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Florida's Pillar Coral (Dendrogyra cylindrus): The Roles of the Holobiont Partners in Bleaching, Recovery, and Disease Processes

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FLORIDA'S PILLAR CORAL (*DENDROGYRA CYLINDRUS*): THE ROLES OF THE HOLOBIONT PARTNERS IN BLEACHING, RECOVERY, AND DISEASE PROCESSES

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Cynthia Fairbank Lewis

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To: Dean Michael R. Heithaus
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This dissertation, written by Cynthia Fairbank Lewis, and entitled Florida's Pillar Coral (*Dendrogyra cylindrus*): The Roles of the Holobiont Partners in Bleaching, Recovery, and Disease Processes, having been approved in respect to style and intellectual content, is referred to you for judgement.

We have read this dissertation and recommend that it be approved

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Florida International University, 2018

DEDICATION

I dedicate this dissertation to my family:

To my parents, Elton Hunt Fairbank and Gloria Guastella Fairbank for instilling in me the deep respect for Nature, the motivation to pursue my education, and the courage to follow my heart.

To my sister, Candice Fairbank Groth, for her unfailing support, giving me the strength to keep my feet moving on this path.

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ABSTRACT OF THE DISSERTATION

FLORIDA'S PILLAR CORAL (*DENDROGYRA CYLINDRUS*):

THE ROLES OF THE HOLOBIONT PARTNERS IN BLEACHING, RECOVERY, AND DISEASE PROCESSES

by

Cynthia Fairbank Lewis

Florida International University, 2018

Miami, Florida

Professor Mauricio Rodriguez-Lanetty, Major Professor

The iconic pillar coral, *Dendrogyra cylindrus*, is one of five Caribbean species listed in 2014 under the US Federal Endangered Species Act because of its extreme low abundance and continued decline in US waters. Until recently, little was known about the demographics or genetic diversity of Florida's *D. cylindrus* population. This study represents the first time two holobiont partners (coral animal and associated photosynthetic algal endosymbionts) have been closely examined, spatially and temporally, in this little-studied species. The aim was to explore the influences of coral animal genotypes, mutualistic photosynthetic algal strains, and hyperthermal stress on bleaching and disease processes, resistance, and recovery through two consecutive hyperthermal events on the Florida Reef Tract (FRT) in 2014 and 2015.

Through geographically stratified, triannual assessments and tissue sampling of *D. cylindrus* colonies across three regions of the FRT from April 2014 to April 2016, I compared genotypic identities of the coral animal to bleaching and disease status and recovery. Additionally, I characterized the algal endosymbionts (Symbiodiniaceae

family) in *D. cylindrus* between regions of the FRT using Illumina amplicon sequencing of the partial chloroplast 23S rDNA Domain V gene and correlated them to differential responses during bleaching and recovery. Finally, I examined the effects of hyperthermal stress on disease prevalence and changes in disease susceptibility in *D. cylindrus* throughout two consecutive hyperthermal events in 2014 and 2015.

Genotypic differences in *D. cylindrus* were associated with full or partial bleaching and/or disease resistance associated with some genets. Additionally, this study characterized unexpected diversity in the Symbiodiniaceae community within *D. cylindrus* and a site-specific, species-level switch in endosymbionts associated with acquired bleaching resistance during the 2015 hyperthermal event. Finally, this study demonstrated that two consecutive hyperthermal events were associated with an increase in prevalence of white plague in *D. cylindrus* and contributed to its susceptibility to black band disease, documented for the first time on the FRT.

Through understanding the response of the *D. cylindrus* holobiont partners to biotic and abiotic stressors, such as hyperthermal bleaching and associated diseases, we gained valuable insights into how this threatened species may respond to a changing climate.

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ABBREVIATIONS AND ACRONYMS

ANOVA	analysis of variance
BBD	black band disease
C-MAN	Coastal-Marine Automated Network
Cp23S	chloroplast 23S gene region
ENSO	El Niño Southern Oscillation
ESA	Endangered Species Act
FDA	Food and Drug Administration
FRT	Florida Reef Tract
ITS-2	internal transcribed spacer 2
IUCN	International Union for the Conservation of Nature
FKNMS	Florida Keys National Marine Sanctuary
FWC	Florida Fish & Wildlife Conservation
FWRI	Fish & Wildlife Research Institute
NDBC	National Data Buoy Center (NOAA)
nMDS	Non-metric multi-dimensional scaling
NOAA	National Oceanographic and Atmospheric Agency
MANOVA	Multivariate analysis of variance
SCTLD	Scleractinian or stony coral tissue loss disease
SWG	State Wildlife Grant
WP	white plague

CHAPTER 1: General Introduction

Coral reefs world-wide have experienced dramatic declines in recent decades because of natural and anthropogenic factors *(1-4)*. An estimated 20% of the world's reefs have been lost in the last 50 years and it is projected that more than 60% of the world's reefs may be gone by 2030 *(5-7)*. These projections have accelerated following the 2014-2016 El Niño Southern Oscillation (ENSO), one of the strongest events on record, which caused staggering losses to coral reefs worldwide as a result of consecutive bleaching events, including within the Greater Caribbean and the Florida Reef Tract *(8)*. In the Caribbean, declines in live coral cover since the 1970s is estimated at more than 80%, leaving less than 5% live coral cover on many reefs *(1, 3, 9, 10)*. Increased frequency and intensity of tropical storms *(10)*, bleaching events *(11-13)*, and disease outbreaks now affecting 33 of the 61 endemic Caribbean coral species have further affected these reefs *(14-18)*. The Florida Reef Tract (FRT), stretching nearly 320 km from Biscayne Bay to the Dry Tortugas, with additional unconsolidated coral habitat extending northward to Martin County, is the only living barrier reef in the continental United States and has experienced alarming declines in live coral cover and diversity *(19-21)*. In light of these dramatic declines in coral reefs, concerns for maintaining and preserving population connectivity and genetic diversity to enhance resilience, as well as reproductive success and potential for adaptation, have become major foci *(5, 22-25)*.

1.1 The importance of the coral holobiont

The existence of coral reef ecosystems worldwide depends on the complex functions of the coral holobiont (coral animal, photosynthetic algae, bacterial community, protozoans, fungi, and viruses) and their critical symbiotic relationships, balancing

cellular respiration, photosynthesis, and nutrient recycling *(26-28)*. The heterotrophic host (coral animal) captures and ingests zooplankton and dissolved organic nutrients from the water column to fuel its own cellular metabolism. By-products of cellular metabolism (ammonium, CO_2) are available to the photoautotrophic symbiotic algae (Symbiodiniaceae), which metabolize ammonium as a nutrient source for their own cellular growth, excreting organic nitrogenous compounds (amino acids) back to the coral animal. Nitrogen recycling between the holobiont partners allows for effective retention of this important nutrient for cellar growth in a typically oligotrophic environment *(29)*. Recent investigations have shown that the coral microbiome also contributes to the function of the holobiont through carbon fixation, nitrogen metabolism, and sulfur cycling, as well as antimicrobial activity *(30)*. Cyanobacteria, especially intracellular types, have been found to fix nitrogen (an energy costly process), converting N_2 to ammonium (NH^{4+}), which is then used by the non-nitrogen fixing endosymbionts for cellular growth *(31, 32)*. Nitrogen availability within the coral animal can affect Symbiodiniaceae growth and density, while disruption of the microbial nitrogen cycling may be linked to coral bleaching and disease *(33)*. Nitrification, the chemo-lithotrophic oxidation of ammonium (NH^{4+}) and nitrite (NO^{2-}) to nitrate (NO^{3-}) by autotrophic bacteria and Archaea, is commonly carried out by members of the coral microbiota. However, ammonium is the preferred source of dissolved inorganic nitrogen by the algal endosymbionts and thus nitrification may compete for the nitrogen available for endosymbiont growth *(34, 35)*. The photosynthetic algal symbionts capture energy from sunlight to oxidize water with CO_2 as the electron acceptor, producing photosynthetically-fixed carbon (glycerol, glucose, and amino acids) as well as O_2 for use

by the coral host for cellular respiration and growth *(36)*. This critical photosynthetic process not only provides all of their own energy needs for the algal symbiont, but often up to 95% of the nutritional needs of their host corals *(37, 38)*. In addition, O_2 produced during photosynthesis enhances coral calcification *(39)* which is critical to reef-building corals and the accretion of coral reefs.

1.2 Symbiodiniaceae diversity and function in hyperthermal stress

Most reef-building corals form an obligate symbiosis with photosynthetic algal endosymbionts (family Symbiodiniaceae). Initially it was thought that these symbiotic dinoflagellates belonged to a single genus (*Symbiodinium* spp.). Work by Rowan and Powers (1991) determined distinct taxonomic lineages or clades within the genus using small ribosomal subunit RNA (18S ss-rRNA) and restriction fragment length polymorphisms *(40-42)* that correlated with differential environmental tolerances *(43-45)*. Thus far, nine phylogenetic clades or lineages of these photosynthetic dinoflagellates have been described *(43, 46, 47)*. Previously designated by clade letters, seven of these clades have recently been described as distinct genera *(48)* (Table 1.1). Buddemeier and Fautin proposed the Adaptive Bleaching Hypothesis *(49)* which posited that endosymbionts respond differently to environmental stressors and that adult corals could acquire new more thermally-tolerant symbionts after bleaching (i.e., expulsion of endosymbionts), allowing the coral host to adapt in the short-term, to environmental changes. Development of additional molecular markers (nuclear ribosomal interstitial transcribed spacers [ITS-1, ITS-2], chloroplast large subunit ribosomal DNA [cp23S rDNA], and mitochondrial DNA [Cyt b]) allowed researchers to further differentiate symbiont types within each newly recognized genus *(50-55)*. It is now accepted that

inter- and intra-generic diversity display clear physiological differences *(45, 56, 57)* and provide differential environmental tolerances and sensitivities to the symbiotic partnership *(58-60)*, including bleaching resistance and susceptibility *(61-63)*. Seminal work by Berkelmans et al. in 2006 *(64)* showed shuffling of symbionts between genera (formerly clades) provided increased thermal tolerance and bleaching resistance. High throughput amplicon sequencing now allows even finer scale resolution of endosymbiont communities within genera, which have also identified additional thermally tolerant symbiont species with improved survivorship during hyperthermal bleaching events *(65, 66)*. Recent studies have also revealed far greater diversity within the cryptic endosymbiont community *(67-71)*, however these cryptic symbionts have been considered transient and assumed to be of little ecological importance thus far *(72)*.

1.3 The role of the coral animal genotype on resistance and resilience to thermal bleaching and disease processes

Biodiversity is considered the cornerstone of healthy ecosystems, buffering and providing resilience in the face of environmental disturbances and stressors, allowing ecosystems to return to their functional integrity *(73-75)*. Additionally, high diversity and species richness at the ecosystem level can ensure diversity of necessary functional groups, providing ecological services to the ecosystem and allowing coral reefs to absorb disturbances and resist unhealthy phase shifts. As a consequence of its biogeographic isolation, the Caribbean has historically been far less diverse than the Great Barrier Reef, containing only 28% of the fish species diversity and 14% of the coral species diversity *(73, 76)* and thus may be more vulnerable to ecosystem disturbances.

At the species level, genetic diversity provides the ability for organisms to adapt to their environment by providing genetic variability for natural selection to act upon. It has been shown that successful sexual reproduction in coral is often enhanced by a diverse genetic population and low diversity has been shown to decrease fertility and subsequent recruitment *(77)*. Loss of genetic diversity can increase the risk and likelihood of gamete incompatibility or self-fertilization within populations, leading to reduced success of sexual reproduction due to low fertility rates and recruitment *(22, 77-79)*. However, it has been shown that larvae produced by surviving genotypes in stressed environments may actually be more thermally tolerant and better able to survive changing conditions. Additionally, it has been shown that some Acroporid genotypes are more thermally tolerant despite having the same dominant endosymbiont type *(80)*, and yet other Acroporid genotypes have been shown to be disease resistant *(81)*. Hence, the importance of retaining diversity at all levels, which may provide yet-unknown resistance and resilience in a changing climate, cannot be overlooked.

1.4 Thermal stress and the disruption of the holobiont

Disruption of the mutualistic relationships within the coral holobiont, as occurs in hyperthermal bleaching and disease processes, can have devastating effects on the stability of the holobiont itself and affect the long-term survival of the reef ecosystem. Collateral damage of heat stress impacts biological, immunological, and physiological functions in the coral animal *(82-85)*. Thermal bleaching and the loss of algal symbionts can deprive the coral animal of necessary nutrients, leaving it in a weakened state and susceptible to diseases *(16, 86-89)*. Additionally, many marine pathogens are known to

proliferate or have increased pathogenicity or virulence at elevated temperatures *(90-92)* and hyperthermal stress has been linked to increases in disease *(16-18, 88, 89, 92-96)*.

Thus far, the mechanisms linking patterns of recurrent bleaching, recovery, and disease remain relatively unexplored, in large part because such studies depend on the contingency of natural bleaching events. Recent recurrent thermally-induced bleaching and subsequent disease events occurring Caribbean-wide presented unique time-sensitive opportunities to explore the holobiont partners and mechanisms underpinning the patterns of recurrent coral bleaching, recovery, and disease.

1.5 Study organism background: *Dendrogyra cylindrus*

The target species for the current research was the iconic but little-studied pillar coral, *Dendrogyra cylindrus* (Ehrenburg, 1834; Figure. 1.1), a slow-growing gonochoric broadcast spawner (male and female colonies), typically found in low abundance throughout its Caribbean range. Recent work *(97)* has shown hermaphrodism in this species to be more prevalent than previously known (i.e., simultaneous hermaphrodism, with male and female gametes released from the same colony, and sequential hermaphrodism, with alternating sexes between years of the same colony). This species is a monospecific member of the family Meandrinidae (Linnaeus, 1758). While widely distributed throughout the Caribbean, *D. cylindrus* is rarely considered an important reef builder, yet its unique columnar growth form provides important vertical structure and habitat complexity wherever it occurs. The columnar growth form of this species predisposes it to asexual fragmentation as pillars break off and are displaced during storms. These broken pillars or ‘ramets’ are often able to stabilize and form a ‘new’ separate colony, yet genetically identical to the parent colony (i.e., same genet).

Dendrogyra cylindrus has been categorized as 'vulnerable' because of its susceptibility to bleaching, disease (especially white plague), and habitat degradation *(98)* under the International Union for the Conservation of Nature (IUCN) Red List since 2008. This coral species is of special concern on Florida's reefs and was federally listed under the US Endangered Species Act as 'threatened' in 2014 *(99)* as a consequence of its rare occurrence and rapidly declining, critically fragmented population on the Florida Reef Tract (FRT). Surveys of the *D. cylindrus* population along the FRT in 2013 and 2014 documented 745 live colonies at 155 sites. More than two-thirds of these sites contained single colonies, often separated by tens of kilometers, further contributing to its low recruitment success (unpublished data). *Dendrogyra. cylindrus* is typically found on shallow, gently-sloping back-reefs (5m to 15m depths) and thus may be particularly sensitive to declining water quality as well as environmental stressors associated with climate change, including thermal bleaching. The dominant endosymbiont thus far reported in *D. cylindrus* is a unique *Breviolum* species (formerly clade B1 phylotype *(48)*; ITS2 type B1-4k; *(52)*) which may contribute to its bleaching sensitivity. *Dendrogyra cylindrus* is also susceptible to white plague (WP), a highly destructive disease effecting more than 40 coral species world-wide *(100, 101)*, and especially the more virulent form white plague type-II (WP-II), which swept through the FRT in 1995 *(102-104)*. These multiple factors (climate change, declining near-shore water quality and susceptibility to bleaching and disease) have contributed to the alarming decline in this critically threatened and little-studied species on Florida's reefs.

It is important to understand the decline of *D. cylindrus* in the context of other corals on Florida's reefs. The FRT has shown marked decline in live coral cover, as well

as declines in species diversity, since the mid-1970s but decline has escalated since the 1997-98 El Nino event as well as other lesser thermal events and severe hurricanes *(20, 105)*. Historically, *Orbicella annularis* was the spatially dominant reef-building coral species on the FRT. By 1999 it accounted for only 1.4% of the live cover, and by 2009 had further declined to 0.6 % *(105)*. *Acropora cervicornis* and *A. palmata*, while not considered reef-building species, historically created the predominant framework on Caribbean reefs *(106, 107)*. Precipitous declines have been documented in these two species since the mid-1970s *(108)*, largely as a result of disease, as well as natural and anthropogenic disturbances *(109)*. By the mid-1990s the acroporid species accounted for 10% to 20% of the coral cover on the FRT but further declined to <2% by 2001 *(110)*. The decline of these and other coral species is typically measured in terms of percent of live coral cover across a reef. By comparison, *D. cylindrus* is rarely if ever captured in survey information, and only by chance, in annual reef-wide monitoring efforts due to its extremely low abundance *(21, 105, 111, 112)*. Its population is measured not on the scale of percent cover on a reef but by the actual number of individual colonies. As mentioned above, in 2014 there were an estimated 745 live *D. cylindrus* colonies on the entire FRT; by 2018 there were fewer than 50 colonies (unpublished data). The catastrophic loss of these colonies in this dwindling population is cause for concern as each colony may represent the last of a unique genotype on the FRT *(113)*. The current research reported here helps to provide a better understanding of resistance and resilience to environmental and biotic stressors, critical to resource managers in their efforts to stabilize and preserve the remaining *D. cylindrus* population and develop future restoration strategies.

1.6 Dissertation objectives, hypotheses and organization

The impetus of the doctoral dissertation was to explore the influences of coral animal genotypes, mutualistic photosynthetic algal strains, and hyperthermal stress on bleaching and disease processes, resistance, and recovery. To achieve this, I focused on the Caribbean pillar coral, *Dendrogyra cylindrus,* to determine if there are differences in resistance or resilience to bleaching or disease within the Florida population and if these differences are associated with specific sites or regions within the Florida Reef Tract (FRT). I had a rare opportunity to closely document patterns of bleaching, recovery, and disease processes within the framework of a natural experiment involving two consecutive mass bleaching events, occurring in August-September 2014 and 2015 *(114)*. I explored two of the holobiont partners (coral animal and algal endosymbionts) to determine their contributions to greater resistance or more rapid recovery from bleaching or disease occurrence. Finally, I examined the cumulative effects of consecutive hyperthermal stress experienced on the Florida Reef Tract in 2014 and 2015 to determine the impact on bleaching resistance and resilience and prevalence of disease in *D. cylindrus*.

Chapter two describes the experimental design and methodology used to answer these research questions in the context of the two consecutive hyperthermal bleaching events on *D. cylindrus* on the Florida Reef Tract (FRT).

Chapter three focuses on the role of coral animal genetics in bleaching susceptibility and recovery and disease resistance and resilience in *D. cylindrus* on the FRT by addressing the following questions and hypotheses:

Do *Dendrogyra cylindrus* genotypes, and their associated physiological differences, affect the response to thermal bleaching, recovery, and disease resistance between corals within a site, between sites, or between regions of the FRT?

- H-3.1 *Dendrogyra cylindrus* genotypes affect the response to thermal bleaching and recovery between colonies within a site, between sites, and/or between regions of the Florida Reef Tract.
- H-3.2 *Dendrogyra cylindrus* genotypes affect the response to disease resistance and resilience within the Florida Reef Tract.

Chapter four focuses on the role of endosymbiotic algae (family Symbiodiniaceae) in bleaching resistance, recovery, and disease resistance. The following questions and hypotheses are addressed:

Are there differences in the baseline pre-bleaching Symbiodiniaceae assemblages between sites or between regions of the Florida Reef Tract? Is the response to hyperthermal stress and bleaching within and between sites and regions along the Florida Reef Tract correlated to the Symbiodiniaceae community within *D. cylindrus*?

- H-4.1 Symbiodiniaceae assemblages in *D. cylindrus* colonies are different between sites and/or between regions of the Florida Reef Tract.
- H-4.2 The response to hyperthermal stress and bleaching observed within and between sites and regions along the Florida Reef Tract are correlated to the Symbiodiniaceae community within *D. cylindrus* colonies.

Chapter five focuses on the relationship between hyperthermal stress on *Dendrogyra cylindrus* and white plague disease prevalence and susceptibility on the Florida Reef Tract (FRT). The following questions and hypothesis are addressed:

How does hyperthermal stress effect the prevalence of white plague in *D. cylindrus*? Are there differences in disease prevalence in *D. cylindrus* between regions of the Florida Reef Tract? Is disease prevalence or incidence in *D. cylindrus* exacerbated by the cumulative effects of consecutive hyperthermal events?

- H-5.1 There are differences between regions of the Florida Reef Tract in baseline pre-bleaching disease prevalence in *D. cylindrus* prior to anomalous hyperthermal events.
- H-5.2 Hyperthermal stress and bleaching in *D. cylindrus* is correlated with increased disease prevalence or incidence, with differences between sites or between regions along the FRT (2014).
- H-5.3 Increases in disease prevalence and/or incidence in *D. cylindrus* colonies is exacerbated by the cumulative effects of consecutive hyperthermal events on the FRT (2014 & 2015).

Chapter six documents for the first time, black band disease in Florida's *Dendrogyra cylindrus* and describes the temporal dynamics of this disease during two consecutive hyperthermal events on the FRT in 2014 and 2015.

Chapter seven provides overall conclusions and synthesis of the research presented here.

1.7 Figures and Tables

Figure 1. The iconic and unique Caribbean pillar coral, *Dendrogyra cylindrus*.

Dendrogyra cylindrus occurs in low abundance throughout the Greater Caribbean. Its unique columnar structure provides important habitat complexity. (photo: C Lewis)

Table 1.1. Seven genera within the family Symbiodiniaceae.

Newly described genera for photosynthetic endosymbiotic dinoflagellates (‘zooxanthellae’) in the family Symbiodiniaceae *(48).*

Genus	**Etymology**	**Former clade designation**
Symbiodinium	living together, whirling	clade A
Breviolum	short & small	clade B
Cladocopium	branch & plenty	clade C
Durusdinium	tough & whirling	clade D
Effrenium	living unrestrained	clade E
Fugacium	ephemeral	clade F
Gerakladium	old & branch	clade G

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CHAPTER 2: Experimental Design, Sampling Strategy, and Characterization of Thermal Profiles Along the Florida Reef Tract (2014-2016)

Chapter two presents in detail the over-arching experimental design and sampling strategy used to address the aims and hypothesis for all subsequent data chapters (chapters three through six). Tri-annual monitoring and assessments (every four months) characterized the broader seasonal fluctuations in Florida's *Dendrogyra cylindrus* population. Monthly assessments and sampling at three sites representative of the three regions of the reef tract (Upper, Middle, and Lower) allowed for more fine-scale inspection of bleaching recovery and disease processes in *D. cylindrus* following two consecutive hyperthermal bleaching events in 2014 and 2015.

2.1 Experimental Design

The questions and goals to be addressed in the current research were answered by rigorous field sampling, designed as a natural experiment, and conducted along the Florida Reef Tract (FRT) over the two-year period (April 2014 to April 2016). The approach allowed for comparisons of bleaching and disease susceptibility and resistance associated with the two hyperthermal events on the FRT. To explore the roles of the coral holobiont partners in the context of these stress events, the three hydrologically distinct regions within the FRT were considered as experimental variables (Figure 2.1: Upper, Middle and Lower regions *(1-4)*). Potential differences among sites and among each region were also analyzed. The first time point, April 2014, was considered the baseline for both bleaching and disease, fully realizing that the FRT is not a pristine reef system and has experienced multiple stressors in recent decades, including previous hyper- and

hypothermal bleaching *(5-7)* and disease events *(8-13)*. The present study marks the first time Florida's *D. cylindrus* populations have been selectively targeted and extensively assessed to establish critical baseline information. Prior to 2014, the most recent hyperthermal bleaching observed in *D. cylindrus* on the FRT occurred in 2011 (pers. obs.). Bleaching was localized and not reef-wide, however *D. cylindrus* observed on recreational dives in the Lower Keys were severely bleached. Normal, beached, and pale status comprised the three categories in the bleaching parameter of the current natural experiment. Coloration was scored categorically using the CoralWatch Coral Health Chart as a reference (Figure 2.2; *(14)*). Additionally, disease status (healthy, diseased, and recovered from disease) was as a second factor within the natural experiment. As a result of consecutive hyperthermal events occurring in 2014 and 2015, differences between the two years were also compared, in the context of cumulative effects of disturbance and potential changes in resistance and resilience in terms of bleaching and disease factors.

2.2 Colony assessment and field sampling

To facilitate the experimental design, 96 *D. cylindrus* colonies were selected and identified for geographically stratified sampling at 18 sites throughout the Upper, Middle and Lower regions of the Florida Keys (32, 30, and 34 colonies respectively) (Figure 2.1). To increase replicates within each region (number of sites and colonies assessed), an additional ten sites (67 colonies) were assessed at each time point, but not sampled to comply with permit restrictions. Selected sites ranged in depth from 4m to 10m. Colonies at all sites were mapped and photographed to create field identification sheets for each

colony to facilitate accurate repeated assessments and coral tissue sampling on subsequent visits.

2.2.1 Tri-annual assessment and sampling of Dendrogyra cylindrus *at 28 sites*

All 28 sites (143 colonies) were assessed three times per year, every four months (April/May, September, January) to capture seasonal variation in response to variables. The selected 96 colonies at 18 sites were also sampled for coral tissue total DNA analysis at each time point. Three of these sites were considered high-density sites defined as more than 20 colonies within a site (PCF: 174 colonies, site area 30m x 200m; Pickles: 107 colonies, site area 25m x 200m; Coffins: 55 colonies, site area 40m x 150m; Figure 2.1). A sub-set of colonies spatially distributed across each of the high-density sites was selected for sampling and marked with numbered plastic tags nailed to the substrate to assure positive identification of the colony for repeat sampling and assessments (PCF n= 26, Pickles n=24, Coffins n=24).

2.2.2 Monthly assessment and sampling during bleaching recovery at three sites

To observe the post-bleaching recovery process at a finer scale between tri-annual assessments, additional sampling time points were added at a sub-set of the original 28 sites (October, November, and December 2014, March, October, November 2015, and April 2016; Table 2.1) focusing on one representative site from each of the three regions of the Florida Keys (Upper: Pickles, Middle: Coffins, Lower: Marker 32; Fig 2.1). A total of 61 colonies were assessed but only 37 colonies were sampled for coral tissue during these targeted assessments to comply with our permits (Pickles n=12, Coffins n=12,

Marker 32 n=13). All originally selected and tagged colonies were assessed for bleaching and disease (Pickles n=24, Coffins n=24, Marker 32 n=13).

2.2.3 Coral tissue sampling for total genomic DNA coordinated in conjunction with tri-annual and targeted monthly assessments

Tri-annual assessments and sampling of the selected *D. cylindrus* colonies commenced in April 2014 and continued through January 2016 (Table 2.1). Targeted monthly assessments and sampling, beginning in October 2014 and October 2015, followed the same assessment and sampling protocols described. Tissue sampling consisted of three to four polyps suctioned from each colony (either healthy, bleached, and/or diseased tissue) with a 35cc syringe as described in the Kemp micro-sampling method *(15)* and modified by Correa *(16)*, to minimize damage to the colonies from repeated sampling over the duration of the study. Samples were transported back to shore on ice, filtered using 3.0µm and 0.2µm glass fiber filters, and preserved in 95% EtOH from which DNA was extracted using the DNeasy Plant Mini kit (Qiagen). All coral samples were collected under Florida Keys National Marine Sanctuary permit #FKNMS-2014-004-A2. Preliminary tests of the syringe micro-sampling technique yielded satisfactory DNA extractions and PCR amplification so that more-invasive core sampling methods were not necessary. Comparative sampling of several colonies and multiple locations within the same colony (top, middle, and base) determined that only a single sample per colony for each tissue status (normal, pale, bleached, healthy, and diseased) would be necessary to capture the DNA signature of each colony *(17)*. Total genomic DNA samples from these 96 sampled colonies was also included in a collaborative

project to determine genetic diversity of Florida's *D. cylindrus* population (State Wildlife Grant SWG-13059: Florida Fish & Wildlife Research Institute; Dr. Iliana Baums, Pennsylvania State University; *(17)*.

At each sampling time point, colonies were also assessed for live coral tissue (visual estimate of percent live, percent old mortality, percent recent mortality). Bleaching status was determined using the CoralWatch Coral Health Chart developed by Siebeck et al. *(14)* (Figure 2.2), scaled from 1 (white) to 6 (heavily pigmented). This colorimetric chart served as proxy for symbiont density and chlorophyll *a* content.

Disease status was determined by visual estimate of percent disease impact on colony. All sites were also assessed for presence of disease or bleaching in other coral species within two meters of each colony, but not quantified at the community level.

2.3 Characterization of thermal profiles at *Dendrogyra cylindrus* sites

Temperature data loggers (Onset HOBO Inc., Bourne, Massachusetts USA), secured to the base of colonies *D. cylindrus* colonies at 12 of the 28 sites along the FRT, recorded hourly temperatures between April 2014 and April 2016. Temperature data were used to calculate mean daily, mean monthly, and maximum weekly sea temperatures. Archived temperature data for 2004-2013 at Molasses Reef C-MAN station MLRF1, *(18)* (Figure 2.1) were used as a proxy to calculate ten-year mean monthly and mean monthly maximum sea temperatures in the Upper region of the FRT. As a result of defunding and lack of maintenance of C-man stations in the Middle and Lower Keys since 2009, continuous data from these sites were unavailable for much of the 2004-2013 time period

used to calculate ten-year means on the FRT. Using the calculated mean monthly maximum temperatures at MRLF1, degree heating weeks (°C-weeks) were calculated for each site for July, August, and September 2014 and 2015 *(19)*.

2.4 Hyperthermal anomalies on the Florida Reef Tract in 2014 and 2015

One of the strongest El Niño Southern Oscillation (ENSO) events on record occurred from May 2014 to June 2016 *(20)*, causing staggering losses to coral reefs worldwide, as a consequence of consecutive bleaching events and subsequent disease outbreaks, including the FRT *(21, 22)*. During August-September 2014 and 2015, unprecedented hyperthermal events occurred along the FRT *(23)* (Figure 2.3), resulting in mass coral bleaching. These events offered a unique opportunity not only to document the cumulative effects of hyperthermal disturbance in the context of spatial and temporal incidence of bleaching, post-bleaching recovery, and disease in the *D. cylindrus* population, but also allowed the exploration of the influences of the coral holobiont partners on differential bleaching and disease susceptibility and subsequent recovery in the context of cumulative disturbances on resistance and resilience.

Florida's reefs exceeded 5°C-weeks (degree heating weeks) during the summers of 2014 and 7°C-weeks in 2015, using NOAA's Coral Reef Watch 50-km Satellite Monitoring *(24, 25)*. Data loggers at study sites recorded sea water temperatures which exceeded the FRT bleaching index of 30.5°C *(23)*, for 8 weeks in 2014 and 11 weeks in 2015. Mean daily sea temperatures at study sites exceeded 29.0°C in early June 2014 and 2015, considered the threshold temperature for many marine pathogens *(26-28)*. By mid-July 2014 and 2015, mean daily sea temperatures exceeded 30.5°C (Figure 2.3A). Mean

monthly temperatures during these two events exceeded the ten-year mean monthly temperatures (± 1 std dev) recorded at Molasses Reef (MLRF-1) 2003-2013 (Figure 2.3B).

The following data chapters will address the observations, patterns, and results of this natural experiment in the context of two consecutive hyperthermal events on the FRT.

2.5 Figures and Tables

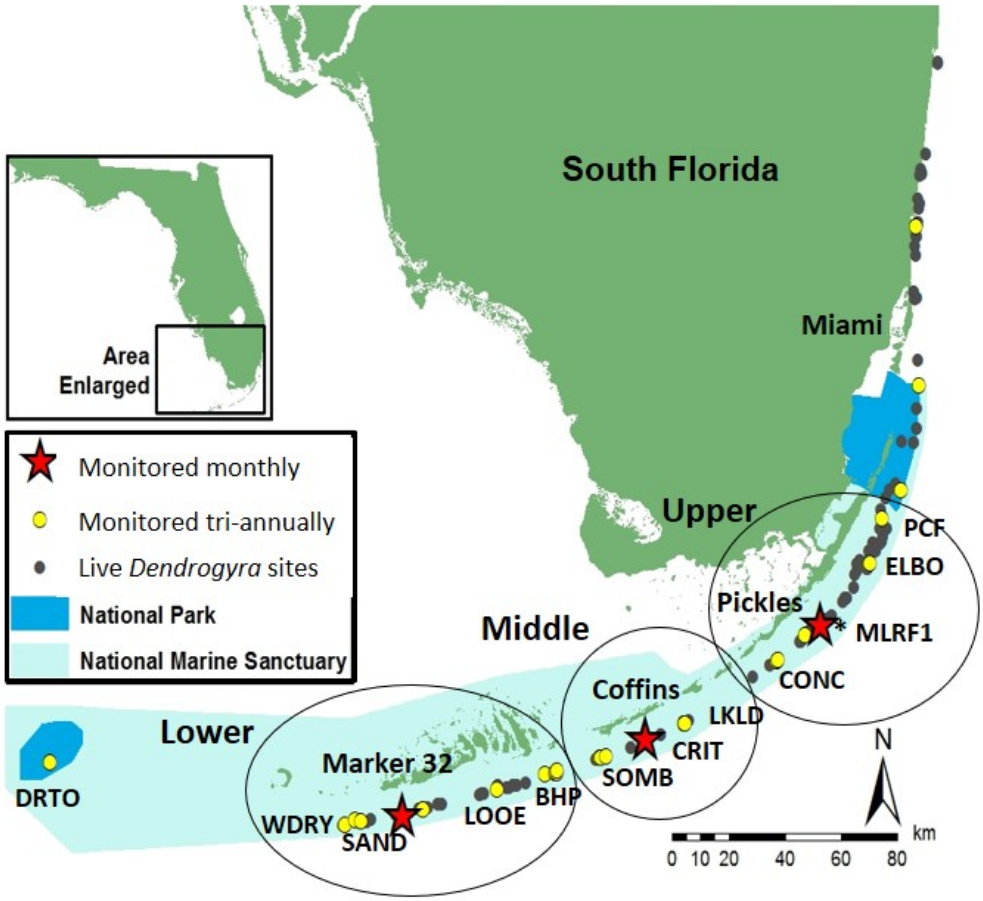

Figure 2.1. Study sites on the Florida Reef Tract where monitoring and sampling were conducted on the pillar coral *Dendrogyra cylindrus*.

