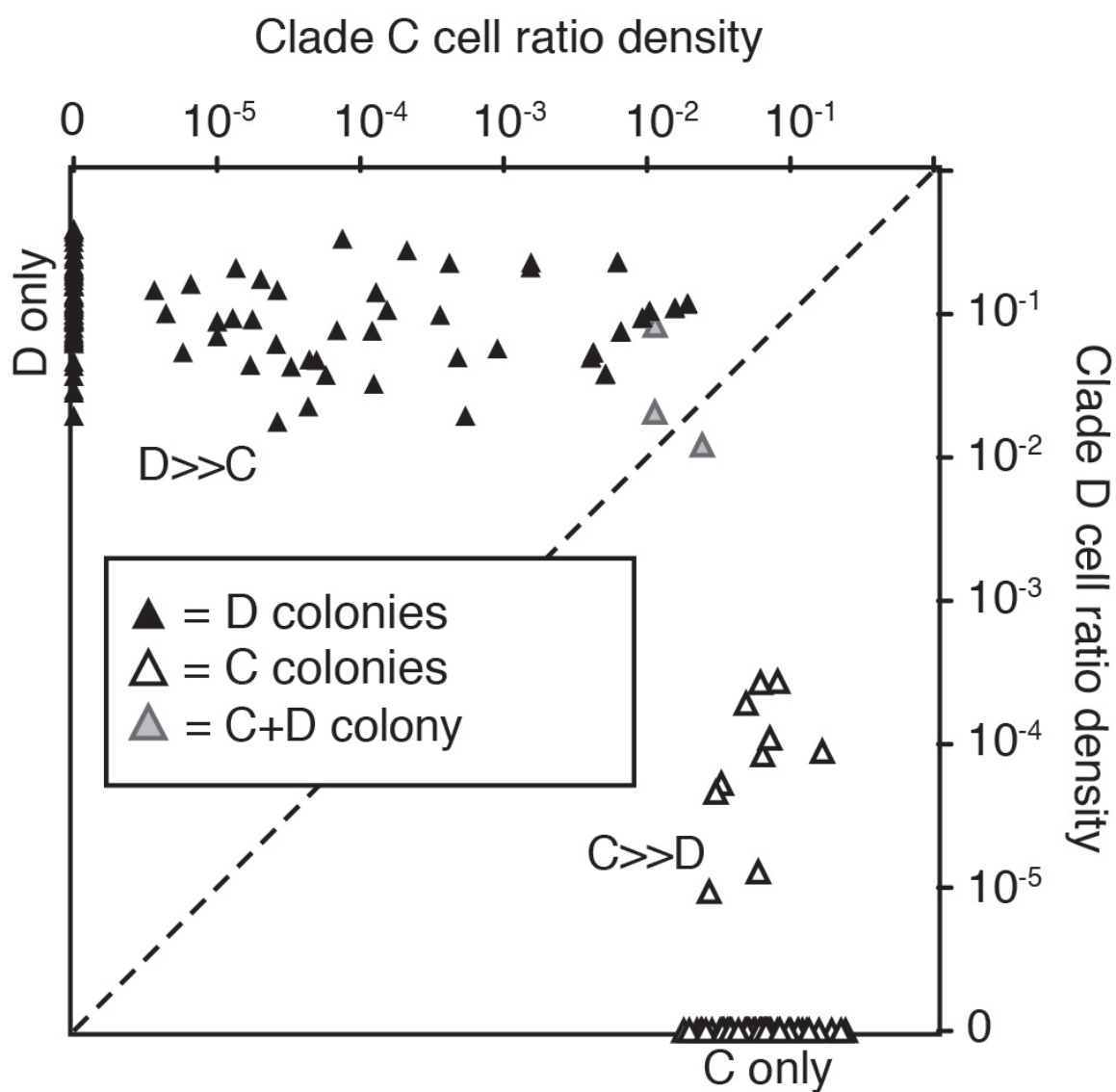


Figure 2.2 Changes in temperature and mean symbiont cell ratio density. Symbiont cell ratio densities (geometric mean \pm 95% confidence interval) in colonies dominated by clade C (open circles and dashed line) and clade D (closed circles and solid line) at each time point during the natural warming period and bleaching event. The mean, quartiles, and total range of water temperatures experienced by the corals over the 14-day period preceding each sampling point are shown with boxplots.

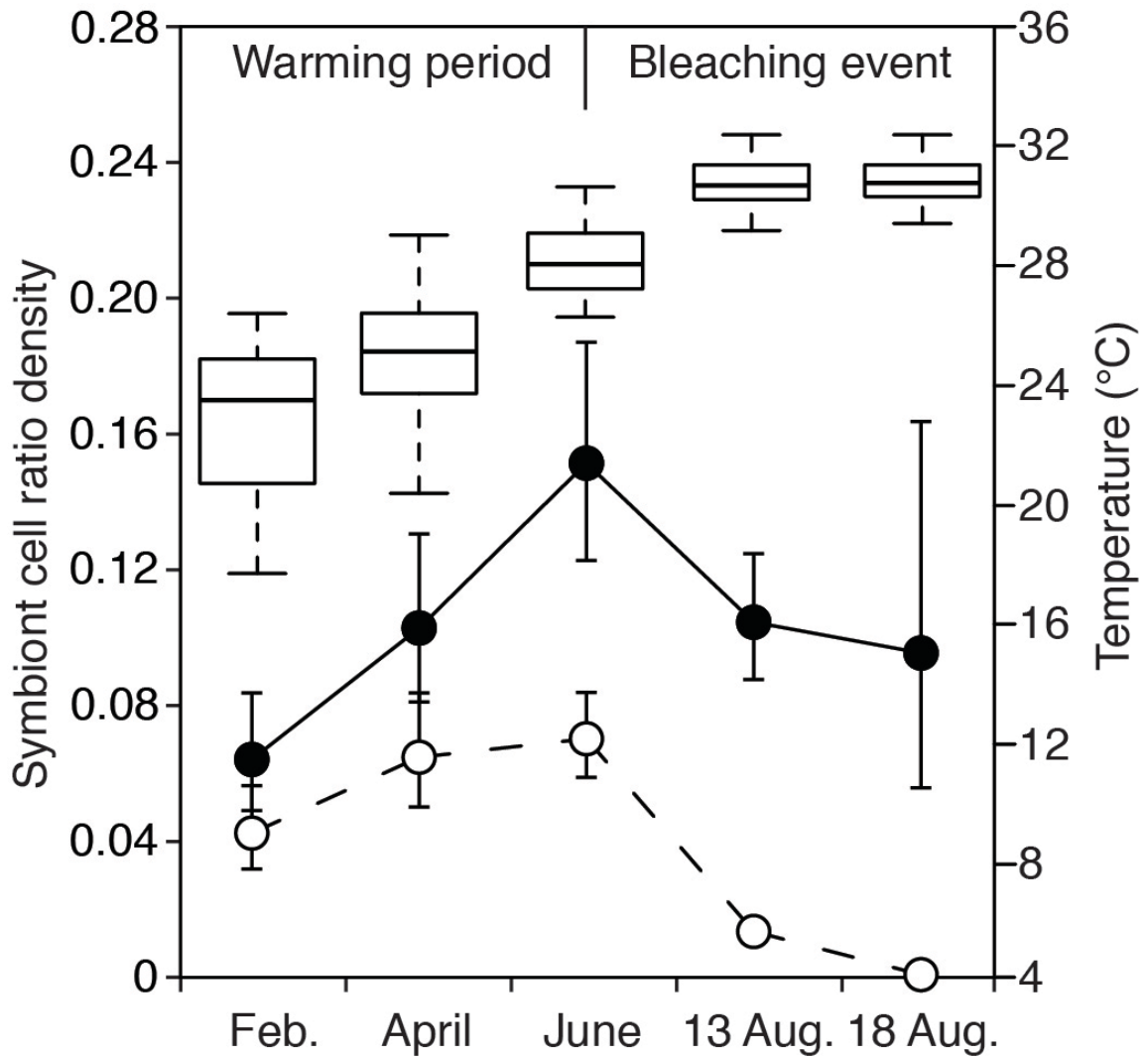


Figure 2.3. Clade- and density-dependence of density changes during warming and bleaching. **(a)** Relationship between total symbiont cell ratio density prior to warming and change in density during warming for each colony. ANCOVA revealed that greater increases occurred in clade D colonies ($p < 0.0001$), and colonies with fewer symbionts ($p < 0.0001$), regardless of clade. The clade D regression ($R^2 = 0.43$) had a similar slope to clade C ($R^2 = 0.66$) but a greater intercept ($p < 0.0001$), indicating greater density increases in clade D colonies. The cell ratio density at which each regression predicts no change over time (indicated by asterisks at x-intercepts, anti-log-transformed to linear scale; clade C = 0.077 clade D = 0.289) may be considered the optimal density favored for the particular symbiont type under these environmental conditions. **(b)** Relationship between total symbiont cell ratio density prior to bleaching and change in density during bleaching for each colony. Linear regressions (clade C $R^2 = 0.46$; clade D $R^2 = 0.41$) had negative slopes ($p < 0.0001$), indicating colonies with more symbionts bleached more severely. The lower intercept for clade C colonies ($p < 0.0001$; ANCOVA) indicates they lost more symbionts than clade D colonies across the range of initial densities. The points at which regression lines for C and D colonies predict zero change may be considered the density thresholds above which the bleaching response is triggered.

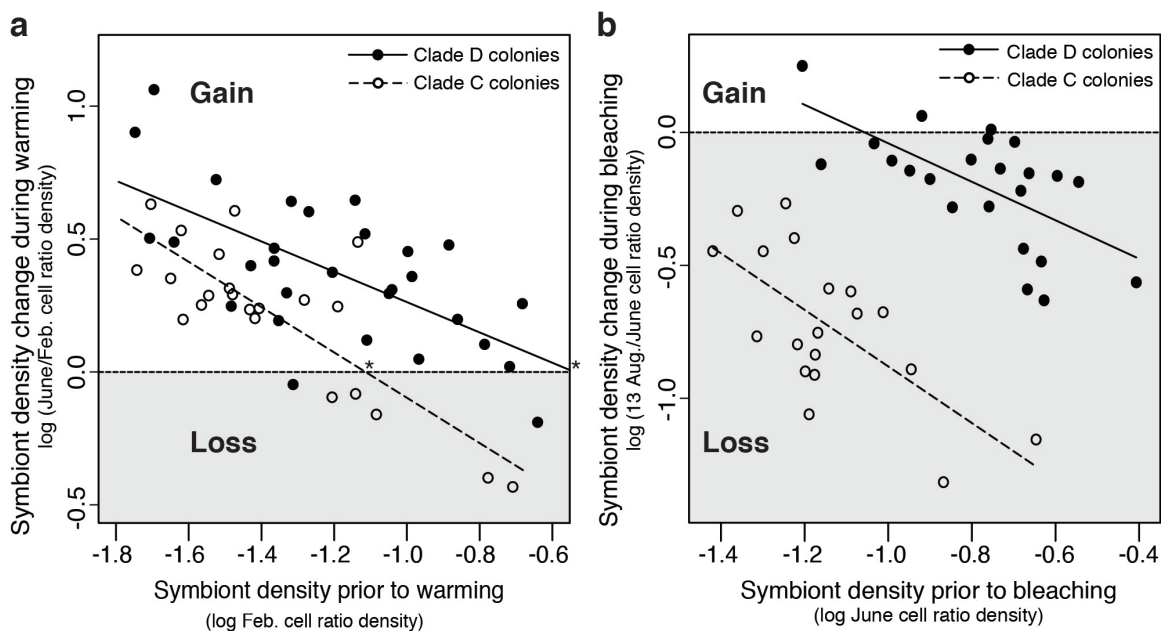


Table 2.1 Cycle threshold values from standard curves of *P. damicornis* assay and *Symbiodinium* clades C and D assays run in simplex and multiplex. Slopes from clades C and D assays are pooled between simplex and multiplex because ANCOVA showed no difference between the slopes of the two standard curves. Amplification efficiencies are calculated as $E=(10^{(-1/\text{slope})}-1)*100$.

log(Copies)	C simplex	C multiplex	D simplex	D multiplex	<i>P. damicornis</i>
5	22.4	22.6	19.7	19.8	22.6
5	22.2	22.2	19.7	19.8	22.8
5	22.4	22.2	19.8	19.8	22.8
5	22.3	22.1	19.8	19.8	23.4
4	26.3	25.1	22.8	22.9	25.6
4	26.0	25.9	22.8	23.0	25.7
4	26.0	25.6	22.9	22.9	26.1
4	25.7	26.2	22.8	22.9	26.7
3	29.7	29.2	26.2	26.4	29.5
3	29.2	29.7	26.3	26.5	29.6
3	29.4	29.4	26.3	26.5	29.6
3	29.1	29.9	26.3	26.5	29.8
2	32.2	33.1	29.3	29.8	33.1
2	33.1	33.3	29.7	29.9	33.0
2	32.3	32.9	29.7	29.7	33.1
2	33.1	33.2	29.5	30.0	32.8
1	35.5	35.8	33.6	33.5	37.5
1	35.6	37.0	33.5	33.2	36.0
1	34.8	35.6	33.6	33.8	35.9
1	37.3	36.6	33.3	34.1	36.8
R-squared	0.99	0.99	0.99	0.99	0.99
Slope		-3.45		-3.44	-3.43
Efficiency		94.9%		95.3%	95.7%

Chapter 3

Continuous variation and context-dependent shifts in algal symbiont community structure drive physiological adaptation in reef corals

Summary

The community of dinoflagellate algal symbionts (*Symbiodinium* spp.) in reef-building corals may critically mediate the impact of global climate change on coral reef ecosystems. Changes in symbiont communities over time may provide corals with an adaptive response to environmental change; in particular, increases in heat tolerant symbionts following thermal bleaching events may reduce future stress susceptibility. However, the relevance and functional significance of symbiont community change in corals are unclear due to conflicting reports of symbiont shuffling and stability. Here, we address this conflict by using new quantitative molecular techniques to investigate the ecological drivers of symbiont shuffling in the Caribbean coral *Orbicella faveolata* and the links between symbiont community composition and host functional traits. We show that both photochemical efficiency and bleaching severity are continuous functions of the proportion of *Symbiodinium* in clade B and D, with more clade D resulting in lower performance but higher stress tolerance. We also show that changes in the proportion of these symbionts after bleaching depend on bleaching severity and recovery temperature, with shuffling to heat tolerant clade D promoted by more severe bleaching and a warmer recovery environment. These findings reconcile conflicting reports over whether corals change their symbionts in response to environmental stress by showing that the magnitude of symbiont shuffling is context-dependent. However, the continuous

relationship between community composition and host function indicates that even small changes in symbiont communities significantly impact coral performance and stress tolerance, facilitating adaptive responses to environmental change without requiring wholesale changes in community dominance.

Background

Coral reefs are threatened by many local and global stressors, the most pressing of which is rising sea temperatures associated with global climate change. Ocean warming is causing more frequent and severe coral reef bleaching events worldwide (Hoegh-Guldberg et al. 2007), and has contributed to the loss of at least 19% of coral reefs (Wilkinson 2008), imperiling the future persistence of these ecosystems.

However, corals' susceptibility to bleaching—the breakdown of their symbiosis with single-celled dinoflagellate algae—is highly dependent on the type(s) of symbiotic algae (genus *Symbiodinium* (Pochon and Gates 2010)) with which they associate. Corals can host different symbiont types that confer varying levels of heat tolerance to the symbiotic partnership. In particular, clade D *Symbiodinium* provide corals with high resistance to bleaching (Glynn et al. 2001; Berkelmans and van Oppen 2006; Stat and Gates 2011). Photosynthetic performance (Rowan 2004; Cantin et al. 2009) and coral growth rates (Little et al. 2004; Jones and Berkelmans 2010) also depend on symbiont identity. While many studies have examined these differences in corals with one symbiont type or another, corals may often contain mixtures of multiple types in varying proportions (Rowan et al. 1997; Silverstein et al. 2012). The functional significance of this variation is poorly understood, as only recently have molecular methods enabled

accurate quantification of mixed assemblages (Mieog et al. 2009; Cunning and Baker 2013). We now aim to link mixed symbiont community structure with responses such as photophysiological performance and bleaching susceptibility, which will dramatically improve our understanding of the functional consequences of community variation (Loram et al. 2007b).

Changes in symbiont communities can alter the functional response of the host, thereby facilitating adaptive responses to environmental change. For example, following a bleaching event, corals can recover with a different symbiont assemblage that may be better suited to the prevailing conditions (Buddemeier and Fautin 1993; Baker et al. 2004), an idea termed the Adaptive Bleaching Hypothesis (ABH). Investigations of the ABH have revealed that although sometimes corals “switch” or “shuffle” their symbionts (Baker 2003), this does not always occur: sometimes they recover with the same symbiont community they had prior to stress (Goulet 2006; Sampayo et al. 2008; Stat et al. 2009a). Numerous conflicting reports have generated controversy over the ABH and hindered understanding of coral responses to climate change.

Symbiont community dynamism may be facilitated or restricted by a variety of factors. Host specificity may limit switching and shuffling (Goulet 2006), although the idea that corals associate with a single symbiont type has been increasingly challenged (Silverstein et al. 2012). Alternatively, symbiont community dynamics may be better understood through an ecological framework in which processes such as changing niche space, competition, and succession (Connell 1978) govern the interactions among diverse symbionts and ultimately drive community organization. In this way, principles of disturbance ecology may predict the circumstances under which communities change,

and to what degree. Indeed, corals need not completely replace one symbiont type with another—the relative proportions of different types may change more subtly. Importantly, subtle changes in the symbiont community may still effect significant changes in host function, and may be a far more likely response than wholesale turnover of these communities.

In this study, we investigate how photophysiological performance and bleaching susceptibility are influenced by varying mixtures of two symbiont types (B1 and D1a) in the Caribbean coral *Orbicella faveolata* exposed to thermal stress. Subsequently, we monitor symbiont community dynamics in corals recovering from low, medium, or high bleaching severity at two different temperatures (24 and 29°C). We test the hypotheses that disturbance severity and recovery environment determine the trajectory of symbiont community reassembly, with the prediction that more severe bleaching and warmer recovery temperature promote shuffling toward clade D-dominated communities. This approach will elucidate the ecological principles governing symbiont community dynamics, help reconcile controversy surrounding the ABH, and evaluate the potential for clade D *Symbiodinium* to increase reef corals' resilience under global climate change.

Materials and Methods

Coral collection and preparation

Three colonies of *Orbicella* (formerly *Montastraea*) *faveolata* were collected at Emerald Reef (25°40.45' N, 80°5.92' W), near Key Biscayne (Florida, USA). Replicate cores were taken from each colony using a seawater-fed drill press equipped with a 2.54 cm diameter core drill bit. Cores (n=87) were mounted on ceramic Reef Plugs (Boston Aqua Farms,

NH, USA) using CorAffix adhesive (Two Little Fishes, Florida, USA) and then placed in experimental aquaria at 24°C for two months of acclimation to the experimental tanks.

Experimental setup

Experimental manipulations were conducted in an indoor semi-recirculating coral culture facility consisting of four 75-gal fiberglass tanks supplied with sand-filtered, UV-treated seawater from Biscayne Bay. Temperature in each tank was set and maintained within $\pm 0.5^\circ\text{C}$ using SeaChill TR-20 heater/chillers (TECO US), and light ($190\text{-}280 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$) was provided by two 400-W metal halide lamps (IceCap Inc., USA) over each tank on a 12-hour light/dark cycle. Corals were fed throughout the experiment 2-3 times per week with Reef Chili (Bulk Reef Supply). These conditions were maintained constant throughout the experiment except for temperature (described below).

Experimental treatments

Corals were experimentally bleached in a single tank maintained at 32°C. Cores from each colony were divided among three bleaching severity treatments and exposed to bleaching conditions for either 7 days, 10 days, or 14 days. Exposures were staggered such that corals were removed from the 32°C tank at the same time. Corals then recovered for 3 months at 24°C or 29°C, with two replicate tanks at each temperature.

Chlorophyll fluorometry

The maximum dark-adapted quantum yield of photosystem II (F_v/F_m) was recorded for each coral core throughout the experiment using an imaging pulse amplitude modulated

(I-PAM) fluorometer (Walz, Effeltrich, Germany). Measurements were taken prior to stress exposure, at the end of the bleaching, and every 1-2 weeks during recovery.

Symbiont identification

Tissue from a single polyp was taken from each coral core using the corner of a new razor blade and placed in 1% SDS. Samples were heated to 65°C for 1.5 hrs, and DNA was extracted using a modified organic extraction protocol (Baker et al. 1997). *Symbiodinium* types were identified from three cores of each colony by sequencing the internal transcribed spacer-2 region of ribosomal DNA (ITS2 rDNA). This gene was amplified using the primers ITSintfor2 and ITS2clamp (LaJeunesse and Trench 2000), and products were separated by denaturing gradient gel electrophoresis (35-75% gradient) using a CBS scientific system. Dominant bands were excised, reamplified, and sequenced in both directions using BigDye Terminator v3.1 cycle sequencing kit and an Applied Biosystems 3130xl Genetic Analyzer.

Symbiont quantification

Each sample was then assayed using quantitative PCR (qPCR) to target specific actin loci in *O. faveolata* and *Symbiodinium* in clade B, C, and D, in order to calculate symbiont density in terms of the symbiont to host (S/H) cell ratio (Mieog et al. 2009; Cunning and Baker 2013). The *O. faveolata* assay included 150 nM OfavActF (5'-CGCTGACAGAATGCAGAAAGAA-3'), 100 nM OfavActR (5'-CACATCTGTTGGAAGGTGGACA-3'), and 250 nM OfavActProbe (5'-NED-TGAAGATCAAGATCATTGC-MGB-3'). The clade B assay included 200 nM BActF

(5'-CGATGGCTTTGCAGCTCAA-3'), 300 nM BActR (5'-TGTCCTCCGGAATTTGTGCAA-3'), and 100 nM BActProbe (5'-FAM-CTGTAAACCCTGTCATTC-MGB-3'). The clade C and D assays were multiplexed, using the same primers and reaction conditions as described in (Cunning and Baker 2013). All reactions were carried out in 10 μ L volumes (with 5 μ L Taqman Genotyping MasterMix and 1 μ L DNA template) on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Thermal cycling conditions consisted of an initial incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Cycle threshold (C_T) values were calculated by the StepOnePlus software package with a set fluorescence threshold of $\Delta R_n=0.01$.

Presence of target DNA was indicated by amplification of two technical replicates and no target detection in negative controls. C_T values for *O. faveolata* and *Symbiodinium* clade B were reduced by 5.74 and 5.41 cycles, respectively, to normalize differences in fluorescent signal intensity among assays, based on the results of standard curves generated following the methods of Cunning and Baker (2013). Adjusted C_T values were then used to calculate S/H cell ratios using the formula $2^{C_T(\text{host})-C_T(\text{symbiont})}$, divided by the symbiont to host ploidy ratio (1/2; Santos and Coffroth 2003), DNA extraction efficiency ratio (0.828; Cunning and Baker 2013), and target locus copy number ratio (see below).

Symbiont copy number estimation

Symbionts were isolated from one *O. faveolata* core containing only clade D symbionts and one containing a mixture of B and D, and counted with a haemocytometer. DNA was extracted from 6 separate aliquots of 100,000 cells from each core, and

template proportional to 2,000 cells was then quantified by qPCR (n=24 technical replicates). Quantities were calculated using standard curves generated from a dilution series of target standards from 10^6 to 10^1 copies per reaction and assuming 95.5% DNA extraction efficiency (Cunning and Baker 2013). From the core containing only clade D cells, copy number for the clade D actin locus was 3.04 ± 0.28 (SEM), and therefore estimated at 3 copies cell⁻¹. Using this value, the number of clade D cells in the mixed sample was then subtracted from the total number of cells in the reaction (2,000) to determine the remaining number of clade B cells. This quantity was used to calculate a copy number of 1.18 ± 0.16 (SEM) for clade B cells, which we estimate to be 1 copy cell⁻¹.

Host copy number estimation

Primers for a single copy marker (“SC”; AY395789) identified in the *Orbicella* species complex (Severance et al. 2004) were designed using Primer Express (Applied Biosystems, Foster City, CA), and tested using a dilution series of *O. faveolata* DNA (OfavscF1 (5'-TCACTTTCGCAGAGCAATGG-3'); OfavscR1 (5'-GGCAATGTTTGTACCCACGAT-3')). The slope of this standard curve was -3.36 (amplification efficiency = 98.4%), indicating the SC assay can be compared to actin assays to estimate actin locus copy numbers. For 12 to 14 samples from each colony, both the single copy and actin loci were then amplified in duplicate 12.5 μ L qPCR reactions using SYBR Green MasterMix and the same thermal cycling conditions (see Methods). The SC assay contained 900nM OfavscF1 and OfavscR1 primers, and the actin assay contained 240 nM OfavActF and 160 nM OfavActR. The ratio of actin:SC was calculated using the formula $2^{[C_T(\text{SC}) - C_T(\text{actin})]}$. The average actin:SC ratios were

13.89±0.36, 7.27±0.42, and 14.43±0.37 (SEM) for the three colonies, and thus values of 14, 7, and 14 copies cell⁻¹ were used in subsequent calculations.

Data analysis

The total symbiont density in a sample was calculated as (clade D S/H cell ratio + clade B S/H cell ratio), and the proportion of clade D was calculated as clade D S/H cell ratio / total density. Multiple regression models were used to analyze the factors affecting F_v/F_m before and after bleaching, symbionts remaining after bleaching, and the proportion clade D after recovery (see Table 1). The significance of each factor was assessed by F-tests comparing full and reduced models. Effects of bleaching duration, proportion clade D, and total density on response variables were analyzed using partial residuals or adjusted means to control for the other factors in the model. Differences among adjusted means were tested using Student's t-tests. For each linear model, observations with residuals of fitted values more than 1.5 times the interquartile range below or above the 1st and 3rd quartiles, respectively, were considered outliers and removed. Symbiont densities were log transformed and the proportion clade D was arcsine squareroot transformed for use in statistical models. All analyses were conducted in R version 2.9.1 (R Development Core Team) with the “effects” package (Fox 2003).

Results

Initial symbiont communities

Sequencing of ITS2 rDNA identified *Symbiodinium* B1 and D1a as the dominant symbionts in each colony, which we refer to as clade B and clade D, respectively. The

initial mean symbiont to host cell ratio in all corals was 0.091, or about one symbiont cell for every 11 host cells (0.074-0.110 95% CI). Cores contained mixtures of clade B and clade D *Symbiodinium* ranging from 1.2-90.1% clade D (except 3 cores in which only clade B was detected). Mean photochemical efficiency (F_v/F_m) was 0.51 ± 0.04 (SD), and was negatively correlated with the proportion of clade D symbionts ($p < 0.0001$; Table 1; Fig. 3.1).

Bleaching phase

Bleaching for 7 (low), 10 (medium), and 14 days (high severity) resulted in mean F_v/F_m of 0.356 ± 0.004 (SE), 0.289 ± 0.004 , and 0.271 ± 0.005 , respectively. F_v/F_m in bleached corals depended on symbiont community composition ($p < 0.01$), with a higher proportion of clade D maintaining higher F_v/F_m (Fig. 3.1). There was also a host genotypic (colony) effect ($p < 0.0001$) on F_v/F_m , with some colonies' symbionts experiencing more photodamage than others.

The number of symbionts remaining in bleached tissues was also influenced by bleaching duration ($p = 0.0003$; Table 1), with low, medium, and high bleaching treatments resulting in symbiont densities (S/H cell ratios) and % symbiont losses of 0.036 (-60.4%), 0.028 (-69.2%), and 0.017 (-81.3%), respectively (Fig. 3.2). Symbiont loss also depended on initial proportion of clade D ($p < 0.001$), and initial symbiont density ($p = 0.0186$; Table 1). Corals bleached more severely (i.e., had fewer symbionts remaining) when they initially had lower proportions of clade D (Fig. 3.3A), or higher total densities (Fig. 3.3B). There was also a host genotypic effect on symbionts remaining, with some colonies retaining more symbionts ($p < 0.0001$).

Recovery phase

Symbiont community composition following recovery was significantly influenced by colony ($p < 0.0001$), the proportion of clade D in bleached tissue ($p < 0.0001$), bleaching severity ($p < 0.0001$), recovery temperature ($p < 0.0001$), and an interaction between bleaching severity and recovery temperature ($p < 0.0001$). Corals recovered with higher proportions of clade D when they bleached more severely and recovered at warmer temperatures (Fig. 3.4). Corals that underwent low severity bleaching and recovered at 24°C had less clade D symbionts (13.8% clade D) than corals that underwent high severity bleaching and recovered at 29°C (88.2% clade D, Fig. 3.4).

Discussion

Our results establish a continuous relationship between symbiont community composition and coral functional traits, which indicates that even small, incremental changes in symbiont communities can impact critical functional outcomes. First, we show, for the first time, that continuous changes in photochemical efficiency in healthy corals result from variation in the relative abundance of different *Symbiodinium* types, with higher proportions of clade B resulting in greater overall efficiency (Fig. 3.1). These results support the hypothesis that clade D is a low-performance symbiont that provides less benefit to its host under non-stressful conditions (Cantin et al. 2009). However, under thermal stress, this trend was reversed: corals with more clade D maintained higher photochemical efficiency (Fig. 3.1), illustrating a tradeoff between performance and stress tolerance in these two symbionts. Importantly, the continuous nature of these relationships indicates that changes in performance and stress tolerance do not require

wholesale turnover of the symbiont community, but will result from even small, incremental shifts in community composition.

In addition to photophysiology, we show that coral bleaching severity is also determined by the proportion of clades B and D *Symbiodinium*. Corals with more clade B bleached more severely, while corals with more clade D did not lose as many symbionts (Fig. 3.3A). While previous studies have shown that corals hosting one symbiont type or another differ in bleaching susceptibility (Glynn et al. 2001; Berkelmans and van Oppen 2006), we show that continuous variation in the proportions of two types predicts the magnitude of symbiont loss in mixed assemblages, such that any incremental increase in clade D will reduce the severity of bleaching. Corals with more symbionts before bleaching also bleached more severely (Fig. 3.3B), confirming previous reports that high densities of algal symbionts exacerbate the bleaching response, possibly due to a greater accumulation of oxidative stress (Cunning and Baker 2013).

While the bleaching phase of this study establishes crucial links between symbiont community composition and holobiont function, it also sets the stage to observe how bleaching severity and recovery conditions influence symbiont community reassembly. During recovery, we found that corals that bleached less severely recovered with more clade B *Symbiodinium*, and corals that bleached more severely recovered with more clade D (Fig. 3.4). This may be driven by contrasting tissue microclimates: less-bleached tissues with more remaining symbionts may be characterized by higher nutrient competition and lower light levels due to self-shading (Enríquez et al. 2005; Wangpraseurt et al. 2012), favoring clade B as a superior competitor. Conversely, severely bleached tissues may be characterized by less competition and higher light,

favoring stress-tolerant clade D. Warmer temperatures also promoted greater recovery with clade D, but only when corals were severely bleached (Fig. 3.4). In severely bleached corals with already high light levels due to less self-shading, high temperatures may give stress-tolerant clade D symbionts an even greater competitive advantage. When corals are less-bleached, or recovering at lower temperatures, the competitive advantage of clade D may be lessened, explaining why these conditions resulted in intermediate proportions of clades B and D.

These differences in recovered community composition due to bleaching severity and recovery temperature can be understood within the context of disturbance ecology and niche differentiation between the two symbiont types (Connell 1978). As our photophysiological data suggest (Fig. 3.1), clade D represents a light-tolerant, superior competitor under stressful conditions, while clade B represents a shade-tolerant, superior competitor under “normal” conditions. After minor disturbance (e.g., low bleaching and 24°C recovery), clade B-dominated communities became established, while major disturbance (e.g., high bleaching and 29° recovery) resulted in clade D domination. Meanwhile, more even mixtures of the two symbionts occurred at intermediate levels of disturbance. Without subsequent disturbance, community succession may lead to dominance by the superior competitor (e.g., clade B). Indeed, some studies have shown that clade D that becomes dominant post-bleaching is gradually lost from symbiont communities over time (Thornhill et al. 2006). Persistence of clade D may therefore require more frequent disturbance or sustained warmer conditions (Stat and Gates 2011).

These findings help reconcile conflict surrounding the ABH. If, as we show here, the symbiont community established in corals after bleaching is determined by the

severity of bleaching and the recovery environment, then differences in these factors may account for some of the variable outcomes reported by numerous studies (Rowan et al. 1997; Toller et al. 2001; Baker 2001; Berkelmans and van Oppen 2006; Jones et al. 2008; Sampayo et al. 2008; Thornhill et al. 2009; LaJeunesse et al. 2009; Stat et al. 2009a; DeSalvo et al. 2010; LaJeunesse et al. 2010; McGinley et al. 2012). In the future, better characterization of bleaching severity and the recovery environment may improve our understanding of symbiont community dynamics and the ABH. Importantly, we show that shuffling does not need to result in complete turnover of symbiont communities in order to have ecological relevance; qPCR-based measurements of the proportions of different symbiont types show that even small changes can have meaningful effects on host function (Fig. 3.1, 3.3).

In conclusion, we show that continuous variation in the composition of *Symbiodinium* communities in corals drives significant changes in critical functional traits, including photochemical efficiency and bleaching susceptibility. In addition, we show that the composition of these communities changes over time in accordance with principles of disturbance and community ecology (Connell 1978). Our findings establish the importance of quantitative symbiont community metrics in understanding host responses, and suggest they should be incorporated into future work. In addition, these findings help reconcile the controversy surrounding the ABH by elucidating the ecological circumstances under which symbiont community shifts are likely to take place. If subtle incremental shifts in the composition of symbiont communities can affect host function, then even small increases in the amount of clade D symbionts may significantly increase the heat tolerance and future persistence of reef corals.

Figure 3.1 Photochemical efficiency (F_v/F_m) as a function of symbiont community composition (proportion clade D) in healthy corals before bleaching (closed circles, $n=87$) and after bleaching (open circles, $n=79$). Values for F_v/F_m are the mean response plus the partial residuals from multiple regression models (see Table 1). Parameter estimates from multiple regression models indicate that the correlation of F_v/F_m with proportion clade D is negative in healthy corals ($p<0.0001$) and positive in bleached corals ($p<0.01$).

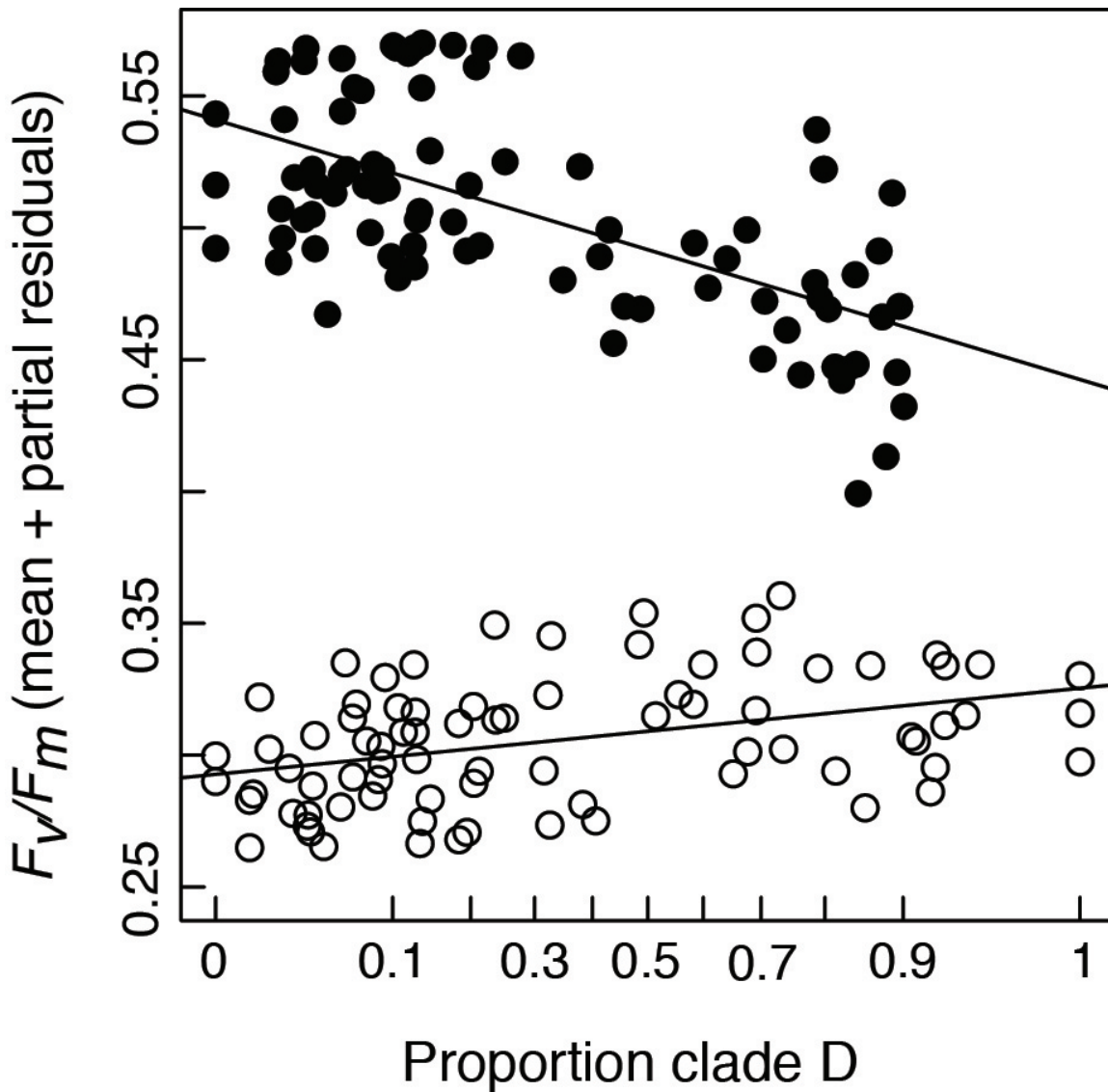


Figure 3.2 Symbiont densities after different bleaching severity treatments. Mean symbiont densities (S/H cell ratio \pm SEM) in corals bleached for 7 (Low, n=26), 10 (Medium, n=36), or 14 days (High severity, n=25) indicate declines of 60.4%, 69.2%, and 81.3% relative to the initial mean density of all corals (n=87). Bleached densities are adjusted means (\pm SEM) from multiple regression models (see Table 1). Columns that do not share a letter are significantly different (Student's t-test; $p < 0.01$).

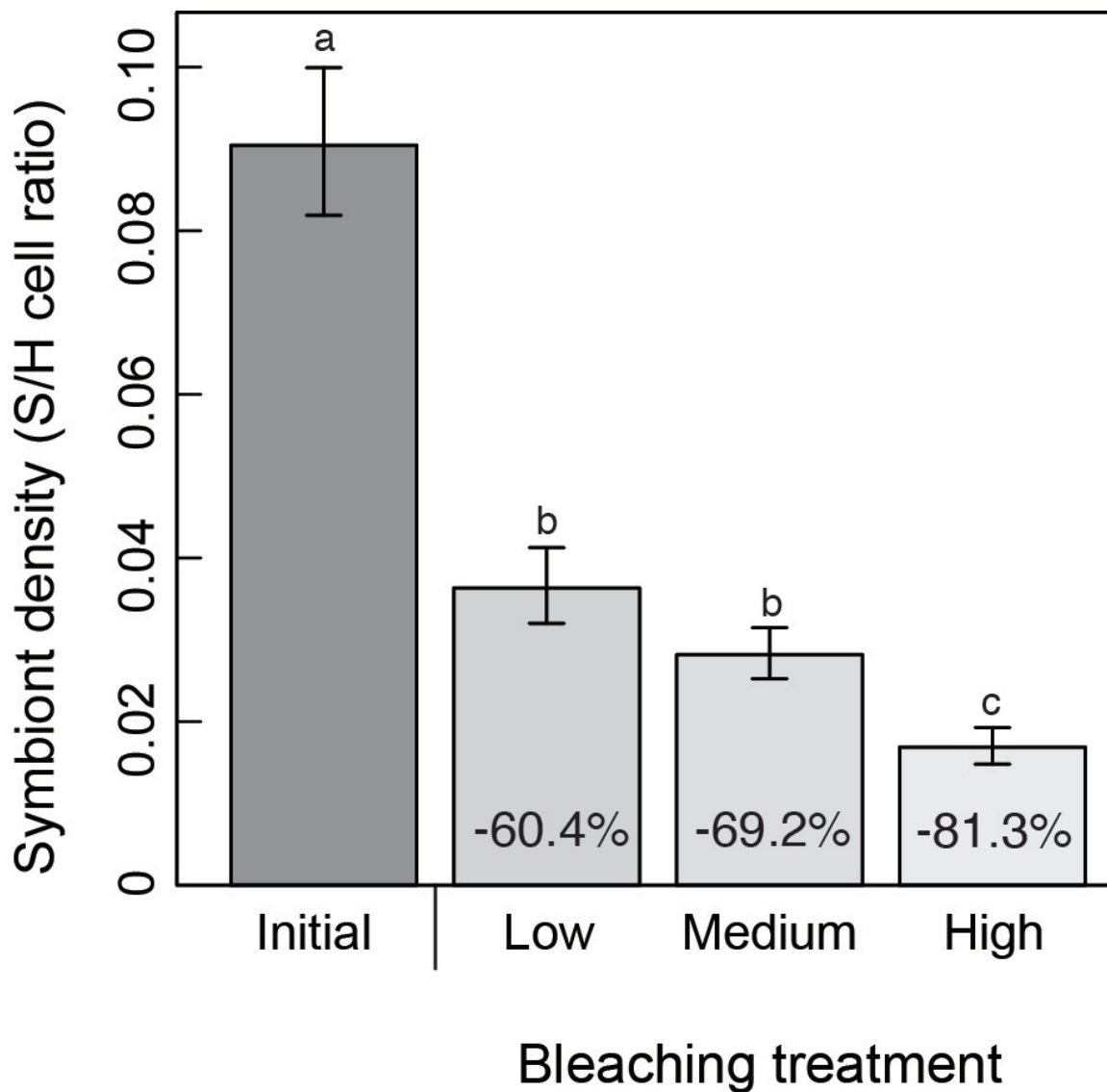


Figure 3.3 Bleaching severity (expressed as the density of symbionts remaining) as a function of (a) initial proportion clade D and (b) total symbiont density. Response values are the mean S/H cell ratio plus partial residuals from multiple regression models (see Table 1). Parameter estimates indicate the density of remaining symbionts after bleaching is positively correlated with the initial proportion of clade D ($p < 0.001$, $n = 87$) and negatively correlated with the initial total symbiont density ($p < 0.05$, $n = 87$).

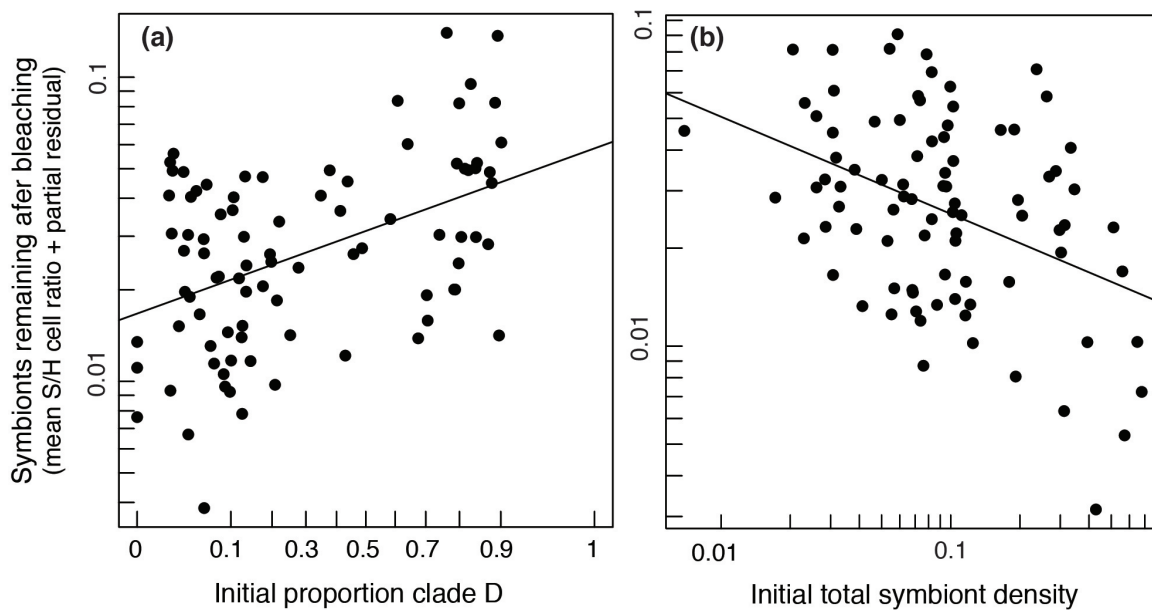


Figure 3.4 Composition of bleached and recovered symbiont communities. The proportion of clade D symbionts after recovery (adjusted mean \pm SEM) was higher in corals that bleached more severely and recovered at higher temperatures, with a significant interaction between these two factors (see Table 1). Columns that do not share a letter are significantly different (Student's t-test, $p < 0.05$, $n = 82$).

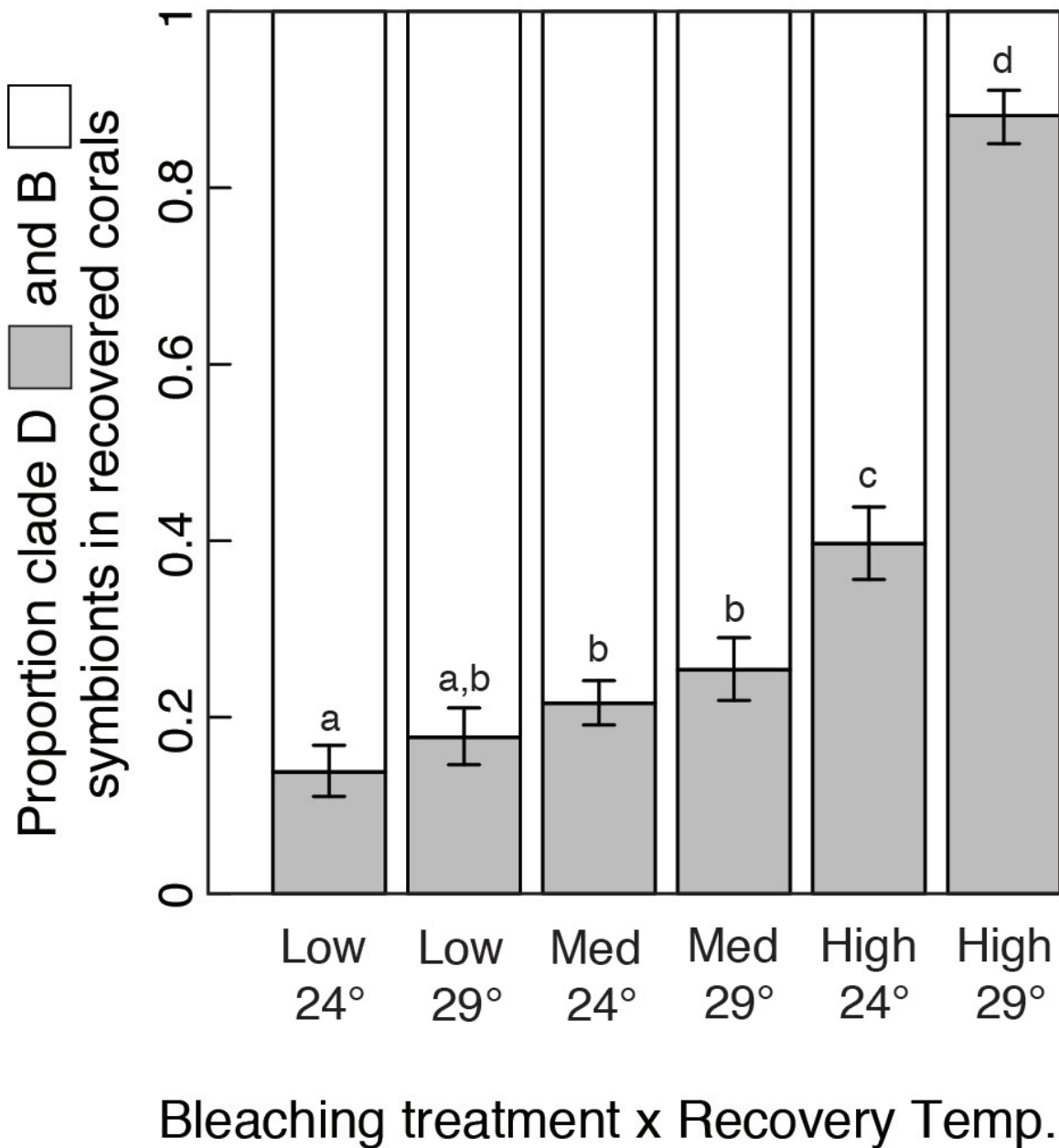


Table 3.1 Multiple regression models constructed for each response variable. Shown are the multiple R-squared values, factors included in each model, and results of partial F-tests comparing the full model with reduced models lacking each factor.

Response	Model R²	Source	Sum of Squares	Partial-F Ratio	P-value
Initial Fv/Fm	0.4415	Colony	0.0061	3.0114	0.0546
		Initial proportion D	0.0380	37.5970	<0.0001
Bleached Fv/Fm	0.7721	Colony	0.0142	13.7550	<0.0001
		Bleaching severity	0.1017	98.7030	<0.0001
		Bleached proportion D	0.0053	10.2920	0.0020
Symbionts remaining after bleaching	0.7599	Colony	8.8171	56.1270	<0.0001
		Bleaching severity	1.4280	9.0902	0.0003
		Initial proportion D	0.9735	12.3950	0.0007
		Initial total density	0.4533	5.7716	0.0186
		Colony	0.7201	16.9750	<0.0001
Proportion D after recovery	0.8794	Bleached proportion D	3.7503	176.814	<0.0001
		Bleaching severity	3.8289	90.2602	<0.0001
		Recovery temperature	0.8529	40.2108	<0.0001
		Bleaching severity*	0.9138	21.5415	<0.0001
		Recovery temperature			

Chapter 4

Symbiont shuffling in reef corals is context- and species-dependent

Summary

Reef corals often associate with multiple types of symbiotic algae (*Symbiodinium* spp.) simultaneously, whose proportions may change over time in a phenomenon known as symbiont “shuffling”. Since different symbiont types are known to confer various physiological properties on their hosts, changes in their relative proportions may have major impacts on holobiont fitness. In particular, increases in the abundance of heat tolerant symbionts (e.g., *Symbiodinium* clade D) may allow corals to acclimatize to rising sea temperatures and resist symbiosis breakdown due to thermal stress (i.e., coral bleaching). However, the capacity for shuffling and circumstances under which it occurs are poorly understood. Here, we address the influence of bleaching severity, recovery temperature, and host-symbiont partnership on symbiont shuffling with a controlled experimental approach. We found that clade D established dominance within the host after mild bleaching in *S. siderea* but not *O. faveolata*. However, after severe bleaching, clade D became the dominant symbiont in both coral species. In addition, warmer temperatures during recovery from bleaching promoted shuffling toward clade D. The degree of symbiont shuffling depends on complex interactions among bleaching stress, recovery environment, and the particular host-symbiont association. Understanding the factors that promote or restrict symbiont shuffling will improve our ability to predict coral responses to climate change.

Background

The persistence of coral reef ecosystems is threatened by local and global stressors including rising temperatures associated with anthropogenic climate change (Baker et al. 2008). Thermal stress causes the breakdown of symbiosis between reef-building corals and *Symbiodinium* algae known as coral bleaching, which often leads to significant coral mortality. However, some corals are able to resist bleaching by hosting thermally tolerant *Symbiodinium* types (e.g., members of clade D (Glynn et al. 2001; Rowan 2004; Berkelmans and van Oppen 2006; LaJeunesse et al. 2010; Cunning and Baker 2013)). Increases in thermotolerant symbionts are often observed following bleaching events (Baker et al. 2004; Jones et al. 2008), suggesting that bleaching provides an opportunity for symbiont community reassembly and rapid acclimatization to environmental change (Buddemeier and Fautin 1993).

The extent to which changes in symbiont communities (i.e., symbiont shuffling) may facilitate coral adaptation is unclear due to many conflicting reports of symbiont shuffling (Rowan et al. 1997; Toller et al. 2001; Baker 2001; Berkelmans and van Oppen 2006; Jones et al. 2008; LaJeunesse et al. 2009; DeSalvo et al. 2010) or stability (Sampayo et al. 2008; Thornhill et al. 2009; Stat et al. 2009a; LaJeunesse et al. 2010; McGinley et al. 2012). However, inconsistencies among the species investigated, environmental stressors applied, and techniques used to assess responses make these studies difficult to compare, and may explain their variable outcomes. In fact, many factors may influence symbiont shuffling, including taxon-specific host and symbiont traits, severity, type, and duration of bleaching stress, and environmental conditions during recovery, but have received little to no experimental investigation. Furthermore,

only recently have molecular techniques been developed to simultaneously identify and quantify different *Symbiodinium* types in corals (Mieog et al. 2009; Cunning and Baker 2013), providing an accurate way of measuring symbiont community change in corals.

In this study, we observed symbiont community dynamics during bleaching in response to different levels of thermal stress and subsequent recovery under different temperatures in the Caribbean coral *Siderastraea siderea*. This experimental design allows us to understand the importance of bleaching severity and recovery environment in driving symbiont community changes in corals. We then compare the results of this study to identical manipulations of the coral *Orbicella faveolata* (Chapter 3) to understand how patterns of symbiont community dynamics change in different host-symbiont associations.

Materials and Methods

Coral collection and preparation

Twelve colonies of *Siderastraea siderea* were collected at Emerald Reef (25°40.45' N, 80°5.92'W), near Key Biscayne (Florida, USA). Replicate cores were taken from each colony using a seawater-fed drill press equipped with a 2.54 cm diameter core drill bit. Cores (n=138) were mounted on ceramic Reef Plugs (Boston Aqua Farms, NH, USA) using CorAffix adhesive (Two Little Fishes, Florida, USA) and then placed in experimental aquaria at 24°C for a two-month acclimation period.

Experimental setup

Experimental manipulations were conducted in an indoor semi-recirculating coral culture facility consisting of four 75-gal fiberglass tanks supplied with sand-filtered, UV-

treated seawater from Biscayne Bay. Temperature in each tank was set and maintained within $\pm 0.5^{\circ}\text{C}$ using SeaChill TR-20 heater/chillers (TECO US), and light ($190\text{-}280\ \mu\text{mol quanta m}^{-2}\ \text{s}^{-1}$) was provided by two 400-W metal halide lamps (IceCap Inc., USA) over each tank on a 12-hour light/dark cycle. Corals were fed throughout the experiment 2-3 times per week with Reef Chili (Bulk Reef Supply). These conditions were maintained constant throughout the experiment except for temperature, which was manipulated as described below.