In April 2014, the Florida population of *D. cylindrus* was comprised of approximately 745 live colonies at 155 known sites along the Florida Reef Tract (FRT; grey and yellow circles). Geographically stratified tri-annual monitoring and sampling at 28 of these sites began April 2014 through April 2016 (yellow circles). Some locations labeled here represent multiple sites: All colonies were sampled at **SAND** – Sand Key SPA & Sand Key north; **Marker 32** – MRK 32-1, MRK 32-2, MRK 32-3; **BHP** – Bahia Honda Pillars 1-5; **SOMB** – Sombrero SPA1 & SPA2, Sombrero Out; **CRIT**; **LKLD** – LKLD-1 & LKLD-2. Three high-density sites were partially sampled: **PCF** (18 of 174 colonies), **Pickles** (PICK3:14 of 107 colonies), **Coffins** (24 of 55 colonies). Pickles (Upper Keys), Coffins (Middle Keys), and Marker 32 (Lower Keys) sites (red stars) were sampled and assessed monthly during bleaching recovery in 2014 and 2015. *MLRF1 – location of Molasses Reef C-man buoy used to calculate 10-year mean monthly temperatures (N25.012 W80.376).

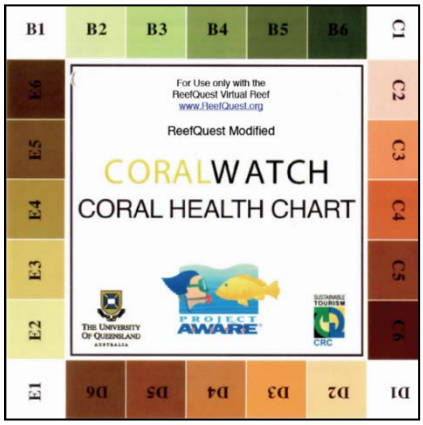

Figure 2.2 Coral Health Chart used to calculate the level of bleaching of *Dendrogyra cylindrus* colonies monitored between 2014 and 2016.

Coloration of live tissue was determined by comparing with the gradient of color on the Coral Health Chart (https://www.projectaware.org) *(14)*. Color scores were further broken down (0.5) if color appeared between two values. Percent of each color value on an individual colony was estimated visually. Chart score (column A) was multiplied by estimated proportion of colony live tissue (column B) to calculate colony score for each chart score value (column C). All colony scores for each coloration value (column C) were added to determine Total Colony Coloration Score.

Calculation example:

Bleach Status	**(A) Coral Health Chart Score**	**(B) Estimated proportion of total live tissue on colony**	**(C) Colony Score** (col A x col B)
Bleached	1.0	.20	1.0 x 0.20 = 0.20
	1.5	0	0
Pale	2.0	.30	2.0 x 0.30 = 0.60
	2.5	0	0
	3.0	0	0
Healthy	3.5	.50	3.5 x 0.50 = 1.75
	4.0	0	0
Total Colony Coloration Score		**1.00**	**2.55**

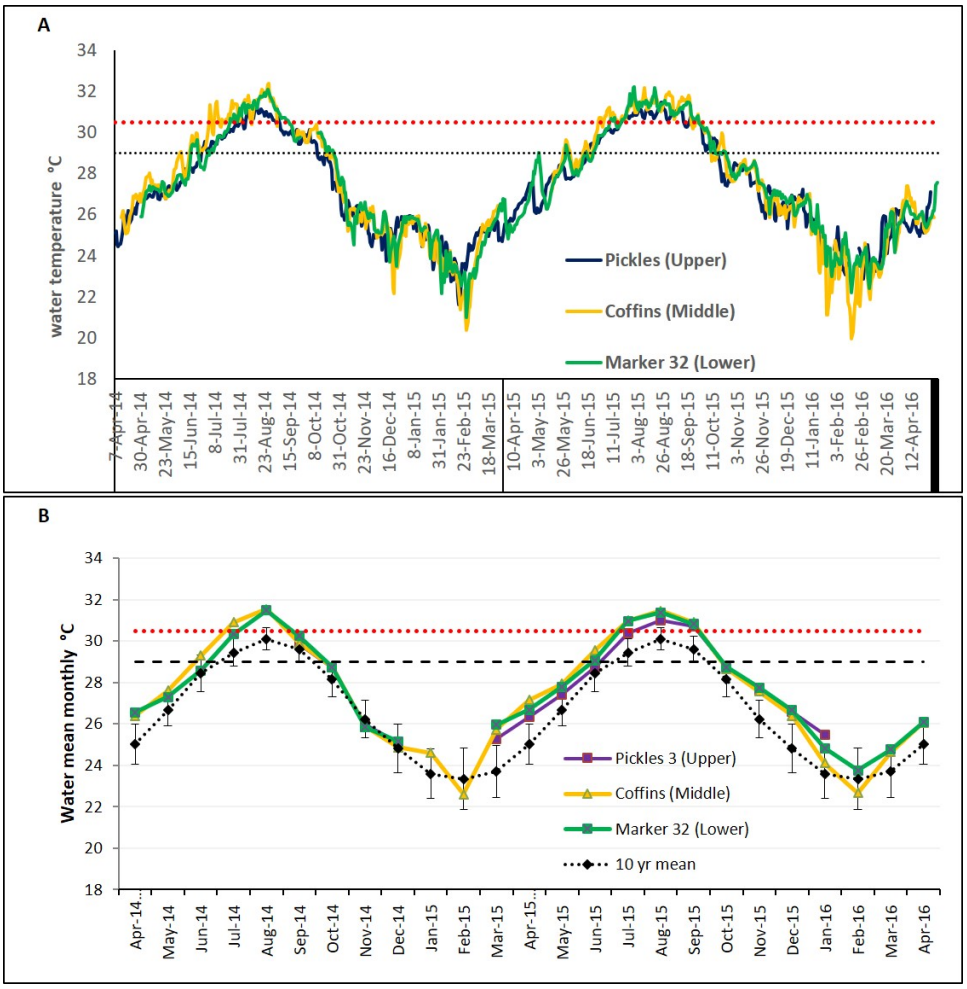

Figure 2.3. Water temperatures at three sites in the Florida Keys recorded from April 2014 to April 2016**.**

(A) Mean daily temperatures from *in situ* HOBO data loggers at three sites **(B)** Mean monthly water temperatures at three sites. Red dotted line (30.5°C) indicates the bleaching threshold for the FRT. Black dashed line (29°C) indicates optimal temperature threshold for many marine pathogens. Black dotted line is calculated 10-year mean monthly water temperatures (± 1 std dev) for Molasses Reef 2004-2013 (MLRF1, National Data Buoy Center). Gaps in data are due to lost or corrupted data loggers.

Table 2.1. Timeline of sampling and assessments conducted from 2014 to 2016.

Tri-annual sampling and assessments (green) of all 96 colonies at 18 sites, plus 10 assessment only sites, across the FRT. Hyperthermal bleaching events (yellow) in August/September 2014 and 2015. Post-bleaching recovery sampling and assessments (orange) at three targeted sites (Marker 32: Lower Keys, Coffins: Middle Keys, Pickles: Upper Keys). A final assessment of the three targeted recovery sites in April 2016.

2014											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
							bleaching				
2015											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
							bleaching				
2016											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec

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CHAPTER 3: The Role of the Coral Animal Genotype in Bleaching and Disease Resistance in Florida's *Dendrogyra cylindrus.*

3.1 Abstract

Population genetic diversity on reef ecosystems may ultimately be the key to survival for many organisms faced with biotic and abiotic stressors in the Anthropocene. Differential physiological performance is often associated with genotypic diversity of the organism. Here we explored the genetic diversity within Florida's *Dendrogyra cylindrus* population and potential associations with resistance to bleaching and disease and potential resilience during recovery, through two consecutive hyperthermal events on the Florida Reef Tract in 2014 and 2015. Florida's *D. cylindrus* population largely consisted of single unique genets not shared between sites, with most sites consisting of fewer than five colonies *(1)*. Few sites contained colonies belonging to more than one unique genet however, three high-density sites (>20 colonies) appeared to be clonal, each comprised of single unique genets, likely a result of asexual fragmentation. A sub-set of 28 *D. cylindrus* sites was assessed every four months for bleaching and disease status. Colonies associated with four genets showed full or partial resistance to both bleaching and disease. Colonies associated with six genets were resistant to disease following both hyperthermal events in 2014 and 2015 but showed variable bleaching resistance in both years. Colonies belonging to two disease-susceptible genets were bleaching-resistant during the second hyperthermal event. It is hoped that identifying genets associated with bleaching and/or disease resistance within the *D. cylindrus* population will help to inform

management strategies for conservation and restoration of this critically threatened species.

3.2 Introduction

Genetic diversity is the cornerstone to ecosystem health and resilience, especially when faced with biotic and abiotic stressors associated with a changing climate *(2-4)*. As coral reefs worldwide are pushed to the limits of their tolerances, it is hoped that pockets of genetic diversity will survive to provide hope for the future of these critical ecosystems *(5)*. As with many marine organisms, genetic diversity in the coral animal is often essential for successful sexual reproduction and recruitment locally, and dependent on connectivity and larval transport for reseeding neighboring reefs after disturbances *(6-10)*. While live coral cover and genetic diversity on many reefs is declining as a result of environmental disturbances, at least some surviving corals may produce more thermotolerant larvae, perhaps better adapted to the changing climate *(11-14)*. Coral animal genotypes have been linked to thermotolerance and bleaching resistance despite having the same dominant endosymbiont type or without changing their photosynthetic algae *(15-17)*. Thermotolerance has also been associated with thermal priming affecting gene expression *(18)*. Additionally, host genotype has played a role in differential disease resistance *(19, 20)*. Thus, it is imperative to maintain or enhance genetic diversity in coral reef ecosystems which may provide a source for yet-unknown resistance and resilience in a changing climate.

The aim of chapter three, building on the work of Chan et. al. which defined the genetic identities and population structure of Florida's *D. cylindrus (1)*, was to determine if some genotypes were associated with bleaching and/or disease resistance. Such

information will be invaluable in future restoration efforts for this species, such as guiding propagation of resistant genets, understanding differential growth rates between genets, and determining genet compatibility for successful sexual reproduction.

3.3 Methods

Experimental design and sampling strategy to achieve the aims of this chapter were described at length in Chapter 2. Specifically, a sub-set of Florida's *D. cylindrus* population (143 colonies at 28 sites distributed along the FRT) were selected for tri-annual assessments of bleaching and disease status beginning in April 2014 (Chapter 2: Figure 2.1). Following hyperthermal bleaching events in August-September 2014 and 2015, three of these sites were selected as representative of the three regions (Upper, Middle, and Lower Keys) where 61 colonies were assessed monthly, in between triannual assessments, to capture bleaching recovery processes at a finer scale (Pickles n=24, Coffins n=24, and Marker 32 n=13; indicated by red stars Figure 2.1). All *D. cylindrus* colonies at selected sites were assessed for bleaching status (normal, pale, bleached) and disease status (healthy, diseased, recovered) as described in chapter 2. Colonies were considered 'recovered' if no signs of active disease were present, but disease had been observed in the previous assessment. Colonies were removed from analysis if more than three assessments were missing from the data set, leaving 131 colonies at 27 sites for analysis (Table 3.1).

3.3.1 Determining genotypic identity and genetic diversity in Florida's Dendrogyra cylindrus *population.*

In conjunction with assessments and monitoring field work, coral tissue was also collected and preserved from 51 sites (n=217 colonies) in a collaborative effort to

determine genetic diversity of Florida's *D. cylindrus* population (Florida Fish & Wildlife Research Institute; Dr. Iliana Baums, Pennsylvania State University) *(1)*, including the 28 tri-annual assessment sites. Coral tissue samples were later collected from the remaining 100 sites to complete the population genetics analysis for Florida's *D. cylindrus*. As described in Chan et al., 11 microsatellite loci were developed using next-generation 454 sequencing of genomic DNA from *D. cylindrus (1)*. Microsatellites and their flanking regions were identified using SciRoKo perl script and primers designed in Primer 3 *(21)*. Samples were screened using multiplex PCR reactions, visualized on the ABI3730 (Applied Biosystems) automated DNA sequencer, and then analyzed using GeneMapper Software 5.0 (Applied Biosystems).

3.3.2 Statistical analysis

Little was known about the genetic diversity or structure of *D. cylindrus* prior to planning experimental design and implementing assessments and sampling for this study. Because of the unexpected nature of the genetic structure of Florida's *D. cylindrus* population *(1)*, analysis of data associated with genotypes often lacked statistical power because of the lack of replication (typically one colony per genet and one genet per site). Thus, results for bleaching and disease resistance or susceptibility for each genet were primarily descriptively interpreted on the basis of repeated monitoring and assessments of individually identified *D. cylindrus* colonies.

3.4 Results

3.4.1 Demographics of Dendrogyra cylindrus *population and genetic diversity along the Florida Reef Tract*

In 2014, Florida's *D. cylindrus* population was comprised of 745 colonies at 155 known sites (Chapter 2: Figure 2.1; and *Neely & Lewis, in prep*). Chan et al. identified 162 unique genets within the population. Most sites consisted of one to fewer than ten colonies and usually represented a single unique genet. The few high-density sites (>20 colonies), located in the Upper and Middle Keys, while not sampled exhaustively to comply with permits, were assumed to be clonal or consisting of single unique genets, likely due to centuries of asexual fragmentation and scattering of ramets from a single parent colony. As described in Chapter 2 Experimental Design, a sub-set of the total Florida *D. cylindrus* population was selected for tri-annual bleaching and disease status assessments from April 2014 through April 2016 (Table 3.1 and Table 3.2; 131 colonies at 27 sites, representing 32 genets). The Lower Keys region consisted of more known *D. cylindrus* sites and therefore more genets (typically one unique genet per site and usually a single colony per site), but all sites had fewer than 12 colonies i.e., no large high-density clonal sites.

3.4.2 Bleaching resistant genets associated with Dendrogyra cylindrus *during two hyperthermal bleaching and recovery events 2014-2016.*

Colonies associated with thirty-two unique *D. cylindrus* genets were observed for differential bleaching responses to hyperthermal events in 2014 and 2015. In September 2014, colonies belonging to 24 genets bleached, however three genets were scored as pale, perhaps indicating partial resistance to thermal bleaching (Figure 3.1A indicated by

[*], Table 3.3). These partially bleaching-resistant genets were each represented by a single *D. cylindrus* colony; two located in the Middle Keys and one in the Lower Keys. These colonies returned to normal coloration by January (Figure 3.1B, Figure 3.3). The same three genets showed differential bleaching responses during the second hyperthermal event in 2015. The colony belonging to genet D1066 bleached while singleton colony D1079 (Looe Key West) was again only pale (Figure 3.1D) and all had once again recovered normal coloration by January and April 2016 (Figure 3.1E & F). Additionally, singleton colonies associated with five genets bleached during the first hyperthermal event in September 2014 (Figure 3.1A, indicated by [^]) and only paled during the second event in 2015 (Figure 3.1D; Table 3.3), returning to normal coloration in January 2016 (Figure 3.1E).

Three high-density sites, each represented by unique genets, also bleached in September 2014 (Figure 3.1A; Coffins, PCF, Pickles). However, colonies belonging to two genets (D1131 Coffins [Middle Keys], D1259 Pickles 3 [Upper Keys]) returned to normal coloration by January 2015 while most colonies belonging to genet D1198 (PCF-Pillar Coral Forrest, Upper Keys) were still pale and one colony was dead (Figure 3.1B). At the April 2015 assessment, many colonies of these genets experienced spring paling or loss of coloration (Figure 3.1C). During the second hyperthermal event in September 2015, most colonies belonging to D1131 (Coffins) were scored as only pale (20 of 24) while 14 colonies associated with genet D1259 (Pickles 3) did not bleach and 10 colonies were scored as only pale (Figure 3.1D). Colonies of genet D1198 (PCF) once again bleached in September 2015 (one colony was pale). By January and April 2016, all genet

D1131 and D1259 had returned to normal coloration, as had most of the genet D1198 colonies (Figures 3.1E & F).

3.4.3 Disease resistant genets associated with Dendrogyra cylindrus *during two hyperthermal bleaching and recovery events 2014-2016.*

A baseline for disease in Florida's *D. cylindrus* population was established in April 2014. Four genets had colonies with active white plague (WP; Figure 3.2 marked with [v]) and occurred in all three regions (Table 3.2; Upper: D1259, D1198; Middle: D1105; Lower: D1170). During the 2014 hyperthermal event on the FRT, ten additional genets had colonies with disease in September 2014 (Table 3.3A), while colonies associated with ten genets remained disease-free through January 2015 (Figure 3.3B, Table 3.3). Colonies associated with six of these genets remained healthy i.e., disease-resistant, through April 2016 (Figure 3.3A-F, marked with [*]). Colonies associated with these disease-resistant genets were located within all regions of the FRT (Upper: D1376; Middle: D1055, D1172; Lower: D1079, D1120, D1194; Table 3.2 and Table 3.3). Some diseased colonies recovered, showing no signs of disease, but had active disease at subsequent assessments, indicating no acquired immunity to WP (Figure 3.3A-F).

Three high-density sites, composed of three unique genets, had some colonies with active disease in September 2014, while some colonies had recovered from disease since April 2014 (Figure 3.3A; D1131: Coffins, D1198: PCF, D1259: Pickles). Disease persisted in colonies at PCF and Pickles (Upper Keys) through April 2016, however all colonies at Coffins were disease free in April 2015 (Figure 3.3C). During the 2015 hyperthermal bleaching and recovery, colonies associate with all three genets at the high-

density sites had active disease in September 2015 (Figure 3.3D) which persisted until April 2016 (Figure 3.3F).

3.4.4 Dendrogyra cylindrus *sites with more than one unique genet showed highly variable bleaching and disease resistance.*

Five sites, located in all three regions of the FRT, contained colonies associated with eleven different genets (Table 3.4). No genets were shared between sites and often only represented by one or two colonies within that site. Evaluating bleaching and disease resistance between genets within a single site allowed some limited comparisons, eliminating site-specific environmental factors. The Crocker Reef 4 site in the Upper Keys had one colony identified as genet D1376 and two colonies identified as genet D1377. Genet D1376 was disease-resistant both in Year 1 and Year 2 however bleaching status was not assessed in September 2014 or 2015 (Figure 3.3A-F, Table 3.4). Genet D1377 was partially disease-resistant, with no disease evident in this colony in Year 1 but active disease in Year 2 after the second hyperthermal event. Sombrero SPA2 site in the Middle Keys had two colonies identified as genet D1172 and one colony identified as D1055 (Table 3.2 and Table 3.4). Genets D1172 and D1055 were disease resistant throughout both hyperthermal events, however, colonies were not assessed in September 2014 (Figure 3.3A) and both colonies bleached in September 2015 (Figure 3.3D, Table 3.4).

Three Lower Keys sites were found to have more than one unique genet within each site. At the Bahia Honda Pillar site, seven colonies were identified as D1028 and a single colony was identified as D1248 (Table 3.2). Genet D1028 was not resistant to bleaching or disease through either hyperthermal event (Figure 3.3A-F, Table 3.4). The

colony identified as genet D1248 bleached both years (Figure 3.3A and 3.3D) and was disease resistant throughout the first hyperthermal recovery 2014 but had active disease during the second hyperthermal event in September 2015 (Figure 3.4A-F). The Sand Key SPA site had 10 colonies identified as genet D1229 and a single colony as genet D1187 (Table 3.2). Genet D1229 and D1187 colonies were not resistant to bleaching or disease in Year 1 or Year 2 (Figure 3.3A-F; Table 3.4). Three unique genets were identified at the Marker 32-3 site (Table 3.2). Two colonies were identified as genet D1170 and three colonies identified as genet D1109 were not resistant to bleaching or disease (Figure 3.3A-F, Table 3.4). Two colonies identified as genet D1120 within this site were not bleaching resistant in either September 2014 or 2015 (Figure 3.3A and 3.3D), however they appeared to be disease-resistant through April 2016 (Table 3.4). It was noted that one of the colonies identified as genet D1120 was dead by April 2015, apparently the result of bleaching, as no signs of disease were observed in this colony (Figure 3.3C).

3.4.5 Bleaching and disease resistance in Dendrogyra cylindrus *colonies associated with some genets on the FRT through two hyperthermal events in 2014 and 2015.*

Colonies associated with six different genets were resistant to disease (WP) through two hyperthermal bleaching and recovery events on the FRT in 2014 and 2015 (Figure 3.3A-F marked by [*]), Table 3.3; Upper: D1376; Middle: D1172, D1055; Lower: D1079, D1120, D1194). Three additional genets were resistant to disease in Year 1 but were not disease-resistant in Year 2 (Upper: 1377; Lower: D1248, D1246). Colonies associated with six genets bleached in September 2014 but were pale in September 2015 (Figure 3.1A and 3.3D marked by [^]; Table 3.3). Four of these partially bleaching-resistant genets were not disease-resistant and occurred across all regions of

the FRT (Upper: D1188, Middle: D1131, Lower: D1109, D1170). One of the six partially bleaching-resistant genets was not disease-resistant in Year 1 but resistant in Year 2 (Lower: D1102) and the sixth partially bleaching-resistant genet was disease-resistant in both Year 1 and Year 2 (Lower: D1194; Table 3.3). And finally, one Lower Keys colony associated with genet D1079 was partially resistant to bleaching and only paled in both Year 1 and Year 2 and was also disease-resistant both years.

Two genets at two high-density sites (Coffins and Pickles) did not show indications of disease resistance in either year yet had very different bleaching status between two consecutive hyperthermal events (Figure 3.5 and Figure 3.3A and 3.3D). Genet D1259 was identified in 24 of the 107 colonies at the Pickles-3 site (Upper Keys). It is likely that all other colonies at this site also belonged to the same genet as a result of asexual fragmentation. While all colonies bleached in September 2014 (Figure 3.1A), half of the assessed colonies were scored as pale (partially bleaching-resistant) and the other half were normal coloration (bleaching-resistant) in September 2015 and two colonies were dead (Figure 3.1D). Genet D1131 was identified in 24 colonies at the Coffins site (Middle Keys). Another clonal site of 55 colonies, all colonies bleached in September 2014 (Figure 3.1A), but most colonies only paled in September 2015 (partially bleaching-resistant) and were more susceptible to disease in Year 2 (Figure 3.1D).

3.5 Discussion

Population demographics and genetic diversity have never before been investigated in *D. cylindrus* because of its historically low abundance throughout its Caribbean range. Chan et al (2018) developed microsatellite markers to determine the genetic structure of Florida's *D. cylindrus* population concurrent with the monitoring and

assessments in the present study. The findings from Chan and collaborators demonstrated a unique single-genet-per-site structure of this population as well as the unique single-genet clonal identities of those few existing high-density sites *(1).* A clearer understanding of how these sites may have been established over centuries of asexual fragmentation and dispersal from the founding parent colony was supported by observations of pillars toppled and displaced up to 50m during a single hurricane (Hurricane Irma - September 2017; pers. obs.).

3.5.1 Evidence of partial bleaching resistance in Dendrogyra cylindrus *colonies associated with some genets during two consecutive hyperthermal events in 2014 and 2015*

Genotypic differences of the host have been associated with thermal tolerance and bleaching resistance in other coral species but not always associated with shifts in their algal symbiont community *(16, 17)*. *Dendrogyra cylindrus* colonies associated with these 32 genets showed highly variable responses to one or both hyperthermal events in 2014 and 2015 and may provide insights into different mechanisms for resistance. Transcriptomic studies have shown evidence of genomic stress response resulting in physiological resilience to thermal stress *(18, 22)*. Other studies have shown that corals exposed to more variable thermal histories may be more resistant to future thermal stress events, in the context of intervening normal years allowing for full recovery *(22-24)*. In scenarios when thermal stress is followed by years of normal temperatures, extended periods of non-stressful conditions may allow the coral animal to respond and recover important biological functions more fully. Annual hyperthermal stress events, as occurred on the FRT in 2014 and 2015, may have exceeded the capacity of the coral to fully

recover, or in fact caused irreparable thermal damage to physiological processes. Here we show that some genets were associated with complete bleaching one or both years, while other genets only paled (partial bleaching-resistance) in Year 1 but bleached in Year 2, supporting the hypothesis that the ability of the coral animal to adapt and recover may have been exceeded with annual hyperthermal stress. In other genets, the bleaching response was reversed, and genets associated with bleaching in Year 1 were partially (i.e., pale) or fully resistant to bleaching (i.e., normal) in Year 2. And finally, one genet was associated with partial bleaching resistance in both years. These observations may indicate acclimatization or resistance to recurrent hyperthermal events.

As none of these bleaching-resistant genets were shared between sites, variable bleaching resistance between years may also have been a result of variable thermal stresses at each site, either intensity or duration i.e., number of days water temperatures exceed the bleaching threshold, or maximum daily temperatures. Temperature loggers were not deployed at many of these single colony sites so direct comparisons between sites could not be made. Additionally, bleaching resistant genets were associated with colonies at sites within all regions of the FRT, although six of the ten genets were located at sites in the Lower Keys region. Other environmental stressors, such as fluctuations in turbidity, water clarity, salinity, and pH were not measured in the present study, but may also have been factors contributing to perceived resistance or susceptibility. Another explanation of variable bleaching resistance may also lie within another member of the holobiont, the algal endosymbiont community, which will be explored further in Chapter four.

3.5.2 Evidence of disease resistance in Dendrogyra cylindrus *colonies associated with some genets during two consecutive hyperthermal events in 2014 and 2015.*

Host genotypic differences have been associated with disease resistance in some coral species *(19, 20).* Muller et al. (2018) demonstrated in a laboratory setting that some *Acropora cervicornis* (staghorn coral) genets may lose their disease resistance with hyperthermal stress and bleaching *(25).* Additionally, it has been reported that *A. palmata* (elkhorn coral) may be more susceptible to disease after a bleaching event *(26).* Conversely, Merselis et al. (2018) have shown that *A. cervicornis* genets that bleached because of thermal stress may actually be more resistant to disease, hypothesizing that immune suppression by the host to maintain symbiosis may be temporarily diverted in bleached corals to enhance immune response and therefore disease resistance *(27).* The innate immune response may also play a role by allowing the coral to adapt to thermal stress to become more disease resistant *(28-30). Dendrogyra cylindrus* colonies associated with these genets again showed highly variable susceptibility to disease following one or both hyperthermal events in 2014 and 2015. Six genets showed no disease-resistance either year. Three genets were initially resistant to disease in Year 1 but became susceptible following the second hyperthermal event in 2015, while six other genets were disease-resistant both years (Table 3.3). Three *D. cylindrus* genets that bleached one or both years were disease-resistant, further supporting the work of Merselis et al. (2018). However, one genet associated with a single Lower Keys colony was both partially bleaching-resistant (i.e., pale) and disease-resistant in both Year 1 and Year 2. As noted above, other environmental parameters may have contributed to these observations. The Lower Keys site with the single bleaching and disease resistant colony

did not have a separate temperature logger to compare water temperatures to other sites. Of note, this single resistant Lower Keys colony was not near other conspecifics to exchange pathogens, either water-borne or by vector transport.

Other factors may contribute synergistically to disease susceptibility. Thermally stressed corals that paled or were partially bleached might not have been as nutritionally compromised as fully bleached coral, which also may contribute to disease-resistance on the part of the coral animal. Consecutive thermal stress events, as experienced on the FRT in 2014 and 2015, may not have allowed sufficient recovery time for the biological functions of the coral animal and its associated protective microbiome to return to pre-bleaching status. Elevated water temperature has been shown to impact pathogenicity of disease-causing organisms, further contributing to disease susceptibility *(31-36)*. Thus, we should expect increased pathogenicity with more frequent scenarios of recurrent or annual hyperthermal events.

3.5.3 Comparisons of differential bleaching and disease resistance within a single site containing multiple unique Dendrogyra cylindrus *genets.*

In a living reef environment, it is often difficult to control for all variables to determine direct cause and effect. As a consequence of the single-genet-per-site structure of the Florida *D. cylindrus* population, confounded by typically single-colony-per-genet, elimination of between site variables was often difficult. A lower Keys site (Marker 32-3) contained corals with three different unique genets (Table 3.4). Two genets were associated with corals that were not bleaching or disease resistant in either 2014 or 2015. However, the third genet was associated with two colonies that were disease resistant in both years despite sensitivity to consecutive bleaching. Disease resistance in these two

colonies at Marker 32-3, in spite of their lack of thermal tolerance, may truly indicate a disease resistant genet, when compared to the other two genets at this site, while all experiencing similar environmental conditions. While the response of the single genet to hyperthermal stress at the Marker 32-3 site may support the findings of Merselis et al. (2018), colonies of the other two genets at this site, sharing the same environmental conditions, did not.

Other factors to consider in disease resistance among these and other genets may also include the age and/or size of the colony. Large and presumably older colonies, may experience senescence and lowered immunity with age, decreasing their ability to resist disease. Younger colonies, if not ramets of an existing genet, may be a result of successful sexual recombination and recruitment, resulting in a genet better adapted to more recent environmental conditions. Baums et al. (2012) suggests that more thermally-tolerant larvae can be the product of surviving parent colonies adapted to more recent conditions *(14).* Younger, smaller colonies may also expose less surface area to pathogens in the water column, providing refuge, and perhaps disease resistance, by size class.

3.5.4. Genets resistant to bleaching and/or disease and the management implications for conservation and restoration of Florida's Dendrogyra cylindrus

Identifying unique genets that show indications of resistance to environmental stressors is of critical importance for future conservation and restoration efforts. The resilience of the colonies associated with such genets is often the cornerstone for establishing a restoration program adapted to a changing reef ecosystem. However, even less-resistant genets are important to maintain and propagate, and thus preserve, genetic

diversity in a population. As there appears to be no clear-cut genotypic winners and losers, at least in the context of recurrent hyperthermal events, each genet may provide some unique and as yet unexplored adaptive advantage to future generations of *D. cylindrus*. Through successful propagation and reintroduction efforts of multiple genets, sexual reproduction can provide a more well-rounded self-sustaining ecosystem. Through genetic diversity, sexual recombination may result in even more tolerant genets better adapted to a changing climate.

3.6 Figures & Tables

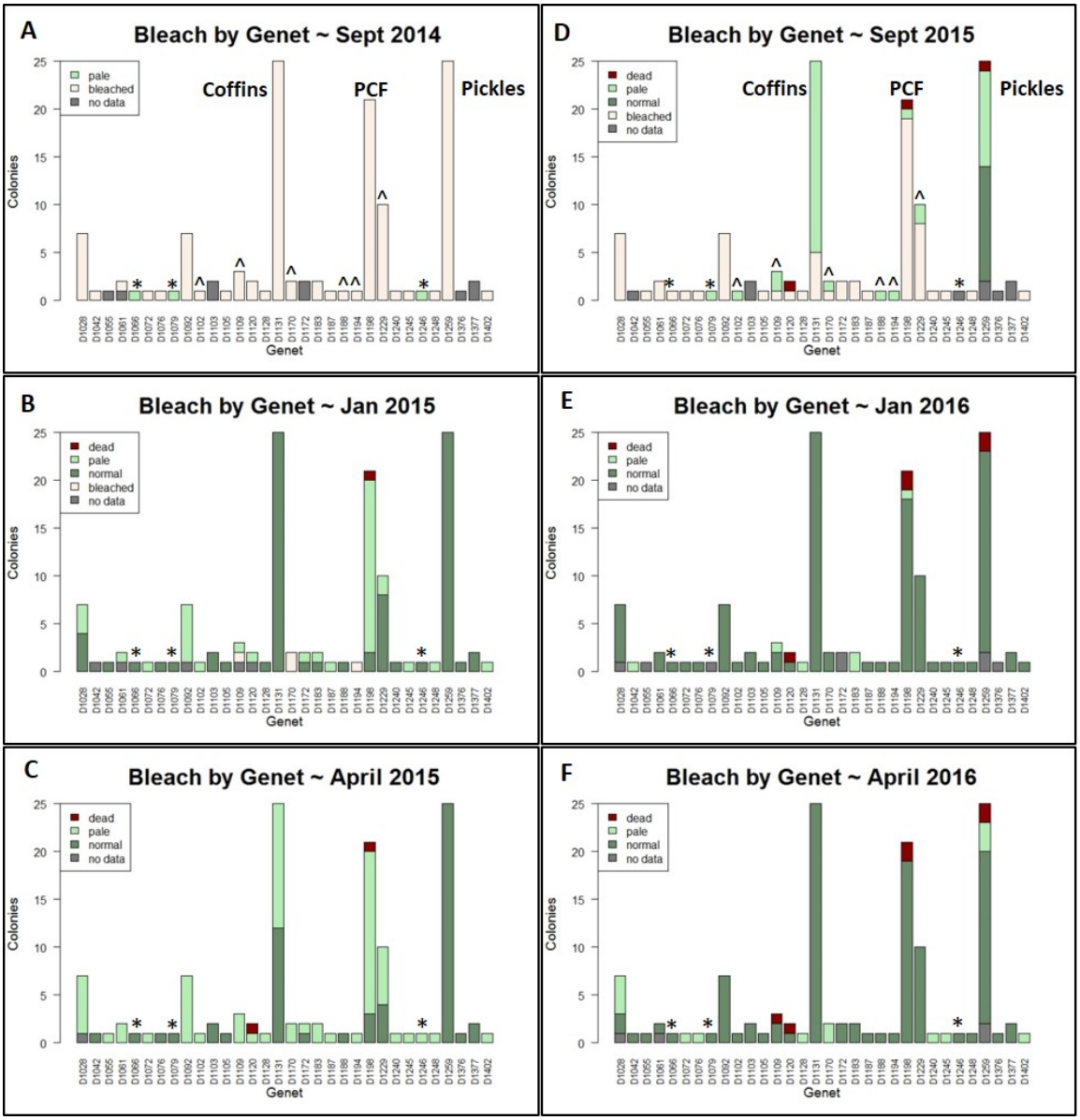

Figure 3.1. Bleaching status of genets associated with *D. cylindrus* colonies through two consecutive hyperthermal events on the FRT 2014-2016.