Experimental treatments

Corals were experimentally bleached in a single tank maintained at 32°C . Cores from each colony were divided among three bleaching severity treatments and exposed to bleaching conditions for either 7 days, 10 days, or 14 days. Exposures were staggered such that all corals were removed from bleaching conditions at the same time. Corals then recovered for 3 months at either 24°C or 29°C , with two replicate tanks at each temperature.

Symbiont community characterization

Tissue from a single polyp was taken from each coral core using the corner of a new razor blade and placed in 1% SDS. Samples were heated to 65°C for 1.5 hrs, and DNA was extracted using a modified organic extraction protocol (Baker et al. 1997). The presence of *Symbiodinium* clades A, B, C, and D was tested in each colony using quantitative PCR (qPCR) assays targeting specific actin loci in each clade (C and D assays following Cunning & Baker (2013), B assay following Chapter 3, and clade A

assay, unpublished). Colonies were found to contain mixtures of *Symbiodinium* in clades C and D, and thus assays targeting actin loci in these two clades as well as *S. siderea* were run for each sample to calculate symbiont density in terms of the symbiont to host (S/H) cell ratio (Mieog et al. 2009; Cunning and Baker 2013). The *S. siderea* assay included 150 nM OfavActF (5'-CGCTGACAGAATGCAGAAAGAA-3'), 100 nM OfavActR (5'-CACATCTGTTGGAAGGTGGACA-3'), and 250 nM OfavActProbe (5'-NED-TGAAGATCAAGATCATTGC-MGB-3'). The clade C and D assays were multiplexed using the same primers and reaction conditions as described in (Cunning and Baker 2013). All reactions were carried out in 10 μ L volumes (with 5 μ L Taqman Genotyping MasterMix and 1 μ L DNA template) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Thermal cycling conditions consisted of an initial incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Cycle threshold (C_T) values were calculated by the StepOnePlus software package with a set fluorescence threshold of $\Delta R_n=0.01$.

Presence of target DNA was indicated by amplification of two technical replicates and no target detection in negative control reactions. C_T values for *S. siderea* and *Symbiodinium* clade C were reduced by 5.74 and 4.07 cycles, respectively, to normalize differences in fluorescent signal intensity among assays, based on the results of standard curves generated following the methods of Cunning and Baker (Cunning and Baker 2013). Adjusted C_T values were then used to calculate S/H cell ratios using the formula $2^{C_T(\text{host})-C_T(\text{symbiont})}$, divided by the symbiont to host ploidy ratio (1/2 (Santos and Coffroth 2003)), DNA extraction efficiency ratio (0.828 (Cunning and Baker 2013)), and target locus copy number ratio (see below).

Symbiont copy number estimation

Symbionts were isolated from four of the *S. siderea* colonies and counted with a haemocytometer. DNA was extracted from 2-3 separate aliquots of 100,000 cells from each core (n=11 extracts), and template proportional to 4,000 cells was quantified by both clade C and D qPCR assays on two separate plates in duplicate. Absolute quantities of C and D copies were calculated using duplicate standard curves of cloned clade C and D target standards from 10^6 to 10^1 copies per reaction on each plate, assuming 95.5% DNA extraction efficiency (Cunning and Baker 2013). The mean number of C and D copies in each colony on each plate was used to construct a system of 8 equations with the structure: C copies/C copy number + D copies/D copy number = 4,000 cells. This over-determined system of equations was solved by singular value decomposition to obtain least squares solutions for the number of actin copies in clade C (23) and D (2) cells.

Host gene copy number estimation

Host actin copy number was estimated by comparison to a *Pax-C* locus, which has been shown to be a single-copy marker in other coral species (van Oppen et al. 2000; 2001). Forward (5'-AGAGATGCTGGAGAAAGAATTTGAG-3') and reverse (5'-GCCTCCGACATGTCAATCTTATTA-3') primers were designed based on an alignment of 12 *S. siderea Pax-C* intron sequences (Nunes et al. 2011) using Primer Express (Applied Biosystems, Foster City, CA), and tested using a dilution series of *S. siderea* DNA. The slope of this standard curve was -3.136 (amplification efficiency = 108.4%), indicating the *Pax-C* assay can be compared to actin assays to estimate actin locus copy numbers. For 2-6 samples from each colony, both the single copy and actin

loci were then amplified in duplicate 12.5 μ L qPCR reactions using SYBR Green MasterMix and the same thermal cycling conditions (see Methods). The *Pax-C* assay contained 900nM each primer, and the actin assay contained 240 nM OfavActF and 160 nM OfavActR. The ratio of actin:*Pax-C* was calculated using the formula $2^{[C_T(Pax-C)-C_T(actin)]}$. The average actin:*Pax-C* ratio was 14.2 ± 0.4 (SEM) and did not differ among colonies (ANOVA, $F > 0.5$), and thus a value of 14 copies cell⁻¹ was used in subsequent calculations.

Data analysis

The total symbiont density in a sample was calculated as (clade D S/H cell ratio + clade C S/H cell ratio), and the proportion of clade D was calculated as clade D S/H cell ratio / total density. Multiple regression models were used to analyze the factors affecting the number of symbionts remaining after bleaching and the proportion clade D after recovery (see Table 4.1). The significance of each factor was assessed by F-tests comparing full and reduced models. Effects of bleaching duration, proportion clade D, and total density on response variables were analyzed using partial residuals or adjusted means to control for the other factors in the model. Differences among adjusted means were tested using Student's t-tests.

Cross-species comparisons of symbiont densities during bleaching were made by Student's t-tests comparing initial means and post-bleaching adjusted means from multiple separate regression models (see Table 1 for *S. siderea* model, see chapter X Table X for *O. faveolata* model). Comparisons of proportion D after recovery were made by fitting a new model to all data with species, bleaching duration, and recovery

temperature as fully-crossed factors, colony nested within species, and initial proportion clade D as a continuous covariate (see Table 4.1). Multiple comparisons of adjusted mean proportion D for each level of species, bleaching duration, and recovery temperature were compared with initial overall proportion D by Student's t-tests. Observations of total S/H cell ratios greater than 0.5 were considered biologically unrealistic and excluded from analysis. Symbiont densities were log transformed and the proportion clade D was arcsine squareroot transformed for use in statistical models. All analyses were conducted in JMP v10.0 and R v2.9.1 (R Development Core Team) with the "effects" package (Fox 2003).

Results

Symbiont loss during bleaching

The density of symbionts remaining in bleached tissues was lower in corals that experienced longer durations of thermal stress ($P < 0.0001$; Fig. 4.1) and that had lower initial proportions of clade D ($P < 0.0001$; Fig. 4.2a). The parameter describing the effect of initial total density was less than 1 ($P < 0.0001$), indicating that corals with higher initial densities lost a greater proportion of symbionts, but was not different from 0 ($P > 0.05$), indicating no effect on absolute density after bleaching (Fig. 4.2b).

Symbiont community recovery

Symbiont community composition in recovered corals depended on bleaching severity and recovery temperature (interaction effect, $F = 5.64$, $P < 0.01$). All treatment groups recovered with 95.8-99.5% clade D except for corals that underwent low

bleaching (7 days) and recovered at 24°C, which had significantly less clade D (74%; $P < 0.0001$; Fig. 4.3).

Cross-species comparisons

In Chapter 3, *Orbicella faveolata* was exposed to the same experimental conditions as *S. siderea* in the present study, enabling direct comparisons between responses of the two species. *Orbicella faveolata* contained mixtures of clade B and clade D symbionts which were maintained at lower total densities than the C and D symbionts in *S. siderea* (0.090 vs. 0.141; Fig. 4.4). After 7 days of bleaching, *S. siderea* had more symbionts remaining than *O. faveolata* (0.050 vs. 0.036), but had lost a greater proportion of its initial community (64% vs. 60%). *Siderastrea siderea* continued to bleach more severely and had fewer symbionts than *O. faveolata* after 10 (0.014 vs. 0.028) and 14 days (0.002 vs. 0.017) of bleaching. After 14 days, *S. siderea* lost 99% of its symbionts, compared to 81% loss in *O. faveolata* (Fig. 4.4).

Recovered symbiont community composition depended on an interaction between species, bleaching severity, and recovery temperature ($F = 15.2$, $df = 2$, $P < 0.0001$), indicating different responses in the two coral species to varying levels of disturbance. With the same initial proportion of clade D, *S. siderea* symbiont communities readily shuffled to clade D-dominance after relatively low disturbance (low bleaching, 24°C recovery), while *O. faveolata* communities only became D-dominated following high disturbance (high bleaching, 29°C recovery; Fig. 4.5).

Discussion

Our results show that symbiont community dynamics during bleaching and recovery depend on the initial community structure, disturbance severity, recovery environment, and the particular host-symbiont partnership. *Siderastrea siderea* bleached more severely when thermal stress lasted longer (Fig. 4.1), as observed in *O. faveolata* (Chapter 3, Fig. 3.2). Corals with more clade D had more symbionts remaining after thermal stress (Fig. 4.2a), providing another example of the thermotolerance conferred by clade D (Glynn et al. 2001; Rowan 2004; Berkelmans and van Oppen 2006; Cunning and Baker 2013).

Corals with higher total densities also experienced greater relative symbiont loss (Cunning and Baker 2013), but ended up with equivalent remnant densities after bleaching (Fig. 4.2b). In contrast, *O. faveolata* cores with higher densities had fewer remnant symbionts (Chapter 3, Fig. 3.3), suggesting high total density was a more important driver of bleaching severity in this species. The relative importance of total density may have been eclipsed in *S. siderea* by other factors associated with this species or its clade C symbiont that drove a more severe overall bleaching response. The greater bleaching severity in *S. siderea* compared to *O. faveolata* (Fig. 4.4) may have been due to higher mean symbiont densities, greater thermal sensitivity of clade C, or other host factors (Baird et al. 2009). While we cannot confirm their relative importance here, each of these factors may be important in determining bleaching severity.

After recovery, *S. siderea* symbiont communities were dominated by clade D (Fig. 4.3, 4.5), although the proportion of clade D depended on bleaching severity and recovery environment. Corals that experienced low bleaching severity and recovered in

cool water (24°C) hosted 63% clade D, while all other corals hosted 92-100% clade D. More severe bleaching and warmer recovery also led to greater clade D dominance in *O. faveolata* (Chapter 3, Figure 3.4), although the degree of D-dominance differed between the two species (Fig. 4.5). When starting with the same amount of clade D (28%), *S. siderea* symbiont communities shifted to nearly complete clade D dominance in response to relatively low disturbance, while much more disturbance was required to elicit the same degree of symbiont shuffling in *O. faveolata*.

The different degrees of shuffling in these species may be a result of different symbionts in competition with clade D (clade C in *S. siderea* vs. clade B in *O. faveolata*). In warmer environments and microhabitats characterizing bleached tissues, clade D may easily outcompete clade C in *S. siderea*, while clade B in *O. faveolata* may still remain competitive until disturbance is very severe. Supporting clade B as a better competitor than clade C is the fact that low disturbance increased the abundance of clade B (Fig. 4.5). An alternative explanation is that host species differentially mediate the competitive dynamics between symbiont types. For example, *O. faveolata* may produce more pigments that reduce irradiance in bleached tissues (Salih et al. 2000) and lessen the competitive advantage of clade D, or *S. siderea* may more actively select for clade D (Yamashita et al. 2011).

Although low disturbance promoted greater increases in clade D in *S. siderea* than *O. faveolata*, severe disturbance led to near complete clade D dominance in both coral species (Fig. 4.5), perhaps because severely bleached tissues represent a niche space inhabitable only by clade D (i.e., as a pioneer species). Over time, successional dynamics may cause further shuffling (Toller et al. 2001) and potentially the loss of clade D in

post-bleaching reversion of symbiont communities (Thornhill et al. 2006).

In summary, we found that symbiont communities shuffle further towards clade D-dominance in corals that bleach more severely and recover at warmer temperatures, although the degree of shuffling varies among different host-symbiont partnerships even under the same abiotic conditions. There are clearly many factors that co-determine the degree to which symbiont communities shuffle or remain stable, and differences in these factors may explain the different outcomes among studies investigating these responses. On reef scales, shuffling may occur in some coral species in response to a mild bleaching event, while severe bleaching may cause shuffling in more species. Future work must control for these factors in order to evaluate the ecological importance of symbiont shuffling in coral responses to global climate change.

Figure 4.1 Symbiont densities before and after different bleaching severity treatments. Adjusted mean S/H cell ratios (\pm SEM) from multiple regression indicate declines of 64%, 90%, and 99% after 7 (n=24), 10 (n=15), or 14 (n=24) days of 32°C thermal stress, respectively, relative to initial mean density (n=77). Columns that do not share a letter are significantly different (Student's t-test; $p < 0.0001$).

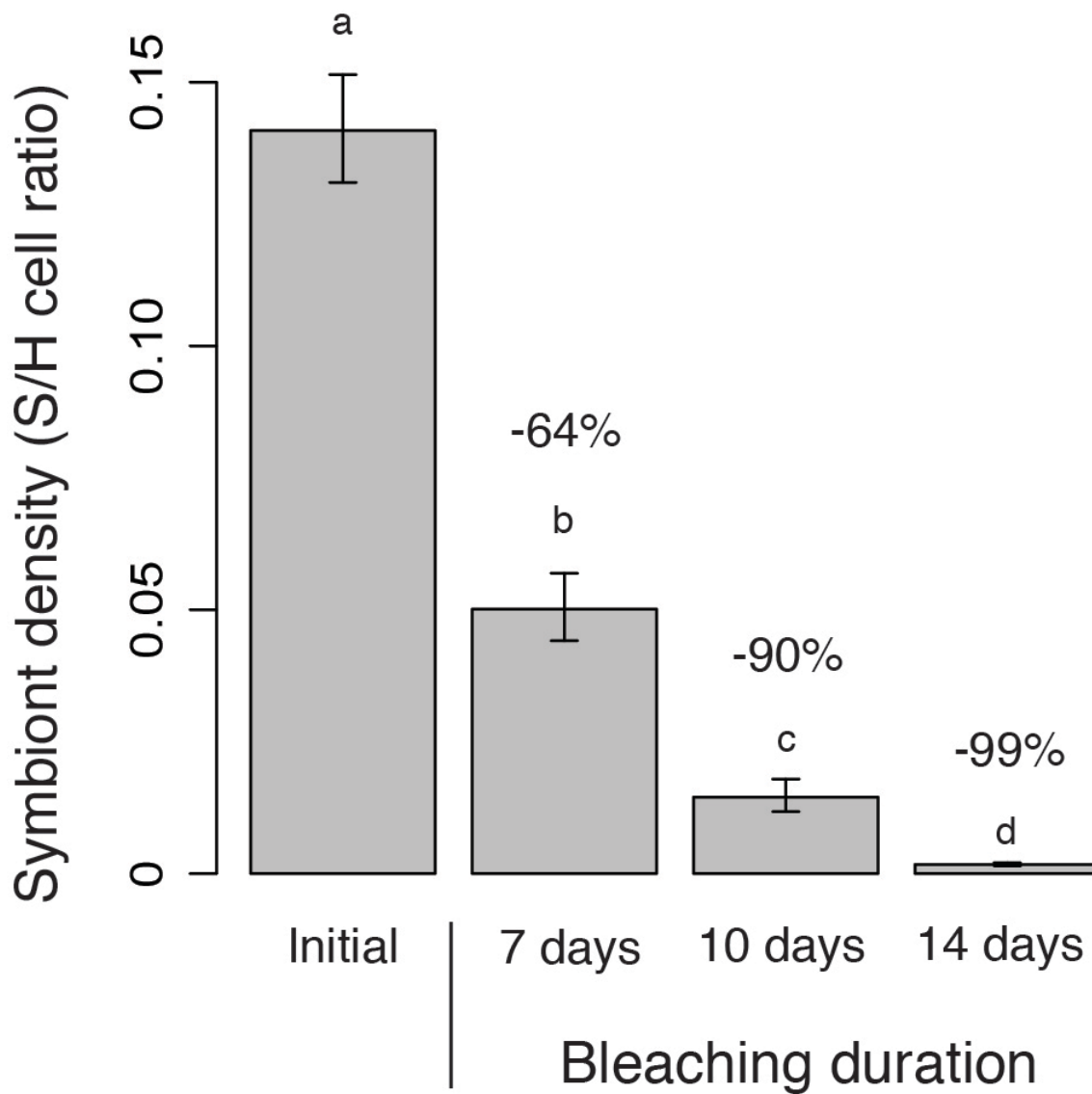


Figure 4.2 Bleaching severity (expressed as the density of symbionts remaining) as a function of initial (a) proportion clade D and (b) total symbiont density. Response values are the mean S/H cell ratio plus partial residuals from multiple regression models (see Table 4.1). The density of remaining symbionts after bleaching is positively correlated with the initial proportion of clade D ($p < 0.0001$, $n = 77$), but equal with respect to initial total density ($p > 0.76$). However, the parameter estimate for initial density is less than 1 (dotted line; $p < 0.0001$), indicating that corals with higher total densities lost a greater proportion of symbionts.

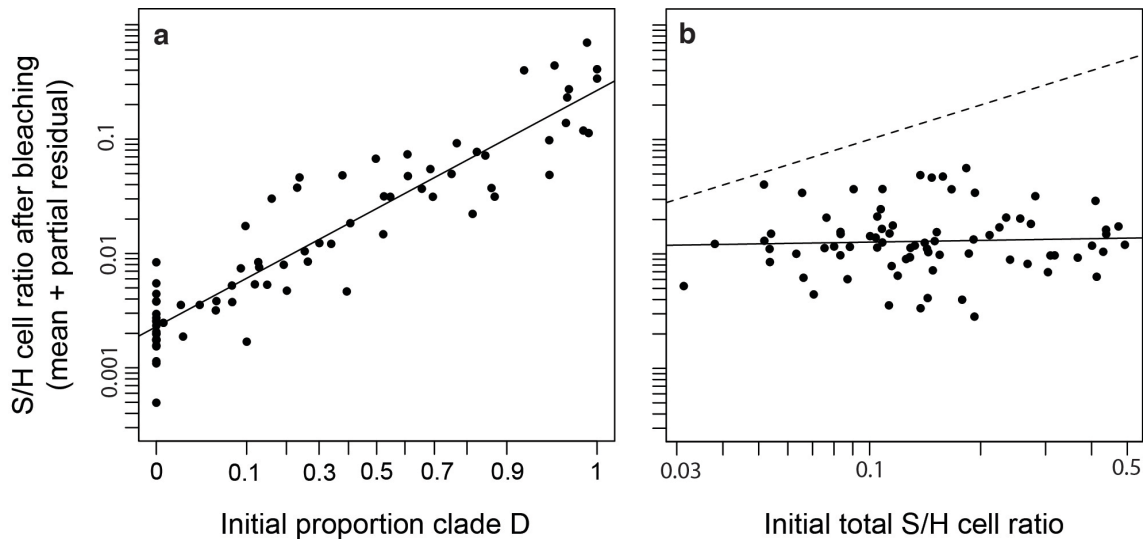


Figure 4.3 Composition of bleached and recovered symbiont communities. The proportion of clade D symbionts after recovery (adjusted mean \pm SEM) was dependent on bleaching severity and recovery temperature (interaction term $P < 0.01$). Columns that do not share a letter are significantly different (Student's t-test, $P < 0.0001$).

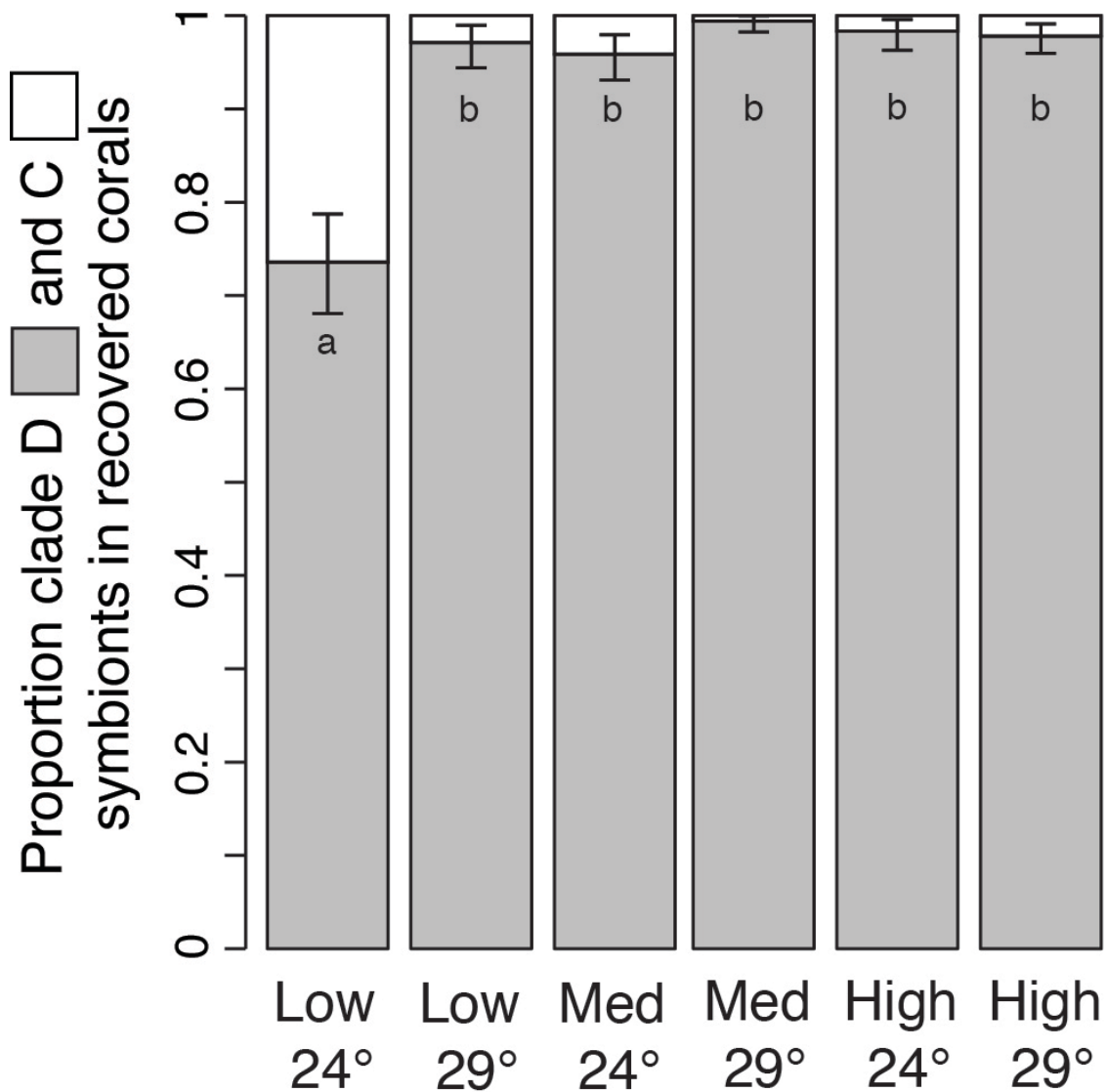


Figure 4.4 Loss of symbionts in *S. siderea* and *O. faveolata* in response to thermal stress. Adjusted means from species-specific statistical models (see Tables 3.1 and 4.1) are compared with initial overall means for each species, showing greater loss of symbionts in *S. siderea*. Points not sharing a letter are significantly different (Student's t-tests, $p < 0.05$).

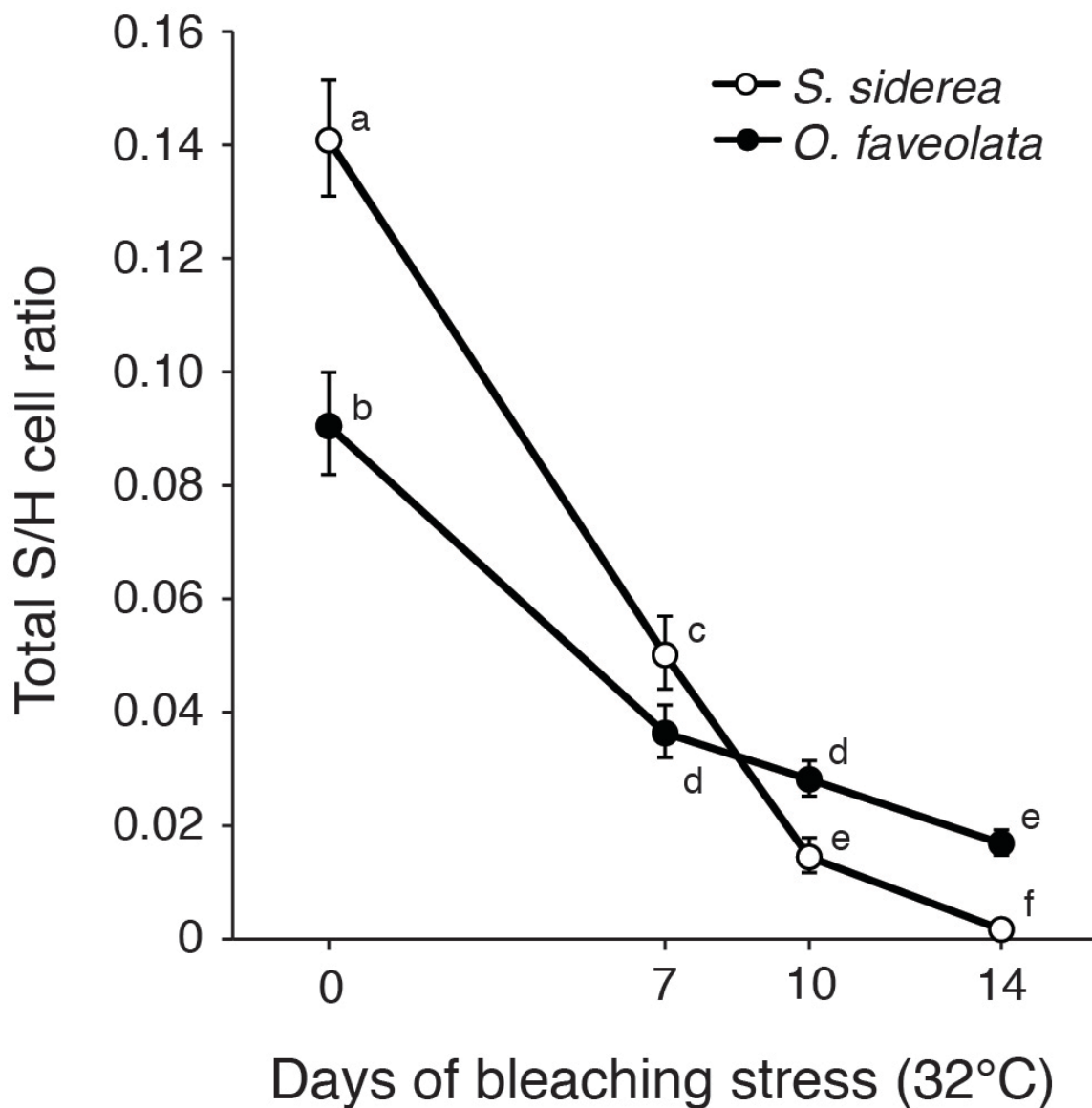


Figure 4.5 Symbiont shuffling in *S. siderea* and *O. faveolata* as a function of bleaching severity and recovery temperature, which are ordered according to increasing disturbance to the symbiont community. Adjusted means (\pm SEM) from a three-way interaction among species, bleaching severity, and recovery temperature ($P < 0.0001$, Table 4.1) indicate that with the same initial proportion of clade D ($\sim 28\%$), *S. siderea* shuffles to clade D-dominance at lower levels of disturbance than *O. faveolata*. Points that do not share a letter are significantly different (Student's t-tests, $P < 0.05$).