All colonies associated with these genets had normal coloration at the time of initial baseline assessments in April 2014 **(A)** The first hyperthermal event occurred in August-September 2014. Colonies associated with 24 genets bleached (cream bars) while colonies associated with three genets were considered pale or partially bleaching resistant (light green bars and marked with [*]). Unique genets found in colonies at high-density sites (Coffins: Middle Keys; PCF and Pickles: Upper Keys) were all bleached. **(B)** In

January 2015, colonies associated with the three partially bleaching-resistant genets (*) had recovered normal coloration (dark green bars), however many colonies remained pale. Colonies associated with three other genets remained bleached. **(C)** In April 2015, several colonies, including one of the three partially bleaching-resistant colonies (*), was again pale. **(D)** The second hyperthermal event occurred in August-September 2015. Singleton colonies associated with six genets were pale or partially bleaching-resistant (light green bar marked with [^]) in September 2015 but bleached in September 2014. One of the partially bleaching-resistant genets in September 2014 (*) was again pale during the second hyperthermal event in 2015. Coffins and Pickles colonies associated with their site-specific genets were partially (light green) or fully bleaching-resistant (dark green) during the 2015 hyperthermal event. Colonies associated with most genets had recovered by **(E)** January 2016 and **(F)** April 2016. Four genets were associated with colony mortality.

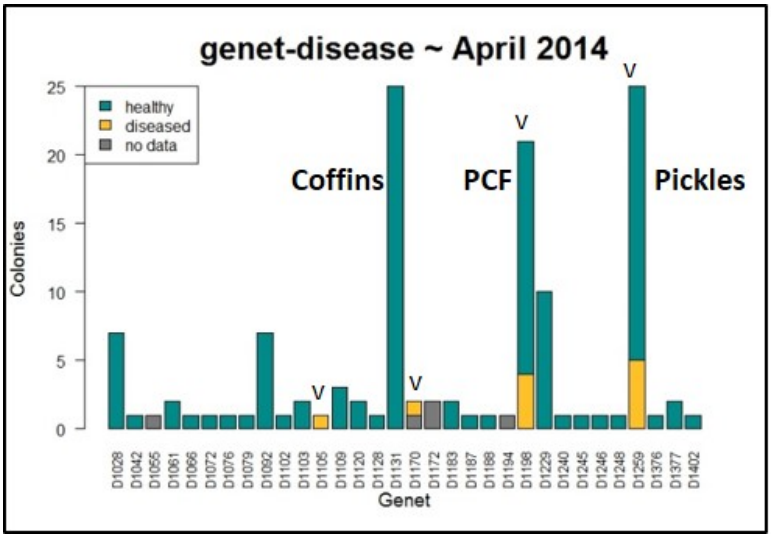

Figure 3.2. April 2014 baseline disease status of genets associated with *D. cylindrus* colonies on the Florida Reef Tract.

Colonies associated with four genets had signs of active white plague (WP) in April 2014 (golden bar marked with [v]). Some colonies sharing single unique genets at each site (PCF and Pickles) showed signs of active disease while other colonies were healthy (dark green bar).

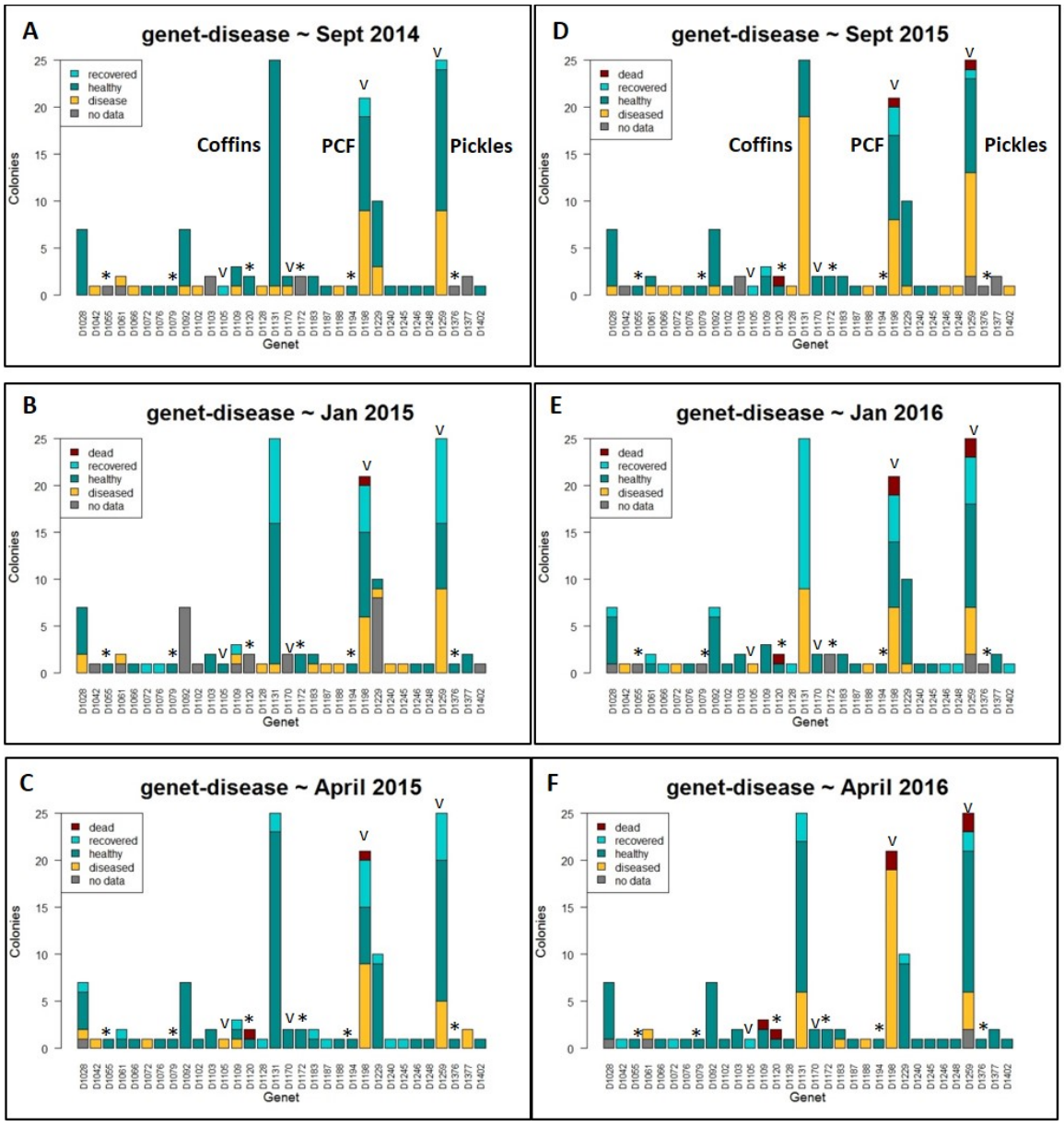

Figure 3.3. Disease status of genets associated with *D. cylindrus* colonies through two consecutive hyperthermal events on the FRT 2014-2016.

Asterisk (*) indicates disease -resistant genets. (V) indicates genets with active disease at the time of April 2014 baseline assessments. **(A)** During the 2014 hyperthermal event, colonies associated with 13 genets showed signs of active disease (golden bar). Some colonies previously diseased in April 2014 (marked with [v]) had recovered (no active disease, light blue bar). Colonies associated with six genets (marked with [*]) remained healthy through **(B)** January 2015 and **(C)** April 2016. **(D)** During the 2015 hyperthermal event, colonies associated with six genets showed signs of disease in September 2015 but were healthy in September 2014 (partial disease resistance). Colonies associated with the

six disease-resistant genets in 2014 remained disease free in **(E)** January 2016 and **(F)** April 2016.

Table 3.1. Summary of thirty-two *D. cylindrus* genets within three regions of the Florida Reef Tract assessed for bleaching and disease resistance.

Thirty-two genets, located at 27 sites across the three regions, were associated with 131 *D. cylindrus* colonies. While 154 colonies at 28 sites were initially selected for assessment, sites and/or colonies were eliminated from analysis if more than three assessment data points were missing.

FRT Region	# Sites	# genets	# colonies
Upper Keys	6	7	53
Middle Keys	7	8	33
Lower Keys	14	17	45
total	27	32	131

Table 3.2. Twenty-seven sites containing thirty-two unique genets identified in *D. cylindrus* colonies, within three regions of the Florida Reef Tract.

A sub-set of Florida's 155 *D. cylindrus* sites were selected based on experimental design. Twenty-seven sites distributed amongst three regions of the Florida Reef Tract (FRT) were sampled and assessed tri-annually. Eighteen of these sites, represented by only one or two colonies, were unique genets. Five sites with three to eleven colonies had multiple genets, unique to each site. Three high-density sites (>20 *D. cylindrus* colonies) were sub-sampled within each site and identified as single genets unique to each site. ([a]) High-density sites that were sub-sampled. ([b]) Sites targeted for monthly assessments October, November, and December during bleaching recovery 2014 and 2015.

Florida Keys Regions	Sites	# colonies at site (# assessed)	Coral Animal Genets	
			# colonies	Genet ID*
Upper Keys	Conch Reef 4	1	1	D1042
	Crocker Reef 3	2	1	D1103
	Crocker Reef 4	3	2	D1377
			1	D1376
	Elbow Reef 3	1	1	D1188
	Pickles Reef 3[a, b]	107 (25)	25	D1259
	Pillar Coral Forrest [a]	174 (21)	21	D1198
Middle Keys	Coffins Patch Pillars [a, b]	55 (25)	25	D1131
	Critter Ridge	1	1	D1105
	Long Key Ledge 1	1	1	D1076
	Long Key Ledge 2	1	1	D1072
	Sombrero Out	1	1	D1240
	Sombrero SPA	1	1	D1066
	Sombrero SPA 2	3	2	D1172
			1	D1055
Lower Keys	Bahia Honda Pillars	8	7	D1028
			1	D1248
	Bahia Honda Pillars 2	2	2	D1061
	Bahia Honda Pillars 3	2	2	D1183
	Bahia Honda Pillars 4	1	1	D1128
	Bahia Honda Pillars 5	1	1	D1245
	Looe Key West	1	1	D1079
	Marker 32-1 [b]	7	1	D1092
	Marker 32-2 [b]	1	1	D1402

	Marker 32-3[b]	7	2	D1170
			3	D1109
			2	D1120
	Middle Sambos	1	1	D1404
	Sand Key North	1	1	D1102
	Sand Key SPA	11	10	D1229
			1	D1187
	Western Dry Rocks	1	1	D1246
	Western Dry Rocks 2	1	1	D1194

*Chan et al. 2019

Table 3.3 Colonies associated with bleaching and disease-resistant genets through two consecutive hyperthermal events in 2014 and 2015 on the Florida Reef Tract.

Colonies associated with 16 genets varied in their response to hyperthermal stress during two consecutive hyperthermal events on the FRT. One genet showed bleaching resistance in both September 2014 and 2015 however one genet showed partial bleaching resistance as its colony paled in 2014 but bleached in 2015. Colonies associated with seven genets bleached in 2014 and only paled in 2015, indicating possible acquired bleaching-resistance. Colonies associated with ten genets were disease-resistant during the first year of recovery from the 2014 hyperthermal bleaching event. Colonies associated with three of the genets showed partial disease-resistance and were no longer disease-resistant following the second hyperthermal event in 2015. Unk - unknown bleach status; Asterisk (*) indicates genets with full or partial bleaching and disease resistance.

				Bleaching Resistant		Disease Resistant	
Genet	Region	Site	# colonies	Sep-14	Sep-15	Year 1	Year 2
D1188	Upper	ELBO3	1	no	partial	no	No
D1259	Upper	PICK3	24/107	no	yes	no	No
D1377	Upper	CROC4	2	unk	unk	yes	no
D1376	Upper	CROC4	1	unk	unk	yes	yes
D1131	Middle	Coffins	24/55	no	partial	no	no
D1066	Middle	SOMB SPA1	1	partial	no	no	no
D1172	Middle	LKLD2	2	unk	no	yes	Yes
D1055	Middle	SOMB SPA2	1	unk	no	yes	Yes
D1109	Lower	MRK 32-3	3	no	partial	no	No
D1170	Lower	MRK 32-3	2	no	partial	no	No
D1248	Lower	BHP	1	no	no	yes	No
D1079*	Lower	LOOE-W	1	partial	partial	yes	Yes
D1120	Lower	MRK 32-3	2	no	no	yes	Yes
D1102*	Lower	SAND-N	1	no	partial	no	Yes
D1194*	Lower	WDRY2	1	no	partial	yes	yes
D1246*	Lower	WDRY1	1	partial	unk	yes	no

Table 3.4 Five sites with multiple *D. cylindrus* genets showed variable bleaching and disease resistance between colonies.

Five sites on the FRT contained colonies associated with eleven unique genets. All genets at these sites showed no indication of bleaching resistance during hyperthermal events in both September 2014 and 2015. Colonies associated with four genets showed disease resistance in both Year 1 and Year 2 of hyperthermal recovery. Colonies associated with two genets were no longer disease resistant following the second hyperthermal event. unk - unknown bleach status.

	Multiple genets per site			Bleaching Resistant		Disease Resistant	
genet	region	Site	# colonies	Sep-14	Sep-15	Year 1	Year 2
D1376	Upper	CROC4	1	unk	unk	yes	yes
D1377			2	unk	unk	yes	no
D1172	Middle	SOM SPA2	2	unk	no	yes	yes
D1055			1	unk	no	yes	yes
D1028	Lower	BHP	7	no	no	no	no
D1248			1	no	no	yes	no
D1229	Lower	SAND SPA	10	no	no	no	no
D1187			1	no	no	no	no
D1170	Lower	MRK 32-3	2	No	no	no	no
D1109			3	No	no	no	no
D1120			2	No	no	yes	yes

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CHAPTER 4: Recurring Episodes of Thermal Stress Shift the Balance from a Dominant Host-Specialist to a Background Host-Generalist Zooxanthella in the Threatened Pillar Coral, *Dendrogyra cylindrus.*

Chapter four consists of the original manuscript submitted to Frontiers in Marine Science, 18 August 2018. This body of work was accepted 23 September 2018, revised 08 January 2019, and published 23 January 2019. The publication is included in the Appendix.

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4.1 Abstract

Most scleractinian corals form obligate symbioses with photosynthetic dinoflagellates (family Symbiodiniaceae), which provide differential tolerances to their host. Previously, research has focused on the influence of symbiont composition and the dynamic processes of symbiont repopulation during single episodes of hyperthermal events, followed by years of less-stressful conditions. In contrast, this study characterized for the first time, the role of Symbiodiniaceae species changes in response to annually recurring hyperthermal events, a scenario soon expected to become the norm. Consecutive hyperthermal events during summer 2014 and 2015 along the Florida Reef

Tract offered a unique opportunity to study bleaching susceptibility and recovery under recurrent annual hyperthermal scenarios (Figure 4.0 graphical abstract). We utilized Illumina amplicon sequencing of the chloroplast 23S DNA region to assess with fine resolution the Symbiodiniaceae diversity associated with pillar coral, *Dendrogyra cylindrus*. Our findings show diverse assemblages of Symbiodiniaceae species and that some cryptic members are not transient associates but persistent and ecologically relevant, especially during recurrent annual warming events. This was evidenced by changes in relative abundance from the typically dominant endosymbiont, *Breviolum dendrogyrum*, to *B. meandrinium* a species common to corals in the family Meandrinidae but occurs at background densities in most colonies of *D. cylindrus*. The rise in abundance of *B. meandrinium* associated strongly with bleaching resistance in the coral host during two consecutive hyperthermal events. In some cases, host-compatible background symbionts can rapidly increase in abundance during episodes of stress and may impart physiological resilience to rapid environmental change; and thus, represents a potentially important ecological process by which symbiotic corals acclimatize to changing ocean conditions.

4.2 Introduction

Coral reefs worldwide have experienced dramatic declines in recent decades due to natural and anthropogenic factors *(1-3)*. Global impacts of climate change, resulting in hyperthermal coral bleaching events (loss of symbiotic photosynthetic algae), have become more frequent in recent decades and are projected to be annual events by 2050, or sooner on some reefs *(2, 4, 5)*. One of the strongest El Niño Southern Oscillation (ENSO) events on record occurred from May 2014 to June 2016 *(6)*, causing staggering

losses to coral reefs worldwide, including the Florida Reef Tract, due to consecutive bleaching events and subsequent disease outbreaks *(3, 7)*.

Most scleractinian corals form obligate symbiotic relationships with photosynthetic dinoflagellates within the family Symbiodiniaceae. This partnership is critical for coral health and vital to enhanced calcification in reef-building corals *(8, 9)*. Understanding the role of Symbiodiniaceae diversity in bleaching susceptibility and recovery, and the physiological constraints and advantages they confer on their coral hosts has become of increasing importance with escalating climate change *(10-12)*. Currently there are seven described genera and a similar number of other divergent lineages requiring generic names *(13)*. Inter- and intra-generic diversity displays clear physiological differences *(14-19)* and provide differential environmental tolerances and sensitivities to the symbiotic partnership *(20-22)*. Seminal work by Berkelmans and van Oppen *(23)* demonstrated experimentally that corals can acquire increased thermal tolerance as a direct result of changes in the symbiont genus dominating their tissues by shuffling existing types already present within the coral host *(24)*.

Molecular approaches to Symbiodiniaceae diversity and community assemblages now allow us to further investigate the functional significance of genetic diversity within genera, thus prompting further questions into the roles of the symbionts during environmental stressors *(18, 25)*. Using higher resolution DNA markers, it has become apparent that there is a very large number of species in this family *(14, 26-31)*. Additionally, advances in high-throughput amplicon sequencing technology have allowed fine-scale exploration of the Symbiodiniaceae community composition by discovering more cryptic, previously undetected symbiont types occurring at abundances less than

0.01% *(32-37)*. While various kinds of Symbiodiniaceae can be detected in trace amounts from host tissues, interpreting the ecological and functional significance of these requires caution *(38)*. Still reef corals are often compatible with more than one symbiont species and differences in their physiological tolerances maybe shift population dynamics allowing for a symbiont at low abundances to proliferate within the coral animal thus changing the composition of the symbiont population to one that is better adapted to prevailing environmental conditions *(23, 24, 39-41)*.

Studies conducted over the last two decades have been fundamental in gauging the response and the ability of corals to acclimatize to increased temperature extremes under scenarios in which isolated thermal anomalies have been followed by years of non-bleaching temperatures. However, the 2014-2016 ENSO phenomenon led to a recurrent thermal stress scenario, resulting in mass coral bleaching which detrimentally impacted coral reefs worldwide, including the Florida Reef Tract, during the summers of 2014 and 2015 *(42)*. These consecutive hyperthermal events, not experienced on Florida's reefs since the 1997-1998 ENSO *(43)*, offered a unique opportunity to document spatial and temporal bleaching and post-bleaching recovery under conditions not frequently seen, but expected to become the norm in the near future based on current predictions *(2, 4, 44)*.

This study targeted the rare and iconic pillar coral, *Dendrogyra cylindrus* (Ehrenburg, 1834), a slow-growing columnar species typically found in low abundance throughout its Caribbean range (Fig. 4.1). This species is currently categorized as 'vulnerable' under the International Union for Conservation of Nature (IUCN) Red List criteria *(45)* and listed as 'threatened' under the US Endangered Species Act *(46)* due to its susceptibility to bleaching, disease, and habitat degradation. We focused on three

representative sites distributed geographically along the Florida Reef Tract (Fig. 4.2). For this study we used Illumina amplicon sequencing of the chloroplast 23S hyper-variable region (cp23S-HV) to characterize for the first time with fine resolution, the Symbiodiniaceae community assemblage associated with *D. cylindrus* colonies at three sites and their temporal dynamics through recurrent hyperthermal events over a two-year period. We detected the presence of low abundance background Symbiodiniaceae genera; and, at one location, observed changes in the symbiont population from the normal host-specialist species, *Breviolum dendrogyrum*, to a sibling species common to corals across the family Meandrinidae. The dramatic increase in abundance of this species corresponded to enhanced colony recovery from bleaching and resistance to consecutive hyperthermal events.

4.3 Methods

4.3.1 Field assessments and sampling

Geographically stratified monitoring and field sampling of 163 *D. cylindrus* at 29 sites across the Florida Reef Tract (FRT), conducted every four months from April 2014 to April 2016, allowed for comparison before, during and after the occurrence of two consecutive hyperthermal bleaching events in 2014 and 2015. Three of these sites (Pickles: Upper Keys, N=107 colonies; Coffins: Middle Keys, N= 55 colonies; Marker 32: Lower Keys, N=16 colonies; represented by stars in Fig 4.2) were selected spatially to represent each region and logistically due to the greater number of *D. cylindrus* colonies present at each site for replication of observations. These sites were targeted for more frequent sampling to more closely observe changes during bleaching and recovery

(2014: September, October, November, and December; 2015: January, March, April, September, October, and November; and 2016: January and April). Sites ranged in depth from 4m to 8m. Colonies at all sites were mapped and photographed to create field identification sheets for each colony to facilitate accurate repeat assessment and sampling. Pendant data loggers (Onset HOBO Inc., Bourne, Massachusetts USA), secured to the base of colonies at Pickles, Coffins, and Marker 32 sites, recorded hourly temperatures between April 2014 and April 2016 (Figure 4.3). Temperature data was used to calculate mean daily, mean monthly, and maximum weekly sea temperatures at each site. Archived temperature data for 2004-2013 at Molasses Reef C-MAN station MLRF1 (Figure 4.2), located in the Upper Keys 4.2 kilometers from Pickles site *(47)*, was used as a proxy to calculate ten-year mean monthly and mean monthly maximum sea temperatures on the FRT (Table 4.1). Using the calculated mean monthly maximum temperatures at MRLF1, degree heating weeks (°C-weeks) were calculated for each site for July, August, and September 2014 and 2015 *(48)*.

At each sampling time point, *D. cylindrus* colonies were assessed for live coral tissue (visual estimate of percent live, percent old mortality, percent recent mortality i.e., bright white, recently-exposed skeleton), and coral bleaching status. Colony color scores were assigned using the CoralWatch Coral Health colorimetric chart developed by Siebeck et al *(49)* and scaled from 1 (white) to 6 (heavily pigmented; Fig. 4.4), served as proxy for symbiont density and chlorophyll *a* content. The CoralWatch Health Chart was not utilized as a color reference until September 2014, therefore, colony coloration scores for April 2014 were estimated after reviewing colony photos. Colonies were sampled at

each time point using a low-impact syringe micro-sampling technique *(50)* to minimize damage to the colonies from repeated sampling (sampling: Pickles n=10 colonies, Coffins n=10 colonies, Marker 32 n=12 colonies). Briefly, tissue from three to five polyps per colony was aspirated using a 30cc syringe, transported back to shore on ice, then filtered through a 13mm Swinnex filter system (Millepore Corporation, Billerica, Massachusetts, USA), using a 3.0 µm filter disk (A/D glass fiber filter, Pall Corporation, Port Washington, New York USA). Each filter disk was preserved in 95% molecular grade ethanol.

4.3.2 Total genomic DNA extraction

DNA was extracted using modified DNeasy Plant Mini kit protocols (Quiagen Corporation, Valencia, California USA) *(26)*. Briefly, half of each sample filter was placed in 400µl supplied lysing buffer and ground with a sterile pestle. Sterile acid-washed glass beads (425-600µm; Sigma-Aldrich, Saint Louis, Missouri, USA) were added and shaken for 3-5 minutes to disrupt symbiont cell walls, followed by the addition of 20µl proteinase K (Promega Corporation, Madison, Wisconsin USA) and incubated at 56°C for 1-2 hours. Standard kit protocols were then followed for the remainder of the extraction.

4.3.3 Cp-23S-HV parallel amplicon sequencing and Symbiodiniaceae community analysis

Amplicon sequencing diversity assays of the Symbiodiniaceae communities was performed on Illumina MiSeq platform with 2x300bp paired-end read capability, utilizing length variation in Domain V of large sub-unit rDNA chloroplast 23S hyper-variable

region (cp-23S-HV *(51)*; Table 4.2) at the Molecular Research LP sequencing facility (MR DNA; Shallowater, Texas UAS). Resulting raw sequence data (Read1 & Read2.fastq file format) were processed using MR DNA pipeline analysis. Briefly, paired-end sequence reads (r1 and r2) were joined after quality control (Q25) trimming of the ends, barcoding was removed, and sequences with <150 base pair overlap were discarded. Remaining sequences were de-noised, OTUs were generated, and chimera sequences were removed. Clustering of OTUs was determined at 97% similarity (3% divergence) across all samples. Resulting OTUs were taxonomically classified using BLASTn against a selected database created from cp23S Symbiodiniaceae sequence data from NCBI (www.ncbi.nlm.nih.gov) to determine relative abundance of Symbiodiniaceae types in *D. cylindrus*. To confirm identities of the most abundant OTUs, a BLAST-search of GenBank was performed (https://www.ncbi.nlm.nih.gov/genbank). Further identification of the two most abundant B1 symbiont type OTUs was independently verified using Symbiodiniaceae microsatellite analysis (B7*Sym*15 primers, Table 4.2) *(52)*. Briefly, selected samples (dominant OTU ≥70% relative abundance) were PCR-amplified using the B7*Sym*15 primers and visualized on ABI 3100 Genetic Analyzer. Peaks were identified in each sample and compared to known Symbiodiniaceae samples in the LaJeunesse Lab *(31)*.

4.3.4 Statistical analysis

Repeated measures analysis of variance (repeated measures two-way ANOVA, α=0.05) was used to compare water temperature profiles between the three sites (Pickles, Coffins, and Marker 32) through two consecutive bleaching and recovery periods (April

2014 through April 2016). CoralWatch bleaching scores for colonies at the three sites were also analyzed, using repeated measures ANOVA (α=0.05; Pickles n=10, Coffins n=10, Marker 32 n=12), to compare bleaching and recovery differences between sites and between years. Symbiodiniaceae community dynamics at the three sites, represented by the relative abundance of OTUs generated by amplicon sequencing of the cp23S-HV region, were also compared to water temperatures between sites and between bleaching events during this same period, using three-way ANOVA (α=0.05; Pickles n=6, Coffins n=6, Marker 32 n=6).

4.4 Results

4.4.1 Baseline Symbiodiniaceae community diversity in Dendrogyra cylindrus

Illumina amplicon sequencing of the cp23S-HV gene region showed a single OTU (or phylogenetic species) within the genus *Breviolum* (formerly clade B; *(13)* to be the dominant Symbiodiniaceae in *D. cylindrus* pre-bleaching (April 2014), ranging from 75% to 81% relative abundance across sites (Table 4.3). Members within *Symbiodinium*, *Cladocopium*, and *Durusdinium* (formerly clades A, C, and D respectively; *(13)* were also detected in cryptic low abundance (<0.1%). Amplicon diversity analysis yielded 266 OTUs (0.97% similarity cut-off). All but fourteen OTUs were taxonomically classified as genus *Breviolum* (formerly clade B *Symbiodinium).* Moreover, the combined relative abundance of all 252 *Breviolum* OTUs represented >99% of the Symbiodiniaceae community at all three representative sites along the Florida Reef Tract (Pickles, Coffins, and Marker 32). Two hundred thirty-two *Breviolum* OTUs were rare members of the Symbiodiniaceae community detected at <0.01% relative abundance. The two most abundant OTUs accounted for >80% of the symbiont community at all three sites. These

two sequences were both identified as sub-genera *Breviolum* type cp-23S B184 (or ITS2 type B1) phylotypes in GeneBank. Using Symbiodiniaceae microsatellite analysis (B7*Sym*15 primers, Table 4.2), the identity of these two sequences was verified as ITS2 types B1-4k and B1-3 (species novo: *Breviolum dendrogyrum* and *B. meandrinium*, respectively; *(13, 31)*. There was no difference in relative abundances of *B. dendrogyrum* or *B. meandrinium* compared between sites in April 2014 prior to bleaching (two-way ANOVA α=0.05 p>0.05).

Ninety-four OTUs belonging to the genus *Symbiodinium* (formerly clade A) were detected in cryptic low abundance at Pickles, Coffins, and Marker 32 in April 2014 (Table 4.3). These OTUs were confirmed by BLAST-search as belonging to *Symbiodinium* strains A2, A3, and A13 (strain A13, putatively *S. necroappetens*). Three cryptic *Cladocopium* OTUs (formerly clade C) were detected only at Pickles and Marker 32 sites. A single OTU, classified as *Durusdinium* (strain D1a, putatively *D. trenchii*), was only detected at Marker 32 in April 2014.

4.4.2 Consecutive hyperthermal bleaching on the Florida Reef Tract in 2014 and 2015

Florida's reefs exceeded 5°C-weeks (degree heating weeks) during the summers of 2014 and 7°C-weeks in 2015, based on NOAA's Coral Reef Watch 50-km Satellite Monitoring *(53, 54)* (Table 4.1B). Data loggers at the three study sites recorded sea water temperatures which exceeded the FRT bleaching index of 30.5°C *(42)* (Fig. 4.3), causing severe bleaching in most coral species across the FRT (pers. obs. and *(7)*. Maximum weekly temperatures in 2014 exceeded the FRT bleaching threshold (30.5°C) 10 weeks at Coffins and 8 weeks at Marker 32 (Figure 4.3, Table 4.1A). Data loggers were lost at the

Pickles site from April to September 2014 and thus meaningful temperature analysis could not be included. In 2015, maximum weekly temperatures exceeded the bleaching threshold 12 weeks at Pickles and 13 weeks at both Coffins and Marker 32. Using mean maximum monthly calculations from MRLF1, degree heating weeks at Coffins (Middle Keys) exceeded 7°C-weeks in 2014 and 5°C-weeks in 2015. Degree heating weeks at Marker 32 (Lower Keys) was nearly 3°C-weeks in 2014 and 4°C-weeks in 2015. Degree heating weeks at Pickles (Upper Keys) as 0°C-weeks in 2015 (no data from 2014). Utilizing the FRT bleaching threshold (30.5°C), calculated DHW was greater at the three sites (Table 4.1A). The number of weeks maximum water temperatures exceeded the bleaching threshold were greater in 2015. Summarizing water temperatures from May through October (i.e., warmest summer months) in 2014 and 2015 shows similar temperature characteristics between sites (Figure 4.4). Interquartile temperature ranges, medians, and maximums were not remarkably different.

4.4.3 Differential bleaching resistance and resilience

The CoralWatch Coral Health colorimetric chart developed by Siebeck et al (2006) was used to determine the bleaching status of colonies. Healthy, non-thermally stressed colonies ranged in color between 3.5 and 4.5 on this chart (Figure 4.5). Colonies with scores between 1.5 and 3.5 were considered pale, while colonies with scores <1.5 were considered bleached.

In response to hyperthermal events in 2014 and 2015, patterns of bleaching and recovery differed between sites as well as between years (Figure 4.6). During the first bleaching event on the FRT (August-September 2014), all *D. cylindrus* colonies,

including at Pickles, Coffins, and Marker 32 sites were severely bleached (Figure 4.6, Table 4.4). Colonies at Marker 32 were the most severely bleached, followed by Coffins colonies and finally Pickles (two-way ANOVA; $p<0.01$ $\alpha=0.05$). Pickles and Coffins colonies regained normal coloration by December 2014 and January 2015. From January to April 2015, colonies at these two sites paled (two-way ANOVA, $p<0.01$ $\alpha=0.05$). Marker 32 colonies recovered slowly, remaining pale through April 2015 (Figure 4.6, Table 4.4).

During the second hyperthermal event in August-September 2015, site-specific differences in bleaching and recovery were observed. Colonies at Coffins and Marker 32 again bleached, although Coffins colonies were less severely bleached than September 2014, indicated by higher coloration scores (two-way ANOVA, $p<0.01$ $\alpha=0.05$; Figure 4.6, Table 4.4). However, unlike these two sites, Pickles colonies were more resistant to bleaching in 2015, with no significant change in colony coloration from April to September 2015 (two-way ANOVA, $p=0.82$ $\alpha=0.05$). While there was not bleaching observed in these colonies during the hyperthermal event, there was an increase of colony coloration from September to October ($p=0.01$ $\alpha=0.05$) which remained constant through January 2016 (Figure 4.6, Table 4.4). Coffins colonies also recovered normal coloration by January 2016. Both Pickles and Coffins colonies again paled during the 2016 winter-spring transition ($p=0.01$ $\alpha=0.05$). Although many colonies at Marker 32 remined pale after September 2015, they appeared to recover slightly when compared with the previous year, indicated by near-normal coloration scores in November 2015 and January 2016 (mean scores: 3.46 ±0.33 and 3.76 ±0.33; two-way ANOVA, $p<0.01$ $\alpha=0.05$).

Colonies at Marker 32 were considered pale again in April 2016 (mean score: 3.01 ±1.13; one-way ANOVA p=0.06 α=0.05; Figure 4.6, Table 4.4).