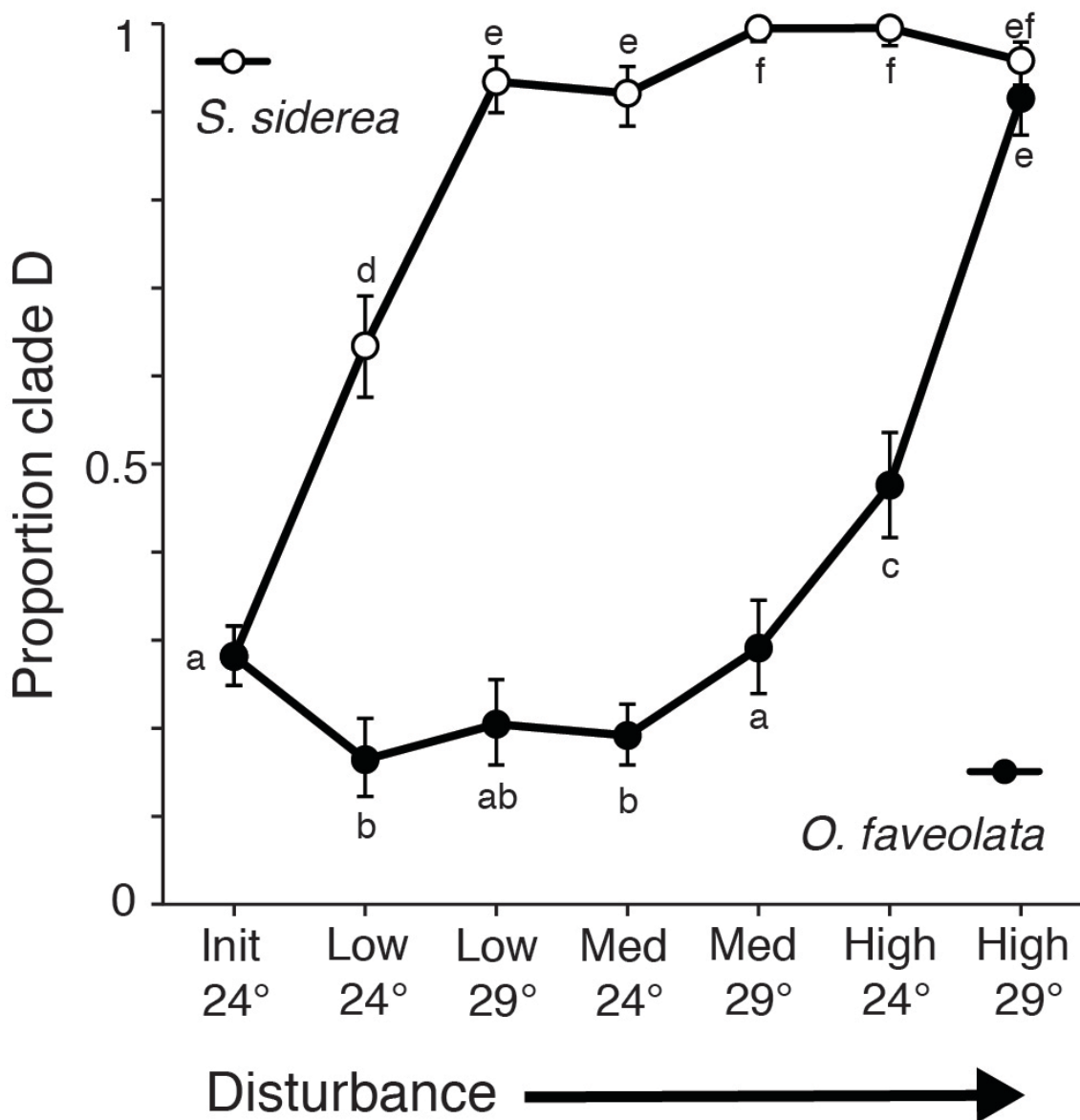


Table 4.1 Multiple regression models constructed for each response variable. Shown are the multiple R-squared values, factors included in each model, and results of partial F-tests comparing the full model with reduced models lacking each factor.

Species: Response	Model R ²	Source	Sum of Squares	Partial-F ratio	P-value
<i>S. siderea</i> : Symbionts remaining after bleaching	0.8887	Colony	4.51	3.96	0.0002
		Bleaching severity	23.48	113.54	<0.0001
		Initial proportion D	0.98	9.51	0.0031
		Bleaching severity: Initial proportion D	6.46	31.25	<0.0001
		Initial total density	0.01	0.09	0.7608
		Colony	0.56	1.13	0.3538
<i>S. siderea</i> : Proportion clade D after recovery	0.5753	Bleached proportion D	0.06	1.31	0.2557
		Bleaching severity	0.61	6.73	0.0022
		Recovery Temperature	0.52	11.45	0.0012
		Bleaching severity: Recovery Temperature	0.51	5.63	0.0054
		Colony[Species]	2.68	5.23	<.0001
		Species	13.19	308.28	<.0001
<i>S. siderea</i> and <i>O. faveolata</i> : Proportion clade D after recovery	0.8666	Bleaching severity	3.73	43.62	<.0001
		Species: Bleaching severity	0.99	11.56	<.0001
		Recovery Temperature	1.20	28.15	<.0001
		Species: Recovery Temperature	0.04	1.00	0.3199
		Bleaching severity: Recovery Temperature	0.02	0.23	0.7942
		Species:Bleaching severity:Recovery Temperature	1.30	15.23	<.0001
Initial proportion D	2.67	62.50	<.0001		

Chapter 5

Dynamic regulation of endosymbionts to maximize interaction benefits for mutualisms in a changing world

Summary

Regulating partner abundance may allow symbiotic organisms to mediate interaction outcomes, facilitating adaptive responses to environmental change. To explore the capacity for adaptive regulation in an ecologically important endosymbiosis, we studied population dynamics of symbiotic algae in reef-building corals under different abiotic contexts. We found that naturally-variable symbiont densities converged under constant conditions and readjusted to new equilibria when conditions changed. To explain these patterns, we modeled interaction costs and benefits as functions of partner density, and show that the observed densities are consistent with maximization of interaction benefit under each set of conditions. Regulating partner density can therefore sustain maximum benefit in a dynamic environment, and represents a mechanism by which diverse symbiotic organisms can adapt to environmental change. While providing a new perspective on reef coral symbioses, this cost-benefit framework has broad utility in understanding the biotic and abiotic context-dependence of interaction outcomes in a variety of mutualisms.

Background

Symbiotic interactions directly or indirectly affect every species on earth (Bronstein et al. 2004). Their outcomes may be positive (mutualism), neutral (commensalism), or negative (parasitism), depending on the interaction costs and

benefits, which may fluctuate based on changes in biotic or abiotic factors (Holland 2002; Neuhauser and Fargione 2004; Agrawal et al. 2007; Holland and Deangelis 2009). In reef coral symbioses, dinoflagellate endosymbionts (genus *Symbiodinium*) benefit their hosts by providing photosynthetically-fixed carbon, which underlies the ecological and evolutionary success of coral reefs (Muscatine and Porter 1977). However, the interaction benefit can be compromised under environmental stress due to the disruption of photosynthesis, leading to the breakdown of symbiosis (coral “bleaching”) that threatens reefs’ future survival (Hoegh-Guldberg et al. 2007). These benefits and costs depend on the genetic identity of the *Symbiodinium* partner, whose physiological performance is mediated by the abiotic environment. For example, clade C *Symbiodinium* provide greater benefit (i.e., photosynthate) than clade D under normal conditions (Cantin et al. 2009), but suffer more photodamage (Rowan 2004) and oxidative stress (McGinty et al. 2012) at higher temperatures, leading to more severe bleaching.

Considerable research has addressed the effects of partner identity on interaction outcomes, and the potential for symbiotic organisms to adapt to environmental change by associating with alternative, better-performing partners (“partner-switching”; Kiers et al. 2010). In reef corals, particular attention has been focused on clade D *Symbiodinium*, which may increase corals’ heat tolerance (Baker et al. 2004; Rowan 2004; Berkelmans and van Oppen 2006), but decrease their growth rates (Little et al. 2004; Jones and Berkelmans 2010). However, while partner identity certainly affects interaction outcomes, recent research has overlooked an even more dynamic aspect of the symbiosis with potentially greater influence over holobiont performance and resilience—partner density.

Symbiont population density can directly influence the costs and benefits of symbiosis (Holland et al. 2002; 2004). In corals, benefits derived from photosynthesis increase with symbiont density but then decline at high levels (Hoogenboom et al. 2010), while the costs of symbiont maintenance and stress continue to increase. This framework predicts that hosts derive maximum net benefit at some “optimal” symbiont density (Hoogenboom et al. 2010; Wooldridge 2012), but obtain less benefit with too many or too few symbionts. Because changes in the abiotic environment continuously alter costs and benefits (Thompson 1988), and thereby the optimal density of symbionts, organisms may be able to sustain maximum benefit by regulating partner density to match a dynamically shifting optimum (Smith 1987).

Regulation of symbiosis has been studied in diverse associations including invertebrates and algae (Falkowski et al. 1993; Davy et al. 2012), insects and *Wolbachia* (Kondo et al. 2005), legumes and *Rhizobium* (Prell et al. 2009), plants and mycorrhizae (Koide and Schreiner 1992), lichens (Hill 1989), and plants and pollinating seed parasite insects (Holland et al. 2004). This work has revealed that hosts can regulate symbiont abundance, although the biotic and abiotic context-dependence of these interactions remains a major research gap in population and community ecology (Agrawal et al. 2007). In particular, the role of symbiont density regulation as a “tuning” mechanism to optimize interactions under changing abiotic conditions is poorly understood.

In order to investigate this type of dynamic regulation in reef coral symbioses, we studied population dynamics of two genetically distinct *Symbiodinium* in clade C (C1b-c) and D (D1) in association with the coral *Pocillopora damicornis* under both constant and seasonally changing conditions. In particular, we tested the hypothesis that population

densities of symbionts are maintained at specific equilibria dependent on their genetic identity and the prevailing environment. To investigate whether changes in symbiont density can represent a strategy to sustain maximum interaction benefit, we modeled density-dependent costs and benefits of symbiosis (*sensu* Holland et al. 2002) under a range of biotic and abiotic contexts and fit this model to the observed data. We suggest that interaction costs and benefits drive host regulation of symbiont populations, and that symbiont density may have an even greater influence on interaction outcomes than symbiont identity. While interspecific population regulation may contribute to the evolutionary stability of mutualism (Holland et al. 2004), it may also, in addition to partner switching (Kiers et al. 2010), provide symbiotic organisms an adaptive ecological response to global environmental change.

Materials and Methods

We conducted two experiments to test how symbiont density equilibria responded to: 1) constant, controlled environmental conditions, and 2) natural seasonal changes in the abiotic environment.

Experiment 1: Constant environmental conditions

Fifty colonies of *P. damicornis* were collected in September 2010 off the island of Uraba, Gulf of Panama, Panama, and transported to the University of Miami Coral Resource Facility (UMCRF). One tissue sample from each colony was taken using bone cutters immediately after arrival in Miami (10 September 2010). Colonies were fragmented, mounted on numbered Eppendorf tubes, and placed in a 2,200 L indoor

semi-recirculating coral culture system, where conditions were maintained constant at 26°C and 100 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ for 10 h day⁻¹ (3.59 mol quanta m⁻² d⁻¹) from Giese Powerchrome T5HO midday and actinic+ lights. One tissue sample from each colony was taken again two years later (6 September 2012).

Experiment 2: Natural fluctuations in environmental conditions

Fifty-two colonies of *P. damicornis* were collected off the island of Taboga, Gulf of Panama, Panama, on 11 February 2009, maintained overnight in running seawater tanks at the Smithsonian Tropical Research Institute on Naos Island, Panama, and transported to the UMCRF the following day. At the UMCRF, corals were maintained for 4 months in a large outdoor raceway tank receiving flow-through ambient seawater from Biscayne Bay, and shaded with neutral density screen to ~30% of ambient irradiance. A HOBO data logger (Onset Corp.) recorded water temperature in the tank throughout the experiment. Small tissue samples were taken from each coral colony at the beginning of the experiment (12 February 2009), after two months (11 April), and after four months (14 June) (Cunning & Baker 2013).

Sample processing and molecular analysis

Coral tissue samples were preserved in DNAB + 1% SDS, and DNA was extracted using a modified organic extraction protocol. These corals hosted ITS-2 types C1b-c and D1 (Cunning and Baker 2013), which we refer to as clade C and clade D. Each sample was analyzed using qPCR assays targeting specific actin loci in *P. damicornis*, and *Symbiodinium* in clades C and D, following the protocol of Cunning and Baker

(2013). Samples from Experiment 1 were assayed following the same protocol modified for 10 μ L qPCR reaction volumes. Modified values for the fluorescence normalization in 10 μ L volume assays were calculated using copy number standards as in Cunning and Baker (2013), which indicated that *P. damicornis* and clade C assays amplified 6.8 and 4.1 cycles later than clade D assays, respectively.

Environmental data

We characterized the environmental conditions corresponding to each sampling time point by calculating mean temperature and irradiance over the 3 weeks prior to sampling. Temperatures in the field were measured hourly by a HOBO data logger at nearby Isla Taboguilla, and an irradiance range was estimated based on previous measurements from the area (D'Croz and O'Dea 2007). Temperatures for Experiment 2 were recorded by a HOBO data logger in the tank, and light levels were estimated using hourly PAR measurements (from a LICOR sensor) from the same 3-week periods in 2010, assuming 70% shading.

Statistical analysis

Differences in symbiont densities were analyzed by ANOVA on log-transformed total symbiont to host cell ratios, with geometric means reported. Bartlett's test was used to test homogeneity of variances, and Welch's ANOVA was used to test differences among means when variances were unequal. Kernel density estimation was used to construct probability density functions for symbiont density, using kernel bandwidth 1.5 times the default value. All analyses were performed in R v2.9.1.

Cost-benefit model description

We modeled the benefits and costs to the coral host as explicit functions of symbiont density (*sensu* Holland et al. 2002). To represent the context-dependence of costs and benefits, we incorporated effects of symbiont type (which determines environmental tolerance, carbon translocation, and cell size), ambient temperature, and irradiance (see below).

We hypothesized that gross benefits are determined by the total amount of photosynthate received from the symbiont population, which initially increases as symbionts become more numerous until it is reduced by density-dependent light limitation. This peak and subsequent reduction of gross benefits is expected, assuming that hosts receive only surplus photosynthate (after symbionts satisfy their own fixed respiratory demands; Muller et al. 2009). This surplus is reduced at high densities due to light-limitation of photosynthesis (i.e., self-shading, which is greater for larger clade C cells; Cunning & Baker 2013), causing a reduction in gross benefits to the host. Photosynthesis was assumed to be inhibited more in stress-sensitive clade C than stress-tolerant clade D in response to heat (Rowan 2004), cold (LaJeunesse et al. 2010), and high irradiance (Ragni et al. 2010). We also assumed that clade C produces twice as much photosynthate as clade D (Cantin et al. 2009).

Gross costs to the host were calculated as the sum of symbiont maintenance costs and oxidative stress costs. Symbiont maintenance costs (including symbiosome maintenance, CO₂-concentration mechanisms (Weis et al. 1989), and scavenging and removal of symbiont waste) were modeled as a linear function of symbiont density, and were assumed to increase with both photosynthetic and metabolic rates (i.e., greater at

higher temperature, and greater for clade C compared to clade D symbionts). The costs of oxidative stress resulting from disruption of photosynthesis were modeled as an exponential function due to the positive feedback between oxidative stress and photodamage (Weis 2008), such that higher symbiont densities exacerbate these costs. Clade D symbionts were assumed to produce less ROS than clade C (McGinty et al. 2012), and therefore have lower oxidative stress costs.

The net benefit was calculated as the difference between gross benefits and costs, and the optimal symbiont density defined as the density that maximizes net benefit. A Monte Carlo adjustment of scaling parameters was utilized to achieve correspondence between model predictions and observational data.

Cost-benefit model formulation

To simulate interaction outcomes under a variety of biotic and abiotic contexts, benefit and cost functions (Table 5.1) include effects of symbiont density (z ; Eq. 1), symbiont type (clade C or D), ambient temperature (15-32°C), and irradiance (1-30 mol quanta $m^{-2} d^{-1}$; these ranges were used to standardize values between 0 and 1). Effects of symbiont type were implemented by assigning clade-specific values for cell size (v ; Eq. 2; Cunning & Baker 2013), optimum temperature (t_{opt} ; Eq. 3), and optimum irradiance (i_{opt} ; Eq. 4), with clade D assumed to be more tolerant of high irradiance (van Oppen et al. 2009). Clade D was also assumed to provide lower carbon translocation (Cantin et al. 2009) but greater environmental tolerance (of heat (Rowan 2004), cold (LaJeunesse et al. 2010), and high irradiance (Ragni et al. 2010)), a tradeoff implemented by the variable f (Eq. 4).

Interaction outcomes are also affected by deviations of ambient temperature and irradiance from optima. These deviations are squared to represent increasing impact with further deviation, and separated piecewise to isolate the effects of low temperature (T_{sub} ; Eq. 6), high temperature (T_{super} ; Eq. 7), low irradiance (I_{sub} ; Eq. 8), and high irradiance (I_{super} ; Eq. 9). Each of these biotic and abiotic influences are subsequently incorporated into benefit and cost functions below. The modeled responses were fit to the data by including scaling multipliers.

Gross benefits as a function of symbiont density ($B_{gross}(z)$; Eq. 10) were modeled using a Ricker curve to represent an initial increase in benefit as symbionts are added (proportional to their productivity (P)), and subsequent decrease due to density-dependent light limitation (D). Symbiont productivity (P ; Eq. 11) is determined by relative carbon translocation (f) and abiotic productivity limitation (L ; Eq. 12). Productivity was assumed to be limited by heat (T_{super}), cold (T_{sub}), and excess light (I_{super}), with f included to represent greater sensitivity of clade C. The density above which benefits of photosynthesis decrease due to self-shading (D ; Eq. 13), is affected by low irradiance (I_{sub}) and symbiont size (v), with larger symbionts and lower irradiance resulting in self-shading occurring at lower densities.

The gross costs of interaction ($C_{gross}(z)$; Eq. 14) were calculated as the sum of symbiont maintenance costs and oxidative stress costs. Symbiont maintenance costs ($C_{maint}(z)$; Eq. 15), which include basic metabolic and respiratory costs such as symbiosome maintenance, CO₂ concentration (Weis et al. 1989), and scavenging of symbiont waste, were modeled as a linear function of symbiont density, with costs

increasing at higher temperatures (due to higher metabolic rates), and for more productive (i.e., clade C) symbionts (which are assumed to have higher metabolic rates).

The oxidative stress costs ($C_{stress}(z)$; Eq. 16) associated with detoxifying reactive oxygen species (ROS) produced by symbionts and repairing oxidative damage, are a product of f (representing higher ROS production in clade C than clade D (McGinty et al. 2012)) and S (ROS production due to environmental stress; Eq. 17). S increases exponentially with heat, cold, and light stress, and is greater for clade C than clade D (McGinty et al. 2012). In addition, $C_{stress}(z)$ includes an exponential component, representing exacerbation of oxidative stress by density-dependent feedback (Weis 2008), which occurs at lower densities (due to S in the denominator) as abiotic stress becomes more severe.

The net interaction benefit to the host as a function of symbiont density ($B_{net}(z)$; Eq. 18) is calculated as the difference between gross benefits and gross costs. The maximum net benefit (b_{max} ; Eq. 19) is the peak of this curve, and the optimal density (z_{opt} ; Eq. 20) is the density at which maximum net benefit occurs.

Results

Symbiont community structure

Corals in both experiments were classified as C- or D-dominated based on the composition of their symbiotic communities. Corals could host mixtures of both symbiont types, although the communities were heavily dominated by one type, with low background populations of the second type. In all experiments, C-dominated corals were $99.9 \pm 0.1\%$ (SE) clade C, and D-dominated corals were $99.3 \pm 0.2\%$ (SE) clade D.

Experiment 1: Constant environmental conditions

Symbiont to host cell ratios immediately following field collection were not significantly different in C- and D-dominated corals (0.0673 vs. 0.0580; $p=0.508$; ANOVA; Fig. 5.1). However, after being maintained for two years at constant temperature (26°C) and irradiance (3.59 mol quanta $m^{-2} d^{-1}$), densities converged to clade-specific equilibria. Densities in clade C corals decreased, compared to initials, to 0.039 (Welch ANOVA; $p=0.0012$), while densities in clade D corals decreased to 0.024 (Welch ANOVA; $p=0.0001$). The final densities in clade D corals were significantly lower than clade C corals ($p<0.0001$; ANOVA), and both clades had lower variance in their symbiont densities compared to the initial samples ($p=0.0046$ and $p=0.0002$ for clades C and D, respectively; Bartlett's test).

Experiment 2: Natural fluctuations in environmental conditions

Symbiont densities increased between February and June, correlated with a 5.3°C increase in mean water temperature (22.7±0.48°C (SEM) to 28.0±0.18°C, averaged over 3 weeks prior to sampling). Symbiont densities during this period were a function of both symbiont clade ($p<0.0001$) and time ($p<0.0001$; two-way ANOVA). Clade C densities increased from 0.042 in February, to 0.070 by June. Clade D colonies began with higher symbiont densities (0.064), and increased to 0.151 by June (Fig. 5.2).

Cost-benefit modeling

The modeled effects of temperature and irradiance on symbiont physiology (light limitation, photosynthate transfer, and oxidative stress; Fig. 5.3) underlie the abiotic

context-dependence of cost and benefit functional responses. In order to visualize the context-dependence of each component of the model output, gross benefit, gross cost, and net benefit functions were plotted for both symbiont types under combinations of low, medium, and high temperature (23, 26, 29°C) and irradiance (5, 10, 15 mol quanta $\text{m}^{-2} \text{d}^{-1}$) (Fig. 5.4). Optimal densities as defined by these functions were then plotted across the entire modeled range of temperature (18-32°C) and irradiance (1-30 mol quanta $\text{m}^{-2} \text{d}^{-1}$) (Fig. 5.5). To demonstrate the model fit, optimal symbiont densities under the particular abiotic conditions experienced by our experimental corals (temperature and light mean \pm SE over 3 weeks prior to sampling, represented by colored boxes in Fig. 5.5) were then plotted together with the probability density functions from observed data (Fig. 5.6).

Discussion

The densities of symbionts in *P. damicornis* were highly dependent on their biotic and abiotic context, supporting the hypothesis that there is an optimal density for a given symbiont in a given environment (Cunning and Baker 2013). Symbiont densities varied widely in field-collected corals, likely due to fine-scale microhabitat variation, confirming previous reports of density variation across reefscapes (Moothien-Pillay et al. 2005). However, when these corals were common-gardened under identical, constant, lab conditions, their symbiont densities converged to symbiont-specific equilibria (Fig. 5.1). Furthermore, our second experiment showed that as abiotic conditions changed seasonally, symbiont densities readjusted to new equilibria (Fig. 5.2). These results show dynamic endosymbiont regulation by the host in response to the abiotic context, and

suggest that seasonal changes, local environments, and microhabitat variation all drive the dynamics and outcomes of these interactions. These changes in partner density suggest that dynamic regulation of symbionts is a critical aspect of the maintenance of healthy symbioses and favorable interaction outcomes.

To explain the observed density shifts, we hypothesize that corals regulate their symbiont populations to maximize interaction benefit in a given environment. To test this hypothesis, we modeled the density-dependent costs and benefits of interaction to the host under variable biotic and abiotic contexts. In our model, costs and benefits are directly influenced by temperature and light, symbiont genetic identity, and density-dependent feedbacks (e.g., light limitation). By incorporating this context-dependence into cost-benefit functions, we improve upon previous models (Hoogenboom et al. 2010) and address a key research gap in population and community ecology (Agrawal et al. 2007). Our cost-benefit model was able to explain the patterns of density change we observed experimentally (Fig. 5.6), suggesting that continuous adjustment of symbiont density would indeed allow corals (and potentially other symbiotic organisms) to match a shifting, context-dependent optimum and sustain maximum interaction benefit in a changing environment.

When corals were moved from the reef to a low-light, indoor aquarium, symbiont densities were reduced (Fig. 5.1), likely to alleviate self-shading (McCloskey and Muscatine 1984). When less light is available, symbiont photosynthesis becomes light-limited at lower densities (Fig. 5.3a), reducing photosynthate transfer and gross benefit to the host. This causes a reduction in the optimal density (Fig. 5.4, 5.5) and explains why densities are reduced in corals under low light (Dustan 1979; McCloskey and Muscatine

1984). Moreover, the cost-benefit framework suggests that the significant loss of symbionts we observed in these corals (42% in clade C and 59% in clade D corals) represents an adjustment to maintain maximum interaction benefit, and not a stress-induced “bleaching” response. This distinction has important implications for the study of these symbioses, as some symbiont reductions interpreted as bleaching (Anthony et al. 2008; Kaniewska et al. 2012) may be better understood as an optimization—rather than a breakdown—of symbiosis.

In our second experiment, symbiont densities increased as temperature and light levels rose (Fig. 5.2). This is also consistent with other studies (Steele 1976; Howe and Marshall 2001; Schlöder and D’Croz 2004; Tremblay et al. 2012; Cunning and Baker 2013), and may reflect host cell catabolism that increases cell ratio density (Cunning and Baker 2013), and enhanced photosynthesis under warmer and brighter conditions (Iglesias-Prieto et al. 1992; Tremblay et al. 2012). Increasing irradiance alleviates light-limitation of photosynthesis (Fig. 5.3a), thereby increasing the benefit of additional symbionts and increasing optimal density (Fig. 5.4, 5.5). However, too much light may cause photoinhibition and oxidative stress (Fig. 5.3b, c), resulting in high costs that limit optimal density (Fig. 5.4). Such stress-related costs in clade C symbionts may explain why their densities did not increase as much as clade D as temperature and irradiance increased from spring to summer (Fig. 5.2). These mechanisms may underlie seasonal changes in symbiont density (Stimson 1997; Fitt et al. 2000), allowing corals to sustain maximum interaction benefit in a fluctuating abiotic environment.

This cost-benefit framework has broad utility in understanding host-symbiont interactions. For example, it highlights how interaction costs and benefits depend on the

physiology of the particular partner, causing different partners to be maintained at different densities even under the same conditions (Schoenberg and Trench 1980; McCloskey et al. 1996; Kondo et al. 2005). The model also illustrates how in certain contexts (e.g., low irradiance), densities are limited by low benefits, while in others (e.g., temperature extremes), they are limited by high costs (Fig. 5.4). The exponential increase in costs for hosts with high symbiont populations brings them closer to the threshold where costs outweigh benefits (i.e., symbiosis breakdown), making them more vulnerable to stress-induced bleaching (Cunning and Baker 2013). Reducing bleaching risk may therefore be an additional advantage of maintaining fewer symbionts that is not explicitly captured by the model, especially for hosts that experience frequent abiotic stress.

This cost-benefit framework also quantifies the net benefit derived from the interaction (Fig. 5.4), which may underlie fitness traits and phenotypes of the host. The decreasing net benefit of superoptimal densities (Fig. 5.4) may explain why excess symbionts (e.g., due to nutrient enrichment) can cause their hosts to grow more slowly (Stambler et al. 1991; Marubini and Davies 1996; McConnaughey 2012). Additionally, the greater maximum net benefit of clade C symbionts compared to clade D under non-stressful conditions (e.g., 26°C, 10 mol quanta m⁻² d⁻¹ PAR; Fig. 5.4) may explain why these holobionts have been observed to grow more quickly (Little et al. 2004; Jones and Berkelmans 2010). However, the model also indicates that under warmer or brighter conditions (e.g., 29°C, 15 mol quanta m⁻² d⁻¹ PAR; Fig. 5.4), clade D provides a greater maximum net benefit than clade C symbionts, suggesting it may be advantageous to coral reefs under climate change scenarios (but see Ortiz et al. 2013).