4.4.4 Breviolum species switched dominance associated with hyperthermal events

The Symbiodiniaceae community in *D. cylindrus* was dynamic in response to hyperthermal bleaching and the subsequent recovery processes following two consecutive bleaching events. Relative abundance of the endosymbionts varied between three representative sites, especially the two species within *Breviolum* described above (Figure 4.7). At the Pickles site (Upper Keys) and associated with the August-September 2014 hyperthermal event, previously cryptic *B. meandrinium* (<10% relative abundance) became the dominant endosymbiont species in October 2014 and persisted through April 2015 while *B. dendrogyrum* declined in relative abundance through December 2014 to 20.60% but then slowly increased to 33.35% through April 2015, (Figure 4.7A, Table 4.3A), but not to pre-bleaching abundance of 75.68% in April 2014. At the Coffins site (Middle Keys), *B. dendrogyrum* remained dominant through bleaching and recovery in 2014. *Breviolum meandrinium* remained at low abundance but showed a slight but significant increase from October to December 2014 (two-way ANOVA p=0.01 α=0.05), reaching maximum 20.15% relative abundance in April 2015 (Figure 4.7B, Table 4.3B). Similar to the Coffins site, *B. dendrogyrum* remained the dominant species at Marker 32 (Lower Keys; Figure 4.7C, Table 4.3C).

In response to the second hyperthermal event in August-September 2015, the two most abundant *Breviolum* species were again dynamic and site-specific. At the Pickles site, fluctuation in dominance was again detected in which *B. dendrogyrum* briefly increased in abundance and re-established dominance by September 2015 (53.41%; two-

way ANOVA, p=0.02, α=0.05) however, this was short-lived. Relative abundance of *B. meandrinium* slowly declined through September 2015 to 30.28% but then quickly increased during the recovery months to persist as the dominant endosymbiont through April 2016 (51.55%). From September 2015 to April 2016, *B. dendrogyrum* slowly declined to 29.97% (Figure 4.7A, Table 4.3A). At the Coffins site, *B. dendrogyrum* remained the dominant species throughout the second bleaching and recovery (2015) and returned to similar abundance observed in April 2014 baseline (Figure 4.7B, Table 4.3B). *Breviolum meandrinium* reached a maximum abundance of 20.39% in September 2015 at this site and declined to 0.12% in January 2016, remaining unchanged through April 2016 (two-way ANOVA p<0.01). And finally, at the Marker 32 site, relative abundance of *B. dendrogyrum* and *B. meandrinium* did not change from April to September 2015 or thereafter, and *B. meandrinium* remained cryptic through April 2016 (Figure 4.7C, Table 4.3C).

4.4.5 Bleaching resistance and resilience

The magnitude of change in relative abundance of dominant *Breviolum dendrogyrum* and cryptic *B. meandrinium* during two consecutive hyperthermal bleaching events was site specific and closely associated with differential resistance and resilience to bleaching and recovery. Colonies at the Pickles site (Upper Keys) bleached severely during August-September 2014 and then recovered by December 2014 (Figure 4.6, Table 4.4). Concurrently, the relative abundance of dominant endosymbiont, *B. dendrogyrum*, declined while cryptic *B. meandrinium* increased to become the dominant symbiont. Relative abundance of these two species did not return to pre-bleaching levels (Figure 4.7A, Table 4.3A). During the second hyperthermal event in August-September

2015, while *B. dendrogyrum* briefly regained dominance by September, *B. meandrinium* once again became the dominant species, persisting through recovery until April 2016. This change was closely associated with the observation that the colonies at Pickles did not bleach during the second hyperthermal event (Figure 4.6, Table 4.4), indicating acquired resistance to annual thermal stress (water temperatures exceeding 30.5°C, Figure 4.3). This resistance was strongly associated with a fluctuation in Symbiodiniaceae species, specifically an increase and persistence in abundance of *B. meandrinium* as it became the dominant species.

Colonies at the Coffins site (Middle Keys) also bleached severely in August-September 2014 (Figure 4.6, Table 4.4). As normal colony coloration returned in December 2014, cryptic *B. meandrinium* slightly increased in abundance, reaching 20.15% by April 2015 (Figure 4.7B, Table 4.3B). Although *B. meandrinium* never became the dominant *Breviolum* species at Coffins, colonies did not bleach as severely the second year and recovered quickly to normal coloration by January 2016 (Figure 4.6, Table 4.4). This strongly suggests at least partial resistance to bleaching associated with an increased relative abundance in cryptic *B. meandrinium*. Colonies at Marker 32 (Lower Keys) bleached severely both in 2014 and 2015 (Figure 4.6, Table 4.4) while *B. meandrinium* remained at cryptic low levels throughout, reaching a maximum abundance of only 2.60% in September 2014 (Figure 4.7C, Table 4.3C).

4.4.6 Symbiodinium, Cladocopium, *and* Durusdinium *persisted at cryptic low abundance*

Although *Breviolum* (formerly clade B) remained the dominant genus in *D. cylindrus* through two consecutive bleaching and recovery events, cryptic Sy*mbiodinium, Cladocopium, and Durusdinium* species (formerly clades A, C, and D, respectively) were

also detected and often persisted through recovery (Table 4.3). *Symbiodinium* spp. were detected in cryptic low abundance (<0.01% to 5.38%) from April 2014 through December 2014 and then not thereafter at Pickles site (Upper Keys). At the Coffins site (Middle Keys), *Symbiodinium* spp. were detected intermittently through both bleaching and recovery events. At Marker 32 (Lower Keys), *Symbiodinium* spp. were detected in April 2014 and persisted at cryptic low abundance though April 2016 (≤0.2%).

Cladocopium spp. were only detected from April to October 2014 and then not after at the Pickles site (Table 4.3). *Cladocopium* spp. were detected at Coffins after the first bleaching in September 2014 and persisted intermittently during recovery until November 2015. At Marker 32, *Cladocopium* spp. were detected at cryptic levels in the April 2014 baseline sampling and persisted through April 2016.

A single OTU, classified as *Durusdinium* sp. and identified by BLAST-search as type D1a (putatively *D. trenchii*, formerly *S. trenchii*), was first detected in cryptic low abundance at Pickles and Coffins sites in October 2014 after the 2014 bleaching event and then intermittently through the second bleaching and recovery period until January 2016 (Table 4.3). At Marker 32, *Durusdinium* sp. was detected pre-bleaching in April 2014 and then intermittently throughout both bleaching and recovery periods through April 2016.

4.5 Discussion

Research over the last two decades has documented the influence of the symbiont composition and the dynamic processes of symbiont repopulation during the coral response to single episodes of hyperthermal stress events, followed by years of normal environmental conditions *(55, 56)*. Our study characterized changes in symbiont species

within some colonies of *Dendrogyra cylindrus* in response to a consecutive hyperthermal event. This study contributes to further understanding how coral-algal mutualisms may respond through shifts in partnerships under long-predicted environmental scenarios (annual bleaching) *(24, 57)*, which coral reefs worldwide are now experiencing *(3)*. Our findings show unexpectedly diverse assemblages of Symbiodiniaceae associated with *Dendrogyra cylindrus*, and that one low abundant host-compatible species was not transient associates but persistent and ecologically relevant symbionts that play a role during thermal stress. Furthermore, site-specific shifts in Symbiodiniaceae species dominance were associated with greater bleaching resistance during consecutive hyperthermal events.

4.5.1 Symbiodiniaceae community dominated mainly by a single symbiont along with a diverse assemblage of cryptic associates

By revealing a Symbiodiniaceae community represented by 266 OTUs, with the majority in very low relative abundances (<0.1%), our findings indicate that past studies have considerably underestimated the actual diversity of endosymbionts associated with *D. cylindrus*, and likely many other coral species, although at least some of these rare and cryptic OTUs may in fact be sequencing artifacts *(32).* Most symbiont types detected in our study (>99%) belonged to the genus *Breviolum* spp. (formerly Clade B). Three other genera, *Symbiodinium*, *Cladocopium*, and *Durusdinium* (formerly clades A, C, and D, respectively) were detected at extremely low levels (0.001% - 5.38%) in *D. cylindrus*. Discovery of the tremendous sequence diversity within the genus *Breviolum* associated with *D. cylindrus* opens new questions regarding whether these symbiont types are the reflection of population variability within species or are indeed independent evolutionary

lineages (i.e., species). Symbiont assemblages across sites and within individual *D. cylindrus* colonies were dominated by a single *Breviolum* species of endosymbiont. Using single copy microsatellite markers (see Methods), it was confirmed that this symbiont corresponds to the recently described *Breviolum dendrogyrum*. Furthermore, low concentrations of *Breviolum meandrinium*, which was also identified and confirmed with the use of diagnostic microsatellites, increased in relative abundance as a function of environmental stress. *Breviolum meandrinium* is common to corals in the family Meandrinidae from shallow habitats (1-10 m) across the Greater Caribbean. *Dendrogyra cylindrus* is one notable exception of the family in that it harbors a unique host-specialist, which appears adapted to associating only with this host. These findings highlight how a host-generalist, *B. meandrinium*, normally rare in *D. cylindrus*, can proliferate in colonies subjected to severe stress.

4.5.2 Changes in balance among host-compatible Symbiodiniaceae during recurrent environmental stress

It has been suggested that most cryptic Symbiodiniaceae are transitory and likely provide minimal ecological significance for their coral hosts *(38)*. However, recent studies have challenged the transitory insignificance and suggested that rare symbionts tend to be non-random clusters of coral host-symbiont communities and may provide environmental resilience for the coral holobiont *(58, 59)*. In agreement with changing views of the importance of the rare symbiont communities, our data show a clear association between a site-specific increase and persistence in relative abundance of the low abundance background *B. meandrinium* and the overall stability of the host–symbiont community during subsequent hyperthermal stress. At the Pickles site in the

Upper Keys region of the Florida Reef Tract, *B. meandrinium* increased in relative abundance during the first hyperthermal bleaching event in 2014 and rapidly switched to become the dominant symbiont during the first seven months of recovery (Figure 4.7A, Table 4.3). After the first hyperthermal event in 2014, the change in the relative abundances of the two species persisted for at least eleven months, but by September 2015 under normal condition, *B. dendrogyrum* displaced *B. meandrinium* as the dominant symbiont. This reversal of endosymbiont assemblages to pre-bleaching abundances is consistent with other studies that have monitored changes before and after non-recurrent hyperthermal events, where changes in symbiont during bleaching episodes reverted to the original state after several months or even years *(18, 24, 60, 61)*. Nevertheless, the reversion to the normal symbiont was short-lived as *B. meandrinium* again become the dominant symbiont species among colonies at the Pickles reef in response to the second hyperthermal event in August-September 2015. These host-symbionts combinations persisted and, at the time of the last sampling for this study in April 2016, *B. meandrinium* remained the dominant species (Figure 4.7A, Table 4.3A). Importantly, corals at this site did not lose color during this second event (Figure 4.6); the relative abundance of *B. meandrinium* was 3-4 times higher during the onset of the 2015 hyperthermal event in comparison to pre-bleaching levels in April 2014. Under scenarios of annual hyperthermal bleaching events, impacted coral communities may not have sufficient time to fully recover their stable host-symbiont pairings.

The Adaptive Bleaching Hypothesis posits that when corals bleach, they expel less thermally tolerant endosymbionts and then acquire new, more favorable endosymbionts, allowing them to acclimatize and adapt to environmental stressors *(62-*

64). Berkelmans & van Oppen (*2006*) demonstrated that thermal stress may induce changes in the dominant symbionts among experimental corals, thus providing thermal tolerance and decreased mortality to the coral animal, supporting the hypothesis that the presence of a thermally tolerant endosymbiont, even in low abundance, may impart an ecological advantage to their coral host *(61)*. Sampayo et al. (*2009*) demonstrated that shifts in dominance between closely related species in the genus *Cladocopium* can also impart differential thermal tolerance and, ultimately, differential colony survival. However, unusual host-symbiont partnerships that emerge during bleaching events revert to the original state after normal environmental conditions return*(65)*.

It appears that during a scenario of hyperthermal stress events, followed by multiple years of less-stressful environmental conditions, new host-symbiont combinations that appear following the bleaching event are short-lived and the Symbiodiniaceae community eventually reverts back to its original state *(18, 23)*. Reverting back to the original pre-bleaching Symbiodiniaceae community is likely as normal host-compatible symbionts are more effective a growing inside their hosts under non-stressful conditions *(20)*. However, under sustained disturbance events, such as annually recurring hyperthermal events, new thermally-tolerant partner pairings may be longer-lived and maintain higher relative proportions within the Symbiodiniaceae community as the period of less-stressful environmental conditions between disturbances becomes shorter *(24)*. Such was the case at the Pickles site where relative abundance of *B. meandrinium* remained near 50% and, while trace amounts of *Durusdinium* persisted through April 2016, *Symbiodinium* sp. and *Cladocopium* sp. disappeared altogether (Table 4.3A). Our data did not show a shift in dominance at the other two sites (Figures

4.7B & 4.7C) and, unlike Pickles colonies, they bleached again during the second hyperthermal event in August-September 2015 (Figure 4.6). However, at the Coffins site (Middle Keys), we detected an increase in relative abundance of *B. meandrinium* to 20.39% during the second hyperthermal event (August-September 2015; Figure 4.7B, Table 4.3B), which was associated with only paling in most colonies at this site (Figure 4.6, Table 4.4). While *B. meandrinium* did not become the dominant Symbiodiniaceae species, perhaps some threshold abundance may also impart at least partial bleaching resistance for the coral animal *(25)*, as was observed at the Coffins site. The Lower Keys site (Marker 32) showed minimal fluctuation in the endosymbiont community but also bleached more severely and recovered more slowly after both bleaching events.

This difference across sites between 2014 and 2015 seems not linked to different temperature profiles during the two hyperthermal events, since all sites experienced similar exposure to elevated temperatures above the 30.5°C bleaching threshold for the FRT (Figures 4.3 and 4.4). While we cannot explain why the shift of Symbiodiniaceae assemblages did not occur across all sites, it is important to note that baseline abundance of the cryptic *B. meandrinium* prior to the first hyperthermal event in April 2014 was higher in Pickles colonies than the other two sites (Table 4.3). Perhaps some critical minimum abundance may be required for a rare symbiont to out-compete dominant symbionts when the opportunity arises, such as during bleaching and recovery. However, the relative abundance of *B. meandrinium* in Coffins colonies was higher (20.15%) prior to the second hyperthermal event and still we did not see a shift in symbiont assemblages during the second bleaching and recovery event at this site. The increase in abundance of *B. meandrinium* in 2015 but no switch in dominance suggests the existence of other site-

specific factors influencing the dynamics and competition processes of symbiont repopulation after environmental stressors. Alternatively, genotypic differences in the coral animal may contribute to the observed differential symbiont flexibility, but their role is thus far unknown. Recent work on genetic diversity in Florida's *D. cylindrus* population indicates that each of these three sites is represented by unique coral genotypes *(66)*. All colonies at the Pickles site comprised one genotype, indicating a high level of clonality at this local. The same pattern of clonality was also detected for colonies at the Coffins site. The Marker 32 site had the most genetic diversity, composed of five different coral animal genotypes, and yet was the population most affected by these hyperthermal events. Continued work on the role of genetic diversity in the coral host and its symbiont requires further investigation.

It has been proposed that colonies exposed to moderate thermal stress are better conditioned to dealing with episodes of severe thermal stress *(67, 68)*. Thermal profiles show a pre-bleaching spike in water temperatures, followed by a recovery period of cooler temperatures, prior to a hyperthermal event, reducing the severity of bleaching. Temperature profiles at the three targeted sites show a similar sub-bleaching spike in water temperatures in June 2014 and May 2015 (Figure 4.3B), however severe bleaching occurred in August-September both years. This may be due to the recovery period being too short or the ensuing hyperthermal stress was too severe (i.e., temperatures exceeding the bleaching threshold for too many weeks, Table 4.1), exceeding the capacity of the thermally-primed corals. As predicted by Ainsworth et al. *(68)*, climate change leading to annual bleaching and excessive thermal stress may indeed disable this protective thermal priming scenario.

4.5.3 Persistent cryptic communities as a source of more thermally-tolerant strains for acclimatization and adaptation

Our data support that under certain circumstances, a low abundant symbiont may emerge during thermal bleaching and/or recovery to enhance acclimatization of the coral host *(56). Durusdinium* sp. known to be opportunistic in certain Caribbean corals exposed to stress *(61)* remained as trace levels (<0.03%) during the 2014 and 2015 bleaching and recovery events on the FRT. Due to the high resolution of this study, this is the first reporting of *Durusdinium trenchii* in *Dendrogyra cylindrus*, suggesting an expansion of this invasive, more thermally tolerant species into another Caribbean coral host. *(21, 69, 69, 70, 70). Durusdinium trenchii* (formerly *Symbiodinium trenchii*) is a stress-tolerant species within the genus *Durusdinium*, commonly found in the Indo-Pacific and known to impart thermal tolerance and bleaching resistance to its coral host *(71).* This species is considered invasive in the Greater Caribbean and has been increasingly found in corals inhabiting marginal habitats or under high environmental stress, particularly after bleaching events *(71).*

One of three cryptic *Symbiodinium* OTUs detected in *D. cylindrus* and identified putatively as *Symbiodinium necroappetens* (strain A13), is considered an opportunist, emerging transiently to associate with thermally stressed or diseased corals *(72)*. Our data show that *Symbiodinium* spp. were part of the cryptic community in *D. cylindrus* even prior to the 2014-2015 thermal events (April 2014, Table 4.3). At sites showing an increased abundance of *B. meandrinium* in response to annual thermal events (Pickles and Coffins), *Symbiodinium* and *Cladocopium* genera were not detected, and may have been displaced as *B. meandrinium* approached 20% relative abundance. Alternatively,

the occurrence and persistence of cryptic symbionts in *D. cylindrus* and other Caribbean corals may also be an indicator of long-term physiological stress due to deteriorating environmental factors, e.g. water quality *(73-75)* and pulsed thermal events (i.e., previous Florida Reef-wide bleaching events in 1987, 1990, 1997, 1998, 2005, as well as other localized bleaching episodes *(76)*.

4.5.4 Conclusions

While it is encouraging to substantiate that coral symbioses respond ecologically and thus "acclimatize" to a changing climate through shifts in the dominant symbiont partner, it may not be enough for their long-term survival in the Anthropocene. As annual thermal bleaching is predicted to become more prevalent on many reefs in the coming decades, the collateral damage to biological, physiological, and immunological functions of the coral holobiont *(77, 78)* may negate their innate ability to acclimatize. It is imperative that we address the issues of environmental stressors in the hopes that at least some reef ecosystems will be able to acclimatize and survive in a changing climate. Even this glimmer of hope for survival may prove to be 'too little, too late' as alarming coral losses due to escalating disease outbreaks in warming oceans may overcome these slow-growing monarchs of the reef more quickly than they can adapt.

4.5.5 Nomenclature

- cp23S-HV primers - utilize length variation in Domain V of large sub-unit rDNA chloroplast 23S hyper-variable gene region
- ENSO - El Niño/ La Niña Southern Oscillation is characterized by oscillating changes from expected sea surface temperatures in the eastern and central equatorial Pacific Ocean (El Niño – warm phase, and La Niña – cool phase)

- FRT – Florida Reef Tract
- Illumina Mi-Seq platform – high resolution parallel amplicon sequencing diversity assay
- Family Symbiodiniaceae *(13)* - photosynthetic endosymbiotic dinoflagellates
    - *Symbiodinium* spp. (living together, whirling) - formerly Clade A
    - *Breviolum* spp. (short & small) – formerly Clade B
    - *Cladocopium* spp. (branch & plenty) - formerly Clade C
    - *Durusdinium* spp. (tough & whirling) – formerly Clade D

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

CL conceived experimental design and sampling strategy, site selection, sampling methodologies for fieldwork, sample processing and data analysis, manuscript preparation including figures and tables, reviewed manuscript drafts

KN contributed site selection, field assessments and dive support, assisted with tables and figures, reviewed manuscript drafts

MRL conceived experimental design and sampling strategy, financial support, manuscript preparation and reviewed manuscript drafts

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4.6 Figures and Tables

Figure 4.0 Graphical abstract

Acquired site-specific bleaching resistance associated with a switch in dominant *Breviolum* species during two consecutive hyperthermal bleaching events (photos: C Lewis).

Figure 4.1. The iconic pillar coral, *Dendrogyra cylindrus.*

This species occurs in historically low abundance throughout the Greater Caribbean. Its unique columnar structure provides important habitat complexity to the reef ecosystem where it does occur. (photo: C Lewis)

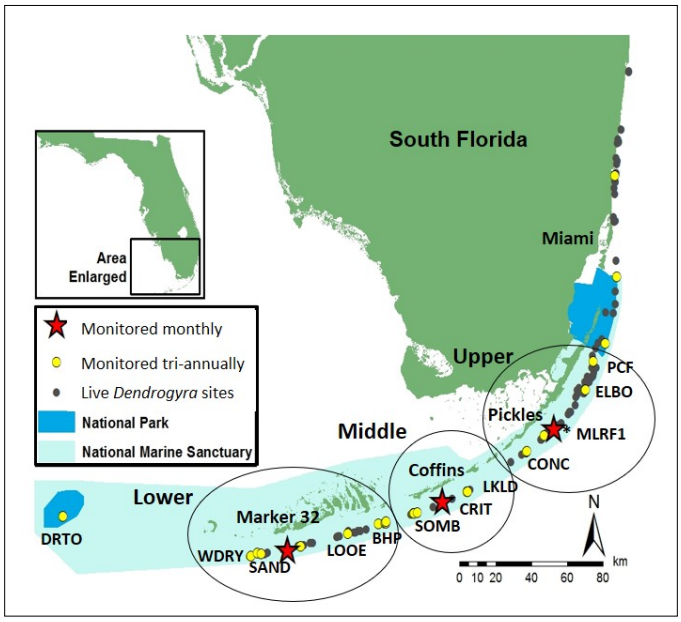


Figure 4.2. Study sites on the Florida Reef Tract where monitoring and sampling was conducted on the pillar coral *Dendrogyra cylindrus*.

Geographically stratified tri-annual monitoring and sampling at 28 sites April 2014 to April 2016 (yellow circles). Pickles (Upper Keys), Coffins Middle Keys), and Marker 32 (Lower Keys) sites (red stars) monthly during bleaching recovery in 2014 and 2015. *MLRF1 – location of Molasses Reef C-man buoy used to calculate 10-year mean monthly temperatures.

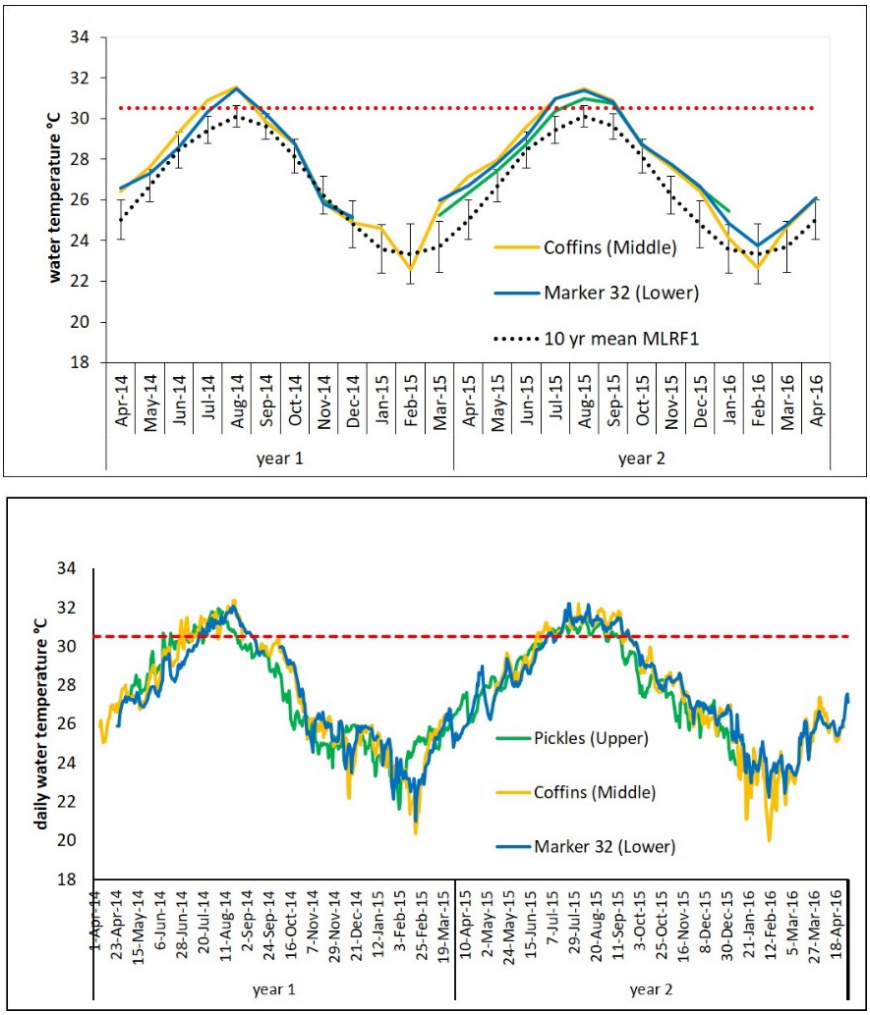


Figure 4.3. Water temperatures at three sites in the Florida Keys recorded between April 2014 to April 2016**.**

The Florida Reef Tract (FRT) in the Florida Keys is divided into distinct regions based on hydrologic influences, represented here by three sites: Pickles - Upper Keys, Coffins - Middle Keys, and Marker 32 - Lower Keys. Red dashed lines indicate the local bleaching threshold for the FRT (30.5°C). Gaps in data are due to lost or damaged temperature loggers. **(A)** Mean monthly water temperature data. Dotted black line is the calculated 10-year mean monthly water temperature (error bars $\pm$ 1 SD) for Molasses Reef 2004-2013 (MLRF1, National Data Buoy Center). **(B)** Mean daily water temperature data.

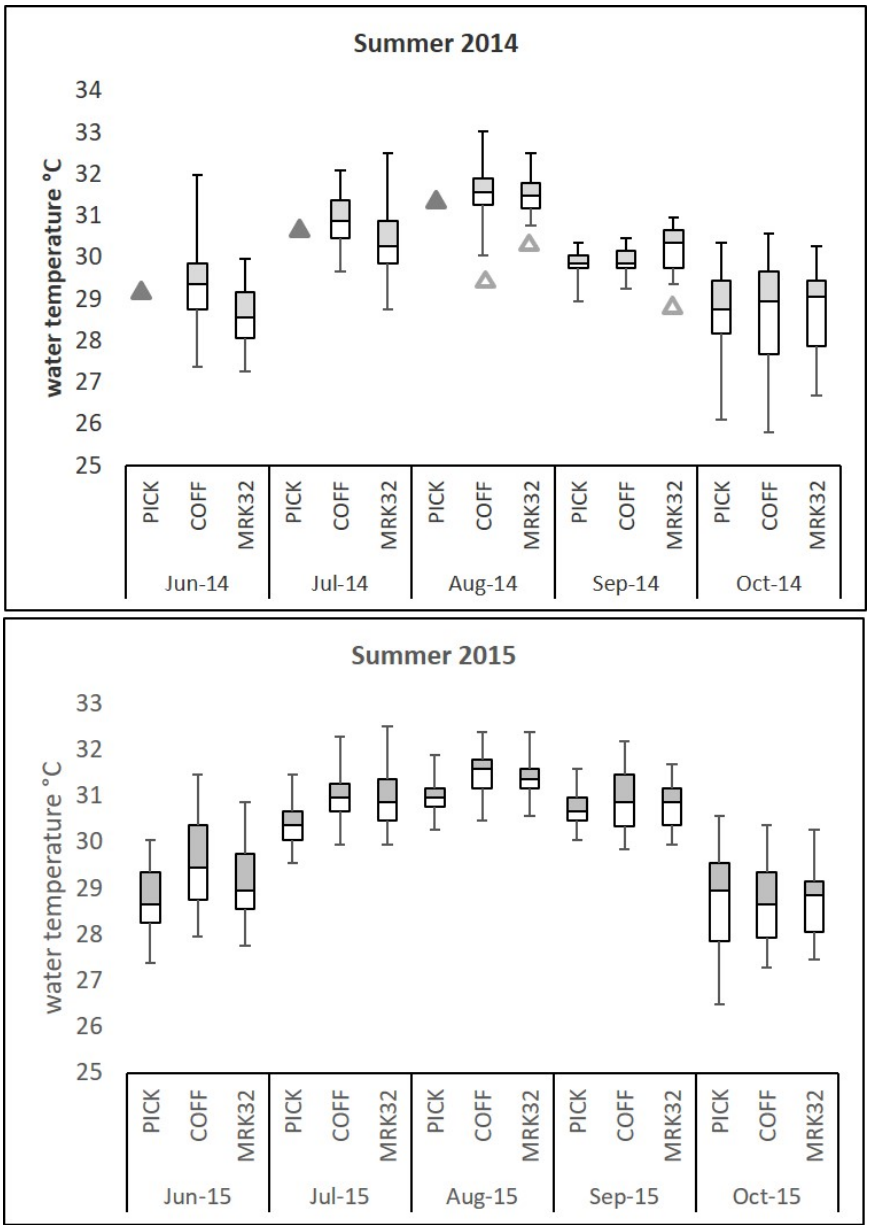
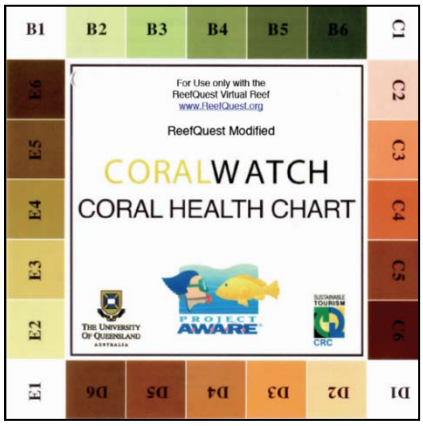


Figure 4.4. Summary of water temperature profiles at three sites during two hyperthermal events on the Florida Reef Tract 2014 and 2015.

Five-number summary of temperature profiles at three sites (Pickles: Upper, Coffins: Middle, Marker 32: Lower) along the Florida Reef Tract. Hourly water temperatures from pendant data loggers were used to create temperature profiles from June through October 2014 and 2015. Box plots indicate interquartile temperature ranges (Q2-Q3) and median temperatures. 'Whiskers' show maximum and minimum water temperature during this time period. Solid grey triangles indicate lost data loggers, outlined grey triangles indicate incomplete temperature profiles due to data logger malfunctions.

Figure 4.5. Coral Health Chart used to calculate the level of bleaching on *Dendrogyra cylindrus* colonies monitored between 2014 and 2016.



Coloration of live tissue was determined by comparing with the gradient of color on the Coral Health Chart (https://www.projectaware.org) *(49)*. Color scores were further broken down (0.5) if color appeared between two values. Percent of each color value on an individual colony was estimated visually. Chart score (column A) was multiplied by estimated percent of colony live tissue (column B) to calculate colony score for each chart score value (column C). All colony scores for each coloration value (column C) were added to determine Total Colony Coloration Score.

Calculation example:

Bleach Status	**A** **Coral Health Chart Score**	**B** **Estimated proportion of total live tissue on colony**	**C** **Colony Score** (col A x col B)
Bleached	1.0	.20	1.0 x 0.20 = 0.20
	1.5	0	0
Pale	2.0	.30	2.0 x 0.30 = 0.60
	2.5	0	0
	3.0	0	0
Healthy	3.5	.50	3.5 x 0.50 = 1.75
	4.0	0	0
Total Colony Coloration Score		**1.00**	**2.55**

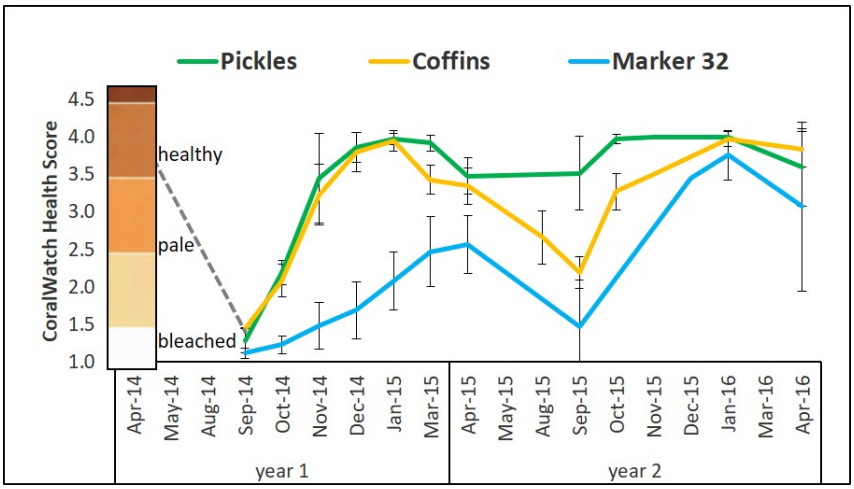


Figure 4.6. Mean bleaching index based on coloration scores of *Dendrogyra cylindrus* colonies at three sites on the FRT between 2014 and 2016.

The coloration scores were determined using the CoralWatch Coral Health Chart for colorimetric reference (version: Project Aware https://coralwatch.org; *(49)*). Coloration ranges for *D. cylindrus* based on triannual assessments of 168 colonies: healthy 3.5-4.0; pale: 2.0-3.0; bleached: 1.0-1.5. Dashed grey line represents trajectory of estimated colony scores from April 2014. Pickles (n=12) Upper Keys; Coffins (n=12) Middle Keys; Marker 32 (n=11) Lower Keys. Error bars ±1 standard deviation.

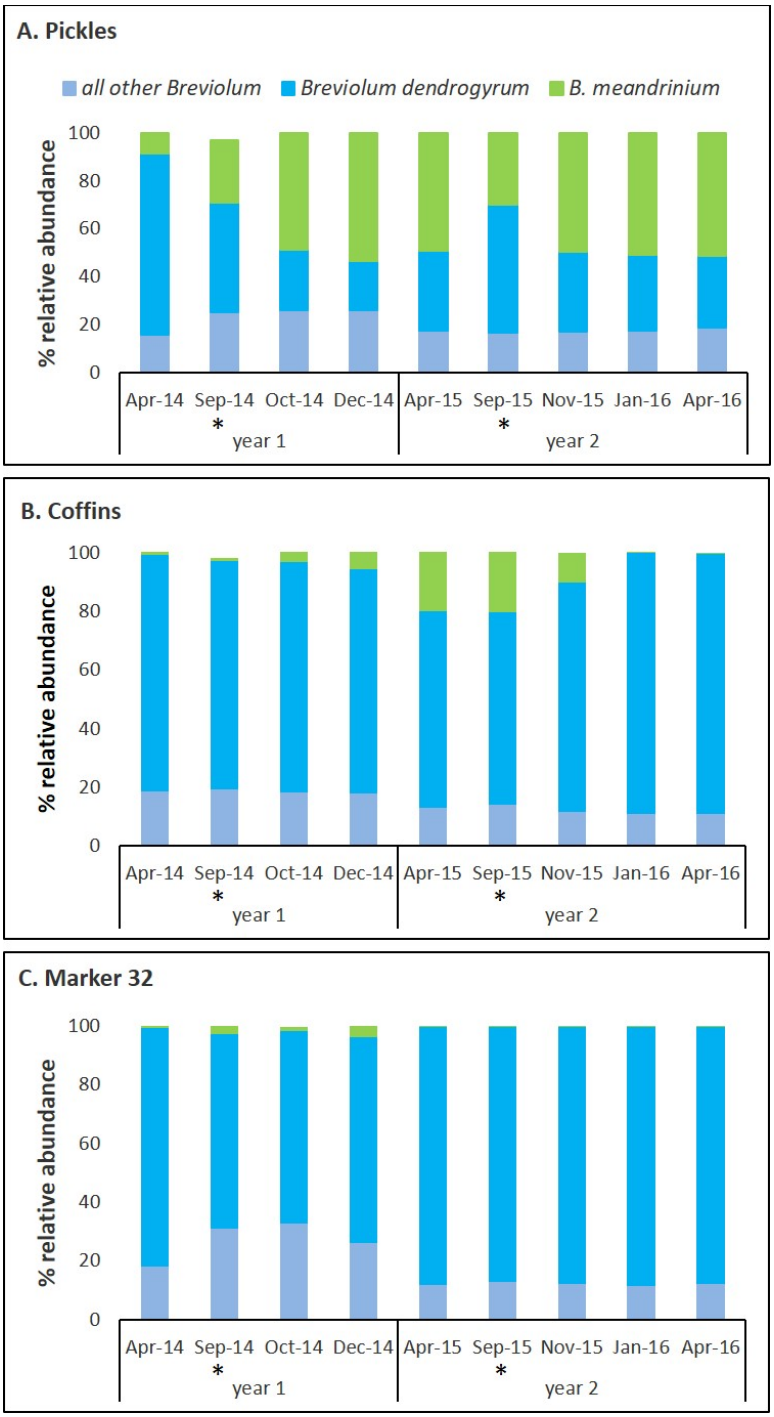


Figure 4.7 Relative abundance of Symbiodiniaceae symbionts from the genus *Breviolum* associated with *Dendrogyra cylindrus* on the Florida Reef Tract between 2014 and 2016.

The dynamics of *Breviolum dendrogyrum* (blue) and *B. meandrinium* (green) between three sites and between years, by percent relative abundance of *Breviolum* spp. OTUs. *B.*

meandrinium increased in relative abundance during bleaching and recovery, becoming the dominant symbiont species at the Pickles site in 2014 and 2015. (**A)** Pickles (n=6) Upper Keys (**B)** Coffins (n=6) Middle Keys (**C)** Marker 32 (n=6) Lower Keys. 'All other *Breviolum*' (blue-grey) represents 250 OTUs combined. Asterisk (*) indicates bleaching months.

Table 4.1. Summary of thermal profiles from three sites on the Florida Reef Tract 2014-2016.

HOBO data loggers recorded hourly water temperatures at three sites (Pickles: Upper, Coffins: Middle, Marker 32: Lower) 2014-2016 along the Florida Reef Tract (FRT). **(A).** Maximum weekly and maximum annual temperatures were recorded at each site. Number of weeks maximum water temperatures exceeded the FRT bleaching threshold (30.5°C) were calculated. for each site. **(B).** Ten-year mean monthly maximum (MMM) water temperatures were calculated for July, August, and September from archived C-man Station data at Molasses Reef (MRLF1) 2004-2013. Degree heating weeks (DHW, °C-weeks) were calculated for each site using the mean monthly maximum temperatures for July, August, and September and compared to NOAA Coral Reef Watch DHW. Degree heating weeks were also calculated at each site using the FRT bleaching threshold for comparison. Weeks where DHW were <1°C-weeks were not counted, as per standard protocols. ND represents insufficient data for meaningful calculations.

A.	2014			2015		
	Pickles*	Coffins	Marker 32	Pickles	Coffins	Marker 32
DHW using MMM	ND	7.48	2.8	0.00	5.21	4.07
DHW -using 30.5°C	ND	13.82	8.65	5.88	13.96	10.59
# weeks >30.5°C	ND	10	8	12	13	13
max temperature °C	30.36	33.01	32.50	31.88	32.39	32.50

*data loggers were lost at Pickles site from 4/1/2014-9/12/2014

B. 10-yr Mean Monthly Maximum 2014-2013 at Molasses Reef (MRLF1)	
Month	temperature (°C)
July	30.80
Aug	31.31
Sept	30.78
FRT bleach threshold	30.50
NOAA Coral Reef Watch 50-km Satellite Degree Heating Weeks (DHW) for FRT	
2014	2015
5°C-weeks	7°C-weeks

Table 4.2. Primers used for cp23S amplicon sequencing and microsatellite genotyping analysis in Symbiodiniaceae.

Primer name	Primer Sequence	Reference
cp23S hyper up (forward)	TCA GTA CAA ATA ATA TGC TG	Santos et al 2003
cp23S hyper down (reverse)	TTA TCG CCC CAA TTA AAC AGT	
B7*Sym*15 forward	CTC ACC TTG AAA TCA GTA GCC A	Pettay & LaJeunesse 2007
B7*Sym*15 reverse	CGT AGC TTC TGA AGG TAC GAC AC	

Table 4.3. Mean relative abundance of Symbiodiniaceae genera in *Dendrogyra cylindrus* at three sites on the Florida Reef Tract 2014-2016.

Percent mean relative abundance (± standard deviation) of operational taxonomic units ($OTU_{0.03}$). Asterisk (*) indicates bleaching months. **(A)** Pickles (n=6 colonies sampled) Upper Keys **(B)** Coffins (n=6 colonies sampled) Middle Keys **(C)** Marker 32 (n=6 colonies sampled) Lower Keys.

(A) Pickles n=6 Upper Keys	**Year 1**				**Year 2**				
	Apr-14	***Sep-14**	**Oct-14**	**Dec-14**	**Apr-15**	***Sep-15**	**Nov-15**	**Jan-16**	**Apr-16**
B. dendrogyrum 1 OTU	75.68 (16.15)	45.47 (25.91)	25.35 (14.73)	20.60 (19.67)	33.35 (14.18)	53.41 (19.15)	33.29 (12.77)	31.63 (14.65)	29.97 (8.02)
B. meandrinium 1 OTU	8.75 (0.88)	29.53 (22.06)	48.74 (3.24)	53.49 (5.79)	49.37 (11.82)	30.28 (13.02)	49.60 (10.35)	51.10 (12.12)	51.55 (6.58)
all other *Breviolum* 243 OTUs	15.56 (1.79)	25.23 (9.47)	25.90 (5.11)	25.90 (4.34)	17.27 (2.38)	16.31 (6.16)	17.11 (2.44)	17.27 (2.54)	18.48 (1.59)
all *Symbiodinium* 94 OTUs	0.01 (0.01)	5.38 (13.85)	0.001 (0.004)	0.002 (0.002)	0	0	0	0	0
all *Cladocopium* 3 OTUs	0.001 (0.001)	0.001 (0.001)	0.01 (0.03)	0	0	0	0	0	0
all *Durusdinium* 1 OTU	0	0	0.001 (0.002)	0	0.001 (<0.001)	0.0003 (0.0007)	0.001 (<0.001)	0.001 (<0.001)	0

(B) Coffins n=6 Middle Keys	**Year 1**				**Year 2**				
	Apr-14	***Sep-14**	**Oct-14**	**Dec-14**	**Apr-15**	***Sep-15**	**Nov-15**	**Jan-16**	**Apr-16**
B. dendrogyrum 1 OTU	80.57 (6.54)	77.60 (5.60)	78.58 (3.68)	76.50 (5.46)	66.78 (0.66)	65.54 (0.56)	78.26 (12.71)	88.87 (0.53)	88.87 (0.17)
B. meandrinium 1 OTU	0.88 (0.12)	0.94 (0.14)	3.24 (1.92)	5.79 (5.05)	20.15 (0.14)	20.39 (0.40)	10.02 (11.42)	0.12 (0.01)	0.15 (0.02)
all other *Breviolum* 243 OTUs	18.52 (6.45)	19.34 (2.91)	18.18 (3.01)	17.71 (2.24)	13.07 (0.62)	14.04 (0.20)	11.58 (1.46)	10.77 (0.53)	10.73 (0.20)
all *Symbiodinium* 94 OTUs	0.02 (0.01)	2.12 (0.01)	<0.001	0.003 (0.01)	0	0	<0.001	0.003 (0.002)	0.003 (<0.001)
all *Cladocopium* 3 OTUs	0	0.003 (0.01)	<0.001	<0.001	0	0	0.14 (0.16)	0	0
all *Durusdinium* 1 OTU	0	0	<0.001	0	<0.001	0.03 (0.06)	0.002 (0.002)	0.004 (0.002)	0.003 (0.003)

(C) Marker 32 n=6 Lower Keys	**Year 1**				**Year 2**				
	Apr-14	***Sep-14**	**Oct-14**	**Dec-14**	**Apr-15**	***Sep-15**	**Nov-15**	**Jan-16**	**Apr-16**
B. dendrogyrum 1 OTU	81.09 (2.24)	66.26 (11.33)	65.58 (15.69)	70.33 (6.90)	*87.82* (0.44)	86.80 (1.05)	87.43 (0.87)	88.34 (0.39)	87.60 (0.30)
B. meandrinium 1 OTU	0.76 (0.08)	2.60 (4.55)	1.44 (0.54)	3.71 (3.04)	0.08 (0.04)	0.17 (0.08)	0.11 (0.05)	0.08 (0.02)	0.06 (<0.01)
all other *Breviolum* 243 OTUs	18.07 (2.21)	31.03 (9.98)	32.76 (15.43)	25.96 (5.34)	11.80 (0.45)	12.72 (1.00)	12.23 (0.88)	11.32 (0.39)	12.12 (0.31)
all *Symbiodinium* 94 OTUs	0.02 (0.01)	0.01 (0.01)	0.20 (0.54)	0.003 (0.005)	0.002 (0.001)	0.007 (0.01	0.003 (0.001)	0.003 (0.001)	0.002 (0.002)
all *Cladocopium* 3 OTUs	0.03 (0.07)	<0.001	0	<0.001	0.29 (0.01)	0.29 (0.01)	0.22 (0.02)	0.25 (0.02)	0.21 (0.02)
all *Durusdinium* 1 OTU	0.002 (0.005)	0	0	0	0.006 (0.003)	0.009 (0.005)	0.005 (0.001)	0.005 (0.001)	0.01 (0.007)

Table 4.4. Mean colony coloration scores at three sites on the Florida Reef Tract 2014-2016.

Mean colony coloration scores (±1 standard deviation) at three sites on the Florida Reef Tract April 2014 to April 2016. CoralWatch Coral Health Chart (version: Project Aware) was used to determine a score for each colony. See Figure 4.5 for calculating colony coloration scores. NA = no data. Colorimetric chart was not utilized during April 2014 assessments; coloration scores were later estimated by reviewing colony photographs (estimated mean coloration scores 3.75).

	Year 1							Year 2					
	Apr-14	**Sep-14**	**Oct-14**	**Nov-14**	**Dec-14**	**Jan-15**	**Mar-15**	**Apr-15**	**Sep-15**	**Oct-15**	**Nov-15**	**Jan-16**	**Apr-16**
Pickles													
n=10		1.29	2.19	3.45	3.87	3.98	3.93	3.48	3.52	3.98	4.00	4.00	3.60
Upper Keys	NA	(0.16)	(0.16)	(0.60)	(0.20)	(0.08)	(0.11)	(0.24)	(0.49)	(0.07)	(<0.01)	(<0.01)	(0.52)
Coffins													
n=10		1.45	2.09	3.23	3.80	3.95	3.43	3.35	2.20	3.28	3.50	3.98	3.84
Middle Keys	NA	(<0.01)	(0.22)	(0.41)	(0.14)	(0.14)	(0.19)	(0.24)	(0.21)	(0.24)	(<0.01)	(0.10)	(0.24)
Marker 32													
n=12		1.12	1.23	1.48	1.70	2.08	2.47	2.57	1.48		3.46	3.76	3.08
Lower Keys	NA	(0.07)	(0.12)	(0.31)	(0.38)	(0.39)	(0.47)	(0.39)	(0.62)	NA	(0.33)	(0.33)	(1.13)

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CHAPTER 5: The Effects of Annual Hyperthermal Stress on White Plague Disease Prevalence and Susceptibility in *Dendrogyra cylindrus.*

5.1 Abstract

Global climate change has led to escalating occurrences of hyperthermal stress on coral reefs worldwide. Increasing frequency and duration of these events have been linked to observations of pathogenicity and virulence contributing to disease expansion into more coral species, as well as increased numbers of newly described diseases. The aim of this chapter was to determine the relationship between hyperthermal stress on *Dendrogyra cylindrus* and white plague (WP) disease prevalence and susceptibility in this coral species on the Florida Reef Tract (FRT).

The FRT experienced consecutive hyperthermal bleaching events in 2014 and 2015 associated with the global El Niño Southern Oscillation (ENSO). Using a geographically stratified design, *D. cylindrus* sites were selected across three regions of the FRT for triannual bleaching and disease assessments. Additionally, three of these sites were selected for monthly assessments to more closely follow recovery after hyperthermal events in 2014 and 2015.

This study establishes a baseline for white plague (WP) disease in Florida's *D. cylindrus* population prior to the 2014 hyperthermal event and shows increased prevalence of WP in *D. cylindrus* subsequent to hyperthermal stress in 2014. Cumulative effects of two consecutive hyperthermal events resulted in greater susceptibility to WP and further increased prevalence of WP in 2015, including a disease epidemic

documented at a Middle Keys site. These findings portend serious ramifications for this critically threatened species under predicted scenarios of ocean warming.

5.2 Introduction

Global impacts of climate change, and especially increases in ocean temperatures, have become of great concern in recent decades *(1-3)*. Hyperthermal bleaching events are projected to become annual occurrences by 2050, or sooner in some regions *(4-6)*. Recent analysis of sea surface temperatures in the Caribbean from 1982 to 2012 reveal an increasing trend, most pronounced in the last 15 years *(7)*. These trends show likely correlation with El Niño and the Southern Oscillation (ENSO). One of the strongest ENSO events on record occurred from May 2014 to June 2016 *(8)*, causing staggering losses to coral reefs worldwide, including the Florida Reef Tract (FRT), due to consecutive bleaching events and subsequent disease outbreaks *(3, 9)*. These hyperthermal events cause collateral damage to biological, physiological, and immunological functions of the coral holobiont *(10)*.

Increases in coral diseases in recent decades have largely been attributed to environmental stressors, especially increasing sea temperatures associated with climate change *(11-17)*. Hyperthermal stress has been linked to increases in disease *(11, 16, 18-25)*. One consequence of thermal stress is often coral bleaching. The resulting loss of algal symbionts can deprive the coral animal of essential nutrients, leaving it in a weakened state and potentially more susceptible to diseases *(11, 20, 22, 26, 27)*. Enhanced pathogenicity has been shown in many marine microbes at temperatures exceeding 29°C *(26, 28, 29)*. Additionally, prolonged elevated sea temperatures, as

occurs during hyperthermal anomalies, may also increase pathogenicity and virulence in the microbial community, further enhancing increases in disease prevalence and epidemics *(12, 16, 30, 31)*.

White plague (WP) disease has been a major contributor to the loss of live coral cover in Florida and throughout the Caribbean (Figure 5.1). It is a highly destructive disease affecting more than 40 coral species worldwide, including Florida's *D. cylindrus (32, 33)*. White plague first appeared on the FRT in the 1970s *(34)* and emerged again, but in epidemic proportions, in June 1995 in the Upper Keys. First appearing in the elliptical star coral (*Dichoceonia stokesi*, also a member of the Meandrinidae family), this more virulent form, termed white plague type-II (WP-II), spread throughout the FRT, effecting 16 of the 43 reef-building coral species, including *D. cylindrus (35, 36)*. Seasonally occurring from June to November, it is typically characterized by a rapidly moving line of freshly exposed coral skeleton adjacent to apparently healthy tissue, advancing at rates up to 2cm per day. A causative gram-negative bacterial pathogen was successfully isolated from *Dichoceonia stokesi* in 1995, satisfying Koch's postulate, and later identified as *Aurantimonas coralicida*, a novel member of *Sphingomonas* (order Rhizobiales, class Alphaproteobacteria) *(37)*.

The aim of chapter five is to examine the role of hyperthermal stress on the incidence and prevalence of WP disease in Florida's pillar coral, *Dendrogyra cylindrus*, focusing on the upper, middle, and lower regions of the Florida Keys Reef Tract. While low background levels of WP disease have become common on many reefs, the biological hypothesis for this chapter is that hyperthermal stress and resulting coral bleaching is correlated to increased prevalence and incidence of disease and exacerbated

by the cumulative effects of consecutive hyperthermal events. First, I characterized WP disease associated with *D. cylindrus* and determine the baseline or background prevalence of disease in the Florida Keys population. Additionally, I determined differences in prevalence between sites and between regions of the FRT. Secondly, I characterized the incidence and prevalence of WP in *D. cylindrus* during hyperthermal bleaching and recovery in 2014 and determine differences between sites or regions. And finally, I explored the cumulative effects of annual hyperthermal stress on WP in *D. cylindrus* during bleaching and recovery in 2015.

5.3 Methods

Experimental design and field sampling methodologies were fully described in chapter two.

5.3.1 White plague prevalence and incidence in Dendrogyra cylindrus *on the Florida Reef Tract*

Baseline white plague (WP) prevalence were established from the first assessment time point in April 2014, prior to the first hyperthermal bleaching in August-September 2014 (but see Chapter two, page 22-23). Prevalence of WP (percentage of diseased colonies) was calculated within sites when applicable (i.e., high-density sites), within regions, and for the overall FRT (Upper, Middle, and Lower regions combined) from April 2014 through April 2016 triannual assessments (n=154 total colonies). Monthly assessments during bleaching recovery periods from October to December 2014 and 2015 at three representative sites (Pickles n=24: Upper Keys, Coffins n=24: Middle Keys n=13; Marker 32: Lower Keys) allowed observation of WP prevalence at greater resolution in *D. cylindrus* in the Florida Keys. Monthly progression of WP through two

consecutive hyperthermal events at the Coffins site was tracked by assessing 24 individually tagged colonies. Incidence of disease (the first occurrence of disease in a colony) following bleaching and during recovery in 2014 and 2015 was also determined from monthly assessment data at the Coffins site for each time point.

5.3.2 Statistical analysis

Fisher's Exact Test for Count (α=0.05) was used to compare baseline prevalence of disease between regions (Upper, Middle and Lower Keys). A pairwise test for Nominal Independence was used to compare healthy and diseased colonies between regions at each time point and, because of the small number of variables, the adjusted Fisher *p*-value was used to determine significance. Pearson's X^2 Test for Independence (α=0.05) was then used to determine significance of observed differences between regions. Differences in disease prevalence between regions and between time points compared between years were determined using Fisher's Exact Test (α=0.05) and X^2 Test (α=0.05). Disease incidence for the FRT was compared between hyperthermal events in Year 1 (April 2014-March 2015) and Year 2 (April 2015-April 2016) using Ch^2 Test (α=0.05).

5.4 Results

Initial April 2014 complete assessments of all 414 *D. cylindrus* at 28 sites were considered to be the baseline or background disease prevalence for the Florida Keys *D. cylindrus* population prior to the hyperthermal stress event in 2014. Background prevalence of WP in April 2014 was 2.7% in all regions combined (Figure 5.2) and was the only disease associated with *D. cylindrus*. Background WP prevalence was greater in

the Upper Keys (17.0%) than either the Middle (3.3%) or Lower Keys (2.4%) in April 2014 (Figure 5.3; Fisher's Exact Test, p=0.009).

5.4.1 Changes in white plague prevalence in Dendrogyra cylindrus *on the Florida Reef Tract over two consecutive hyperthermal events*

During the first hyperthermal bleaching event in 2014, WP prevalence on the FRT increased from baseline background levels of 2.7% to 18.8% in September 2014 (n=154; Figure 5.2; X^2 α=0.05, p=0.001). At the three targeted sites, disease prevalence increased to 32.0% in November 2014 (n=61) and then declined to 3.9% in March 2015 (n=61 colonies; Figure 5.2). At the triannual assessment in April 2015, WP prevalence was 9.4% (n=154), nearly four times greater than the pre-bleaching baseline in April 2014 (*p<0.001*, Table 5.1). With the onset of the second hyperthermal bleaching in September 2015, disease prevalence for all regions increased to 21.3% (p=0.001, Figure 5.2, Table 5.2), primarily driven by a white plague outbreak in the Middle Keys and significantly higher than September 2014 (18.8%, X^2 α=0.05; p=0.016). White plague reached greatest prevalence at the three targeted sites in October 2015 (25.0%, n=61), one month earlier than the previous year (Figure 5.2). During bleaching recovery from September 2015 to January 2016, disease prevalence declined over all on the FRT to 13.9% (p=0.001), especially in the Middle and Lower regions (Figure 5.2 and Figure 5.3; p=0.032 and 0.017 respectively, Table 5.2). After two consecutive hyperthermal events, WP prevalence in April 2016 did not return to April 2014 baseline levels and was nearly ten times greater (Figure 5.2; X^2 α=0.05; p<0.001, Table 5.1).

5.4.2 Differences in white plague prevalence among regions of the Florida Reef Tract 2014-2016.

To determine differences between regions of the FRT, prevalence of WP was calculated from triannual assessments at 28 sites through two consecutive hyperthermal events (Upper Keys: 12 sites, n=56 colonies; Middle Keys: 6 sites, n=32 colonies; Lower Keys: 10 sites, n=44 colonies). Within regions, disease prevalence increased in the Upper and Lower Keys (Figure 5.3; p=0.006 and p=0.008 respectively; Table 5.2) but remained relatively unchanged in the Middle Keys. White plague prevalence did not significantly change within regions during bleaching recovery from September 2014 to January 2015 prevalence (p>0.2, Table 5.2), remaining elevated in the Upper and Lower regions (Figure 3.3). Additionally, disease prevalence in the Upper Keys region increased from 28.0% in January 2016 to 47.1% in April 2016 (X^2 α=0.05; p=0.048; Figure 5.3).

5.4.3 Progression and dynamics of white plague during recurrent hyperthermal bleaching and recovery at a Middle Keys site

All 55 *D. cylindrus* colonies at the Coffins site (Middle Keys) were mapped and assessed in April 2014, showing no signs of active WP disease (Figure 5.4). Microsatellite analysis of 24 of these 55 colonies targeted for assessments determined this site was likely a single unique genotype (Chapter 3, *(38)*, due to centuries of asexual fragmentation and displacement of pillars from the original parent colony, establishing new genetically identical colonies or ramets along this patch reef). Florida's reefs exceeded 5°C-weeks (degree heating weeks) during the summers of 2014, based on NOAA's Coral Reef Watch 50-km Satellite Monitoring *(39, 40)* (Table 4.1B). Data

loggers at assessment sites recorded sea water temperatures which exceeded the FRT bleaching index of 30.5°C *(41)* (Fig. 4.3) and all *D. cylindrus* were severely bleached (presented in Chapter 4). White plague was first observed in a single *D. cylindrus* colony at the southernmost end of the Coffins site in September 2014 (Figure 5.5). One month later, seven more colonies, also on the southern end of the reef, showed active WP. By November, an additional four *D. cylindrus* colonies on the northern end of the reef had active WP (Figure 5.5). Colony assessments in January, March, and April 2015 showed no signs of active WP at the Coffins site (Figure 5.6A). However, following the second hyperthermal event in August-September 2015, when the FRT experienced seven DHW *(40)*, monitoring in September 2015 revealed 20 of the 24 regularly assessed *D. cylindrus* colonies throughout the Coffins site with signs of active WP (Figure 5.7). While all *D. cylindrus* colonies were severely bleached in September 2014, eight of the colonies interspersed throughout the site appeared resistant to the active WP in nearby colonies (Figure 5.5). However, in 2015, with the recurrence of hyperthermal stress in August-September, all nine apparently-resistant colonies became diseased following the second hyperthermal event. Prevalence of WP in the 24 assessed colonies reached 88% in October 2015 (Figure 5.6A). As with the previous year (November 2014) and concurrent with cooling winter water temperatures, active disease declined in November 2015 to 13% prevalence in assessed colonies. Deviating significantly from January 20 15, white plague remained active in eight of the assessed colonies in January 2016 (p=0.004*)* and seven colonies in April 2016 (p=0.002) at this Middle Keys site (Figure 5.6A and Figure 5.7).

Incidence of WP at the Coffins sites (number of new colonies with active disease each month) showed a different profile between hyperthermal events in 2014 and 2015 (Figure 5.6B). White plague appeared in the first *D. cylindrus* colony in September 2014 (Figure 5.5). In October 2014, seven new colonies had active disease and in November 2014, seven additional new colonies were actively diseased. White plague persisted in many of these colonies between months through December 2014 (Figure 5.6A). During the second hyperthermal event in August-September 2015, the first two colonies with active disease were observed in August, a month earlier than in 2014. In September 2015, 16 new colonies had active disease (Figure 5.6B and Figure 5.7). Unlike in December 2014, WP continued to appear in new colonies at this site into January 2016, but active disease persisted in many colonies until April 2016.

5.5 Discussion

The increasing frequency and intensity of global thermal events have had profound impacts on marine ecosystems *(3)*. The biological stresses associated with these occurrences, impacting cellular and immunological functions, gene expression, and the microbial community, are often the precursors to increased disease susceptibility, incidence, prevalence and epidemics *(16, 22, 24)*.

5.5.1 Greater prevalence and incidence of white plague disease in Dendrogyra cylindrus *during recurrent hyperthermal events*

Recurrent hyperthermal stress and associated bleaching events in 2014 and 2015 on the Florida Reef Tract (FRT) was associated with greater WP prevalence in *D. cylindrus* within the Florida Keys, although some differences were observed among

regions and between years. While all regions of the FRT experienced similar prolonged elevated sea temperatures exceeding the bleaching threshold for the FRT *(42)* (Figure 4.4), localized differences between sites may have also contributed to differential disease susceptibility (i.e., salinity, turbidity, pH, dissolved nutrients not tested for in this study). White plague prevalence on the FRT remained elevated through recovery April 2016 and did not return to baseline disease prevalence (April 2014), highlighting another aspect of cumulative effects of annual hyperthermal events. It has been shown that recovery from bleaching events may require years for the coral holobiont to re-establish normal endosymbiont assemblages *(43-46)* as well as their microbial community *(47)*. Annually recurring hyperthermal events may not allow the holobiont adequate time to fully recover before being faced with additional stressors, further increasing their susceptibility to pathogens. These findings further substantiate links between hyperthermal stress and subsequent increases in disease prevalence *(11, 20, 22, 24)*.

Unexpectedly, the Lower Keys region showed a decline in disease prevalence during the second hyperthermal bleaching and recovery (Figure 5.3). The non-significant increase in disease prevalence from April 2015 to September 2015 (X^2 test α=0.05, p=0.918) was followed by a significant decline in disease prevalence from September 2015 to January and April 2016 (p=0.017). The Lower Keys region also experienced higher mortality due to severe bleaching loss and disease (pers. obs., Neely and Lewis, unpublished data) and this may have contributed to the perceived decline in disease prevalence by eliminating previously diseased colonies from later assessments.

Monthly monitoring during recovery at three representative sites in 2014 and 2015, which tracked individual marked colonies during recurrent hyperthermal events, allowed finer resolution of WP disease dynamics and seasonal fluctuations in disease prevalence. Highest disease prevalence was in November 2014 following the first hyperthermal event and in October 2015 following the second hyperthermal event, peaking a month earlier during the second year. Additionally, regular and frequent monitoring and assessments of the *D. cylindrus* population determined a more accurate seasonal incidence and prevalence of WP in *D. cylindrus* following consecutive hyperthermal events.

5.5.2 Progression and dynamics of white plague during recurrent hyperthermal bleaching and recovery

Recurrent thermal stress, which resulted in severe annual bleaching in 2014 and 2015, was strongly associated with an outbreak of WP at a Middle Keys site. White plague progressed through a large stand of *D. cylindrus* colonies at the Coffins site during the 2014 hyperthermal bleaching and post-bleaching recovery, appearing again during the 2015 hyperthermal bleaching and post-bleaching recovery. These findings support the findings that bleaching may contribute to disease outbreaks and cause the loss of disease resistance *(21, 23)*. Due to the high density of this site dominated by 55 *D. cylindrus* colonies, this is especially relevant to studies showing an association with disease and live coral cover *(48)*. Disease appeared to advance from offshore at this site, spreading from one colony to the next, but leaving some colonies untouched in its progression. Observations of disease-resistant colonies during the 2014 hyperthermal bleaching event

support work by Merselis et al *(49)* indicating that loss of algal symbionts during bleaching may actually allow the coral animal to divert energies to enhanced disease immunity. However, these eight colonies became susceptible to WP following the second hyperthermal event in August-September 2015, suggesting the immune system was unable to accommodate consecutive hyperthermal stress events. Alternatively, work by Muller et al. showed that some disease-resistant genotypes lost their disease resistance after bleaching *(50)*. Initially it was thought that the eight colonies resistant to disease belonged perhaps to more disease-resistant genotypes, however it was later determined that all assessed colonies, and perhaps the entire site, was clonal, likely due to centuries of asexual fragmentation, and comprised of a single unique genotype *(38)*. The lack of genetic diversity in this large clonal stand of *D. cylindrus* may have contributed further to its susceptibility to WP under thermal stress. Alternatively, the disease progression suggests the pathogen may be water-born, however daily tides, currents, and wave action are too variable at this site to support this hypothesis. Due to its apparent linear progression through the site, the pathogen may also be transmitted by a vector, such as common corallivores, including the ubiquitous butterfly fish (family Chaetodontidae) and sergeant majors (*Abudefduf saxatilis*, a species of damselfish) frequently found nesting on portions of old dead colony skeletons. It has previously been shown that gall crabs (family Cryptochiridae) and corallivorous snails (*Coralliophila abbreviata)* can be potential vectors for WP *(51, 52)*. Butterfly fish have been associated with the spread of BBD *(19)*. Concern for divers as vectors of disease cannot be overlooked as well. Caution was exercised to avoid unnecessary contact with colonies and rigorous biosecurity protocols included disinfecting all dive gear and sampling syringes between uses.

As was observed at several other *D. cylindrus* sites on the FRT, active WP ceased during the cooler winter and spring months at the Coffins site in 2014-2015 *(36, 53-55)*. With the onset of a second hyperthermal bleaching event in August-September 2015, 20 of 24 assessed colonies at Coffins immediately showed active WP in August and September 2015, compared with only one colony the previous year (Figure 5.7). Additionally, WP remained active at this site throughout the cooler winter months (December 2015, January 2016) until April 2016. This strongly suggests cumulative effects of annual thermal stress and increased disease susceptibility associated with the second hyperthermal event in 2015. It is also likely that the microbial community did not have time to fully recover from the 2014 hyperthermal event and had not regained its typical probiotic assemblages, making the coral holobiont more susceptible to disease with annually occurring thermal stress *(56)*. Increased pathogenicity of the WP-causing organism(s) due to prolonged elevated water temperatures and consecutive hyperthermal stress events may also have contributed to increased disease susceptibility and prevalence. Additionally, the pathogen load may have persisted in the local environment near threshold levels (sediments, algal turf, etc.) as well as within the coral holobiont microbial community in very low numbers, allowing WP to activate more quickly during the second hyperthermal event when conditions became more favorable for the pathogens and the corals were again stressed.

5.5.3 First indications of the new scleractinian tissue loss disease in Dendrogyra cylindrus *in the Upper Keys in early spring 2016*

Marine diseases have been associated with seasonal changes in water temperatures, often subsiding during the cooler winter and spring months. However, data from this research show increased prevalence of WP following a second hyperthermal event in 2015, extending through the winter months to April 2016 (Figure 5.2). Especially noteworthy is the increase in disease prevalence from January 2016 to April 2016 in the Upper Keys region (Figure 5.3). This is likely the first indicator of the 'new' scleractinian (stony coral) tissue loss disease (SCTLD) extending into the Upper Keys and south along the Florida Reef Tract *(9)*. This disease of unknown etiology was first observed in the Miami area during Fall 2014 and spread north more than 160 kilometers by Summer 2017 *(57)*. Disease assumed to be this new tissue loss disease was reported in Biscayne National Park in Fall 2015. At this writing, it has now spread along the FRT into the Lower Keys (Summer 2018) effecting 22 different reef-building coral species. This disease is characterized by rapid tissue loss associated with a rapidly progressing band of exposed dead coral skeleton, not unlike white plague. It also can manifest in some species as multifocal areas of sloughing tissue that coalesce over time. *Dendrogyra cylindrus* is considered one of the most susceptible species to the disease and its Meandrinidae family members (*Meandrina meandrites* and *Dichoceonia stokesi*) are often the first species to show signs of this disease on the reef. Stony coral tissue loss disease, associated with recent hyperthermal stress events on the FRT, has greatly contributed to the near total collapse of Florida's *D. cylindrus* population (pers. obs.,

Neely and Lewis unpublished data) with the few remaining colonies located only in the Middle and Lower Keys.