While future work must confirm these direct fitness effects, this cost-benefit framework suggests that symbiont density is a prime determinant of interaction outcomes in corals and other symbiotic organisms. Although much research has focused on the effects of partner identity on interaction outcomes, the effects of partner density may be equally, if not more, important (but see Freeman et al. 2013). Moreover, while host-symbiont specificity may restrict the potential for partner-switching, changes in partner density may be prevalent in all symbioses, and should therefore be an important component of continuing symbiosis research.

By integrating the effects of partner genotype, density, and the abiotic environment, our model illustrates the importance of context-dependence in symbiotic interactions. Future work should consider the effects of additional abiotic factors, such as eutrophication and ocean acidification. Although more complexity likely underlies the dynamics of these interactions, modeling elemental costs and benefits in reef coral symbioses nevertheless explains observed patterns of symbiont density equilibria, suggesting that costs and benefits are indeed driving the regulation of the symbiosis. Regulating partner density may allow symbiotic organisms to mediate the outcome of these interactions to maintain maximum benefit in response to environmental change.

Figure 5.1 Symbiont density convergence under constant conditions. Symbiont densities measured in clade C (n=27) and D colonies (n=21) of *P. damicornis* in experiment 1 immediately after collection (“field”, gray) and again after two years under identical, constant conditions (“lab”, orange). Boxplots at each time point show the mean, quartiles, and range of data. Probability density functions were constructed by kernel density estimation using a bandwidth of 1.5 times the default value in R.

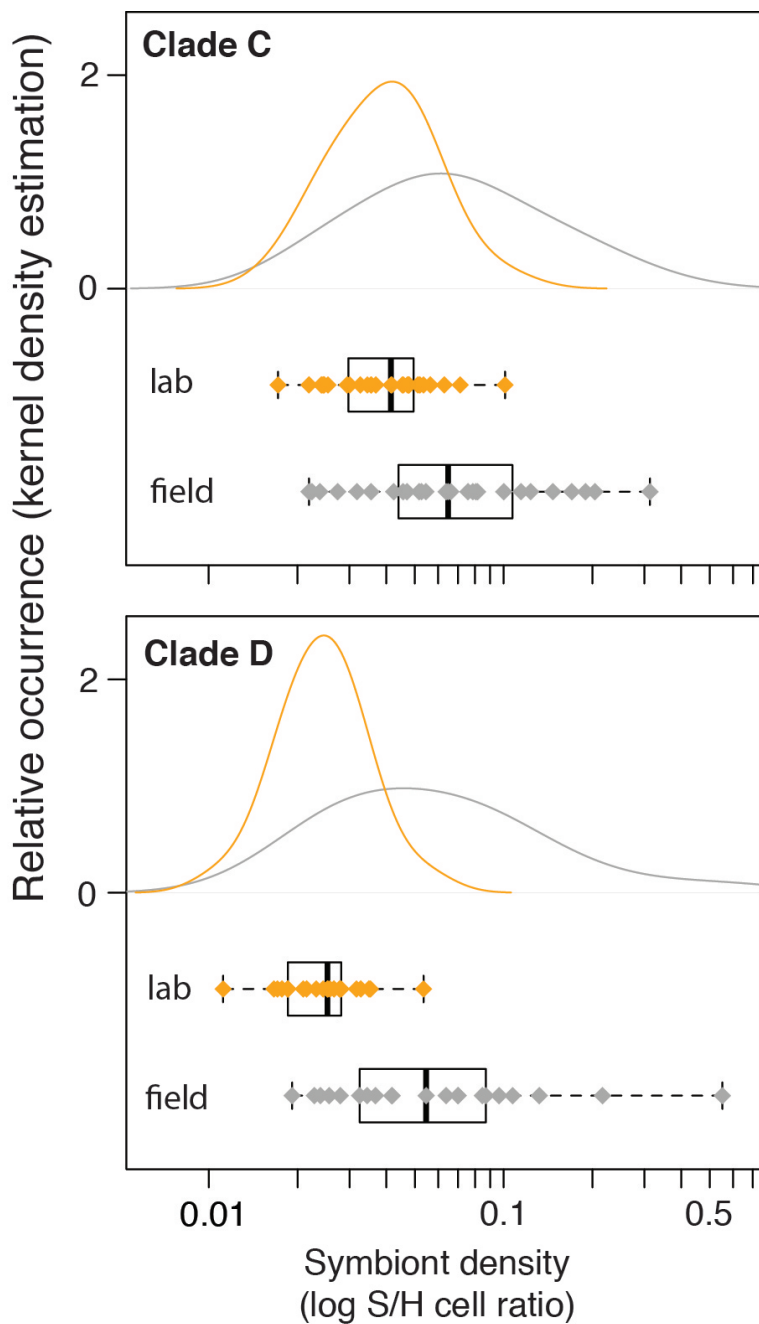


Figure 5.2 Symbiont density regulation during seasonal environmental change.

Symbiont densities measured in clade C (n=22) and D colonies (n=30) of *P. damicornis* in experiment 2 in February (immediately after collection; blue), April (green) and June (red). Boxplots at each time point show the mean, quartiles, and range of data. Probability density functions were constructed by kernel density estimation using a bandwidth of 1.5 times the default value in R.

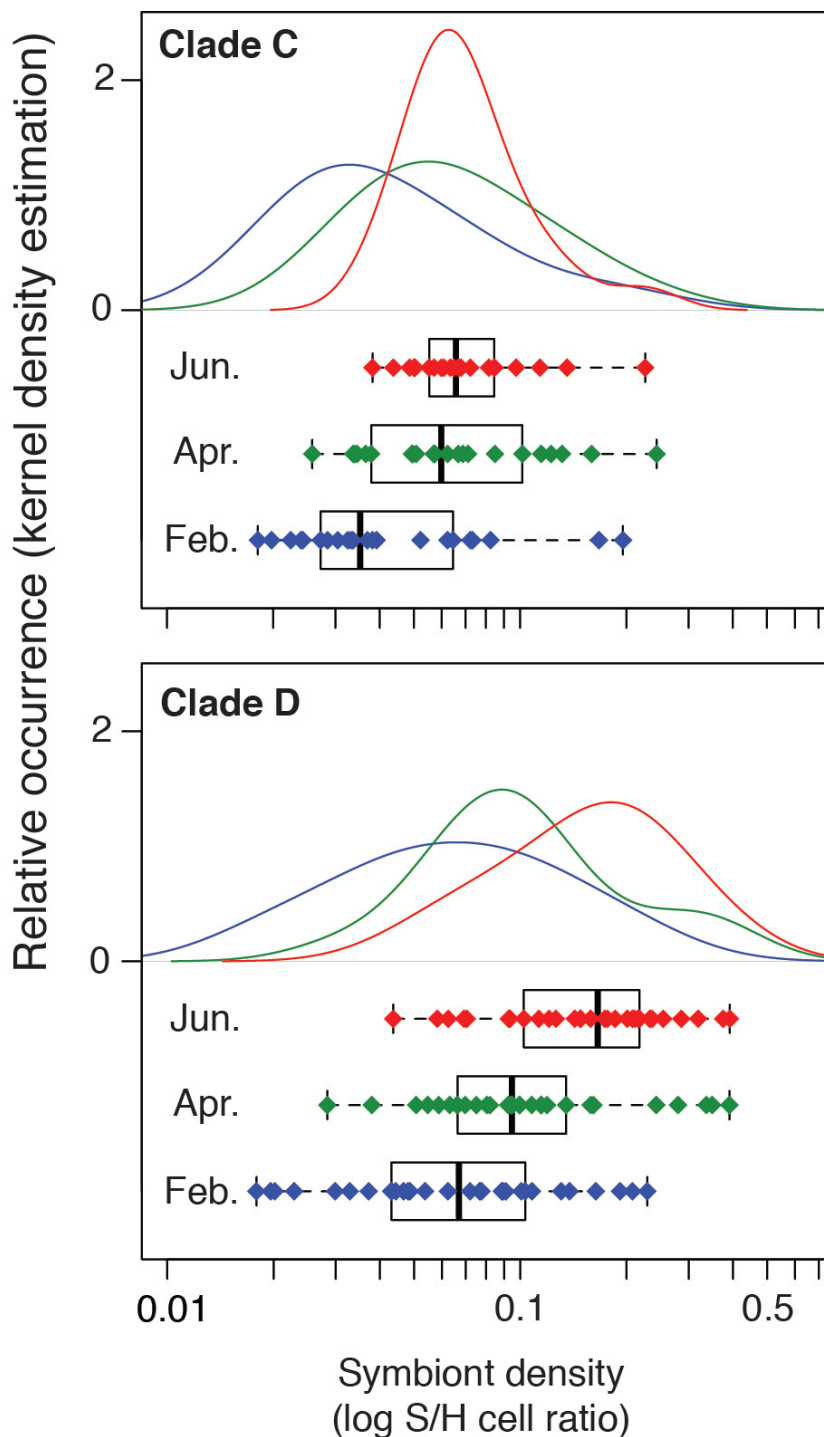


Figure 5.3 The influence of abiotic factors on the primary drivers of modeled benefits and costs. These include (A) the effects of irradiance and symbiont density (self-shading) on total light capture; (B) the effects of irradiance and temperature on symbiont productivity and (C) oxidative stress. These abiotic influences, combined with the effect of symbiont identity, determine the density-dependent costs and benefits to the host under a given set of environmental conditions.

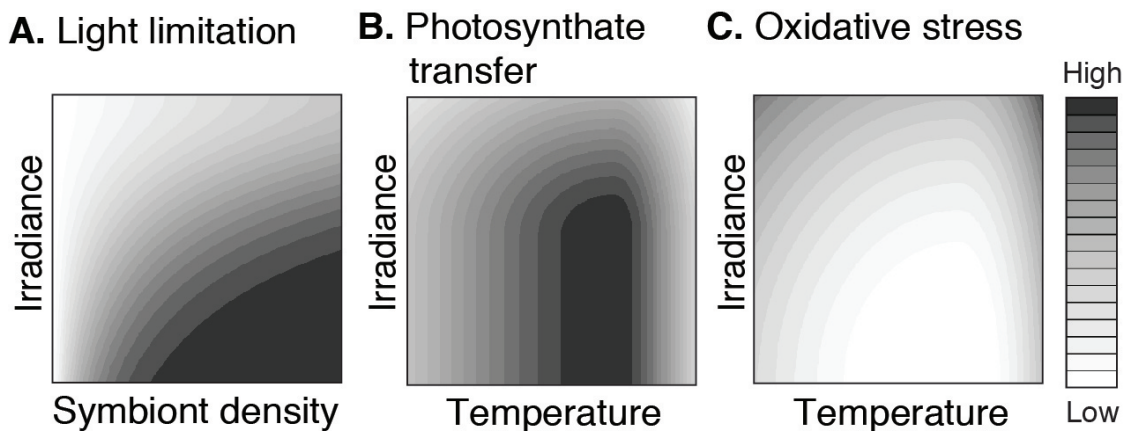


Figure 5.4 Density-dependent costs and benefits for (A) clade C and (B) clade D corals for a range of environmental conditions, including low, medium, and high levels of temperature (23, 26, 29°C) and irradiance (5, 10, 15 mol quanta $m^{-2} d^{-1}$ PAR). Each panel shows gross benefit (green line), gross cost (red line), and net benefit (blue line) as a function of symbiont density. Dotted lines indicate the maximum net benefit (on the y-axis) and optimal density (on the x-axis) under each scenario.

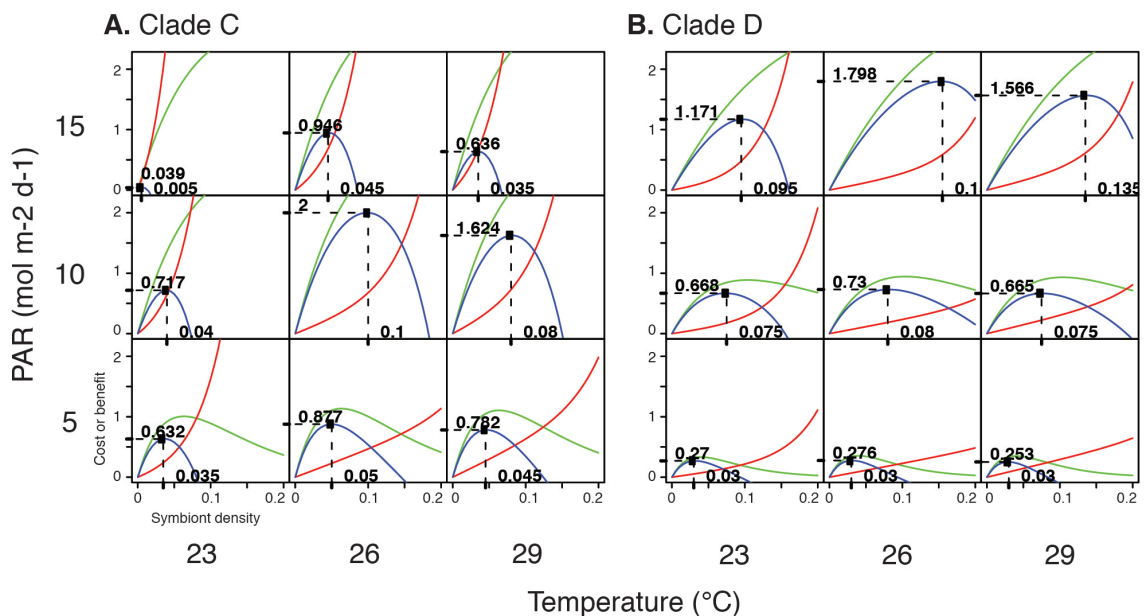


Figure 5.5 Optimal densities of clade C and D *Symbiodinium* in *P. damicornis* over a range of temperature and irradiance conditions. Colored boxes overlaying optimal density contours indicate the environmental conditions (temperature and irradiance mean \pm 1 SE over the 3-week period prior to each sampling) experienced by the corals in our two experiments. The modeled optimal density ranges contained within each of these environmental “windows” are used to construct the shaded regions in Fig. 5.6.

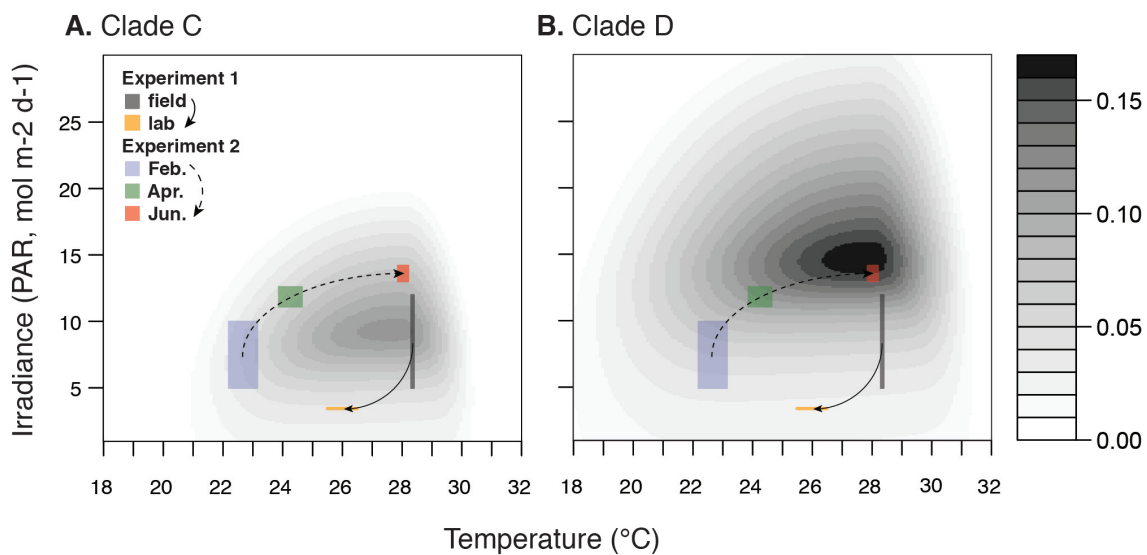


Figure 5.6 Modeled and observed symbiont densities. Observed symbiont densities (probability density functions) in **(A)** clade C and **(B)** clade D corals in experiments 1 and 2 are shown with the corresponding modeled optimal densities (shaded regions) for each environmental “window” (see Fig. 5.5). The capacity of the cost-benefit model to fit multiple datasets strongly supports the hypothesis that symbiont densities are regulated to maximize interaction benefit.

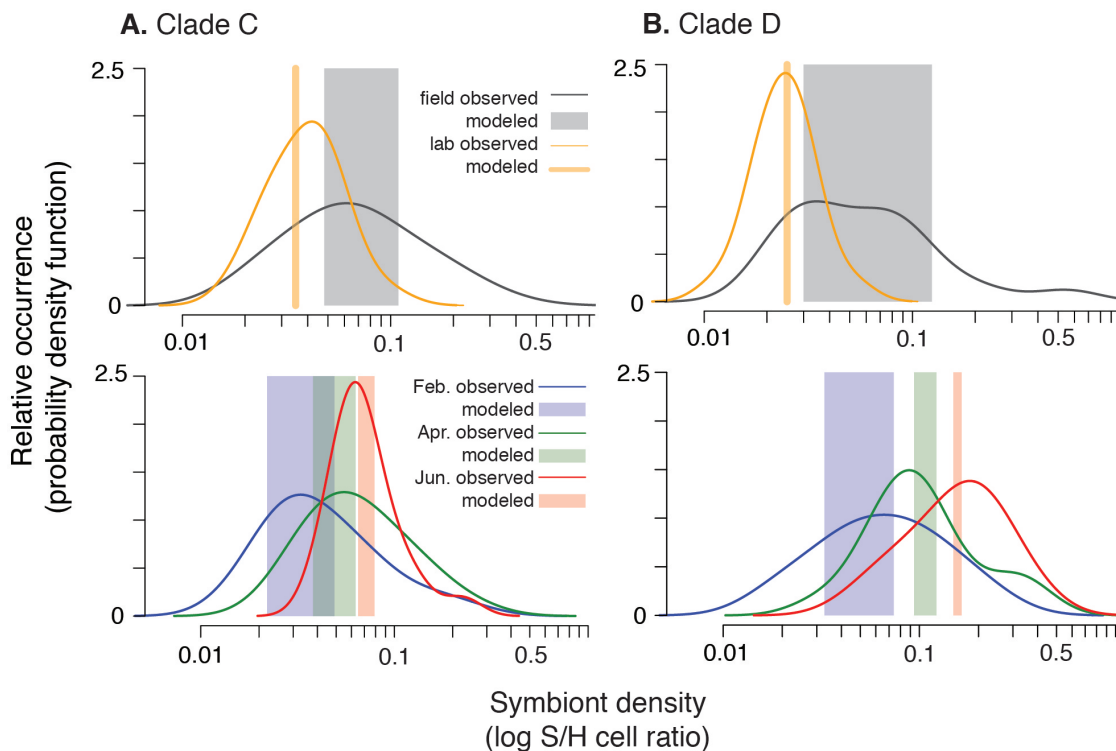


Table 5.1 Equations used to model cost-benefit outcomes.**Biotic factors**

$$\text{Symbiont density } z \quad (1)$$

$$\text{Relative cell volume } v = \begin{cases} 1, & \text{clade C} \\ 0.478, & \text{clade D} \end{cases} \quad (2)$$

$$\text{Temperature optimum } t_{opt} = 28 \quad (3)$$

$$\text{Irradiance optimum } i_{opt} = \begin{cases} 10, & \text{clade C} \\ 16, & \text{clade D} \end{cases} \quad (4)$$

$$\text{Carbon translocation / environmental sensitivity } f = \begin{cases} 1, & \text{clade C} \\ 0.5, & \text{clade D} \end{cases} \quad (5)$$

Abiotic factors

$$\text{Low temperature } T_{sub} = \begin{cases} (t_{amb} - t_{opt})^2, & t_{amb} < t_{opt} \\ 0, & t_{amb} > t_{opt} \end{cases} \quad (6)$$

$$\text{High temperature } T_{super} = \begin{cases} 0, & t_{amb} < t_{opt} \\ (t_{amb} - t_{opt})^2, & t_{amb} > t_{opt} \end{cases} \quad (7)$$

$$\text{Low irradiance } I_{sub} = \begin{cases} (i_{amb} - i_{opt})^2, & i_{amb} < i_{opt} \\ 0, & i_{amb} > i_{opt} \end{cases} \quad (8)$$

$$\text{High irradiance } I_{super} = \begin{cases} 0, & i_{amb} < i_{opt} \\ (i_{amb} - i_{opt})^2, & i_{amb} > i_{opt} \end{cases} \quad (9)$$

$$\text{Gross benefit } B_{gross}(z) = Pz e^{-Dz} \quad (10)$$

$$\text{Symbiont productivity } P = \frac{50f}{L} \quad (11)$$

$$\text{Productivity limitation } L = 1 + \left(f(1.8T_{sub} + 15T_{super}) + I_{super} \right) \quad (12)$$

$$\text{Light-limiting density } D = 6v(1 + 55I_{sub}) \quad (13)$$

$$\text{Gross cost } C_{gross}(z) = C_{maint}(z) + C_{stress}(z) \quad (14)$$

$$\text{Maintenance cost } C_{maint}(z) = 7ft_{amb}z \quad (15)$$

$$\text{Oxidative stress cost } C_{stress}(z) = fSze^{20\left(z - \frac{0.32}{S}\right)} \quad (16)$$

$$\text{ROS production } S = e^{10\left(f(1.8T_{sub} + 15T_{super}) + 0.08\left(\frac{i_{amb}}{i_{opt}}\right)^2\right)} \quad (17)$$

$$\text{Net benefit } B_{net}(z) = B_{gross}(z) - C_{gross}(z) \quad (18)$$

$$\text{Maximum benefit } b_{max} = \max B_{net}(z) \quad (19)$$

$$\text{Optimal density } z_{opt} = \arg \max_z B_{net}(z) \quad (20)$$

Chapter 6

Growth disadvantage for corals with thermotolerant symbionts is ameliorated in warmer oceans

Summary

The growth and survival of reef corals are influenced by their symbiotic algal partners (*Symbiodinium* spp.), which may be flexible in space and time. Tradeoffs among partnerships exist such that corals with thermotolerant symbionts (e.g., clade D) resist bleaching but grow more slowly, making the long-term ecosystem-level impacts of different host-symbiont associations uncertain. However, much of this uncertainty is due to limited data regarding these tradeoffs and particularly how they are mediated by the environment. To address this knowledge gap, we measured growth and survival of *Pocillopora damicornis* with thermally-sensitive (clade C) or -tolerant (clade D) symbionts at three temperatures. While clade D corals grew 35-40% slower at cooler temperatures (26°C), warming of 1.5-3°C reduced and eliminated this growth tradeoff, suggesting clade D may enhance survival at no cost to growth in warmer oceans. Understanding these genotype-environment interactions can help improve modeling efforts and conservation strategies for reefs under global climate change.

Background

The persistence of coral reef ecosystems under climate change scenarios depends on the ability of reef-building corals to survive and grow in warmer oceans. Rising seawater temperature is a major threat facing coral reefs, as thermal stress causes collapse of the symbiosis between corals and their symbiotic algae (*Symbiodinium* spp.), a

phenomenon known as coral bleaching. In association with climate warming, coral reef bleaching events worldwide are becoming more frequent and severe, often leading to mass coral mortality (Hoegh-Guldberg et al. 2007; Baker et al. 2008).

Susceptibility to bleaching varies widely among corals, in part due to differences in the type of *Symbiodinium* they host. Corals that associate with clade D *Symbiodinium* are more resistant to bleaching (Glynn et al. 2001; Berkelmans and van Oppen 2006), suggesting these corals have an advantage under climate change scenarios. Other corals may “switch” or “shuffle” (Baker 2003) their algal partners to associate with more clade D *Symbiodinium* as a mechanism to rapidly adapt to rising temperatures (Buddemeier and Fautin 1993; Baker 2001; Berkelmans and van Oppen 2006; Jones et al. 2008).

However, the increased thermal tolerance provided by clade D *Symbiodinium* may come at a cost of reduced carbon translocation (Cantin et al. 2009), leading to slower coral growth (Little et al. 2004; Jones and Berkelmans 2010). This tradeoff raises questions about whether *Symbiodinium* in clade D will ameliorate the effects of climate change on reefs by reducing bleaching, or contribute to the long-term decline of reefs by reducing coral growth (Stat and Gates 2011; Ortiz et al. 2013b). Recent modeling work has predicted that coral cover on Caribbean reefs may actually decline faster in the presence of thermally tolerant symbionts, suggesting that clade D symbionts will have a deleterious ecosystem-level effect (Ortiz et al. 2013b). However, due to a paucity of data on the growth effects of different symbiont types, current models are constrained to assume a fixed negative growth effect of clade D (e.g., 50-60% reduction (Ortiz et al. 2013a; 2013b)) that is environment-independent. In reality, the tradeoffs associated with different symbiont types may be mediated by numerous environmental variables.

To address this critical research gap, we conducted two experiments to evaluate growth of the major Indo-Pacific reef-building coral *Pocillopora damicornis* harboring either clade C (type C1b-c) or clade D (type D1) *Symbiodinium* at three temperatures (26, 27.5, and 29°C). In particular, we tested the hypothesis that the growth disadvantage associated with thermally tolerant symbionts may be reduced as temperature increases. Understanding the interactive effects of symbiont type and environment on coral growth will improve our ability to model and predict coral reef futures under global climate change.

Materials and Methods

Experiment 1: Growth at 26°C

P. damicornis colonies were collected from Uraba, Panama in August, 2010, and transported to the University of Miami's Coral Resource Facility (CRF). Approximately 20 fragments (~2-5 cm) were taken from each colony and mounted on labeled, weighted microcentrifuge tubes using hot glue. From August 2010 until March 2013, fragments were grown in a single indoor semi-recirculating 233 L tank maintained at $26 \pm 0.5^\circ\text{C}$. Recirculating seawater was treated by a UV-sterilizer, protein skimmer, and 10 μM canister filter, while continuously supplemented with 1 μM -filtered seawater pumped directly from Biscayne Bay at a rate of 1 L h^{-1} . Light was provided by two 80 W Gieseemann T5 fluorescent bulbs delivering approximately $100 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ PAR on a 14h:10h light:dark cycle. Growth was monitored over a 55-week period by taking buoyant weight measurements for $n=349$ fragments on 1 June 2011 and 19 June 2012.

Experiment 2: Effect of temperature on growth

In March 2013, 6-20 additional fragments were created from each of 58 colonies that had been maintained under the conditions described for Experiment 1. Fragments were transferred to a set of 4 outdoor semi-recirculating experimental tank systems at the CRF (see Gillette 2012) where they recovered from fragmentation and acclimated to new conditions for 30 days (at 26°C) prior to temperature manipulations. Each tank system consisted of a 350 L fiberglass tank used for experimentation mounted above a 500 L covered fiberglass sump tank used for temperature control, with seawater continuously recirculating between tanks. Temperature was maintained within 0.1°C by submersible titanium heaters and heat exchangers supplied with chilled (~18°C) fresh water. Each tank received a constant input of 5 µM-filtered seawater (0.2 L min⁻¹) from Biscayne Bay. Seawater was also recirculated within each upper tank to provide continuous flow around coral fragments. Shadecloth over the tanks reduced ambient irradiance by ~90% (~50 µmol quanta m⁻² s⁻¹ midday irradiance), and AT-Films Super 4 Agricultural Foil blocked ~90% of UVA and UVB.

After 30 days of acclimation at 26°C, corals were divided by genotype among three temperature treatments: 26°C, 27.5°C, and 29°C. Water temperature was increased in the warmer treatments at a rate of 0.5°C day⁻¹ so that final temperatures were reached on 26 April 2013. Temperatures treatments were maintained for 125 days and rotated among the four independent tank systems every 5-7 days in order to control for tank effect. HOBO data loggers (Onset Corp.) recorded water temperature in each tank throughout the experiment.

Symbiont community characterization

One tissue sample was taken from each coral colony in August 2010 and September 2012 for qPCR analysis of *Symbiodinium* communities. DNA was extracted using an organic extraction protocol (Baker et al. 1997) and qPCR assays targeting actin loci in *Symbiodinium* clades C and D were performed as described in Cunning & Baker (2013). The proportion of clades C and D were calculated following Cunning et al. (2013) to categorize colonies as C- or D-dominated (i.e. “C colonies” or “D colonies”).

Coral growth measurements

Coral growth was measured by buoyant weights of coral fragments at the beginning and the end of each experiment. Corals were suspended in seawater beneath an analytical balance and buoyant weight recorded to the nearest milligram. To account for the positive buoyancy of the caps to which each coral was mounted, 65 mg was added to each measurement (mean weight of n=5 caps; corals whose mass did not exceed this positive buoyancy were excluded). In Experiment 2, bleaching (bleached vs. non-bleached) and mortality (total and partial) were assessed visually for each fragment at intervals of 54, 95, and 125 days.

Data analysis

For Experiment 1, growth was analyzed by regression of (log-transformed) final weight against (log) initial weight, with symbiont type as a fixed factor and coral colony as a nested factor. Only coral fragments that were not overtopped, did not fuse to other fragments, and did not experience partial mortality during the experiment were included

in the analysis (n=283 fragments). For Experiment 2, growth was analyzed by regression of (log-transformed) final buoyant weight against (log) initial weight, with symbiont type and temperature as fully crossed fixed factors and coral colony as a nested factor. Only coral fragments that survived to the final time point and had not suffered major partial mortality (>50% tissue loss) were included in the analysis (n=590 fragments). The significance of each factor in these models was tested by partial F-tests comparing the full model to a reduced model lacking each factor. Percent growth rates were calculated by comparing adjusted mean weights (\pm SEM) for each group of corals to initial mean weight of all corals. Differences among treatment groups were assessed *post-hoc* by two-tailed Student's t-tests with $\alpha = 0.05$.

Mortality at the end of Experiment 2 (3 levels: dead, partial mortality, or healthy) was analyzed by a nominal logistic model with temperature and symbiont type as fully-crossed fixed factors and colony as a nested effect (n=664 fragments). Significance of each factor was assessed with Likelihood-ratio Chi-square tests comparing the full model to reduced models lacking each factor. Fragments that experienced mortality early in the experiment (within the first interval) were assumed to have been affected by fragmentation and transplantation stress rather than temperature, and were therefore excluded from the analysis. All analyses were conducted in JMP v10.0.

Results

Symbiont community characterization

All corals were dominated by *Symbiodinium* in clade C (>98.1%) or clade D (>99.5%), although 23% of colonies contained background levels of the other clade.

Several studies have identified the particular clade C and D symbionts hosted by *Pocillopora* in the eastern Pacific as ITS2 types C1b-c and D1, respectively (LaJeunesse et al. 2007; 2008; Cunning and Baker 2013). The dominant symbiont did not change in colonies between Aug. 2010 (before Experiment 1) and Sep. 2012 (before Experiment 2) and was therefore assumed to be stable throughout both experiments. Fragments from the same colony were assumed to have the same dominant symbiont, as intracolony variation in the dominant symbiont type is rare in *Pocillopora* (LaJeunesse et al. 2007).

Experiment 1: Growth at 26°C

Coral growth at 26°C was significantly influenced by symbiont clade ($F = 161.3$, $df = 1$, $P < 0.0001$). Relative to clade C corals, growth of clade D corals was reduced by 39% (Fig. 6.1).

Experiment 2: Effect of temperature on growth

Overall, coral growth decreased at warmer temperatures ($F = 29.9$, $df = 2$, $P < 0.0001$). However, the reduction in growth was more severe for corals with clade C than clade D (symbiont:temperature interaction, $F = 3.59$, $df = 2$, $P < 0.05$). Compared to 26°C, growth of clade C corals at 27.5 and 29°C was reduced by 38% and 61%, respectively, while growth of clade D corals was reduced by 29% and 45% (Fig. 6.2). Within-temperature comparisons indicate that relative to clade C corals, growth of clade D corals was reduced at 26°C by 36%, while at 27.5°C by only 27%. At 29°C, clade D and C corals grew the same amount (Fig. 6.2). When bleached fragments were excluded, growth at 29°C was still not different between C and D corals ($t = -1.2$, $P > 0.2$).

Experiment 2: Mortality and bleaching

Mortality in response to temperature was significantly different between corals with clade C and D symbionts (symbiont:temperature interaction, $\chi^2 = 24.4$, $df = 4$, $P < 0.0001$). Mortality occurred mostly among clade C corals at 29°C, of which 39% were dead and 24% suffered major partial by the end of the experiment (Fig. 6.3). Among the clade C corals at 29°C that remained alive, 59% were bleached. Bleaching was not observed in clade C corals at 26 or 27.5°C, or in any clade D corals. These corals also had low rates of total and partial mortality (< 6% overall; Fig. 6.3) that could not be distinguished statistically.

Discussion

In Experiment 1, we found that clade D corals grew 39% slower than clade C corals at 26°C (indoors under artificial lighting; Fig. 6.1). Similar reductions in growth associated with clade D symbionts were observed in adult *Acropora millepora* (29-38% (Jones and Berkelmans 2010) or 50% reduction (Mieog et al. 2009)), with even greater reductions in juveniles (Little et al. 2004). Reduced growth may be caused by decreased carbon translocation by clade D symbionts (Cantin et al. 2009), and has been cited as a tradeoff to the thermal tolerance clade D confers on its hosts (Jones and Berkelmans 2010; 2011; Stat and Gates 2011). In our second experiment, we investigated how this tradeoff is affected by changes in the environment (e.g., temperature).

In Experiment 2, clade D corals grew 36% slower than clade C corals at 26°C. Although differing conditions preclude comparison of absolute growth, the 36% reduction in D corals closely matches that of 39% in Experiment 1 at the same

temperature. At warmer temperatures, growth rates decreased for all corals, indicating they were above their current thermal growth optimum (Marshall and Clode 2004). However, warming also alleviated the growth disadvantage of clade D: at 27.5°C, clade D reduced growth by only 27%, while at 29°C, clade D did not reduce growth at all, indicating a significant genotype-environment interaction. In contrast to this finding, Jones and Berkelmans (2010) found no effect of temperature (23 vs. 29°C) on growth of *A. millepora* with clade C or D *Symbiodinium*, and no interaction between temperature and symbiont type. These different findings may be explained by unique Gaussian relationships between temperature and growth (Marshall and Clode 2004) for different host-symbiont associations, highlighting the need for additional research to elucidate these complex interactions (Smith et al. 2008; Mieog et al. 2009). Nevertheless, our findings show that warming reduces growth of *P. damicornis* more severely when hosting clade C, causing their growth advantage over clade D corals to be lost after 1.5-3°C of warming, which is expected before the end of this century (Meehl et al. 2007).

Importantly, the loss of a growth advantage in clade C corals is only relevant for those corals that survive. We found that less than half of clade C corals at 29°C escaped complete or partial mortality, and less than half of those remained unbleached. The significant bleaching and mortality that occurred among (and only among) clade C corals at 29°C (Fig. 6.3) reflects the well-documented thermal sensitivity of *Pocillopora* with clade C (Glynn et al. 2001; LaJeunesse et al. 2007; 2010; Cunning and Baker 2013). However, some clade C corals at 29°C (16%) still remained healthy, suggesting that at least some corals with thermally-sensitive symbionts may still survive, possibly due to local adaptation (Howells et al. 2011) and/or host-derived thermal tolerance (Baird et al.

2009). However, even the 16% of healthy clade C corals at 29°C grew no faster than clade D corals, showing that the lost growth advantage is not simply due to bleaching. Furthermore, the growth advantage of clade C corals was significantly reduced at 27.5°C although no bleaching occurred.

Taken together, these bleaching, mortality, and growth data suggest that warming will have a major impact on the relative success of corals with clade C and D partnerships. While clade C corals have a significant growth advantage in the absence of thermal stress, warmer oceans may cause significant mortality among these thermally sensitive partnerships while eliminating the growth advantage of those that survive. By contrast, clade D partnerships may greatly enhance thermotolerance and survival at little to no cost in growth. Although other tradeoffs to hosting clade D may still exist (e.g., reproductive output (Jones and Berkelmans 2011)), these trends suggest that clade D symbionts may have beneficial ecosystem-level effects on reefs under climate change scenarios. Switching or shuffling toward clade D-dominated symbiont communities may allow corals to rapidly acquire these advantages and acclimatize to warmer environments (Berkelmans and van Oppen 2006; Jones et al. 2008; Correa and Baker 2011).

In summary, this work reveals that genotype-environment interactions may be important in driving many coral traits, and further work to quantify these interactions may directly inform modeling efforts to predict the fate of coral reefs (Ortiz et al. 2013a; 2013b), and conservation strategies to protect them (Pandolfi et al. 2011). Nevertheless, the slower growth of all corals at higher temperatures, regardless of symbiont type, suggests that reefs are being pushed beyond their thermal growth optima and that warming must be curbed to ensure the future persistence of these ecosystems.

Figure 6.1 Growth of clade C and D corals indoors under artificial lighting at 26°C (Experiment 1). Clade D corals grew 39% slower than clade C corals (student's t-test, $P < 0.0001$).

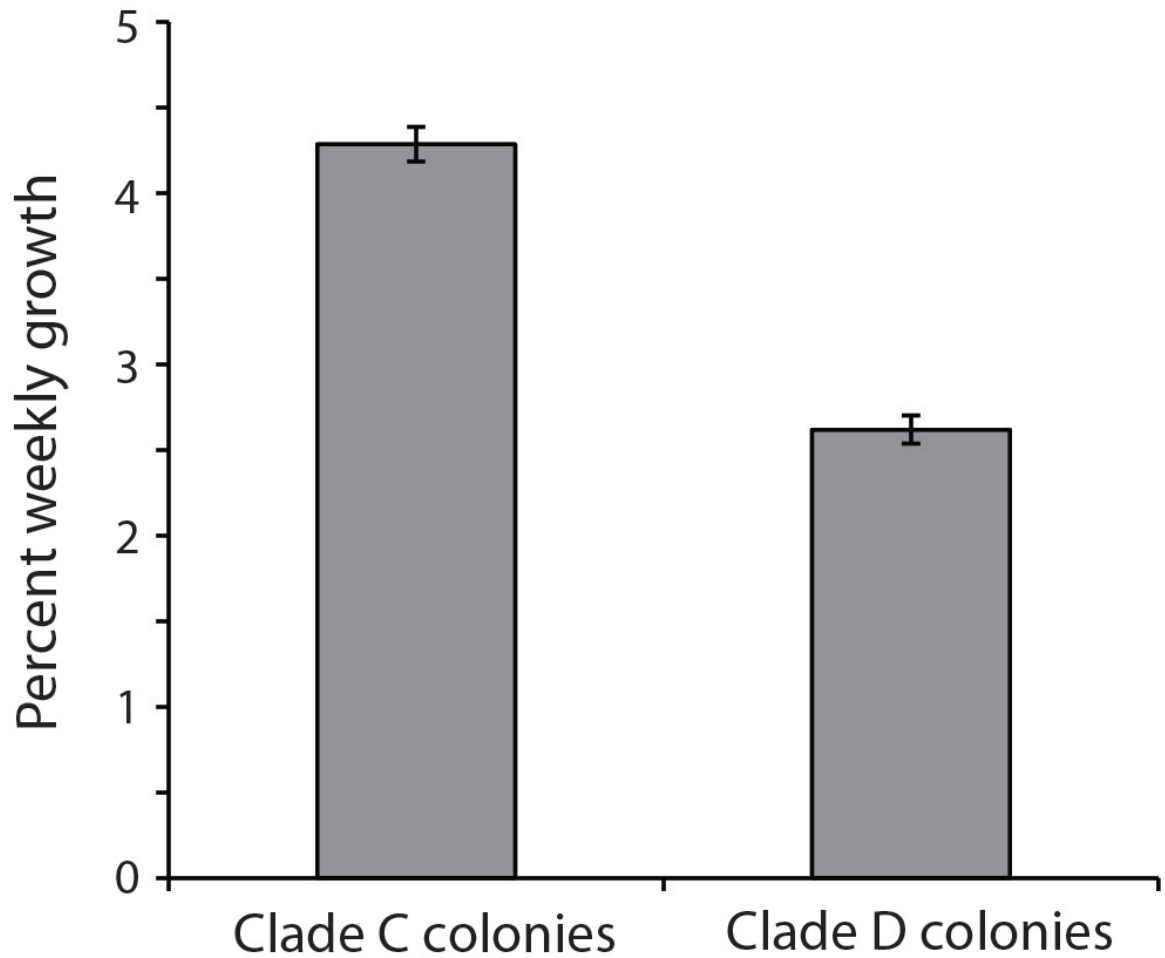


Figure 6.2 Growth of clade C and D corals at different temperatures (Experiment 2). Group means that do not share a letter are significantly different ($\alpha = 0.05$).

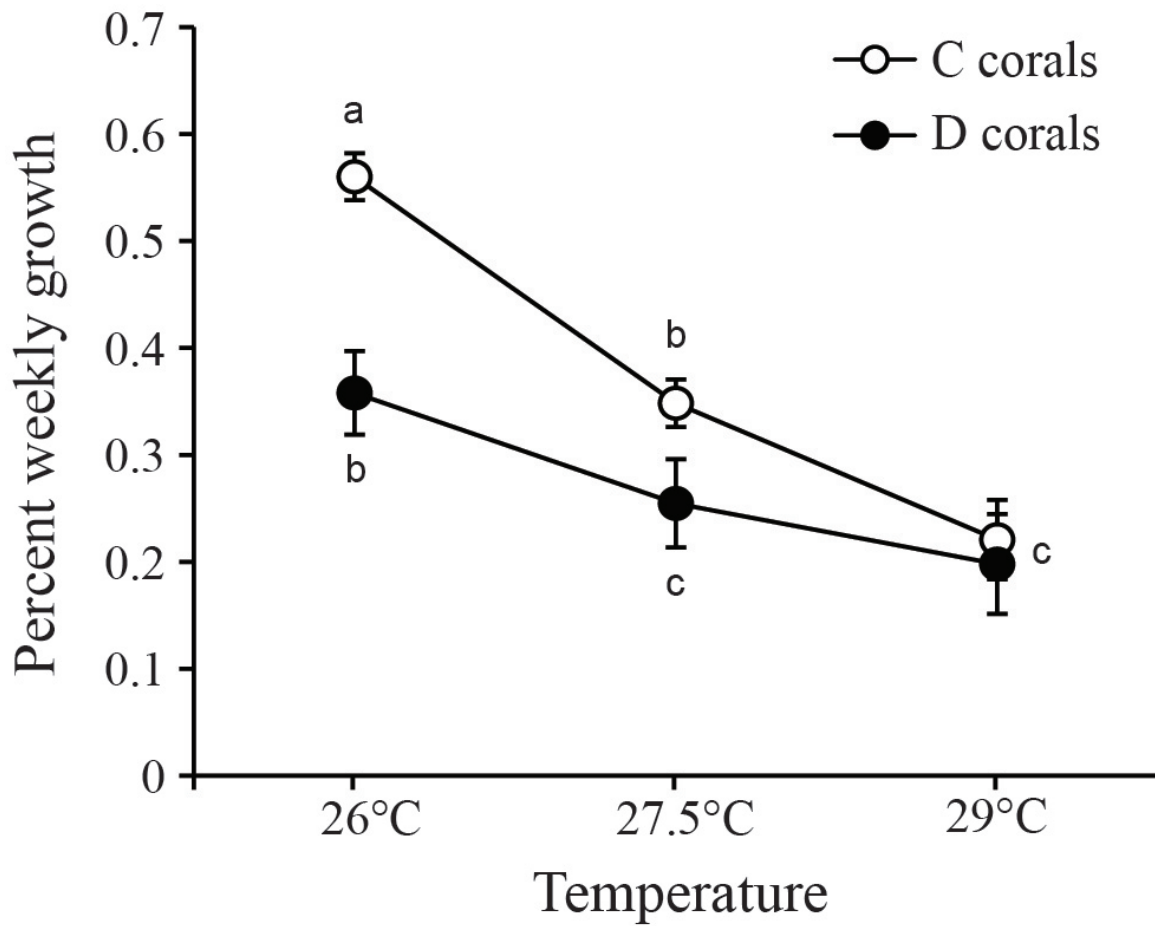
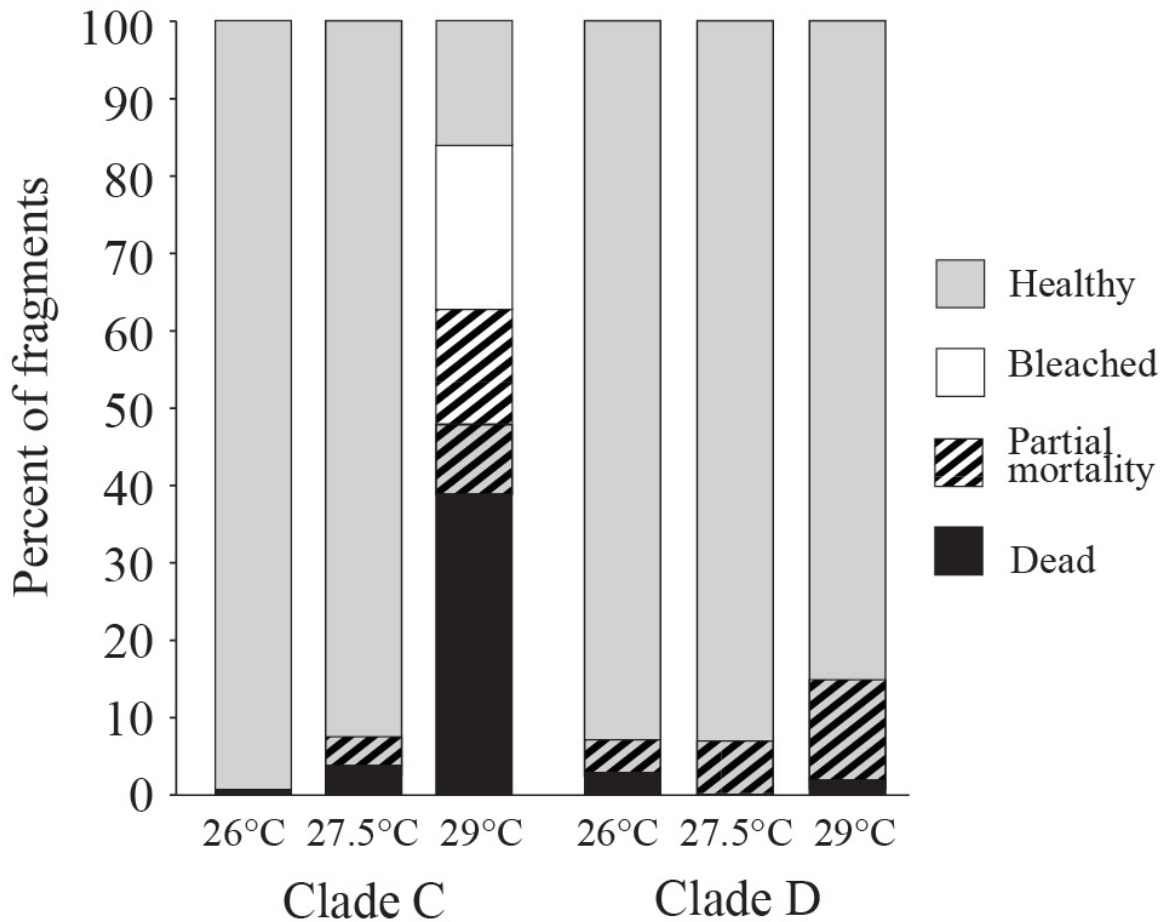


Figure 6.3 Bleaching and mortality of clade C and D corals at different temperatures. After 125 days, living coral fragments in each treatment group were visually assessed to record significant partial mortality (>50% tissue loss) and bleaching (white tissue).



Chapter 7

Flexible associations between *Pocillopora* corals and *Symbiodinium* limit utility of symbiosis ecology in defining species²

Summary

Corals in the genus *Pocillopora* are the primary framework builders of eastern tropical Pacific (ETP) reefs. These corals typically associate with algal symbionts (genus *Symbiodinium*) in clade C and/or D, with clade D associations having greater thermal tolerance and resistance to bleaching. Recently, cryptic “species” delineations within both *Pocillopora* and *Symbiodinium* have been suggested, with host-symbiont specificity used as a supporting taxonomic character in both genera. In particular, it has been suggested that three lineages of *Pocillopora* (types 1-3) exist in the ETP, of which type 1 is the exclusive host of heat-tolerant *Symbiodinium* D1. This host specificity has been used to support the species name “*Symbiodinium glynni*” for this symbiont. To validate these host-symbiont relationships and their taxonomic utility, we identified *Pocillopora* types and their associated *Symbiodinium* at three sites in the ETP. We found greater flexibility in host-symbiont combinations than previously reported, with both *Pocillopora* types 1 and 3 able to host and be dominated by *Symbiodinium* in clade C or D. The prevalence of certain combinations did vary among sites, showing that a gradient of specificity exists which may be mediated by evolutionary relationships and environmental disturbance history. However, these results limit the utility of apparent host-symbiont specificity (which may have been a result of undersampling) in defining species boundaries in either corals or *Symbiodinium*. They also suggest that a greater diversity of corals may benefit from the thermal

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tolerance of clade D symbionts, affirming the need to conserve *Pocillopora* across its entire geographic and environmental range.

Background

Corals in the genus *Pocillopora* are the primary builders of coral reefs throughout the eastern tropical Pacific, ETP (Glynn et al. 1972; Glynn and Wellington 1983). Recently, molecular genetic evidence has called into question the validity of current morphological species delineations in this genus, suggesting that distinct molecular lineages exist that may not correspond with morphology (Combosch et al. 2008; Flot et al. 2008; Souter 2010; Pinzón and LaJeunesse 2011; Combosch and Vollmer 2011). Understanding species boundaries in this genus is necessary to understand the ecology and evolution of these critical ecosystem engineers, and to implement effective conservation and management objectives.

Elevated temperatures associated with climate change and El Niño-Southern Oscillation (ENSO) events have had major impacts on ETP reefs, causing mass bleaching and mortality of *Pocillopora* (Glynn 1988; Podesta and Glynn 2001; Glynn et al. 2001) and other reef builders. However, there is great variability in the severity of these impacts both within and among colonies: some colonies, or parts of colonies, bleach completely, while neighboring colonies or tissues appear unaffected (Glynn et al. 2001). For *Pocillopora* in Panama, much of this variability can be attributed to different algal symbionts (genus *Symbiodinium*), with one type of symbiont, D1, conferring increased thermal tolerance on their coral hosts. *Pocillopora* colonies hosting thermally tolerant D1 are much more resistant to bleaching than those hosting other types of *Symbiodinium*, principally in clade C (Glynn et al. 2001; LaJeunesse et al. 2010).

Flexibility of coral-algal partnerships has been suggested as a mechanism by which corals may be able to adapt to environmental change (Buddemeier and Fautin 1993; Baker 2001). By changing its assemblage of symbiotic algae, a coral may increase its fitness in response to altered environmental conditions. However, there is also evidence that corals exhibit strong specificity for a certain type of algal symbiont and may not be able to change partners (Goulet 2006; Goulet et al. 2008; LaJeunesse et al. 2008). Although *Pocillopora* is known to regularly associate with *Symbiodinium* in both clades C and D (and rarely with clades A (Stat et al. 2009b) and B (LaJeunesse et al. 2010)), and thus appears to be symbiotically flexible (Glynn et al. 2001; LaJeunesse et al. 2007, 2010), recent evidence suggests that these associations may be much more specific, with certain morphologically cryptic *Pocillopora* taxa only associating with certain symbiont types (Pinzón and LaJeunesse 2011).

Recently, Pinzon and LaJeunesse (2011) used several molecular markers to define three distinct *Pocillopora* lineages (types 1, 2, and 3) in the ETP with differing geographic distribution and symbiosis ecology. They suggested that each of these lineages associates with specific *Symbiodinium* types, and that symbiont specificity further supports their designation as distinct coral species. Additionally, they suggest that only type 1 *Pocillopora* can associate with thermally tolerant *Symbiodinium* D1, and that host specificity also supports species designation of the symbiont as “*S. glynni*” (*nomen nudum*) (LaJeunesse et al. 2010). If these relationships are true, this would suggest that type 1 *Pocillopora* might be better able to survive more frequent and severe episodes of coral bleaching predicted under continued climate change. Lineages that are unable to host *Symbiodinium* D1 (i.e., *Pocillopora* types 2 and 3) will be less likely to survive under these conditions.

In order to validate these species and host-symbiont relationships, we studied *Pocillopora* colonies (with morphology characteristic of *P. damicornis*) from three different sites in the ETP and analyzed the relationships among geographic locations, *Pocillopora* types, and algal symbionts. The delineation of species among corals and their symbionts, and the flexibility of associations between these partners, is critical in understanding the ecology and evolution of coral holobionts and the capacity they may have to adapt and survive climate change.

Materials and Methods

Samples

Samples of *P. damicornis* were collected from Isla Taboga, Panama (Gulf of Panama, Feb. 2009; 2-6m depth; n=52), Uva Island, Panama (Gulf of Chiriqui, March 2010; 2-28m depth; n=44) in March 2010, and the Galapagos Islands (Marchena, Santa Cruz, Floreana, Santa Fe, 1998; 2-8m depth; n=43). Small branch tips were cut from colonies using bone clippers and preserved in 1% SDS or ethanol. DNA was extracted using a modified organic extraction protocol (Baker et al. 1997).

Genotyping of coral hosts

To rapidly screen samples and differentiate among *Pocillopora* types 1-3, we designed restriction fragment length polymorphism (RFLP) assays based on mitochondrial open reading frame (ORF) sequences. The ORF was PCR-amplified using the primers FATP6.1 and RORF (Flot et al. 2008) and digested using the enzyme *HhaI* (Promega, Madison, WI, USA), which differentiates type 1 sequences (two fragments) from types 2 and 3 (three fragments). A second digestion with *EcoRV* was used to distinguish type 2 (two fragments) and type 3 sequences

(undigested). RFLPs were analyzed on a 2.5% agarose gel and used to classify corals as either type 1, 2, or 3 *Pocillopora*.

To validate this RFLP assay, a subset of samples (n=14; 7 of each RFLP pattern), were sequenced in both directions using BigDye Terminator v3.1 cycle sequencing kit and an Applied Biosystems 3130xl Genetic Analyzer (Foster City, CA, USA). Sequences were inspected using Geneious Pro, and unique haplotypes were aligned with ORF sequences from (Pinzón and LaJeunesse 2011) (HQ378758–HQ378761). Phylogenetic relationships were determined by a neighbor-joining consensus tree with 1000 bootstrap replicates.

Detecting symbionts using qPCR

Symbiodinium in clades C and D were quantified by actin-targeted quantitative PCR (qPCR) assays developed for these particular symbionts (Cunning and Baker 2012), which also match *Symbiodinium* C1 and D1 in a recent actin phylogeny (Pochon et al. 2012). A symbiont clade was considered present in a sample with positive amplification of two technical replicates and no amplification of no-template controls. The proportion of clade D in each colony was then calculated as D cells/(C cells + D cells), with the number of cells of each clade calculated as $2^{(40-\text{avg. } C_T)}$ divided by the gene copy numbers (Cunning and Baker 2012).