The present study demonstrates a strong association between hyperthermal stress and disease in Florida's *D. cylindrus*. There appear to be cumulative effects of consecutive hyperthermal events causing increased disease prevalence and susceptibility impacting this species through April 2016. While WP is considered seasonal and typically associated with warmer water temperatures of summer and early fall in the Florida Keys, no previous monitoring of *D. cylindrus* has ever been conducted at this scale to determine background levels of disease in non-bleaching years for comparison or to fully understand the dynamics and impacts of disease on Florida's *D. cylindrus*. Through the present research, we have gained a better understanding of the impacts of hyperthermal stress associated with a changing climate, leading to the catastrophic collapse of Florida's *D. cylindrus* population, which may result in its local extinction within the next decade.

5.6 Figures and Tables

Figure 5.1. *Dendrogyra cylindrus* colony with active white plague

Classic signs of white plague in *D. cylindrus*, demonstrating the rapidly-advancing disease line of freshly-exposed white coral skeleton adjacent to apparently healthy live tissue (upper left, golden-brown). Dark, overgrown coral skeleton (lower right) is old dead, from months to years. Insert: 30cc sampling syringe for size reference, 5cc ~ 1cm. (photos: C Lewis)

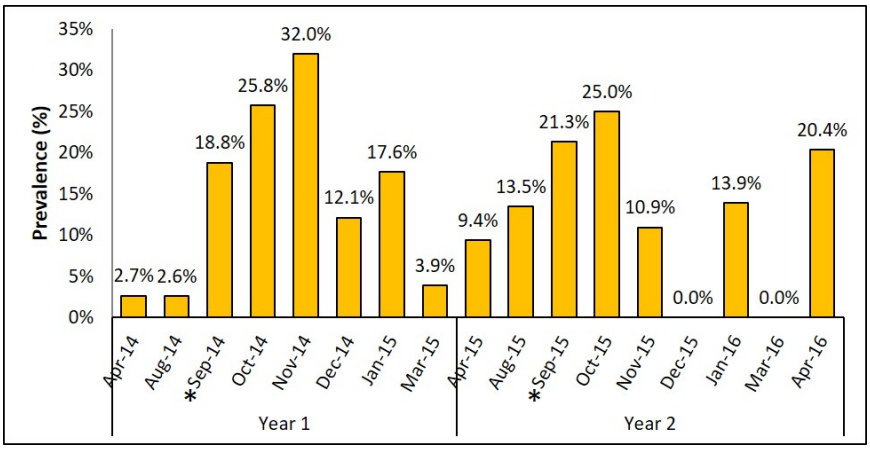
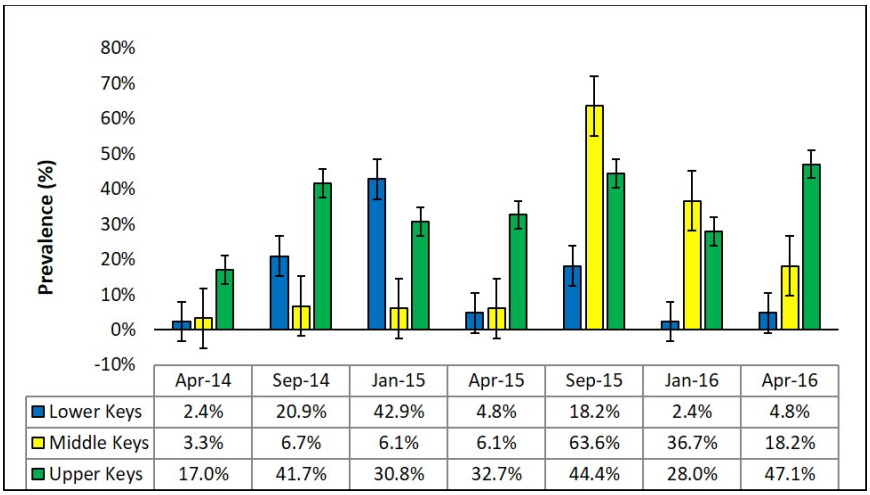


Figure 5.2. Prevalence of white plague in *Dendrogyra cylindrus* in the Florida Keys.

White plague (WP) prevalence was determined in *D. cylindrus* at 28 sites in the Florida Keys (Upper, Middle, and Lower regions combined) during two consecutive hyperthermal events (August-September 2014 and 2015). April 2014 was considered the initial baseline assessment of 414 colonies at 28 sites to determine 'normal' background disease levels. The remaining tri-annual assessments (September, January, April) evaluated 154 *D. cylindrus* colonies at these 28 sites. Additional assessments at a sub-set of three representative sites (Pickles, Coffins, Marker 32; n=61 total colonies) occurred in October, November, December 2014 and March, October, November, December 2015. (*) indicates hyperthermal bleaching events.



	Apr-14	Sep-14	Jan-15	Apr-15	Sep-15	Jan-16	Apr-16
Lower Keys	2.4%	20.9%	42.9%	4.8%	18.2%	2.4%	4.8%
Middle Keys	3.3%	6.7%	6.1%	6.1%	63.6%	36.7%	18.2%
Upper Keys	17.0%	41.7%	30.8%	32.7%	44.4%	28.0%	47.1%

Figure 5.3. Regional differences in white plague prevalence in *Dendrogyra cylindrus* in the Florida Keys.

White plague (WP) prevalence in *D. cylindrus* was compared between three regions of the Florida Reef Tract through two consecutive hyperthermal events on the Florida Reef Tract (2014 and 2015). Upper Keys (12 sites, 56 colonies), Middle Keys (6 sites, 32 colonies), Lower Keys (10 sites, 44 colonies). Bars indicate standard error.

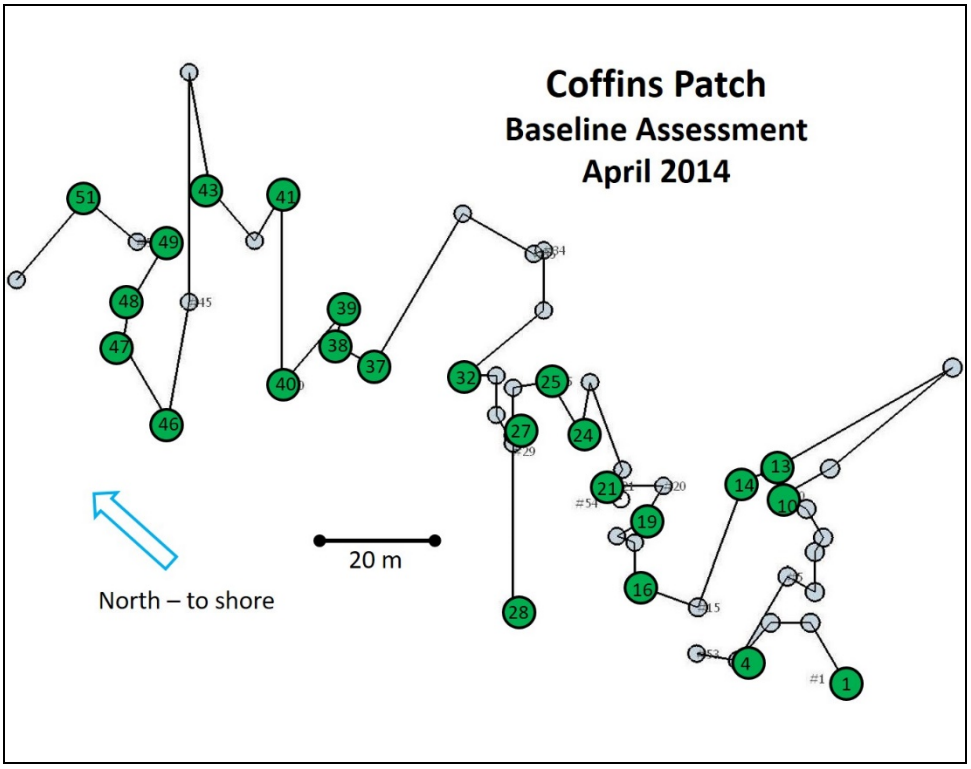


Figure 5.4. Spatial distribution of *Dendrogyra cylindrus* at the Coffins site (Middle Keys).

Fifty-five *D. cylindrus* colonies were mapped at the Coffins site, located within the Coffins Patch Special Preservation Area in the Florida Keys National Marine Sanctuary. This large stand of 55 *D. cylindrus* colonies is the dominant species along this patch reef. In April 2014, baseline assessment of all 55 colonies showed no signs of disease. Green circles indicate the colonies that were selected and marked for assessment (green = no disease). Small grey circles mark the location of other *D. cylindrus* colonies at this site not included in assessments.

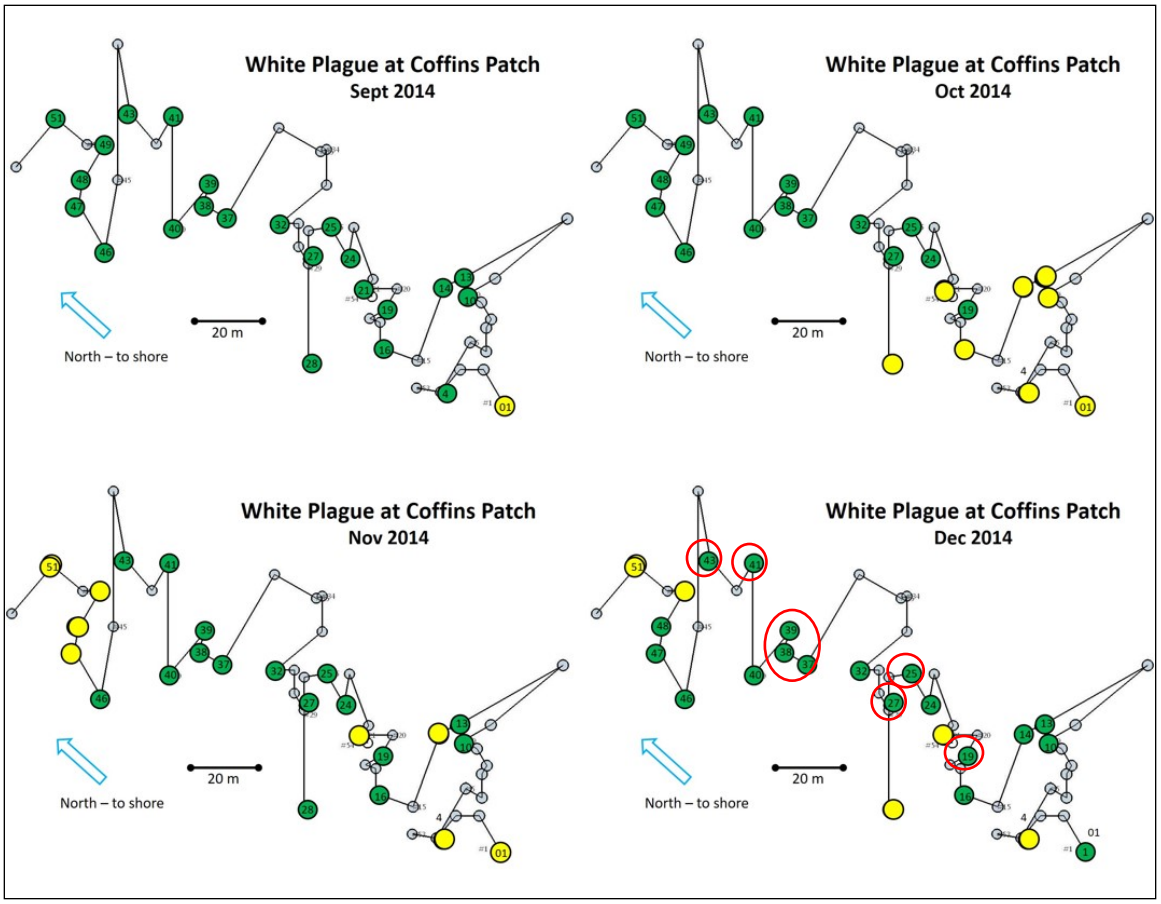


Figure 5.5. Monthly progression of white plague at the Coffins site from September through December 2014.

Twenty-four of 55 *D. cylindrus* were monitored for disease each month (green circles – assessed but no disease; small grey circles – unassessed colonies). White plague (WP) first appeared in a single colony (yellow circle, colony-04) at the southernmost extent of the Coffins site in September 2014, during the first hyperthermal bleaching event. All other *D. cylindrus* colonies appeared healthy. White plague progressively spread through this site in October, reaching the northernmost colonies by November 2014 and persisted in many colonies through December 2014. There were no signs of disease during January, March, and April 2015 assessments. Red circles indicate eight colonies that did not show signs of active disease in 2014 however, were all later identified as the same genet unique to this site *(38)*.

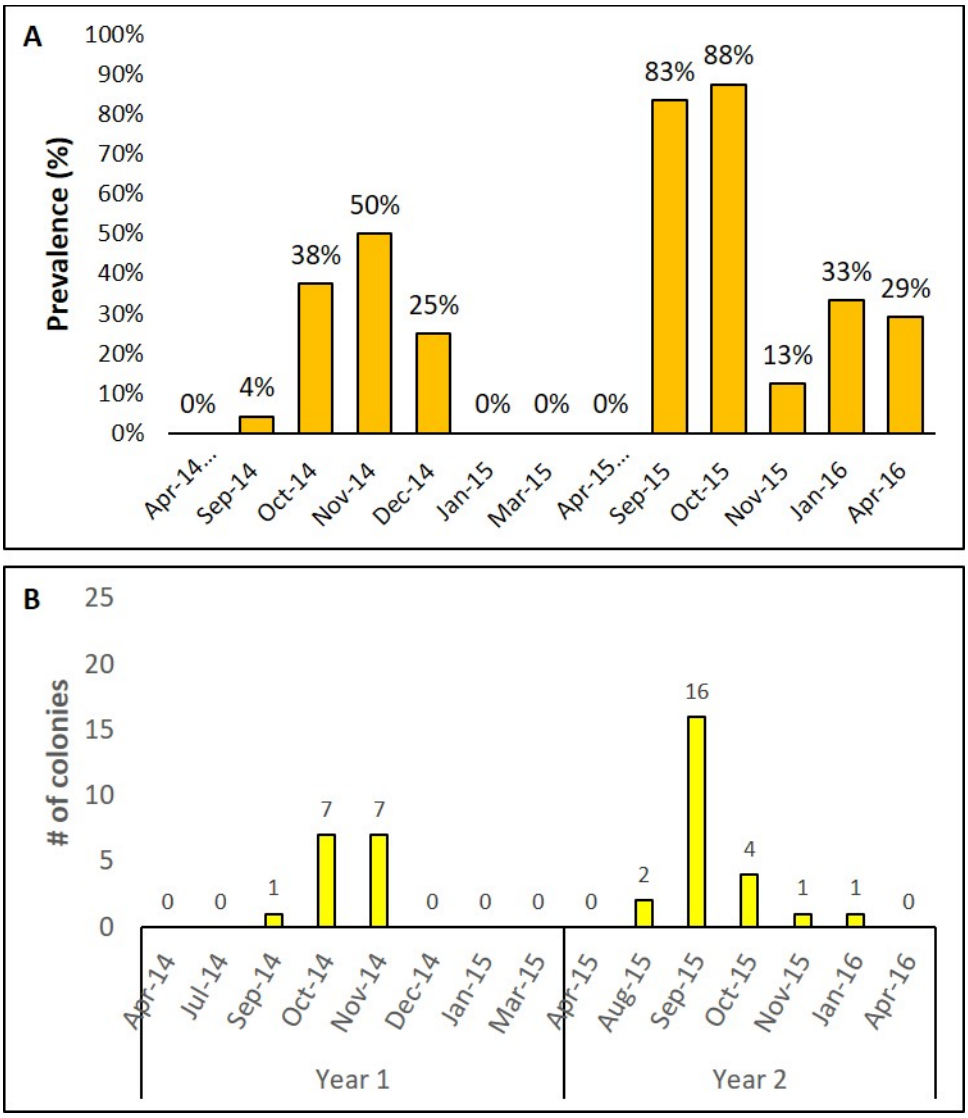


Figure 5.6. Prevalence and incidence of white plague in *Dendrogyra cylindrus* at Coffins site 2014-2016.

(A) Changes in prevalence of WP in *D. cylindrus* colonies at Coffins site (Middle Keys) on the FRT, beginning in April 2014, prior to the August-September hyperthermal event. *D. cylindrus* colonies at this site were assessed monthly during recovery from thermal bleaching. No active disease was observed from January through April 2015 at this site. Prevalence increased in September and October 2015 after the second hyperthermal event and persisted through Aril 2016. **(B)** Incidence of disease in 24 of the 55 marked colonies at the Coffins site was recorded through two hyperthermal events (new colonies with disease each month within each year). The first signs of disease were observed one month earlier (August 2015) during the second hyperthermal event. Incidence of disease was greater, and disease continued to appear in new colonies through January 2016.

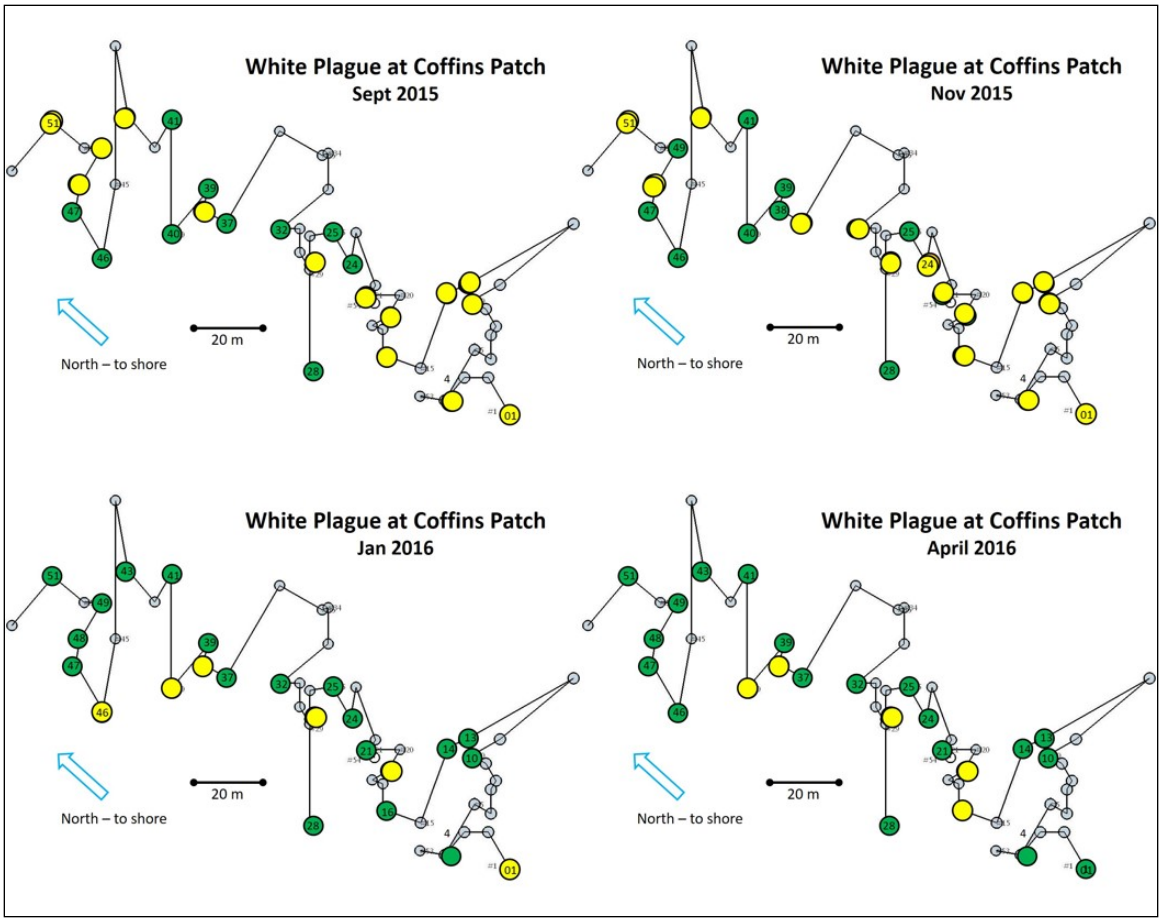


Figure 5.7. Progression of white plague at the Coffins site September 2015 through April 2016.

A second hyperthermal bleaching event occurred on the Florida Reef Tract in August-September 2015. Twenty of the 24 targeted *D. cylindrus* distributed throughout the site showed signs of active white plague (WP) at the September 2015 assessment (yellow circles). While some colonies appeared to recover by November 2015 and January 2016 (green circles), active WP persisted throughout the site through April 2016. Small grey circles – unassessed colonies, green circles – no disease at that assessment.

Table 5.1. Pearson's X^2 test *p*-values for disease prevalence in *Dendrogyra cylindrus* between years on the Florida Reef Tract 2014-2016.

Pearson's X^2 test of independence (α=0.05) for changes in disease prevalence in *D. cylindrus* on the FRT and by region, comparing months and years. Green *p*-values indicate significant increase in disease between months. Red *p*-values indicate significant decrease in disease between months.

Pearson's X^2 Test		***p*-values**			
df=1		All Regions n=130	Upper n=44	Middle n=33	Lower n=53
Apr-14	Apr-15	0.000	0.070	0.555	0.557
Apr-15	Apr-16	0.000	0.135	0.131	1.000
Apr-14	Apr-16	0.000	0.001	0.046	0.557
Sep-14	Sep-15	0.000	1.000	0.000	0.787
Jan-15	Jan-16	0.000	0.666	0.005	0.007

Table 5.2. Pearson's X^2 test *p*-values for changes in disease prevalence between months regions of the Florida Reef Tract 2014-2016.

Pearson's X^2 test of independence (α=0.05) for disease prevalence in *D. cylindrus* on the FRT and by region, comparing changes in disease between assessment months. Green *p*-values indicate significant increase in disease between months. Red *p*-values indicate significant decrease in disease between months.

Pearson's X^2 Test		***p*-values**			
df=1		all regions n=130	Upper (n=44)	Middle (n=33)	Lower (n=53)
Apr-14	Sep-14	0.001	0.006	0.554	0.008
Sep-14	Jan-15	0.980	0.257	0.922	0.256
Jan-15	Apr-15	0.093	0.833	1.000	0.329
Apr-15	Sep-15	0.001	0.235	0.000	0.918
Sep-15	Jan-16	0.001	0.095	0.032	0.017
Jan-16	Apr-16	0.447	0.048	0.099	0.557

Table 5.3. Pearson's X^2 test *p*-values comparing white plague prevalence in *Dendrogyra cylindrus* at Coffins site 2014-2016.

Pearson's X^2 test of independence (α=0.05) comparing white plague prevalence in 24 of 55 assessed *Dendrogyra cylindrus* at Coffins site (Middle Keys) between Year 1 and Year 2. Green *p*-values indicate significant increase in disease between months. Red *p*-values indicate significant decrease in disease between months.

Pearson's X^2 Test (df=1)		
Year 1	**Year 2**	***p*-values**
Apr-14	Apr-15	0.004
Sep-14	Sep-15	0.000
Oct-14	Oct-15	0.000
Nov-14	Nov-15	0.005
Jan-15	Jan-15	0.002
Apr-15	Apr-16	0.004

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CHAPTER 6: Temporal Dynamics of Black Band Disease Affecting Pillar Coral (*Dendrogyra cylindrus)* Following Two Consecutive Hyperthermal Events on the Florida Reef Tract.

Chapter six consists of the manuscript accepted for publication in *Coral Reefs*. The manuscript was submitted 8 April 2017; accepted for publication 8 January 2017; published online 30 January 2017 (DOI 10.1007/s00338-017-1545-1). This publication appears as published in the Appendix.

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6.1 Abstract

Black band disease (BBD) affects many coral species worldwide and is considered a major contributor to the decline of reef-building coral. On the Florida Reef Tract BBD is most prevalent during summer and early fall when water temperatures exceed 29 °C. BBD is rarely reported in pillar coral (*Dendrogyra cylindrus*) throughout the Caribbean, and here we document for the first time the appearance of this disease in this species on Florida reefs. The highest monthly BBD prevalence values in the *D. cylindrus* population were 4.7% in 2014 and 6.8% in 2015. In each year, BBD appeared immediately

following a hyperthermal bleaching event, which raises concern as hyperthermal seawater anomalies become more frequent.

6.2 Introduction

The increase in coral diseases in recent decades has largely been attributed to environmental stressors, especially increasing sea temperatures associated with climate change *(1-5)*. One such coral disease, black band disease (BBD), is now found worldwide, affecting 42 coral species *(6)*. In the Greater Caribbean, 19 scleractinian species, in particular massive reef-building forms, and six octocoral species are known to be susceptible *(7)*. The background prevalence of BBD throughout the Caribbean is typically between 1% and 4% at the community level but can be higher for individual species *(1, 8, 9)*.

BBD is a complex polymicrobial disease dominated by cyanobacteria and is characterized by a migrating mat or dark band that moves across infected corals at rates 3–10 mm d^{-1} *(10)*. Sulfide produced within the band acts synergistically with the cyanotoxin microcystin to cause lysis of coral tissue *(11, 12)*. Environmental factors, including elevated temperatures, nutrients, and light intensity, affect the rate of BBD progression *(13-15)*. Although it can persist year-round in some areas of the Caribbean *(8, 16)*, on Florida reefs, BBD tends to be seasonal and most active during the warmer summer and early fall months, especially once temperatures exceed 29 °C *(10, 17-19)*.

In 2014 and 2015 the Florida Reef Tract (FRT) experienced sustained hyperthermal sea temperatures that exceeded the FRT bleaching index of 30.5 °C *(20)* for 8 and 11 weeks respectively (Fig. 6.1). Mean monthly temperatures during these two events exceeded the 10-yr mean monthly temperatures (±1 SD) recorded at Molasses

Reef (MLRF-1) from 2003 to 2013. NOAA's Coral Reef Watch sea surface temperature models reported five degree heating weeks for the summers of 2014 and 2015 on reefs of the Florida Keys (http://coralreefwatch.noaa.gov). Both hyperthermal anomalies resulted in consecutive bleaching events *(21)*, severely affecting many species of coral along the FRT, including the pillar coral *Dendrogyra cylindrus* (Ehrenburg, 1834).

Dendrogyra cylindrus is a slow-growing gonochoric broadcast-spawner typically found in low abundance throughout its Caribbean range. While this species is rarely considered an important reef builder, its unique columnar growth form provides important vertical structure and habitat complexity. It was categorized as 'vulnerable' in 2008 under the IUCN Red List criteria because of its susceptibility to bleaching, disease (especially white plague), and habitat degradation *(22)*. *Dendrogyra cylindrus* was federally listed in the US as 'threatened' in 2014 *(23)* due to its rare occurrence and rapidly declining, critically fragmented population. Surveys of the *D. cylindrus* population along the FRT in 2013–2014 documented fewer than 600 live colonies at 106 sites, with two-thirds of these sites consisting of single colonies, often separated by tens of kilometers, contributing to low recruitment success. *Dendrogyra cylindrus* was once anecdotally reported to have BBD throughout its Caribbean range *(24)*. Here we document for the first time the occurrence of BBD in *D. cylindrus* along the FRT in the context of the hyperthermal bleaching events of 2014 and 2015.

6.3 Materials and Methods

Between April 2014 and April 2016, 163 *D. cylindrus* colonies at 28 sites located along the FRT (Fig. 6.2) were assessed tri-annually (April/September/January) for health status. Data loggers (Onset HOBO Inc., Bourne, MA, USA) were secured at each

assessment site to record hourly temperatures. During the monitoring period, two hyperthermal bleaching events occurred (August/September 2014 and 2015). After the 2014 event, 64 of the 163 colonies were selected from three of the 28 geographically stratified sites (Pickles, Coffins, and Marker 32; Fig. 6.2) to document the dynamics of bleaching recovery. Recovery monitoring was carried out in October, November, and December (2014 and 2015) and March 2015 to quantitatively track bleaching, disease, and recovery. Assessments tracked individual *D. cylindrus* colonies for bleaching and recovery using the CoralWatch Coral Health Chart *(25)*. Disease presence and progression were also documented for each colony. The presence of bleaching and disease were noted, but not quantified, for other coral species at each site. Mean daily and monthly water temperatures and number of days per month that mean daily sea temperatures exceeded 29.0 °C and 30.5 °C were calculated for each site. Archived temperature data for 2004–2013 at Molasses Reef C-MAN station MLRF1 (25.012 N 80.376 W; NOAA National Data Buoy Center; http://www.ndbc.noaa.gov/station_page.php?station=mlrf1) were used to calculate 10-yr mean monthly sea temperatures.

6.4 Results and Discussion

Active BBD was first observed on a single *D. cylindrus* colony at an Upper Florida Keys site in August 2014 (marked with a star in Fig. 6.2). During the September tri-annual survey, we observed three additional colonies with BBD among the 28 sites. Subsequent, repeated monitoring in 2014 and 2015 documented increased BBD prevalence in both years (Fig. 6.3), with maximum values of 4.7% (7 of 163 colonies) in 2014 and 6.7% (11 of 163 colonies) in 2015. BBD was observed to progress 15 cm in

five weeks (October to November 2014) on one closely monitored colony (Fig. 6.4). Monitoring also revealed that nearly all 163 *D. cylindrus* colonies were severely bleached in 2014, scoring C1 on the Coral Watch Coral Health Chart, while in 2015, 64% of colonies were severely bleached and the remainder were partially bleached or pale. Bleaching events occurred during each year, in both cases two weeks after the temperature maximum for that year was reached (Fig. 6.1). During the larger tri-annual assessment conducted in January 2015, active BBD was no longer evident on any *D. cylindrus* colony; however, one *Montastraea cavernosa* colony at Coffins remained infected. BBD was not observed in *D. cylindrus* or any other susceptible species in March, April, May or December 2015.

Background prevalence of BBD throughout the Caribbean is typically <4% in susceptible species and is normally present during the warmer months of each year *(1, 8, 9)*. Because BBD is rarely, if ever, reported in *D. cylindrus* throughout the wider Caribbean *(24)*, this species may be relatively resistant to BBD. BBD was not observed in *D. cylindrus* during the initial fieldwork to locate live colonies at 106 sites on the FRT leading up to this monitoring effort (summer 2013 to spring 2014). 2013 was a non-bleaching year on these reefs. The quantitative documentation of zero BBD signs on the 163 *D. cylindrus* colonies identified in 2013, together with the fact that prior to 2014 there were no reports of BBD on *D. cylindrus* on these reefs while BBD was reported in other species, may serve as a tentative baseline for BBD in this species on the FRT. Furthermore, the increase from zero BBD in the *D. cylindrus* population in 2013 compared to BBD prevalence values of 4.7% and 6.7% in the following years, immediately after two hyperthermal events, suggests a relationship between anomalously

elevated water temperatures (and associated thermal stress), bleaching, and disease for *D. cylindrus*. Differences in other water quality parameters at each site, not measured in this study, may also be driving the apparent susceptibility to BBD of *D. cylindrus*. Additionally, coral animal genotypes, the *D. cylindrus* microbiome, and the microbiota in the surrounding environment may play roles in differential BBD susceptibility and/or resistance.

The impacts of bleaching are known to include a nutritionally compromised health status of the affected corals due to the loss of their *Symbiodinium*-derived nutrients *(26, 27)*. Potential synergy of thermal and nutritional stress may have contributed to the vulnerability of *D. cylindrus* to BBD pathogens *(1, 28, 29)*. However, it was not possible to separately address these stressors in a natural setting. Additionally, prolonged elevated sea temperatures may have increased the pathogenicity of the polymicrobial community associated with BBD. Enhanced pathogenicity occurs in these and many other marine microbes at temperatures exceeding 29 °C *(10, 17-19)*.

BBD has rarely been reported in *D. cylindrus*, perhaps due to the relatively low abundance of this little-studied species throughout its Caribbean range or perhaps also due to its relative resistance to this particular disease. This study presents the results of the first quantitative monitoring of *D. cylindrus* on the FRT for health, bleaching status, and disease, and includes the first report of BBD for this species in this region. This data set is the first step in potential management of this recently listed threatened species. The observed persistent advance of BBD (progressing up to 0.5 cm d^{-1}; Fig. 5.4) on this slow-growing coral, the pattern of increased BBD prevalence following two consecutive hyperthermal events, and escalating environmental stressors due to predicted climate

change, all suggest that BBD may play a more prominent role in the decline of *D. cylindrus* and other susceptible reef-building species, lending urgency for management and restoration efforts.