Detecting clade D using DGGE

Symbiont communities in a subset (n=9) of *Pocillopora* type 3 samples in which clade D was detected by qPCR were assayed by denaturing gradient gel electrophoresis (DGGE) of the internal transcribed spacer-2 (ITS2) region of nuclear ribosomal DNA (nrDNA) in order to determine whether clade D was also detectable by this method. The ITS2 region was amplified

using the primers “ITSintfor2” and “ITS2clamp” (LaJeunesse and Trench 2000), and amplification products were separated by DGGE (35-75% gradient) using a CBS Scientific system. Dominant bands on the gel were excised, re-amplified, and sequenced as above. We did not attempt to identify or catalogue ITS2 sequences in every sample due to the problematic nature of its ability to define *Symbiodinium* diversity (Stat et al. 2011; LaJeunesse and Thornhill 2011), and because the clade-level data obtained by qPCR are sufficient for the purpose of this study, which was to determine which *Pocillopora* types are capable of hosting clade D *Symbiodinium*.

Statistical analysis

Pearson’s χ^2 test was used to test for differences in proportions of *Pocillopora* ORF types among locations, and differences in the probability of C- or D-dominance among locations and between *Pocillopora* ORF types. Fisher’s exact test was used post-hoc to test the hypothesis that D-dominance was more frequent in type 1 than type 3 corals. All statistical analyses were performed in JMP v9.0.2.

Results

Identification of coral hosts

Mitochondrial ORF sequences revealed two unique haplotypes (JX888898-9), which we compared with existing *Pocillopora* ORF sequences. One of these haplotypes was a 100% match to *Pocillopora* type 1 as defined by (Pinzón and LaJeunesse 2011), which we refer to as type 1 in this manuscript. The second haplotype differed by a single base deletion or two base substitutions, respectively, from *Pocillopora* type 3 and type 3b (Pinzón and LaJeunesse 2011),

and we refer to this haplotype as type 3. *Pocillopora* type 2 (Pinzón and LaJeunesse 2011), which to date has only been reported from Clipperton Atoll, was not found in our study. The phylogenetic relationships among the haplotypes identified here and in (Pinzón and LaJeunesse 2011) are depicted in Figure 7.1a.

Consistent with restriction enzyme mapping of ORF sequences, type 1 sequences were digested by the enzyme *HhaI* into two fragments (~400bp and >600bp), while type 3 sequences were digested into three fragments (~400bp and two >300bp fragments, which appeared as a single band; Fig. 7.1b). Because type 2 sequences would have the same *HhaI* RFLP as type 3 sequences, an *EcoRV* digest was used to differentiate these types. No sequences were digested by *EcoRV*, confirming they were all type 3 sequences. These RFLP patterns allowed all samples to be categorized as type 1 or 3 *Pocillopora*.

Based on this analysis, 96 samples (69%) were type 1, and 43 (31%) were type 3. The distribution of these types differed among locations ($p < 0.0001$; Pearson's χ^2 ; Figure 7.2). Type 1 comprised 100% of colonies sampled in the Galapagos, and was more prevalent than type 3 at Uva Island (Gulf of Chiriqui, Panama: 77% vs. 23%), while at Taboga (Gulf of Panama, Panama), most colonies were type 3 (63% vs. 37%).

Symbiont communities

Corals hosted *Symbiodinium* in both clades C and/or D, as detected by qPCR. Mixed communities (both clades) were found in over half of all colonies (53%) and were prevalent in each location (Galapagos=70%, Taboga=46%, Uva=43%). Based on their symbiont community composition, colonies were classified as either C-dominated (C only (100% C) or C>D (53.5-99.9% C, median 99.8%)) or D-dominated (D only (100% D) or D>C (83.1-99.9% D, median

99.8%). Both type 1 and type 3 colonies could host (and be dominated by) either symbiont clade (Fig. 7.3), although the frequency of C- vs. D-dominance depended on ORF type ($p < 0.01$; Pearson's χ^2) and location ($p < 0.0001$; Pearson's χ^2). At Taboga and Uva, type 1 colonies were mostly dominated by clade D (100% and 74% of colonies, respectively), but in the Galapagos, they were mostly dominated by clade C (81%). Type 1 colonies were also more likely to be D-dominated than type 3 colonies ($p < 0.0001$, Fisher's exact).

Since type 3 colonies were not previously found to associate with clade D when analyzed by ITS2 DGGE (Pinzón and LaJeunesse 2011), a subset of type 3 colonies in which clade D was detected by qPCR were also analyzed by ITS2 DGGE to determine whether clade D was still detectable with this less sensitive technique. For all colonies ($n=9$), *Symbiodinium* in clade D were clearly detected (Fig. 7.4).

Discussion

Analysis of coral host DNA revealed that all samples analyzed from Panama and the Galapagos were either *Pocillopora* type 1 or type 3, consistent with the findings of Pinzon and LaJeunesse (Pinzón and LaJeunesse 2011). These types have also been found in Hawaii (Flot et al. 2008), and type 3 has been found in the western Indian Ocean (Souter 2010), suggesting these *Pocillopora* lineages are widely distributed. However, the frequency of occurrence of these types was different among our three sites (Fig. 7.2). At Taboga (Gulf of Panama), most colonies (63%) were type 3, while at Uva (Gulf of Chiriqui), most colonies were type 1 (77%). In the Galapagos, all colonies were type 1. We hypothesize that these geographic differences in the relative abundance of type 1 and 3 colonies may be due to the different history of bleaching and mortality among these locations. As type 1 colonies tended to host heat-tolerant clade D

symbionts more than type 3 colonies (Fig. 7.3), they may be more resistant to bleaching, thereby increasing in relative abundance following major bleaching and mortality events, such as those that have occurred due to ENSO. This would result in greater dominance of type 1 colonies in locations that have experienced more recent and/or severe bleaching and mortality. Indeed, during the major 1982-83 ENSO event, the Galapagos experienced significantly greater mortality (97%) than the Gulfs of Chiriqui and Panama (75-85%) (Glynn 1990). During the 1997-98 ENSO, the Galapagos again suffered greater mortality than the Gulf of Chiriqui (26.2% vs. 13.1% mortality), and the Gulf of Panama experienced 0% bleaching and mortality due to seasonal cool water upwelling which kept temperatures below bleaching thresholds (Glynn et al. 2001). If type 1 colonies preferentially host clade D and selectively survive these events, then this geographic pattern of bleaching and mortality may explain the greater relative proportion of type 1 colonies in the Galapagos (100%) than in the Gulf of Chiriqui (Uva; 77%) and the Gulf of Panama (Taboga; 37%). Greater abundance of type 1 colonies in the Galapagos compared to Taboga was also found by Pinzon & LaJeunesse (Pinzón and LaJeunesse 2011).

However, although type 1 colonies hosted clade D more often than type 3 colonies, both types were able to host and be dominated by clade D. Indeed, we found that both *Symbiodinium* clade C and clade D can associate with, and even be the dominant symbiont type in, both *Pocillopora* types 1 and 3. This directly contrasts with the results of Pinzón and LaJeunesse (2011), who found clade D only in association with *Pocillopora* type 1. One major difference between these two studies that may account for this discrepancy is the technique used to identify symbionts. Here, we used qPCR, which has been shown to be up to 1000x more sensitive than DGGE (Mieog et al. 2007), the technique used by Pinzon and LaJeunesse. We were, however, able to detect clade D in type 3 *Pocillopora* using ITS2 DGGE (Fig. 7.4), so a priori

methodological constraints cannot explain the contrasting results. Rather, patchy distributions and undersampling are more likely to explain the discrepancy between the two studies.

Clonality and large genetic patch sizes on ETP reefs may result in type 3 colonies with clade D being aggregated in certain areas but absent from others (Combosch and Vollmer 2011; Pinzón et al. 2012). Distributions may also be influenced by variable environmental conditions or microhabitats across the reefscape that may favor certain host-symbiont combinations over others. Thus, discrete and limited distributions of taxa and sampling effort may determine which hosts and symbiont types are found. For example, we did not find *Pocillopora* type 3 in the Galapagos, while Pinzon and LaJeunesse (2011) did. We do not suggest that type 3 does not, or cannot, exist in the Galapagos, but rather that our sampling did not overlap its distribution, which may be severely limited in the Galapagos. Similar limited distributions may also explain why clade D was not found in type 3 colonies by Pinzon and LaJeunesse (2011).

Extensive sampling over the biogeographic and environmental ranges of many coral species previously assumed to be specific in their symbiotic associations has revealed additional symbiont diversity and flexibility (Baker and Romanski 2007; Silverstein et al. 2012), and our results provide another case of greater symbiotic flexibility than previously reported. As symbiont specificity has never been shown to be absolute for any coral species (and theoretically cannot be, as no coral can be exhaustively sampled across its entire range), apparent or perceived specificity from limited sampling is not a good criterion for delineating species boundaries in scleractinian corals (Pinzón and LaJeunesse 2011).

By the same reasoning, *Symbiodinium* distributions in coral hosts are not good criteria for identifying and naming *Symbiodinium* “species” (LaJeunesse et al. 2010, 2012). Many symbionts associate with a wide variety of host taxa, and a recent study using qPCR revealed that symbiont

clades A-D associate with a much broader range of host taxa than previously reported (Silverstein et al. 2012). Furthermore, although the process of identifying *Symbiodinium* “species” using molecular criteria has begun (LaJeunesse et al. 2012), it remains controversial due to difficulties in interpreting sequence diversity in commonly-used genetic markers (Stat et al. 2011; Pochon et al. 2012), and delineating species boundaries among taxa (Correa and Baker 2009; Sampayo et al. 2009). Because species boundaries in *Pocillopora* are also not clearly identified (Flot et al. 2008; Combosch and Vollmer 2011; Stat et al. 2012), apparent specific associations between poorly-defined symbiont and host taxa should not be used to define *Symbiodinium* “species”.

Although our data add to the accumulating evidence that absolute specificity in coral-algal symbioses is not supported, they do show that different hosts nevertheless vary in their tendency to host particular symbionts (i.e., type 1 colonies were dominated by clade D more frequently than type 3 colonies). Instead of absolute (strict) specificity, it is more likely that a gradient of specificity exists over which corals vary in the frequency with which they are dominated by a specific symbiont type (Baker 2003; Silverstein et al. 2012).

In conclusion, the symbiotic flexibility of coral hosts continues to be greater than reported, with both types 1 and 3 *Pocillopora* hosting *Symbiodinium* in clade C and/or clade D, following increased sampling across their geographic range. Symbiont specificity is therefore not a good criterion for delineating species boundaries in these corals, and its utility as a taxonomic marker for any scleractinian coral species is probably very limited (Silverstein et al. 2012). Additionally, *Symbiodinium* distributions in coral host species may not be good criteria for identifying (and naming) new *Symbiodinium* “species”. Previous reports of the apparent specificity of *Symbiodinium* D1 (*S. glynni*) to *Pocillopora* type 1 are probably the result of

sampling a restricted or patchy distribution. Greater symbiotic flexibility suggests that a greater diversity of corals (not just type 1 *Pocillopora*) will benefit from the thermally tolerant characteristics of D1 symbionts, giving them a potential mechanism to respond to future climate warming. Additionally, due to the potential patchy distribution of these associations, this provides additional management stimulus to protect these corals across their entire geographic and environmental ranges.

Figure 7.1 (a) Phylogenetic reconstructions of *Pocillopora* types identified in the current study (\wedge) and in Pinzon and LaJeunesse 2011(*) using the mitochondrial open reading frame (ORF). **(b)** The distinct RFLP patterns of the ORF of types 1 and 3 *Pocillopora* digested by the enzyme *Hha*I, used to identify the ORF type of corals in this study.

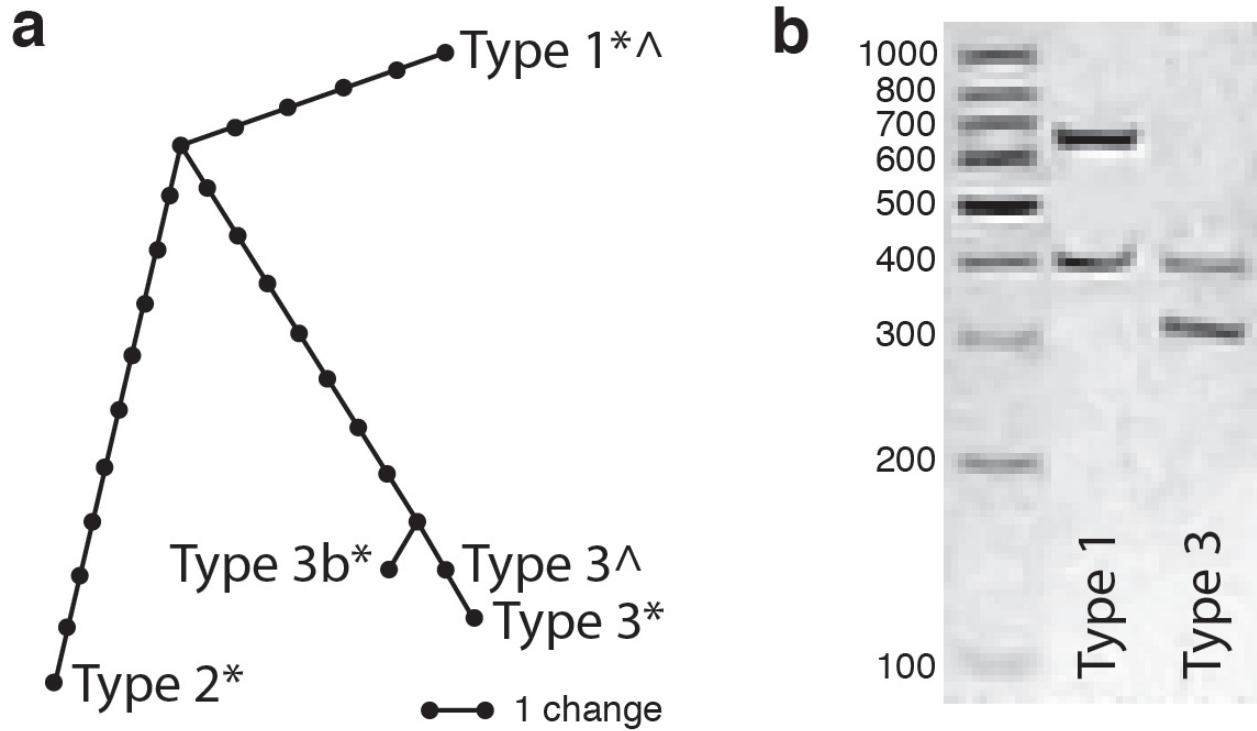


Figure 7.2. Distribution of type 1 and 3 *Pocillopora* at each sampling location. Diameters of pie charts are scaled to samples size.

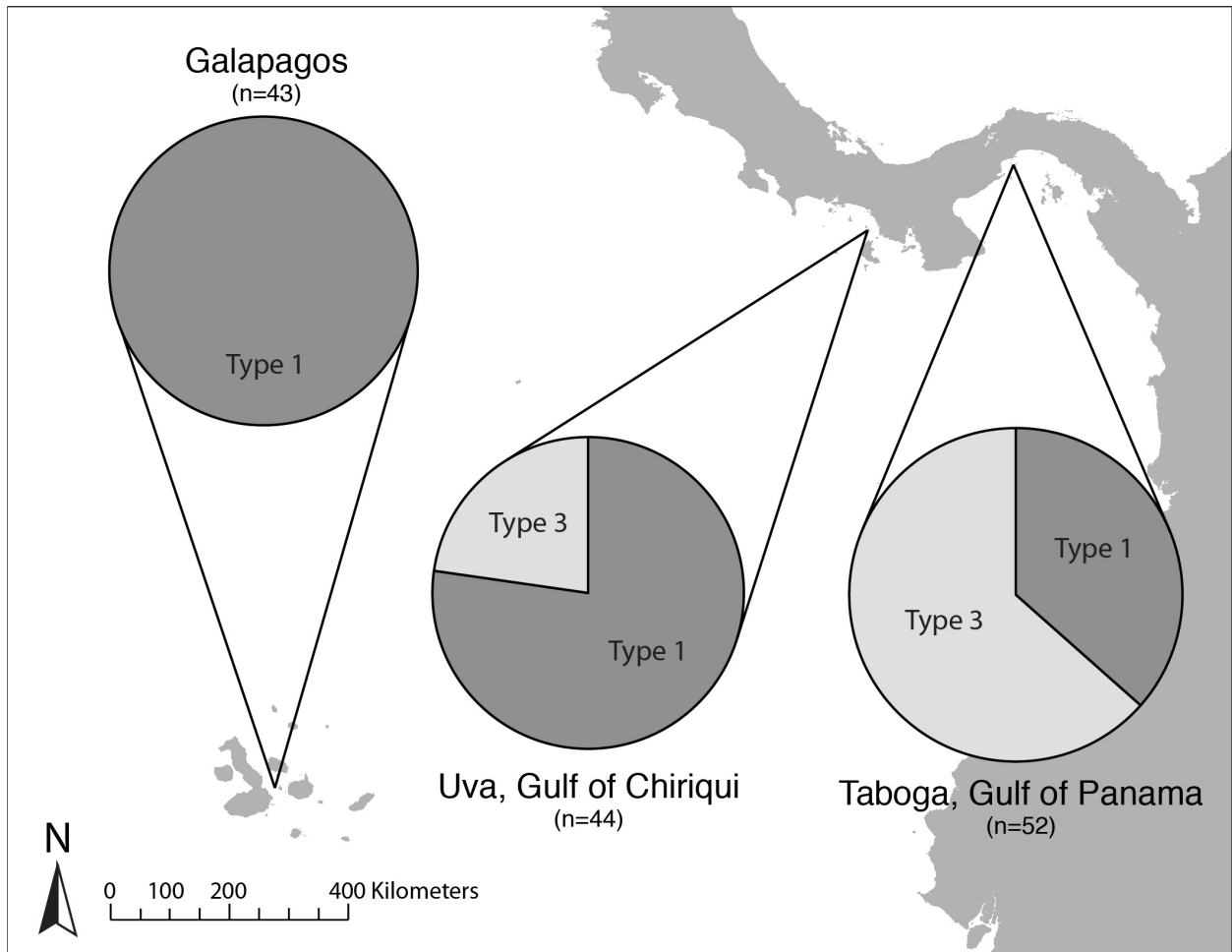


Figure 7.3. Distribution of *Symbiodinium* in clades C and D in *Pocillopora* types 1 and 3 among all samples, and at each location.

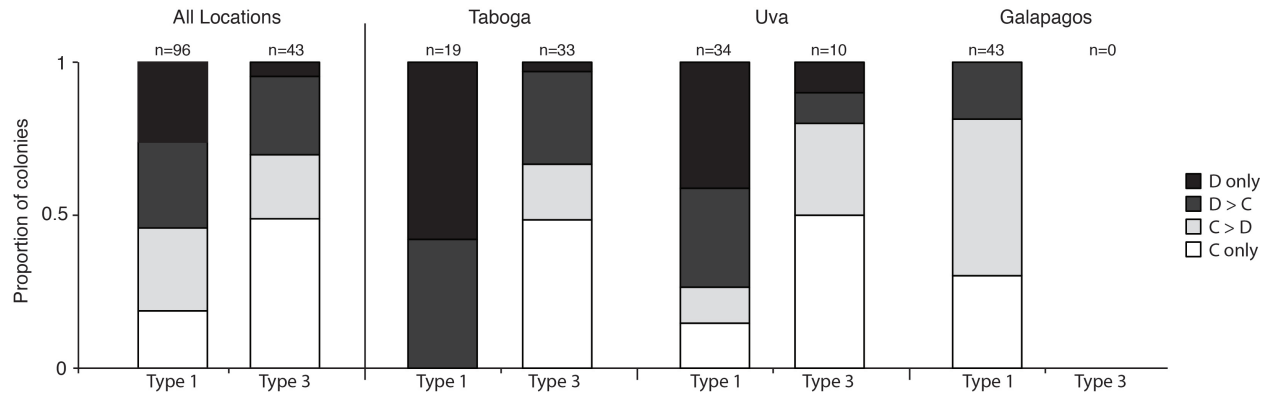
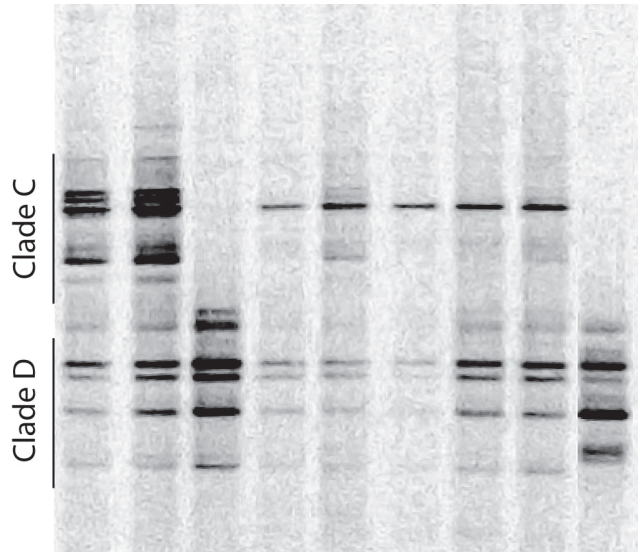


Figure 7.4. Denaturing Gradient Gel Electrophoresis (DGGE) gel of *Symbiodinium* ITS2 rDNA from 9 colonies of type 3 *Pocillopora* in which clade D was detected by qPCR. In each sample, clade D was clearly detected, even at the relatively lower level of numerical resolution provided by DGGE.



Chapter 8

Not just who, but how many: the importance of symbiont abundance in reef coral symbioses

Background

Reef-building corals engage in mutualistic symbioses with single-celled dinoflagellate algae in the genus *Symbiodinium*, from which they acquire photosynthetic products that support over 90% of their energetic requirements (Muscatine and Porter 1977). Coral “bleaching”—the breakdown of symbiosis that can lead to mass coral mortality—is predicted to occur with greater frequency and intensity due to rising sea surface temperatures (Hoegh-Guldberg et al. 2007; Baker et al. 2008). Thus, the integrity of coral-algal symbioses underpins the future persistence of coral reef ecosystems under anthropogenic climate change.

The function of these symbioses is highly dependent on the genetic identity of the algal symbionts. Different taxa within the genus *Symbiodinium* (Pochon and Gates 2010) are functionally distinct, conferring different physiological properties on their coral hosts. Certain taxa, particularly members of clade D, are heat-tolerant (Rowan 2004), conferring increased resistance to thermal stress on their hosts (Rowan et al. 1997; Glynn et al. 2001; Berkelmans and van Oppen 2006; LaJeunesse et al. 2010; McGinley et al. 2012; Cunning and Baker 2013). Other types, including members of clade C, may provide corals with more fixed carbon (Cantin et al. 2009), enabling faster growth (Little et al. 2004; Jones and Berkelmans 2010). Symbiont taxa also differ in their ability to acquire inorganic nutrients (Baker et al. 2013b) and combat oxidative stress (McGinty et al. 2012). These differences may significantly impact symbiosis function, contributing to

variation in growth, stress susceptibility, and other functional responses among corals hosting different symbiont types.

Coral colony-level phenotypes, however, are a product of not only the genetic identity of algal symbionts, but also their abundance within the coral. Symbiont population density may directly influence the costs and benefits of all symbiotic interactions (Holland et al. 2002; 2004), thereby determining the interaction outcome. In corals, symbiont abundance varies in both space and time, although the drivers and consequences of these dynamics are poorly understood. Nevertheless, symbiont abundance may influence nutrient cycling (Wooldridge 2009), light absorption (Enríquez et al. 2005), stress response (Nesa and Hidaka 2009; Nesa et al. 2012; Cunning and Baker 2013), and indeed most aspects of coral physiology. Therefore, symbiont abundance is likely to be a critical determinant of many coral functional responses such as growth rate and bleaching susceptibility.

Despite the potential importance of symbiont abundance, its specific role in determining coral responses is poorly understood and often overlooked. This may be due in part to methodological constraints that make it difficult or time-consuming to accurately measure symbiont abundance. Moreover, different ways of normalizing abundance (e.g., per unit area, mass, volume, protein, or cell) may not be equally relevant to understanding physiological attributes of the symbiosis, potentially confusing or obscuring important relationships (Edmunds and Gates 2002). Furthermore, recent work on coral symbioses has focused largely on the effects of symbiont genetic identity, detracting attention from symbiont abundance and other potentially important factors.

Older literature that focused more on basic symbiosis physiology in *Hydra* or *Aiptasia* evaluated symbiont abundance to understand how these symbioses were regulated and controlled. Symbiont population regulation in corals has been the subject of several seminal studies (Muscatine and Pool 1979; Falkowski et al. 1993; Jones and Yellowlees 1997). However, these studies focused on the mechanisms that regulate abundance, rather than how symbiont abundance affects coral physiology. Some studies have evaluated the influence of symbiont abundance on photosynthesis and respiration (Hoegh-Guldberg and Smith 1989; Hoogenboom et al. 2010), and others have explored its potential physiological impacts within conceptual (Wooldridge 2013) and modeling frameworks (Anthony et al. 2009; Terán et al. 2010; Chapter 5), suggesting it has fundamental impacts on symbiosis function. However, most studies utilize symbiont abundance simply as diagnostic for coral bleaching. We suggest that the role of symbiont abundance is much greater than a simple stress indicator; it both affects, and is affected by, coral physiology, and represents a fundamental metric of holobiont physiology.

This review discusses the different ways of measuring symbiont abundance in reef coral symbioses and how these metrics may relate to various physiological attributes of the symbiosis. It then discusses how symbiont abundance may vary across coral taxa, life history stages, ecological gradients, and time, and how symbiont population dynamics may influence the responses of these symbioses to environmental change.

Measuring symbiont abundance

Many techniques and metrics have been employed by researchers to measure the abundance of algal symbionts in coral hosts. The most frequently used metric is the

number of symbiont cells per unit surface area of coral skeleton (cells cm⁻²). This measurement involves extracting intact *Symbiodinium* cells from living coral (e.g., using a Water Pik (Johannes and Wiebe 1970) or airbrush), counting them under a microscope using a hemocytometer, and normalizing cell numbers to skeletal surface area. This method is inexpensive but labor-intensive and requires sacrificing at least several square centimeters of coral tissue. Furthermore, the accuracy and precision of these metrics depend on complete extraction of symbionts from the skeleton, the breakup of coral mucus to ensure an even distribution of symbionts in the hemocytometer counting field, and accurate measurement of skeletal surface area, all of which can be difficult to achieve (Johannes and Wiebe 1970; Edmunds 1994; Veal et al. 2010).

Areal symbiont abundance also provides no information about the coral host, even though coral tissue biomass (per unit surface area) is known to vary considerably among coral species, colonies, and over time (Fitt et al. 1993; Brown et al. 1999; Fitt et al. 2000; Edmunds and Gates 2002; Thornhill et al. 2011). Therefore, although different corals may host very similar numbers of symbionts on an areal basis (per square centimeter), these symbionts may be contained within a very different biomass (or volume) of coral host tissue. Consequently, normalizing symbiont abundance by area may obscure important functional variation among symbioses related to differences in host tissues (Edmunds and Gates 2002). In studies of symbiosis, there is a need for metrics that reflect the abundance (or size) of both interacting partners, i.e., a “symbiont to host ratio” (Douglas 1985). For this reason, other metrics normalize symbiont abundance to host-associated biological units instead of area.