Acknowledgements

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6.5 Figures and Tables

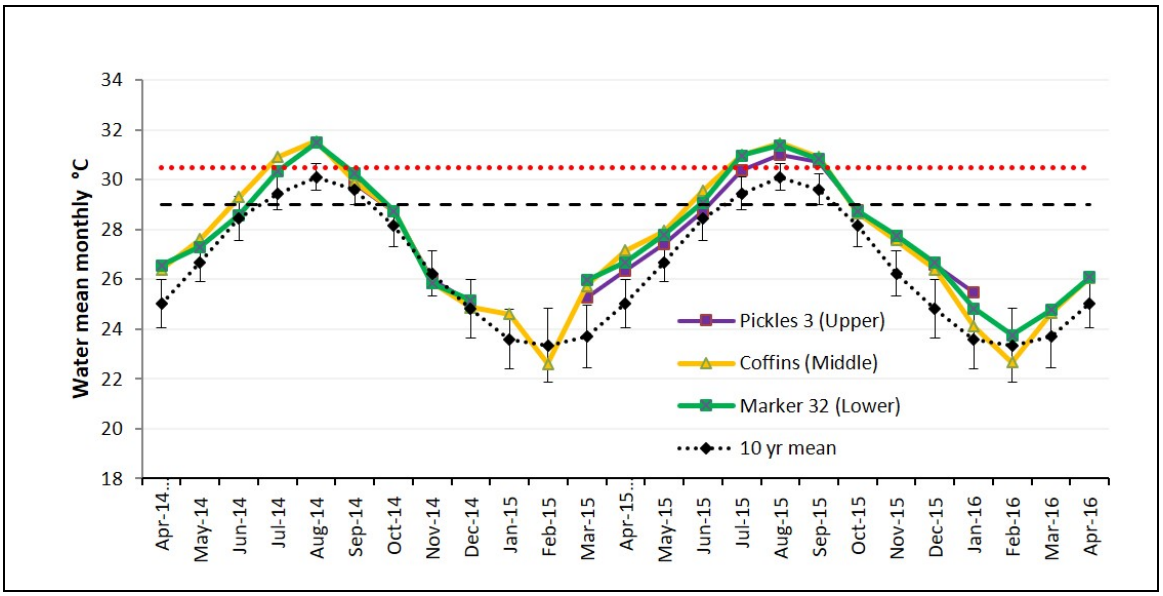

Figure. 6.1 Mean monthly sea temperature profiles from April 2014 to April 2016 at three sites: Pickles, Coffins, and Marker 32.

Dotted black line represents mean monthly water temperatures (± SD) recorded at Molasses Reef 2003–2013 (National Data Buoy Center, MLRF1). *Dotted red line* indicates bleaching threshold for the Florida Reef Tract (30.5 °C). *Dashed black line* indicates optimal temperature for black band disease microbial community (29.0 °C). Gaps in data are due to lost or broken HOBO data loggers.

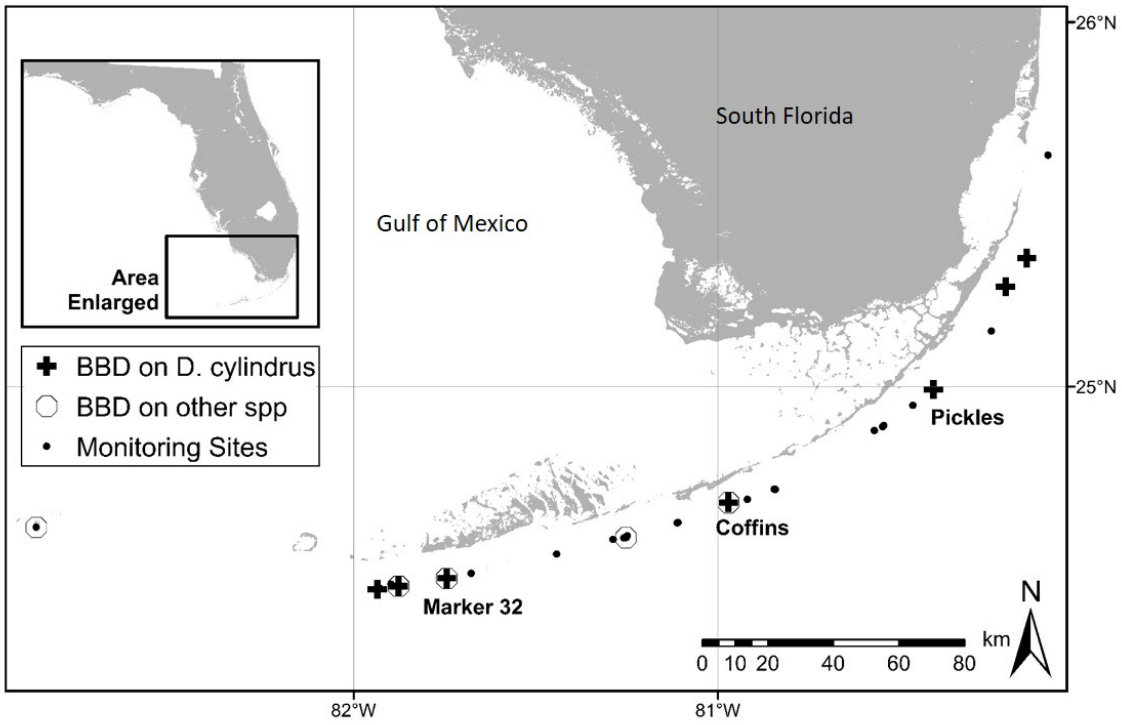

Fig. 6.2 *Dendrogyra cylindrus* tri-annual assessment sites along the Florida Reef Tract.

Black dots: *D. cylindrus* sites with no observations of black band disease (BBD). *Black star*; site where BBD was first observed in *D. cylindrus*. *Black crosses*: all other sites where BBD was observed on *D. cylindrus* between April 2014 and April 2016 (includes three bleaching recovery sites: Marker 32, Coffins, and Pickles). *Open circles*: sites where BBD was observed on other coral species at *D. cylindrus* assessment sites.

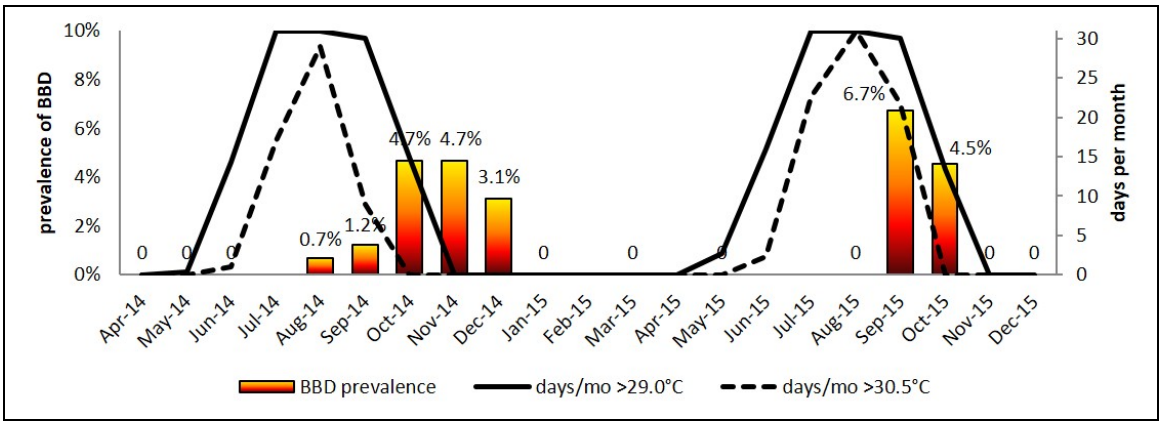

Fig. 6.3 Prevalence of black band disease (BBD) in *Dendrogyra cylindrus* on the Florida Reef Tract (FRT) from April 2014 to December 2015.

Tri-annual assessments (*) occurred in April/May, September 2014, and January, April/May and September 2015. Additional assessments at three sites occurred in October, November, December 2014 and March, October, November, December 2015 to document the dynamics of bleaching recovery after the hyperthermal events in August/September 2014 and 2015. *Solid black line*: number of days per month that mean daily sea temperatures exceeded 29.0 °C, the optimal temperature for active BBD. *Dashed black line*: number of days per month that mean daily sea temperatures exceeded 30.5 °C, the bleaching threshold for the FRT.

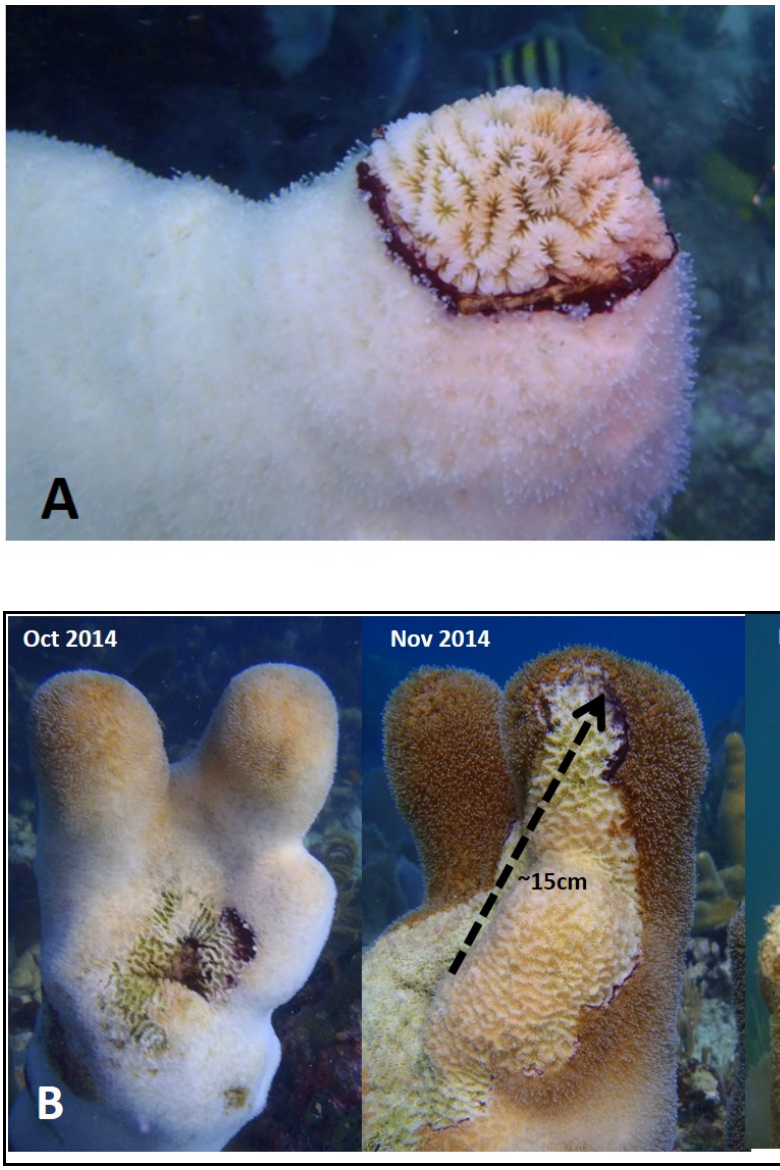

Fig. 6.4 Black band disease (BBD) in *Dendrogyra cylindrus*.

(A) BBD on bleached *D. cylindrus* demonstrating the characteristic dark band and adjacent freshly-denuded coral skeleton. **(B)** Progression of BBD on a single *D. cylindrus* pillar—BBD was not observed on this colony in September 2014, although the colony was severely bleached, but first appeared in October 2014, displaying a BBD lesion approximately 2 cm in diameter. By November 2014, the active band had progressed upwards approximately 15 cm and expanded laterally (calculated 0.5 cm d^{-1}). Four weeks later, the active band had slowly progressed approximately 5 cm to the top of the pillar. Active BBD was no longer visible in January 2015 but reoccurred on the same pillar in September 2015. Photos: C Lewis.

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CHAPTER 7: Final Conclusions and Synthesis

The research aim of this dissertation was to gain better understanding of the coral holobiont partners, specifically the coral animal and the mutualistic photosynthetic algal, and their contributions to the processes of bleaching, recovery, and disease resistance. The present study focused on the threatened pillar coral, *Dendrogyra cylindrus*, on the Florida Reef Tract (FRT). Through rigorous field monitoring and sampling, the cumulative effects of hyperthermal stress were evaluated in the context of two consecutive hyperthermal events which occurred in the summers of 2014 and 2015 on the FRT.

Embarking on a collaborative effort to map Florida's *D. cylindrus* and determine the population structure, Chan et al. *(1)* established the genetic framework of the *D. cylindrus* population along the FRT, which allowed me to associate coral host genotypes with observations of bleaching, recovery, and disease processes. Although coral animal genotypes primarily consisted of single colonies representing single unique genets at most sites, it was observed that some genets exhibited partial bleaching resistance and while other genets showed resistance to disease (i.e., white plague) either as a result of inherent variability or perhaps adaptation to thermal stress at the genomic level of the coral animal *(2, 3)*. It was also hypothesized that resistance observed in isolated colonies was related to site-specific environmental factors or individual adaptations to unique micro-habitats at each site, rather than host genotype. Alternative hypotheses were that these lone *D. cylindrus* colonies, often separated by many kilometers from con-specifics, were not exposed to sufficiently high pathogen loads in the water column to cause disease or that these colonies did not experience the same thermal stress that caused severe

bleaching at other sites along the FRT. Further sequencing of archived *D. cylindrus* tissue samples from these sites may reveal changes in the Symbiodiniaceae community to also explain these observations.

Regardless, the potential for thermal tolerance and/or disease resistance in some *D. cylindrus* has important implications for future restoration efforts of this species. Propagation of these and other genets of interest through asexual fragmentation will allow us to verify bleaching or disease resistance through controlled *ex situ* experiments. Future propagation and out-planting of resilient genets offers some hope of restoring *D. cylindrus* to the Florida Reef Tract in the Anthropocene. Ongoing efforts to enhance sexual recombination of resilient genets using controlled breeding will result in new genets perhaps more suited to survival under future environmental conditions and guide restoration efforts towards establishing a self-sustaining *D. cylindrus* population on Florida's reefs.

Understanding the resilience of the endosymbiotic algal community within *D. cylindrus* is also vital to understanding the complex coral holobiont. The present research revealed far greater diversity in the cryptic Symbiodiniaceae assemblages than previously known *(4)*, which has implications as a potential source for stress-tolerant endosymbiont species or strains capable of allowing the coral holobiont to acclimatize and adapt to changing conditions *(5-10)*. This capability was demonstrated by the site-specific switch from the host-specialist symbiont, *Breviolum dendrogyrum*, to the host-generalist, *B. meandrinium*, which was associated with observations of bleaching resistance when exposed to consecutive hyperthermal events *(4)*. At this time, neither of these algal endosymbiont species have been successfully cultured to allow further exploration of the

tolerances they may bring to the partnership. To that end, further attempts are currently being made to culture these algal endosymbiont species. It is also unknow what trade-offs in physiological processes may occur with a switch in dominant species. Endosymbiont communities in *D. cylindrus* did not return to pre-bleaching baseline abundances prior to the onset of the second hyperthermal event, supporting other findings that have shown it may require several years for the coral holobiont to recovery from a single hyperthermal event *(11-13)*. Under sustained disturbance events, such as annually recurring hyperthermal events, new thermally-tolerant holobiont assemblages may be longer-lived and maintain higher relative proportions within the Symbiodiniaceae community as the period of less-stressful environmental conditions between disturbances becomes shorter. Replacing the closely co-evolved symbiosis between the coral host *Dendrogyra cylindrus* and its algal symbiont partner *Breviolum dendrogyrum*, with another more thermally-tolerant photosynthetic algal species, may have long-term detrimental impacts on growth, reproduction and/or disease resistance. Understanding the physiological tolerances and limitations of the tightly co-evolved *D. cylindrus/B. dendrogyrum* partnership, as well as the sibling species *B. meandrinium*, will help to clarify their roles in bleaching and disease resistance and resilience. With the recent alarming decline and collapse of Florida's *D. cylindrus* population since 2014, also comes the reality that we may be witnessing the local extinction of not one but two unique species (*D. cylindrus* and *B. dendrogyrum*), within the next five years.

Hyperthermal bleaching events are projected to become annual occurrences by 2050, or sooner in some regions *(14-16)*. It would appear from this body of work that

Florida's reefs may already be experiencing this annual phenomenon of thermal stress and bleaching. Persistent hyperthermal events can cause collateral damage to biological, physiological, and immunological functions of the coral holobiont *(17-20)* and have been linked to increases in disease *(21-30)* and enhanced pathogenicity *(31-33).* Establishing a baseline disease prevalence in Florida's *D. cylindrus* population, such as the April 2014 population assessments in the present study, is critical to managing this species, although it is likely that *D. cylindrus* has already experienced decades of chronic environmental stress. White plague was first described in the 1970's *(34)* but emerged again in 1995 in the Upper Keys and spread throughout the reef tract affecting 16 reef-building species, including *D. cylindrus (35, 36).* In the present study, the Upper Keys sites initially had a higher prevalence of white plague than the other two regions which may be related to land-based sources of pollution (i.e., proximity to South Florida's rapidly expanding population), but increased prevalence was observed in all regions following the first hyperthermal event in August/September 2014, including new susceptibility to black band disease *(37).* The cumulative effects of annual hyperthermal events was demonstrated by further increases in disease prevalence in the *D. cylindrus* population following the second hyperthermal event in 2015, including an outbreak of white plague at a Middle Keys site. These data suggest that the ability of the coral holobiont to acclimatize to thermal stress was outpaced by the compounded damages caused by recurrent hyperthermal events. It is likely that the hyperthermal events of 2014 and 2015 acted synergistically with decades of declining water quality on the Florida Reef Tract, associated with the increase in land use development and population of South Florida and the Florida Keys since the 1980's *(38, 39).* Inadequate sewage treatment, agricultural and

urban run-off, and reduced flow of fresh water to Florida Bay have all contributed to the chronic stress on the reef ecosystem.

The current research presented here has contributed significantly to the body of knowledge for this unique and little-studied coral species, *Dendrogyra cylindrus*. It has also advanced a better understanding of the complex coral holobiont and its response to hyperthermal stress, bleaching, recovery and disease processes, applicable to corals worldwide. Future work will include the characterization of the microbial community associated with *D. cylindrus* to gain insights into how these communities respond to bleaching and disease processes.

Many aspects of the present research have led directly to management decisions to preserve and restore this critically threatened coral. The lack of smaller *D. cylindrus* colonies within the population signaled decades of poor reproductive success and recruitment and the conclusion that Florida's population of *D. cylindrus* is likely already reproductively extinct as a result of the declining and fragmented population. Regular monitoring and assessments beginning in 2014 revealed the dramatic decline and accelerated loss in the population following consecutive hyperthermal bleaching events in 2014 and 2015. With science to support recommendations to management, the *D. cylindrus* Genetic Rescue Project was proposed and implemented in January 2016, creating a living genetic bank in temperature-controlled *ex situ* nurseries (Keys Marine Laboratory, Florida Aquarium, Mote Marine Laboratory, Coral Restoration Foundation). Collaboration with partners at NOAA's National Ocean Service Coral Health and Disease facility in Charleston, NC, led to successful disease treatments (white plague) in

the race to preserve *D. cylindrus* genets from extinction. With the approval of state and federal agencies (FWC, NOAA, FKNMS, FDA Center for Veterinary Medicine/Minor Use Minor Species,), additional collaborations have led to the implementation of pilot disease treatment trials in the wild *D. cylindrus* population, in hopes of forestalling the local extinction of this species.

Concurrent with the 2014 and 2015 hyperthermal events, a new disease (scleractinian or stony coral tissue loss disease, SCTLD) appeared on Florida's reef-building corals. The disease of unknown etiology now affects 22 of the 43 reef-building coral species found on the Florida Reef Tract, spreading quickly and with high mortality. *Dendrogyra cylindrus* has proven to be one of the most-susceptible of these species and often the canary-in-the-mine-shaft marking the progression of SCTLD along the Florida Reef Tract. Successful disease treatments first developed for *D. cylindrus* as a direct result of the present dissertation research, have been further tested in other reef-building coral species affected by SCTLD and approved for application on Florida's reefs since May 2018 with encouraging success rates.

In large part due to the comprehensive information on the population genetics of Florida's *D. cylindrus (1)*, we have been able to rescue a total of 88 of the original 162 Florida genets and currently house nearly 400 fragments at five different facilities. Asexual propagation techniques are being tested and refined for *D. cylindrus* to prepare for future restoration efforts. Genets of special interest, particularly bleaching and/or disease resistance, will be further tested in controlled laboratory settings. New research in the coming months will explore genotypic differences in growth rates *ex situ*,

contributing valuable insights for restoration efforts. With so many *D. cylindrus* genets safely held in *ex situ* seawater systems, it has been possible to facilitate successful spawning, fertilization and settlement at the Keys Marine Laboratory, resulting in more than 150 new *D. cylindrus* recruits in 2018, now held at Florida Aquarium Center for Conservation (Apollo Beach, FL) to add to the genetic diversity of the genetic bank. In conjunction with the 2018 spawning event, partners from the South-East Zoo Alliance for Conservation (SEZARC) attempted cryopreservation of *D. cylindrus* sperm for the first time in 2018. As these techniques are refined it is hoped that cryopreserved sperm can be banked for the future to perpetuate *D. cylindrus* and other coral species.

From the present body of work comes the hope for the future of this unique and iconic species we affectionately refer to as ‘unicorns’, referring to the columnar growth form as well as their uncommon and elusive nature. We have become the Guardians of the Last Unicorns.

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APPENDIX: Publications

Publication 1: Temporal dynamics of black band disease affecting pillar coral (*Dendrogyra cylindrus)* following two consecutive hyperthermal events on the Florida Reef Tract

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Publication 2: Recurring Episodes of Thermal Stress Shift the Balance from a Dominant Host-Specialist to a Background Host-Generalist Zooxanthella in the Threatened Pillar Coral, *Dendrogyra cylindrus*

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NOTE

Temporal dynamics of black band disease affecting pillar coral (*Dendrogyra cylindrus*) following two consecutive hyperthermal events on the Florida Reef Tract

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Abstract Black band disease (BBD) affects many coral species worldwide and is considered a major contributor to the decline of reef-building coral. On the Florida Reef Tract BBD is most prevalent during summer and early fall when water temperatures exceed 29 °C. BBD is rarely reported in pillar coral (*Dendrogyra cylindrus*) throughout the Caribbean, and here we document for the first time the appearance of the disease in this species on Florida reefs. The highest monthly BBD prevalence in the *D. cylindrus* population were 4.7% in 2014 and 6.8% in 2015. In each year, BBD appeared immediately following a hyperthermal bleaching event, which raises concern as hyperthermal seawater anomalies become more frequent.

Keywords Black band disease · Coral bleaching · *Dendrogyra cylindrus* · Florida Reef Tract

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Introduction

The increase in coral diseases in recent decades has largely been attributed to environmental stressors, especially increasing sea temperatures associated with climate change (Croquer and Weil 2009; Harvell et al. 2009; McLeod et al. 2010; Hoegh-Guldberg 2012; Randall and van Woesik 2015). One such coral disease, black band disease (BBD), is now found worldwide, affecting 42 coral species (Sutherland et al. 2004). In the Greater Caribbean, 19 scleractinian species, in particular massive reef-building forms, and six octocoral species are known to be susceptible (Weil et al. 2006). The background prevalence of BBD throughout the Caribbean is typically between 1 and 4% at the community level but can be higher for individual species (Kuta and Richardson 1996; Bruckner and Bruckner 1997; Croquer and Weil 2009).

BBD is a complex polymicrobial disease dominated by cyanobacteria and is characterized by a migrating mat or dark band that moves across infected corals at rates 3–10 mm d^{-1} (Rützler and Santavy 1983). Sulfide produced within the band acts synergistically with the cyanotoxin microcystin to cause lysis of coral tissue (Viehman et al. 2006; Richardson et al. 2009). Environmental factors, including elevated temperatures, nutrients, and light intensity, affect the rate of BBD progression (Kaczmarsky et al. 2005; Voss and Richardson 2006; Boyett et al. 2007). Although it can persist year round in some areas of the Caribbean (Edmunds 1991; Kuta and Richardson 1996), on Florida reefs, BBD tends to be seasonal and most active during the warmer summer and early fall months, especially once temperatures exceed 29 °C (Rützler and Santavy 1983; Kushmaro et al. 1997; Ben-Haim et al. 2003; Richardson and Kuta 2003).

In 2014 and 2015 the Florida Reef Tract (FRT) experienced sustained hyperthermal sea temperatures that exceeded the FRT bleaching index of 30.5 °C (Manzello et al. 2007) for 8 and 11 weeks, respectively (Fig. 1). Mean monthly temperatures during these two events exceeded the 10-yr mean monthly temperatures (±1 SD) recorded at Molasses Reef (MLRF-1) from 2003 to 2013. NOAA's Coral Reef Watch sea surface temperature models reported five degree heating weeks for the summers of 2014 and 2015 on reefs of the Florida Keys (http://coralreefwatch.noaa.gov). Both hyperthermal anomalies resulted in consecutive bleaching events (Precht et al. 2016), severely affecting many species of coral along the FRT, including the pillar coral *Dendrogyra cylindrus* (Ehrenburg, 1834).

Dendrogyra cylindrus is a slow-growing gonochoric broadcast spawner typically found in low abundance throughout its Caribbean range. While this species is rarely considered an important reef builder, its unique columnar growth form provides important vertical structure and habitat complexity. It was categorized as 'vulnerable' in 2008 under the IUCN Red List criteria because of its susceptibility to bleaching, disease (especially white plague), and habitat degradation (Aronson et al. 2008). *Dendrogyra cylindrus* was federally listed in the US as 'threatened' in 2014 (NOAA Fisheries 2014) due to its rare occurrence and rapidly declining, critically fragmented population. Surveys of the *D. cylindrus* population along the FRT in 2013–2014 documented fewer than 600 live colonies at 106 sites, with two-thirds of these sites consisting of single colonies, often separated by tens of kilometers, contributing to low recruitment success. *Dendrogyra cylindrus* was once anecdotally reported to have BBD throughout its Caribbean range (Ward et al. 2006). Here we document for the first time the occurrence of BBD in *D. cylindrus* along the FRT in the context of the hyperthermal bleaching events of 2014 and 2015.

Materials and methods

Between April 2014 and April 2016, 163 *D. cylindrus* colonies at 28 sites located along the FRT (Fig. 2) were assessed tri-annually (April/September/January) for health status. Data loggers (Onset HOBO Inc., Bourne, MA, USA) were secured at each assessment site to record hourly temperatures. During the monitoring period, two hyperthermal bleaching events occurred (August/September 2014 and 2015). After the 2014 event, 64 of the 163 colonies were selected from three of the 28 geographically stratified sites (Pickles, Coffins, and Marker 32; Fig. 2) to document the dynamics of bleaching recovery. Recovery monitoring was carried out in October, November, and December (2014 and 2015) and March 2015 to quantitatively track bleaching, disease, and recovery. Assessments tracked individual *D. cylindrus* colonies for bleaching and recovery using the CoralWatch Coral Health Chart (Siebeck et al. 2006). Disease presence and progression were also documented for each colony. The presence of bleaching and disease were noted, but not quantified, for other coral species at each site. Mean daily and monthly water temperatures and number of days per month that mean daily sea temperatures exceeded 29.0 and 30.5 °C were calculated for each site. Archived temperature data for 2004–2013 at Molasses Reef C-MAN station

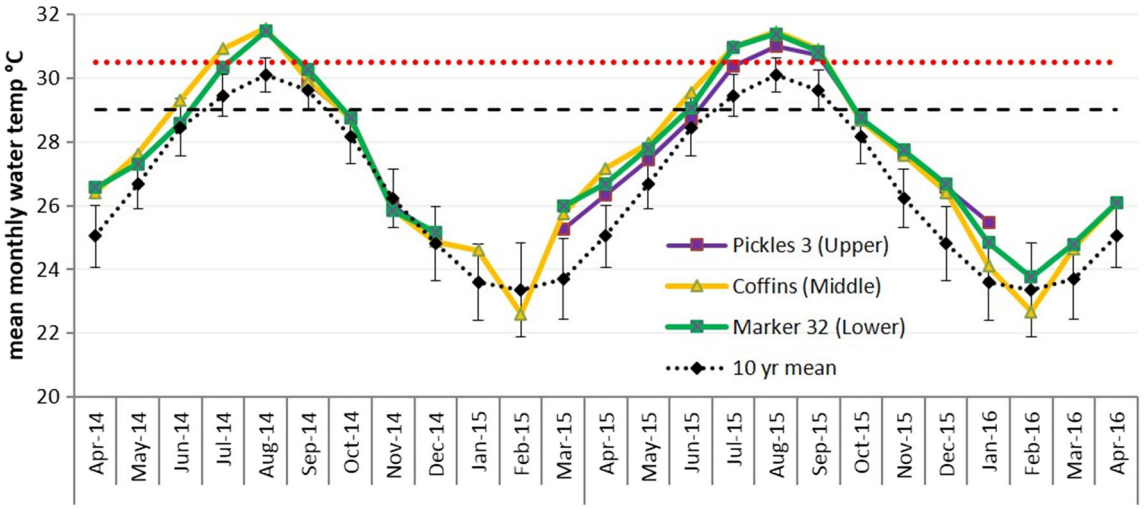

Fig. 1 Mean monthly sea temperature profiles from April 2014 to April 2016 at three sites: Pickles, Coffins, and Marker 32. *Dotted black line* represents mean monthly water temperatures (±SD) recorded at Molasses Reef 2003–2013 (National Data Buoy Center, MLRF1). *Dotted red line* indicates bleaching threshold for the Florida Reef Tract (30.5 °C). *Dashed black line* indicates optimal temperature for black band disease microbial community (29.0 °C). Gaps in data are due to lost or broken HOBO data loggers

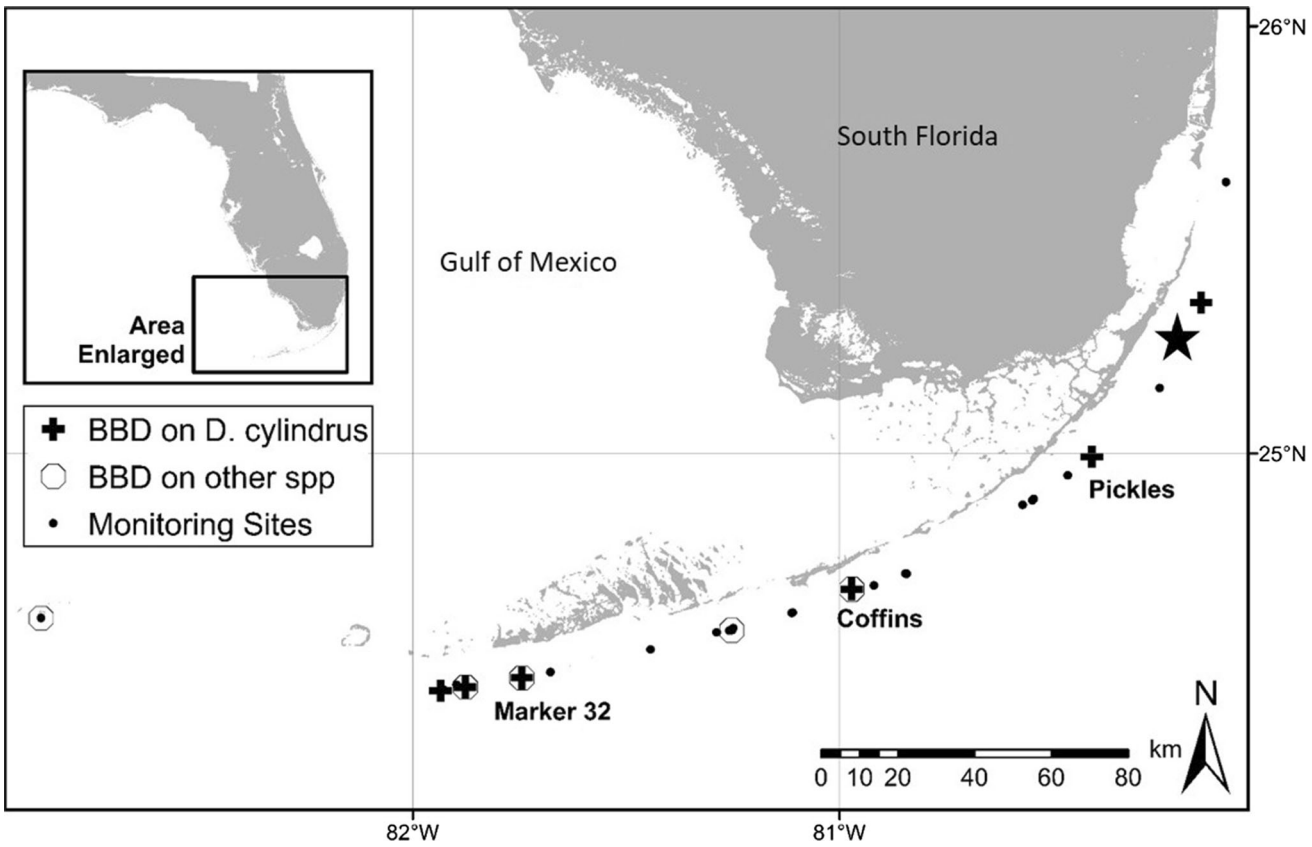

Fig. 2 *Dendrogyra cylindrus* tri-annual assessment sites along the Florida Reef Tract. *Black dots*: *D. cylindrus* sites with no observations of black band disease (BBD). *Black star*; site where BBD was first observed in *D. cylindrus*. *Black crosses*: all other sites where BBD was observed on *D. cylindrus* between April 2014 and April 2016 (includes three bleaching recovery sites: Marker 32, Coffins, and Pickles). *Open circles*: sites where BBD was observed on other coral species at *D. cylindrus* assessment sites

MLRF1 (25.012 N 80.376 W; NOAA National Data Buoy Center; http://www.ndbc.noaa.gov/station_page.php?station=mlrf1) were used to calculate 10-yr mean monthly sea temperatures.