The number of polyps has been occasionally used to normalize symbiont abundance (Muscatine et al. 1991; Jones and Yellowlees 1997), although differences in polyp size, structure, and density among coral taxa may prevent useful comparisons of symbiont abundance per polyp (Edmunds and Gates 2002). Other metrics that are more comparable across taxa include symbiont cells normalized to mass (Fitt 1982) or, more commonly, protein. For protein normalization, researchers either measure total (animal and algal) protein (Saunders and Muller-Parker 1997; Shick et al. 1999; Edmunds and Gates 2002; Anthony and Hoegh-Guldberg 2003; Hoogenboom et al. 2010), or physically separate animal and algal fractions to measure only animal protein (Muller-Parker 1985; Muller-Parker et al. 1994; Hawkins et al. 2013). Protein is then quantified using the Bradford Assay (Bradford 1976) and used to calculate symbiont abundance (from cell counts, as above) as cells per mg protein. While this metric provides information about both algal and coral partners, it also has associated drawbacks. First, a total protein denominator does not provide a true symbiont to host ratio, as this includes algal-derived protein (~10-13% in anemones (Saunders and Muller-Parker 1997) and corals (Douglas 1985)). Using only animal protein as a denominator theoretically overcomes this issue, although common procedures for separating algal and animal tissues (e.g., centrifugation) are not fully effective (Douglas and Smith 1983), leading to considerable error in these metrics. Moreover, these techniques remain hampered by issues of incomplete tissue removal from the skeleton, which may be even greater for corals with thicker tissue (Edmunds 1994).

Symbiont abundance has also been measured by volume (e.g., algal volume as a percent of host cell volume or per mg protein (Douglas and Smith 1983; 1984)) in green

Hydra symbioses. However, these techniques are not amenable to coral symbioses because symbionts often occupy nearly 100% of the host cell volume (Muscatine et al. 1998). Moreover, volume estimation relies on assumptions of cell shape and size that are likely incorrect (Douglas 1985).

To overcome problems associated with volume ratios and separation of algal and host tissues discussed above, the total amount of chlorophyll a per unit protein ($\mu\text{g } \mu\text{g}^{-1}$) of intact tissues has been proposed as a useful metric of the symbiont to host ratio for diverse invertebrate-algal symbioses (Douglas 1985). A similar metric of chlorophyll a normalized to tissue ash-free dry weight (AFDW) has been used for corals (Grottoli et al. 2004; 2006). However, because symbionts comprise 5-12% of coral AFDW (Porter et al. 1989) and chlorophyll a content varies widely per symbiont cell (Chang et al. 1983), this metric may not reflect symbiont abundance so much as the photosynthetic capacity of the symbiosis. As such, it may still provide useful information, and has the advantages of being rapidly and reliably calculated, requiring only small amounts of tissue, and being comparable across diverse symbiotic associations (Douglas 1985).

Finally, symbiont abundance has been normalized to host cell numbers. In *Hydra*, symbiont abundance is often quantified as the mean number of symbiont cells within a single host digestive cell (Douglas and Smith 1984). A similar cell-specific density (CSD) metric was developed to quantify the average number of symbionts within a symbiont-containing gastrodermal cell (i.e., cells without symbionts are not counted), which typically has a mean value between 1 and 2 (Muscatine et al. 1998). CSD in corals may increase with nutrient enrichment (Muscatine et al. 1998), UV exposure (Shick et al. 1999), and elevated pCO_2 (Reynaud et al. 2003), suggesting symbionts are proliferating

faster than their host cells and possibly indicating destabilization of the symbiosis.

However, because CSD does not include cells that do not contain symbionts, it is clearly decoupled from colony-level phenotypes; an increase in CSD can occur simultaneously with major declines in overall symbiont abundance (cells mg protein⁻¹; Shick et al. 1999).

More recently, the abundance of symbionts relative to the total number of host cells at the tissue or colony level has been measured using quantitative PCR (qPCR) (Mieog et al. 2009; Cunning and Baker 2013). This technique involves amplification of target gene loci in both the symbiont and the host to calculate a ratio of the total number of symbiont to host cells (S/H cell ratio) within a sample. For this analysis, DNA can be extracted from an intact coral fragment, which overcomes the problems of incomplete tissue extraction and fractionation that introduce inaccuracy in other metrics that rely on mechanical separation of tissues (see above). Moreover, very small tissue samples (0.25 cm² or less) can be used for this analysis, enabling repeated sampling of the same coral colony over time. Most importantly, because this technique does not rely on recognizing (and counting) symbionts based on their visual appearance, which is fairly uniform across *Symbiodinium* taxa, it can also be used to identify (and quantify) different symbiont types. Genetic sequence variation allows qPCR to simultaneously quantify different symbiont types in mixed communities at any level of taxonomic resolution. This is of fundamental importance, because the overall function of a symbiont community depends quantitatively on its composition (Loram et al. 2007b; Chapter 3). Finally, standardizing the abundance of symbionts using host cell numbers is an appropriate metric for the “symbiont to host ratio” (*sensu* Douglas 1985), because the cell is the fundamental unit of biological organization.

Another technique called “FISH-Flow” also quantifies the abundance of different symbiont types in mixed assemblages by utilizing fluorescence in-situ hybridization and flow cytometry in tandem (McIlroy et al. 2013). However, this technique has only been used to quantify relative proportions of different symbiont types, and does not allow normalization of symbiont abundance that is achieved by S/H cell ratios.

Drawbacks associated with the S/H cell ratio include higher variability among technical replicates than is associated with areal metrics (Mieog et al. 2009) due to the logarithmic error inherent in qPCR. In addition, calculation of absolute S/H cell ratios from qPCR data requires normalizing fluorescence intensity if different reporter dyes are used and estimating DNA extraction efficiency and gene copy numbers for target loci (Mieog et al. 2009; Cunning and Baker 2013). Primer and probe sequences must also be carefully designed to match target sequences while mismatching all non-target sequences (Cunning and Baker 2013). However, once assays have been developed and validated, they enable high-throughput data collection relative to methods that rely on cell counts and estimates of surface area. To date, assays have been developed to quantify *Symbiodinium* in clades B (Chapter 3), C, and D (Chapter 2, Mieog et al. 2009, Cunning and Baker 2013) in several coral host species (Chapters 2, 3, 4, Silverstein 2012), which can be easily adapted for use in any laboratory with a qPCR platform.

Implications of different metrics of symbiont abundance

Depending on which metric is used to quantify symbiont abundance, different aspects of symbiosis structure and function may be revealed (or obscured). For example, Muller-Parker et al. (1994) found that nutrient enrichment increased the number of

symbiont cells per cm^2 while cells per mg protein remained constant. In contrast, in response to low light, Anthony and Hoegh-Guldberg (2003) found no change in cells per cm^2 but more than double the number of cells per mg protein. Similarly, Edmunds and Gates (2002) found that different coral colonies had the same number of symbionts per cm^2 , but significantly different abundances normalized to protein.

Differences among these metrics may be the result of a dynamic vs. fixed quantity in the denominator. When symbionts are normalized to a dynamic unit (host protein, cells, etc.), their abundance is also influenced by changes in these units. To illustrate how this may produce different patterns in different metrics, consider seasonal changes in coral symbioses from winter into summer. Higher light and temperature in the summer is associated with decreases in symbiont cells per cm^2 (Stimson 1997; Brown et al. 1999; Fagoonee et al. 1999; Fitt et al. 2000), but increases in the symbiont to host cell ratio (Cunning and Baker 2013). These opposite trends may be explained by overall changes in coral tissue architecture (Fig. 8.1). Tissues become thinner in the summer (Brown et al. 1999; Fitt et al. 2000; Thornhill et al. 2011), which may involve a net loss of both symbiont and host cells on an areal basis. However, a greater net loss of host cells will increase the S/H cell ratio. Factors that may promote such changes include decreased heterotrophy in summer months (Ferrier-Pagès et al. 2011) that may reduce numbers of host cnidocyte and mucocyte cells, and increased respiration that may drive host cell catabolism. Reductions in host cells relative to symbionts increases the overall S/H ratio, a phenomenon that has also been observed in *Hydra* symbioses (Douglas and Smith 1984).

Since these metrics provide different information, it is important for researchers to select the most informative and relevant metric for their particular research. If the research focuses primarily on a physical interaction (e.g., the interception of light by symbionts), then it may be useful to normalize symbiont abundance to a physical unit of area. Because light is always measured on an areal basis (e.g., $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, or $\text{W m}^{-2} \text{s}^{-1}$), an areal metric of symbiont abundance may be most appropriate for understanding the capacity of symbionts to collect light. However, because coral tissues and light fields are also three-dimensional, the abundance of symbionts per unit volume may be even more informative (Terán et al. 2010), which would require information on host tissue thickness.

For research focused primarily on biological interactions between symbionts and hosts, it may be more useful to normalize symbiont abundance to a host-related biological unit (i.e., a “symbiont to host ratio” (Douglas 1985)). The currency of host-symbiont interactions are metabolic products and cellular signaling molecules, which are produced and received by cells as fundamental biological units. Therefore, measuring the abundance of symbionts relative to host cells (or other biological units, e.g., biomass, protein) may be more informative and relevant for research concerned with these interactions. For example, in one study of bleaching and recovery, symbiont abundance per unit area recovered to pre-bleaching levels within months, but tissue biomass, proteins, and lipids per unit area remained lower than pre-bleaching levels (Fitt et al. 1993). In this case, recovered corals might be expected to function differently from their pre-bleaching state, although areal symbiont abundance metrics would not reveal any

difference. Meanwhile, symbiont abundance normalized to a biological parameter would reveal important differences indicative of functional variation.

In phototrophic symbioses such as corals, the physical interactions between symbionts and light, and the biological interactions between symbionts and hosts are fundamentally linked. Therefore, measuring the number of symbionts normalized to both physical and biological units would provide the most comprehensive information regarding both the physical and biological aspects of symbiosis function.

These issues demonstrate the importance of normalizing data in a way that is relevant to the research question and the response variable of interest. In agreement with Edmunds and Gates (2002), we suggest that normalizing symbiont abundance to dynamic biological units, rather than static physical units, is more generally relevant to the physiology and function of coral-algal symbioses.

Effect of symbiont abundance on coral symbiosis function

Partner abundance is likely to influence the overall costs and benefits of symbiosis (Holland et al. 2002; 2004), which may affect coral phenotypes in a number of important ways. The amount of light received by individual *Symbiodinium* cells is of fundamental importance given their role as photosymbionts. The light environment they experience is significantly influenced by surrounding symbionts due to self-shading (Enríquez et al. 2005; Terán et al. 2010). When symbiont abundance is low, each symbiont receives more light. As their abundance increases, the average irradiance they experience declines (Terán et al. 2010), with symbionts that are deep within coral tissue sometimes receiving only 10% of those on the colony surface (Kaniewska et al. 2011;

Wangpraseurt et al. 2012). Because light absorption takes place within a 3-dimensional coral tissue matrix, light field modification is likely a function of symbiont abundance per unit volume, and has been implemented in this way (as cells per mm^3) in modeling these dynamics (Terán et al. 2010).

The amount of light received by symbionts in part determines the amount of carbon they can fix and the amount of photodamage they experience (Powles 1984), which has important implications for symbiosis function. However, while the amount of light received is directly influenced by symbiont abundance due to self-shading (Enríquez et al. 2005), how that light is absorbed and quenched involves additional layers of symbiont photobiology. Moreover, how symbiont photobiology in turn affects symbiosis function is further mediated by interactions between symbiont and host.

Nevertheless, these complex biological responses may still be dependent on symbiont abundance. For example, because each symbiont provides some photosynthate to the coral, increasing symbiont abundance should increase total amount of photosynthate translocated (i.e., the gross benefit of the interaction, Fig. 8.2). However, at high abundances, both self-shading and carbon-limitation may reduce photosynthesis in each cell, causing gross photosynthate transfer to decline (Fig. 8.2). This relationship is supported empirically by a study of P:R ratios in corals that showed an initial increase as a function of symbiont abundance (per mg protein) followed by a decline (Hoogenboom et al. 2010). Importantly, the benefit to the coral is determined by the amount of photosynthate translocated relative to the amount of coral tissue to which it is transferred. Therefore, symbiont abundance normalized to biological parameters (protein, cell) may be the best measure of benefits to the coral host.

Another important correlate of symbiont abundance is the energetic cost of maintaining symbionts (Douglas and Smith 1983). These costs include the provision of space within host cells for symbiont occupation (Douglas and Smith 1983), the creation and maintenance of host-derived symbiosome membranes (Peng et al. 2010), the active concentration of carbon dioxide for symbiont photosynthesis (Weis et al. 1989; Meyer and Weis 2012), the detoxification of oxygen radicals, and the repair of macromolecular damage caused by symbiont photooxidative stress (Lesser 2006). Therefore, because each additional symbiont has an associated cost, the gross costs of symbiosis should increase with symbiont abundance (Fig. 8.2). At high abundance, costs may increase exponentially, as carbon-limitation of symbiont photosynthesis may exacerbate photodamage and oxidative stress (Wooldridge 2009, Chapter 5, Fig. 8.2). Importantly, the energetic cost to the host also depends on the amount of coral tissue supporting this cost. Therefore, the costs of symbiosis may also be better predicted by adopting a symbiont to host ratio approach.

Thus, both the costs and benefits of symbiosis may be related to the abundance of symbionts within coral tissues. The gross benefit minus the gross cost represents the net benefit to the coral (Fig. 8.2), the magnitude of which may subsequently correlate with aspects of coral performance such as growth and reproductive output, which may influence coral fitness. One example of this is the effect of nutrient enrichment: nutrients elevate symbiont abundance in corals, which is associated with reduced coral growth (Marubini and Davies 1996; Fabricius 2005). This may reflect an increase in symbiont abundance beyond the optimum that reduces the net benefit of the association (Fig. 8.2), resulting in reduced growth rates.

Effect of symbiont abundance on coral symbiosis breakdown

Symbiont abundance may also influence corals' susceptibility to bleaching. Because bleaching is triggered by photodamage and production of reactive oxygen species (ROS) in symbionts (Weis 2008), this response may depend on their abundance. This relationship was demonstrated for the first time in Chapters 2, 3, and 4, which show that corals with more symbionts (measured by S/H cell ratios) bleach more severely. Higher S/H cell ratios are linked to greater bleaching severity in *Pocillopora damicornis* (Chapter 2), *Montastraea cavernosa* (Silverstein 2012), *Orbicella faveolata* (Chapter 3), and *Siderastraea siderea* (Chapter 4), suggesting this is a general phenomenon in corals. Although counter to previous suggestions that corals with more symbionts (per cm²) may be more resistant to bleaching (Stimson et al. 2002; Enríquez et al. 2005), this finding fits within the mechanistic framework for bleaching by suggesting that the severity of the response is related to the total amount of ROS produced by the symbionts, relative to the host's capacity to detoxify it. In this way, greater cumulative ROS production by a larger symbiont population may trigger a more severe bleaching response. Because the primary source and sink of oxidative stress are symbiont and host cells, respectively, the S/H cell ratio may be the best indicator of this functional relationship in connection with bleaching severity.

In fact, areal symbiont abundance is suggested to have the opposite influence, such that fewer symbionts per unit area may lead to greater light-driven ROS production per cell due to reduced self-shading (Enríquez et al. 2005; Terán et al. 2010). However, total ROS accumulation is a product of the per-cell rate of production and the total number of symbiont cells. Therefore, the effect of changing symbiont abundance on both

these factors must be evaluated simultaneously to determine cumulative ROS production and its potential relationship with bleaching severity.

The relationship between symbiont abundance and local irradiance (which may drive the rate of ROS production per cell) has been identified using both empirical and modeling approaches as being nonlinear, such that pigments (Enríquez et al. 2005) or symbionts (Terán et al. 2010) may decline by ~80% before the internal light environment is significantly amplified. These findings suggest that large changes in symbiont abundance may take place without impacting light-driven ROS production per cell. Meanwhile, 80% fewer symbionts would reduce cumulative ROS production by 80%. Therefore, changes in symbiont abundance may influence the sum total ROS production more than the rate per cell, indicating that cumulative oxidative stress and bleaching may be more severe in corals with more symbionts, not less. However, enhanced ROS production per cell may become relatively more important if symbiont populations are reduced below a threshold (i.e., due to partial bleaching) where the internal light environment becomes exponentially enhanced (Enríquez et al. 2005; Terán et al. 2010). This positive feedback associated with reduced symbiont abundance may exacerbate bleaching in already-bleached corals, although their initial susceptibility may be exacerbated by greater symbiont abundance (Cunning and Baker 2013). Importantly, because the hypothesized mechanism underlying this relationship is fundamentally a host-symbiont interaction, it may only be detected by metrics of symbiont abundance normalized to host biological parameters.

These conclusions are supported by a study in which Edmunds and Gates (2002) used both area- and protein-normalized metrics to assess changes in symbiont abundance

in two colonies of *Montastraea franksi* transplanted to a high light environment. Initial symbiont abundance per cm² did not differ between colonies, but symbionts per mg protein differed by ~60%. Only the coral with more symbionts per mg protein bleached when transplanted to the high light environment, supporting the hypothesis that excess symbionts cause more severe bleaching. Even though these corals showed different functional responses, areal symbiont density measurement failed to identify any difference between them, showing how certain metrics can mask or obscure important functional variation. This provides another illustration of how metrics that incorporate both symbiont and host information may be more relevant to physiology and better predict symbiosis functional outcomes.

Symbiont abundance regulation and dynamics

Understanding the natural variability in symbiont abundance over space and time is important due to the many ways it may influence symbiosis costs and benefits, coral performance, and stress susceptibility. Symbiont abundance is known to change in different environments, and the level at which symbionts are maintained was observed early on to be partly determined by environmental conditions in *Hydra* (Douglas and Smith 1984) *Aiptasia* (Steele 1976) and corals (Dustan 1979). In particular, these early studies showed that specific feeding and light regimes led to specific abundances of symbionts within the host. This apparent host regulation was well-studied in *Hydra*, and involved arrested growth and expulsion (Douglas and Smith 1984). Corals may also actively regulate their symbiont populations, evidenced by the continuous expulsion of symbionts (Hoegh-Guldberg et al. 1987; Baghdasarian and Muscatine 2000; Yamashita

et al. 2011), and the higher growth rates observed in *Symbiodinium* living outside the host (Chang et al. 1983). Various mechanisms of host control of the symbiont population have been investigated, including nutrient limitation (Falkowski et al. 1993), expulsion (Baghdasarian and Muscatine 2000), apoptosis (Dunn and Weis 2009), symbiophagy (Downs et al. 2009), or other mechanisms (Gates et al. 1992).

However, the underlying factors that determine precisely what abundance of symbionts is maintained are not well understood (Douglas and Smith 1984; Smith 1987). It has been hypothesized that spatial or volumetric limitations determine the abundance of symbionts in a coral (Jones and Yellowlees 1997), although several lines of evidence suggest that symbionts are not (or at least are not often) hosted at space-limited maxima: symbiont abundance frequently changes over time on seasonal and even diel scales, and nutrient addition typically increases symbiont abundance above levels that are typically found in corals. Therefore, symbiont abundance is likely to be determined by mechanisms other than space-limitation (Davy et al. 2012).

One hypothesis for why corals actively regulate the size of their symbiont population is that it may help maximize the net benefit of the symbiosis (Chapter 5). Because costs and benefits may vary as a function of symbiont abundance (see above), there may be a specific optimum abundance at which the net benefit to the coral is maximized (Fig. 8.2). These costs, benefits, and optima may also be influenced by the environment. For example, more light may increase photosynthetic capacity and gross benefits, although very high light may cause photodamage and increased costs. High and low temperatures may also increase costs associated with photooxidative stress. As these costs and benefits fluctuate, the optimal abundance of symbionts for a coral to maintain

also varies. Furthermore, symbiont types may differ in the costs and benefits they confer to their hosts, such that there exists an optimal symbiont abundance for a given symbiont type under a given set of environmental conditions.

The regulation of symbiont abundance to match this optimum may explain the variation observed in space and time. High spatial variability in abiotic factors, even over reefal scales (Brakel 1979), may drive corresponding variation in optimal symbiont abundance, explaining the high variability observed among corals on the same reef at any given time (Moothien-Pillay et al. 2005). Seasonal changes in light, temperature, and other factors may also shift these optima, leading to dynamic regulation by coral hosts to match these shifting optima. In this way, symbiont abundance may be continuously regulated as a mechanism for corals to sustain maximum interaction benefit in a dynamic environment. Among *P. damicornis* colonies sampled under a range of different environmental conditions over time, a cost-benefit model (Chapter 5) could be parameterized to explain variability in S/H cell ratios, suggesting that symbiont abundance is indeed regulated by corals to sustain maximum interaction benefit.

Other factors influencing symbiont abundance

While the primary abiotic factors influencing symbiont abundance are thought to be light and temperature, other factors such as salinity, nutrient levels, dissolved oxygen (Brown et al. 1999; Fagoonee et al. 1999), and pCO₂ may also play an important role. These factors may also influence symbiont abundance by altering the costs and benefits of symbiosis. For example, several studies have shown declines in areal symbiont densities in response to elevated pCO₂, which has been interpreted as acidosis-induced

coral bleaching (Anthony et al. 2008; Kaniewska et al. 2012). However, instead of acute bleaching, this response may represent a host-controlled reduction of symbiont abundance to maximize interaction benefit in a high-pCO₂ environment. In this way, symbiont reductions under elevated pCO₂ may represent an optimization—rather than a breakdown—of symbiosis. More research is needed to understand how symbiont abundance changes in response to different abiotic factors, which can then validate mechanistic frameworks (e.g., costs and benefits, host regulation) that may drive these changes.

Certain abiotic factors may interfere with mechanisms of symbiosis regulation, causing the symbiont population to escape host control. For example, elevated nutrients increase symbiont abundance by releasing them from their normal state of nutrient limitation (Falkowski et al. 1993). This may have detrimental impacts on the host (see above; Marubini and Davies 1996; Fabricius 2005), suggesting that, in this case, the increase in symbionts does not occur as a result of host regulation to maximize benefit. Therefore, an important goal for future research is to understand which changes in symbiont abundance represent host regulation, and which reflect a loss of host control.

Other biological factors that may also affect symbiont abundance in corals include intrinsic differences in tissue architecture among coral species. For example, corals with thinner tissues which may correlate with generally higher symbiont abundance relative to host tissue. In Chapter 4, we found significant differences in symbiont abundance between *O. faveolata* and *S. siderea* under identical conditions, and even differences among colonies of the same species. There may also be significant intra-colony variation

in symbiont abundance depending on the position and angle of coral tissues, which affects incident light fields (Kaniewska et al. 2011).

Reproductive status and heterotrophy may also influence symbiont abundance relative to host cells. For example, corals feeding heterotrophically may upregulate cnidocyte and mucocyte cells to aid in prey capture. An increase in these aposymbiotic ectodermal cells would reduce tissue-level S/H cell ratios. Similarly, production of egg and sperm cells without symbionts would also decrease the overall S/H cell ratio, indicating that reproductive cycles (and lunar cycles) may also impact symbiosis dynamics. Lesions such as parrotfish bites may also affect symbiont regulation, as symbiont abundance is reduced in tissues surrounding lesions (Palmer et al. 2011). Diseases may also significantly impact the abundance of zooxanthellae in coral tissues (Toller et al. 2001).

Ecological implications of symbiont abundance

Any biotic or abiotic factor that affects symbiont abundance in corals may have consequences for coral performance and stress susceptibility as a result of changes in the costs, benefits, and overall function of the symbiosis. Importantly, changes in either symbiont or host tissues affects symbiont to host ratios. Due to the relationship between symbiont abundance and bleaching susceptibility, corals in high-pCO₂ environments with fewer symbionts may actually be less susceptible to thermal bleaching. This suggests that corals in naturally acidic areas, or at high latitudes where acidification may occur before warming, may actually be more resistant to climate change-induced bleaching than corals in non-acidified conditions. If true, this would have important implications for survival

trajectories of corals facing the combined effects of high temperature and pCO₂. In contrast, corals exposed to elevated nutrients that have enlarged symbiont populations may be more susceptible to thermal stress. This indicates that efforts to reduce nutrient pollution on coral reefs may help corals be more resistant to climate change-related stressors (Wooldridge and Done 2009; Wiedenmann et al. 2013; Cunning and Baker 2013).

Differences in symbiont abundance may also help explain differences in bleaching susceptibility across depth gradients (Dustan 1979), at different times of the year (e.g., summer, when S/H cell ratios are higher (Cunning and Baker 2013)), among coral species (McClanahan et al. 2004), and even within coral colonies (Rowan et al. 1997).

Conclusions

While much of the focus of recent research has been on the influence of symbiont identity, symbiont abundance must also be considered as a crucial factor influencing the function of coral-algal symbioses. Therefore, it is necessary for researchers to obtain some metric of symbiont abundance in order to properly evaluate coral responses to various environmental conditions and experimental treatments. Because symbiont abundance may critically mediate coral responses, accounting for this factor may help explain variable outcomes and observations. Ideally, knowledge of both symbiont identity and abundance (with respect to both physical and biological units) would provide the most comprehensive information on the state of the symbiosis, but we suggest that taxon-specific symbiont to host cell ratios are the most biologically relevant and

efficiently obtainable metrics. These metrics have shown consistent functional relationships with photosynthetic performance and bleaching severity, and may also be good predictors of the overall costs and benefits of symbiosis. We suggest that the use of more relevant metrics and a greater appreciation for importance of symbiont abundance will greatly advance our understanding of the biology of coral-algal symbioses, and their responses to environmental change.

Figure 8.1. Theoretical changes in coral and symbiont tissue architecture in winter and summer. Black rectangles represent coral host cells (comprising two tissue layers) and brown circles represent symbiont cells within the host gastrodermal cells. Although in summer both symbiont and host cells are reduced on an areal basis, a greater net loss of host cells causes the S/H cell ratio to increase.

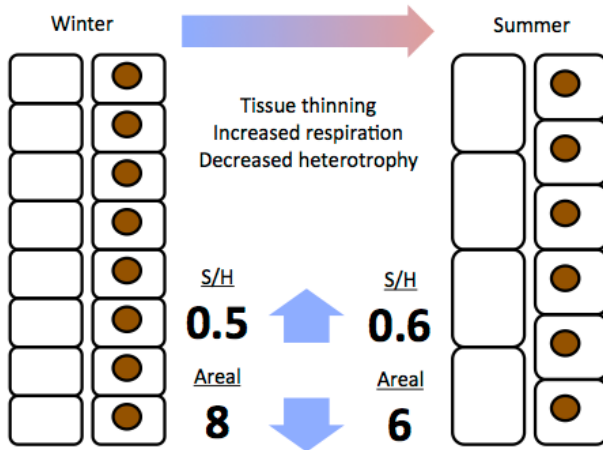
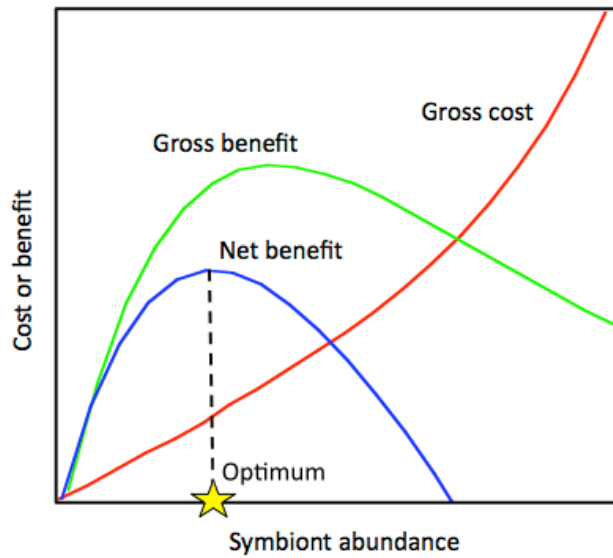


Figure 8.2. Theoretical costs and benefits to the coral host as a function of symbiont abundance. See text for justification of shapes of gross cost and benefit curves. Net benefit is gross benefit minus gross cost. The point at which the net benefit is maximized is hypothesized to represent the optimal symbiont abundance for the coral.



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