Results and discussion

Active BBD was first observed on a single *D. cylindrus* colony at an Upper Florida Keys site in August 2014 (marked with a star in Fig. 2). During the September tri-annual survey, we observed three additional colonies with BBD among the 28 sites. Subsequent, repeated monitoring in 2014 and 2015 documented increased BBD prevalence in both years (Fig. 3), with maximum values of 4.7% (7 of 163 colonies) in 2014 and 6.7% (11 of 163 colonies) in 2015. BBD was observed to progress 15 cm in five weeks (October to November 2014) on one closely monitored colony (Fig. 4). Monitoring also revealed that nearly all 163 *D. cylindrus* colonies were severely bleached in 2014, scoring C1 on the Coral Watch Coral Health Chart, while in 2015, 64% of colonies were severely bleached and the remainder were partially bleached or pale. Bleaching events occurred during each year, in both cases two weeks after the temperature maximum for that year was reached (Fig. 1). During the larger tri-annual assessment conducted in January 2015, active BBD was no longer evident on any *D. cylindrus* colony; however, one *Montastraea cavernosa* colony at Coffins remained infected. BBD was not observed in *D. cylindrus* or any other susceptible species in March, April, May or December 2015.

Background prevalence of BBD throughout the Caribbean is typically <4% in susceptible species and is normally present during the warmer months of each year (Kuta and Richardson 1996; Bruckner and Bruckner 1997; Croquer and Weil 2009). Because BBD is rarely, if ever, reported in *D. cylindrus* throughout the wider Caribbean (Ward et al. 2006), this species may be relatively resistant to BBD. BBD was not observed in *D. cylindrus* during the initial fieldwork to locate live colonies at 106 sites on the FRT leading up to this monitoring effort (summer 2013 to spring 2014). 2013 was a non-bleaching year on these reefs. The quantitative documentation of zero BBD signs on the 163 *D. cylindrus* colonies identified in 2013,

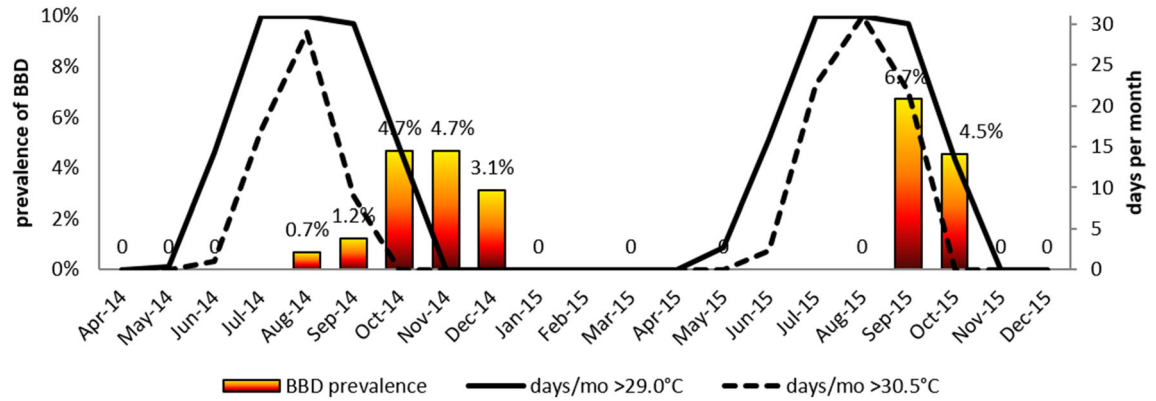

Fig. 3 Prevalence of black band disease (BBD) in *Dendrogyra cylindrus* on the Florida Reef Tract (FRT) from April 2014 to December 2015. Tri-annual assessments (*) occurred in April/May, September 2014, and January, April/May and September 2015. Additional assessments at three sites occurred in October, November, December 2014 and March, October, November, December 2015 to document the dynamics of bleaching recovery after the hyperthermal events in August/September 2014 and 2015. *Solid black line*: number of days per month that mean daily sea temperatures exceeded 29.0 °C, the optimal temperature for active BBD. *Dashed black line*: number of days per month that mean daily sea temperatures exceeded 30.5 °C, the bleaching threshold for the FRT

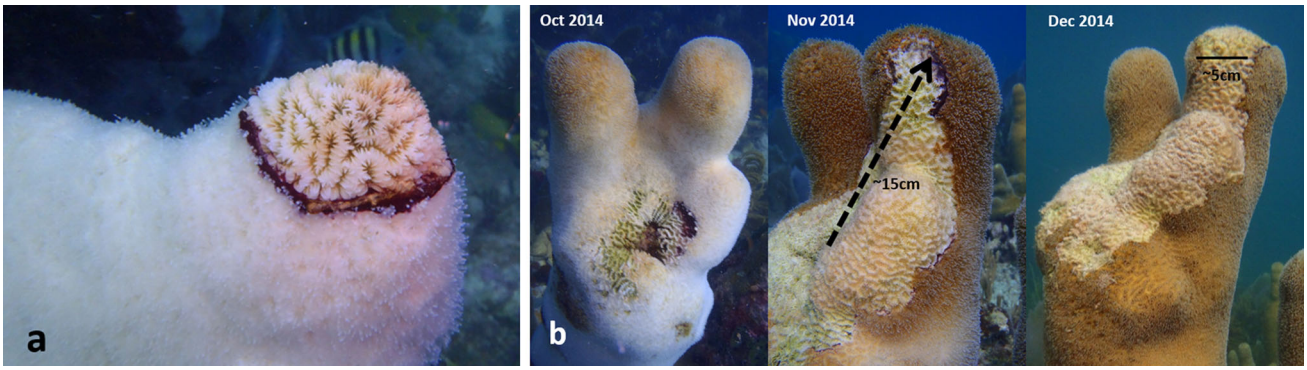

Fig. 4 Black band disease (BBD) in *Dendrogyra cylindrus*. **a** BBD on bleached *D. cylindrus* demonstrating the characteristic dark band and adjacent freshly denuded coral skeleton. **b** Progression of BBD on a single *D. cylindrus* pillar—BBD was not observed on this colony in September 2014, although the colony was severely bleached, but first appeared in October 2014, displaying a BBD lesion approximately 2 cm in diameter. By November 2014, the active band had progressed upwards approximately 15 cm and expanded laterally (calculated 0.5 cm d^{-1}). Four weeks later, the active band had slowly progressed approximately 5 cm to the top of the pillar. Active BBD was no longer visible in January 2015 but reoccurred on the same pillar in September 2015

together with the fact that prior to 2014 there were no reports of BBD on *D. cylindrus* on these reefs while BBD was reported in other species, may serve as a tentative baseline for BBD in this species on the FRT. Furthermore, the increase from zero BBD in the *D. cylindrus* population in 2013 compared to BBD prevalence values of 4.7% and 6.7% in the following years, immediately after two hyperthermal events, suggests a relationship between anomalously elevated water temperatures (and associated thermal stress), bleaching, and disease for *D. cylindrus*. Differences in other water quality parameters at each site, not measured in this study, may also be driving the apparent susceptibility to BBD of *D. cylindrus*. Additionally, coral animal genotypes, the *D. cylindrus* microbiome, and the microbiota in the surrounding environment may play roles in differential BBD susceptibility and/or resistance.

The impacts of bleaching are known to include a nutritionally compromised health status of the affected corals due to the loss of their *Symbiodinium*-derived nutrients (Muscatine and Porter 1977; Muller et al. 2009). Potential synergy of thermal and nutritional stress may have contributed to the vulnerability of *D. cylindrus* to BBD pathogens (Croquer and Weil 2009; Rogers et al. 2009; Kuehl et al. 2011). However, it was not possible to separately address these stressors in a natural setting. Additionally, prolonged elevated sea temperatures may have increased the pathogenicity of the polymicrobial community associated with BBD. Enhanced pathogenicity occurs in these and many other marine microbes at

temperatures exceeding 29 °C (Rützler and Santavy 1983; Kushmaro et al. 1997; Ben-Haim et al. 2003; Richardson and Kuta 2003).

BBD has rarely been reported in *D. cylindrus,* perhaps due to the relatively low abundance of this little-studied species throughout its Caribbean range or perhaps also due to its relative resistance to this particular disease. This study presents the results of the first quantitative monitoring of *D. cylindrus* on the FRT for health, bleaching status, and disease, and includes the first report of BBD for this species in this region. This data set is the first step in potential management of this recently listed threatened species. The observed persistent advance of BBD (progressing up to 0.5 cm d^{-1}; Fig. 4) on this slow-growing coral, the pattern of increased BBD prevalence following two consecutive hyperthermal events, and escalating environmental stressors due to predicted climate change, all suggest that BBD may play a more prominent role in the decline of *D. cylindrus* and other susceptible reef-building species, lending urgency for management and restoration efforts.

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Recurring Episodes of Thermal Stress Shift the Balance From a Dominant Host-Specialist to a Background Host-Generalist Zooxanthella in the Threatened Pillar Coral, *Dendrogyra cylindrus*

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Most scleractinian corals form obligate symbioses with photosynthetic dinoflagellates (family Symbiodiniaceae), which provide differential tolerances to their host. Previously, research has focused on the influence of symbiont composition and the dynamic processes of symbiont repopulation during single episodes of hyperthermal events, followed by years of less-stressful conditions. In contrast, this study characterized for the first time, the role of Symbiodiniaceae species changes in response to annually recurring hyperthermal events, a scenario soon expected to become the norm. Consecutive hyperthermal events during summer 2014 and 2015 along the Florida Reef Tract offered a unique opportunity to study bleaching susceptibility and recovery under recurrent annual hyperthermal scenarios. We utilized Illumina amplicon sequencing of the chloroplast 23S DNA region to assess with fine resolution the Symbiodiniaceae diversity associated with pillar coral, *Dendrogyra cylindrus*. Our findings show diverse assemblages of Symbiodiniaceae species and that some cryptic members are not transient associates but persistent and ecologically relevant, especially during recurrent annual warming events. This was evidenced by changes in relative abundance from the typically dominant host-specialist endosymbiont, *Breviolum dendrogyrum,* to *B. meandrinium* a host-generalist species common to corals in the family Meandrinidae but occurs at background densities in most coral colonies of *D. cylindrus*. The rise in abundance of *B. meandrinium* associated strongly with bleaching resistance in the coral host during two consecutive hyperthermal events. In some cases, host-compatible background symbionts can rapidly increase in abundance during episodes of stress and may impart physiological resilience to rapid environmental change and thus, represents a potentially important ecological process by which symbiotic corals acclimatize to changing ocean conditions.

Keywords: pillar coral, bleaching resistance, host-specialist zooxanthella, *Dendrogyra cylindrus*, Florida Reef Tract, Symbiodiniaceae

BACKGROUND

Coral reefs worldwide have experienced dramatic declines in recent decades due to natural and anthropogenic factors (Gardner et al., 2003; Van Hooidonk et al., 2016; Hughes et al., 2017). Global impacts of climate change, resulting in hyperthermal coral bleaching events (loss of symbiotic photosynthetic algae and/or chlorophyll), have become more frequent in recent decades and are projected to be annual events by 2050, or sooner on some reefs (Hoegh-Guldberg et al., 2007; Van Hooidonk et al., 2015, 2016). One of the strongest El Niño Southern Oscillation (ENSO) events on record occurred from May 2014 to June 2016 (Lian et al., 2017), causing staggering losses to coral reefs worldwide, including the Florida Reef Tract, due to consecutive bleaching events and subsequent disease outbreaks (Precht et al., 2016; Hughes et al., 2017).

Most scleractinian corals form obligate symbiotic relationships with photosynthetic dinoflagellates within the family Symbiodiniaceae. This partnership is critical for coral health and vital to enhanced calcification in reef-building corals (Muscatine and Porter, 1977; Mallela, 2013). Understanding the role of Symbiodiniaceae diversity in bleaching susceptibility and recovery, and the physiological constraints and advantages they confer on their coral hosts has become of increasing importance with escalating climate change (Cunning and Baker, 2012; Silverstein et al., 2012; Logan et al., 2014). Currently there are seven described genera and a similar number of other divergent lineages requiring generic names (LaJeunesse et al., 2018). Inter- and intra-generic diversity displays clear physiological differences (Rodriguez-Lanetty et al., 2004, 2006; Rodriguez-Lanetty, 2003; Rowan, 2004; Warner et al., 2006; Abrego et al., 2008; Sampayo et al., 2008; Fitt et al., 2009) and provides differential environmental tolerances and sensitivities to the symbiotic partnership (Jones and Berkelmans, 2010; LaJeunesse et al., 2014; Hume et al., 2015). Seminal work by Berkelmans and Van Oppen (2006) demonstrated experimentally that corals can acquire increased thermal tolerance as a direct result of changes in the symbiont genus dominating their tissues by shuffling existing types already present within the coral host.

Molecular approaches to Symbiodiniaceae diversity and community assemblages now allow us to further investigate the functional significance of genetic diversity within genera, thus prompting further questions into the roles of the symbionts during environmental stressors (Sampayo et al., 2008; Bay et al., 2016). Using higher resolution DNA markers, it has become apparent that there is a very large number of species in this family (Rodriguez-Lanetty et al., 2001, 2004; Rodriguez-Lanetty, 2003; LaJeunesse, 2002; Sampayo et al., 2009; LaJeunesse and Thornhill, 2011; Thornhill et al., 2014; Wilkinson et al., 2015; Lewis et al., 2018). Additionally, advances in high-throughput amplicon sequencing technology have allowed fine-scale exploration of the Symbiodiniaceae community composition by discovering more cryptic, previously undetected symbiont types occurring at abundances less than 0.01% (Arif et al., 2014; Green et al., 2014; Quigley et al., 2014; Thomas et al., 2014; Boulotte et al., 2016; Cunning et al., 2017). While various kinds of Symbiodiniaceae can be detected in trace amounts from host tissues, interpreting the ecological and functional significance of these requires caution (Lee et al., 2016). Still reef corals are often compatible with more than one symbiont species and differences in their physiological tolerances may shift population dynamics allowing for a symbiont at low abundances to proliferate within the coral animal thus changing the composition of the symbiont population to one that is better adapted to prevailing environmental conditions (Berkelmans and Van Oppen, 2006; LaJeunesse et al., 2009a; Baums et al., 2014; Grottoli et al., 2014; Parkinson and Baums, 2014).

Studies conducted over the last two decades have been fundamental in gauging the response and the ability of corals to acclimatize to increased temperature extremes under scenarios in which isolated thermal anomalies have been followed by years of non-bleaching temperatures. However, the 2014–2016 ENSO phenomenon led to a recurrent thermal stress scenario, resulting in mass coral bleaching which detrimentally impacted coral reefs worldwide, including the Florida Reef Tract, during the summers of 2014 and 2015 (Manzello, 2015). These consecutive hyperthermal events, not experienced on Florida's reefs since the 1997-1998 ENSO (Causey, 2001), offered a unique opportunity to document spatial and temporal bleaching and post-bleaching recovery under conditions not frequently seen, but expected to become the norm in the near future based on current predictions (Hoegh-Guldberg et al., 2007; Altizer et al., 2013; Van Hooidonk et al., 2016).

This study targeted the rare and iconic pillar coral, *Dendrogyra cylindrus* (Ehrenburg, 1834), a slow-growing columnar species typically found in low abundance throughout its Caribbean range (**Figure 1**). This species is currently categorized as 'vulnerable' under the International Union for Conservation of Nature (IUCN) Red List criteria (Aronson et al., 2008) and listed as 'threatened' under the US Endangered Species Act (NOAA Fisheries, 2014) due to its susceptibility to bleaching, disease, and habitat degradation. We focused on three representative sites distributed geographically along the Florida Reef Tract (**Figure 2**). For this study we used Illumina amplicon sequencing of the chloroplast 23S hyper-variable region (cp23S-HV) to characterize for the first time with fine resolution, the Symbiodiniaceae community assemblage associated with *D. cylindrus* colonies at three sites and their temporal dynamics through recurrent hyperthermal events over a 2-year period. We detected the presence of low abundance background Symbiodiniaceae genera and, at one location, observed changes in the symbiont population from the normal host-specialist species, *Breviolum dendrogyrum,* to a sibling species, *B. meandrinium,* common to corals across the family Meandrinidae. The dramatic increase in abundance of this species corresponded to enhanced colony recovery from bleaching and resistance to consecutive hyperthermal events.

MATERIALS AND METHODS

Field Assessments and Sampling

Geographically stratified monitoring and field sampling of 163 *D. cylindrus* at 29 sites across the Florida Reef Tract (FRT),

FIGURE 1 | The iconic pillar coral, *Dendrogyra cylindrus*. This unique species occurs in historically low abundance throughout the Greater Caribbean. Its unique columnar structure provides important habitat complexity to the reef ecosystem where it does occur (Photo: C. Lewis).

conducted every 4 months from April 2014 to April 2016, allowed for comparison before, during and after the occurrence of two consecutive hyperthermal bleaching events in 2014 and 2015. Three of these sites (Pickles: Upper Keys, N = 107 colonies; Coffins: Middle Keys, N = 55 colonies; Marker 32: Lower Keys, N = 16 colonies; represented by stars in **Figure 2**) were selected spatially to represent each region and logistically due to the greater number of *D. cylindrus* colonies present at each site for replication of observations. These sites were targeted for more frequent sampling to more closely observe changes during bleaching and recovery (2014: September, October, November, and December; 2015: January, March, April, September, October, and November; and 2016: January and April). Sites ranged in depth from 4 to 8 m. Colonies at all sites were mapped and photographed to create field identification sheets for each colony to facilitate accurate repeat assessment and sampling. Pendant data loggers (Onset HOBO Inc., Bourne, MA, United States), secured to the base of colonies at Pickles, Coffins, and Marker 32 sites, recorded hourly temperatures between April 2014 and April 2016 (**Figure 3**). Temperature data was used to calculate mean daily, mean monthly, and maximum weekly sea temperatures at each site. Archived temperature data for 2004–2013 at Molasses Reef C-MAN station MLRF1, located in the Upper Keys 4.2 km from Pickles site (*NOAA National Data Buoy Center, 2016*), was used as a proxy to calculate 10-year mean monthly and mean monthly maximum sea temperatures on the FRT (**Supplementary Table 1B**). Using the calculated mean monthly maximum temperatures at MRLF1, degree heating weeks (°C-weeks) were calculated for each site for July, August, and September 2014 and 2015 (Liu et al., 2006) (**Supplementary Table 1A**).

At each sampling time point, *D. cylindrus* colonies were assessed for live coral tissue (visual estimate of percent live, percent old mortality, percent recent mortality, i.e., bright white, recently exposed skeleton), and coral bleaching status. Colony color scores were assigned using the CoralWatch Coral Health colorimetric chart developed by Siebeck et al. (2006) and scaled from 1 (white) to 6 (heavily pigmented; **Supplementary Figure 1**), served as a proxy for symbiont density and chlorophyll *a* content. The CoralWatch Health Chart was not utilized as a color reference until September 2014, therefore, colony coloration scores for April 2014 were estimated after reviewing colony photos. Colonies were sampled at each time point using a low-impact syringe micro-sampling technique (Kemp et al., 2008) to minimize damage to the colonies from repeated sampling (sampling: Pickles n = 10 colonies, Coffins n = 10 colonies, Marker 32 n = 12 colonies). Briefly, tissue from three to five polyps per colony was aspirated using a 30 cc syringe, transported back to shore on ice, then filtered through a 13 mm Swinnex filter system (Millepore Corporation, Billerica, MA, United States), using a 3.0 μm filter disk (A/D glass fiber filter, Pall Corporation, Port Washington, NY, United States). Each filter disk was preserved in 95% molecular grade ethanol.

Total Genomic DNA Extraction

DNA was extracted using modified DNeasy Plant Mini kit protocols (Quiagen Corporation, Valencia, CA, United States) (LaJeunesse, 2002). Briefly, half of each sample filter was placed in 400 μl supplied lysing buffer and ground with a sterile pestle. Sterile acid-washed glass beads (425–600 μm; Sigma-Aldrich, Saint Louis, MO, United States) were added and shaken for 3–5 min to disrupt symbiont cell walls, followed by the addition of 20 μl proteinase K (Promega Corporation, Madison, WI, United States) and incubated at 56°C for 1–2 h. Standard kit protocols were then followed for the remainder of the extraction.

Cp-23S-HV Parallel Amplicon Sequencing and Symbiodiniaceae Community Analysis

Amplicon sequencing diversity assays of the Symbiodiniaceae communities was performed on Illumina MiSeq platform with 2 × 300 base pairs paired-end read capability, utilizing length variation in Domain V of large sub-unit rDNA chloroplast 23S hyper-variable region (cp-23S-HV, Santos et al., 2003; **Supplementary Table 2**) at the Molecular Research LP sequencing facility (MR DNA; Shallowater, TX, United States). Resulting raw sequence data (Read1 & Read2.fastq file format) were processed using MR DNA pipeline analysis. Briefly, paired-end sequence reads (r1 and r2) were joined after quality control (Q25) trimming of the ends, barcoding was

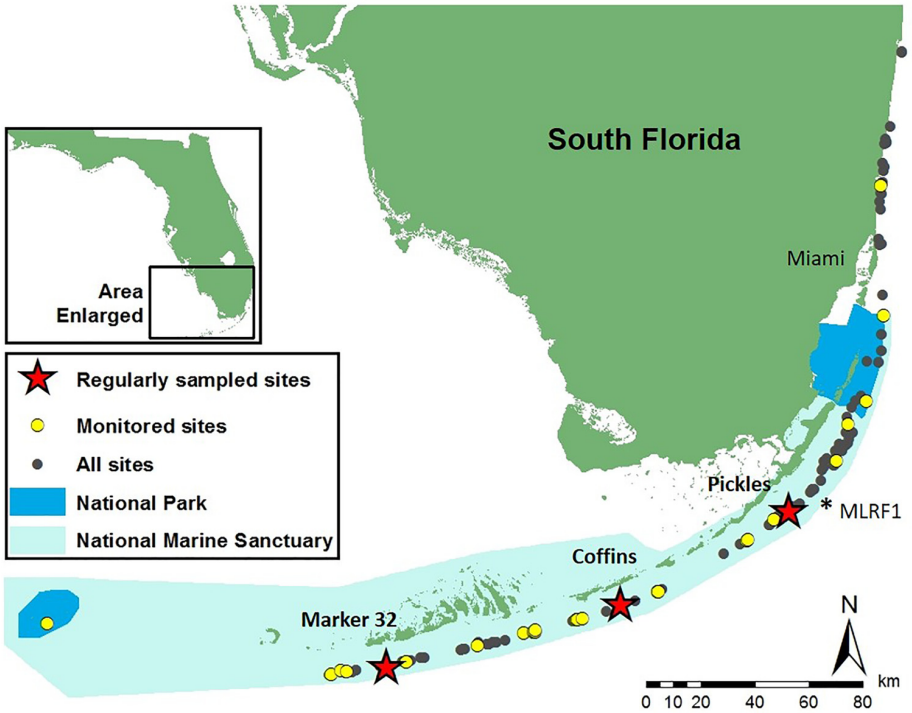

FIGURE 2 | Study sites on the Florida Reef Tract where monitoring and sampling was conducted on the Pillar Coral *Dendrogyra cylindrus*. Geographically stratified tri-annual monitoring and sampling occurred at 28 sites April 2014 to April 2016 (yellow circles). Pickles (Upper Keys), Coffins (Middle Keys), and Marker 32 (Lower Keys) sites (red stars) were monitored and sampled monthly during bleaching recovery in 2014 and 2015. *MLRF1 – location of Molasses Reef C-man buoy used to calculate 10-year mean monthly temperatures.

removed, and sequences with <150 base pair overlap were discarded. Remaining sequences were de-noised, OTUs were generated, and chimera sequences were removed. Clustering of OTUs was determined at 97% similarity (3% divergence) across all samples. Resulting OTUs were taxonomically classified using BLASTn against a selected database created from cp23S Symbiodiniaceae sequence data from NCBI[1] to determine relative abundance of Symbiodiniaceae types in *D. cylindrus*. To confirm identities of the most abundant OTUs, a BLAST-search of GenBank was performed[2]. Further identification of the two most abundant B1 symbiont type OTUs was independently verified using Symbiodiniaceae microsatellite analysis (B7*Sym*15 primers, **Supplementary Table 2**) (Pettay and Lajeunesse, 2007). Briefly, selected samples (dominant OTU ≥ 70% relative abundance) were PCR-amplified using the B7*Sym*15 primers and visualized on ABI 3100 Genetic Analyzer. Peaks were identified in each sample and compared to known Symbiodiniaceae samples in the LaJeunesse Lab (Lewis et al., 2018).

Statistical Analysis

Repeated measures analysis of variance (repeated measures ANOVA, $\alpha = 0.05$) was used to compare water temperature profiles between the three sites (Pickles, Coffins, and Marker 32) through two consecutive bleaching and recovery periods April 2014 through April 2016. CoralWatch bleaching scores for colonies at the three sites were also analyzed, using repeated measures ANOVA with *post hoc* Tukey and Bonferroni correction ($\alpha = 0.05$; Pickles $n = 10$, Coffins $n = 10$, Marker 32 $n = 12$), to compare bleaching and recovery differences between sites and between years. To determine the relationships between coral bleaching and changes in the Symbiodiniaceae community between sites, multivariate analysis of variance (MANOVA $\alpha = 0.05$) was used to compare colony bleaching index (colony scores) to the relative abundance of OTUs generated by amplicon sequencing of the cp23S-HV region (Pickles $n = 6$, Coffins $n = 6$, Marker 32 $n = 6$).

RESULTS

Baseline Symbiodiniaceae Community Diversity in *Dendrogyra cylindrus* Before 2014 and 2015 Hyperthermal Events

Illumina amplicon sequencing of the cp23S-HV gene region showed a single OTU (or phylogenetic species) within the genus *Breviolum* (formerly clade B; (LaJeunesse et al., 2018) to be the dominant Symbiodiniaceae in *D. cylindrus* pre-bleaching (April 2014), ranging from 75 to 81% relative abundance across sites (**Supplementary Table 3**). Members within *Symbiodinium*, *Cladocopium*, and *Durusdinium* (formerly

[1]www.ncbi.nlm.nih.gov

[2]https://www.ncbi.nlm.nih.gov/genbank

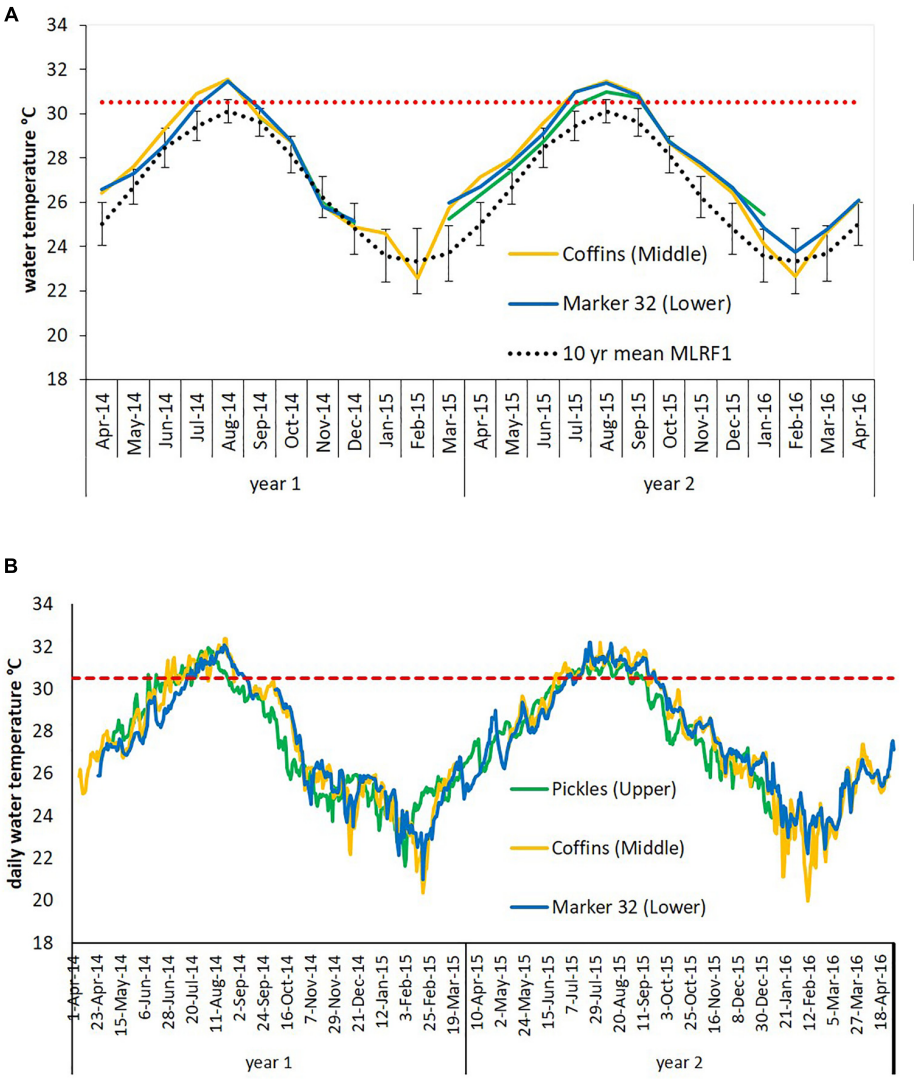

FIGURE 3 | Water temperatures at three sites in the Florida Keys recorded between April 2014 and April 2016. The Florida Reef Tract (FRT) in the Florida Keys is divided into distinct regions based on hydrologic influences, represented here by three sites: Pickles, Upper Keys; Coffins, Middle Keys; and Marker 32, Lower Keys. Red dashed lines indicate the local bleaching threshold for the FRT (30.5°C). Gaps in data are due to lost or damaged temperature loggers. **(A)** Mean monthly water temperature data. Dotted black line is the calculated 10-year mean monthly water temperature (error bars $\pm$1 SD) for Molasses Reef 2004-2013 (MLRF1, National Data Buoy Center). **(B)** Mean daily water temperature data.

clades A, C, and D respectively; (LaJeunesse et al., 2018) were also detected in cryptic low abundance (<0.1%). Amplicon diversity analysis yielded 266 OTUs (0.97% similarity cut-off). All but fourteen OTUs were taxonomically classified as genus *Breviolum* (formerly clade B *Symbiodinium*). Moreover, the combined relative abundance of all 252 *Breviolum* OTUs represented > 99% of the Symbiodiniaceae community at all three representative sites along the Florida Reef Tract (Pickles, Coffins, and Marker 32). Two hundred thirty-two *Breviolum* OTUs were rare members of the Symbiodiniaceae community detected at <0.01% relative abundance. The two most abundant OTUs accounted for >80% of the symbiont community at all three sites. These two sequences were both identified as sub-genera *Breviolum* type cp-23S B184 (or ITS2 type B1) phylotypes in GeneBank. Using Symbiodiniaceae microsatellite analysis (B7*Sym*15 primers, **Supplementary Table 2**), the identity of these two sequences was verified as ITS2 types B1-4k and B1-3 (species novo: *Breviolum dendrogyrum* and *B. meandrinium,* respectively; LaJeunesse et al., 2018; Lewis et al., 2018). There was no difference in relative abundances of *B. dendrogyrum* or *B. meandrinium* compared between sites in April 2014 prior to bleaching (two-way ANOVA $\alpha = 0.05$, $p > 0.05$).

Ninety-four OTUs belonging to the genus *Symbiodinium* (formerly clade A) were detected in cryptic low abundance at Pickles, Coffins, and Marker 32 in April 2014 (**Supplementary Table 3**). These OTUs were confirmed by BLAST-search as belonging to *Symbiodinium* strains A2, A3, and A13 (strain A13, putatively *S. necroappetens*). Three cryptic *Cladocopium* OTUs

(formerly clade C) were detected only at Pickles and Marker 32 sites. A single OTU, classified as *Durusdinium* (strain D1a, putatively *D. trenchii*), was only detected at Marker 32 in April 2014.

Consecutive Hyperthermal Bleaching on the Florida Reef Tract in 2014 and 2015

Florida's reefs exceeded 5°C-weeks (degree heating weeks) during the summers of 2014 and 7°C-weeks in 2015, based on NOAA's Coral Reef Watch 50-km Satellite Monitoring (NOAA Coral Reef Watch, 2000)*, updated twice-weekly*; NOAA OSPO, 2018) (**Supplementary Table 1A**). Data loggers at the three study sites recorded sea water temperatures which exceeded the FRT bleaching index of 30.5°C (Manzello, 2015) (**Figure 3**), causing severe bleaching in most coral species across the FRT (personal observation and Precht et al., 2016). Maximum weekly temperatures in 2014 exceeded the FRT bleaching threshold (30.5°C) 10 weeks at Coffins and 8 weeks at Marker 32 (**Figure 3** and **Supplementary Table 1B**). Data loggers were lost at the Pickles site from April to September 2014 and thus meaningful temperature analysis could not be included. In 2015, maximum weekly temperatures exceeded the bleaching threshold 12 weeks at Pickles and 13 weeks at both Coffins and Marker 32. Using mean maximum monthly calculations from MRLF1, degree heating weeks at Coffins (Middle Keys) exceeded 7°C-weeks in 2014 and 5°C-weeks in 2015 (**Supplementary Table 1B**). Degree heating weeks at Marker 32 (Lower Keys) was nearly 3°C-weeks in 2014 and 4°C-weeks in 2015. Degree heating weeks at Pickles (Upper Keys) was 0°C-weeks in 2015 (no data from 2014). Utilizing the FRT bleaching threshold (30.5°C), calculated DHW was greater at the three sites (**Supplementary Table 1B**). The number of weeks maximum water temperatures exceeded the bleaching threshold were greater in 2015. Summary of water temperatures from June through October (i.e., warmest summer months) in 2014 and 2015 show differences in temperature characteristics between sites and between years (**Figure 4**; repeated measures ANOVA $p < 0.04$), except for July 2015 ($p = 0.076$). While all sites experienced temperatures exceeding the bleaching threshold, Coffins experienced the highest temperatures in August 2014, however, Marker 32 was warmer (maximum and median temperatures) than the other two sites in September 2014 ($p < 0.01$). During the second hyperthermal event in 2015, Coffins and Marker 32 sites experienced warmer temperatures than Pickles site (maximum and median temperatures; $p < 0.01$).

Differential Bleaching Resistance and Resilience

The CoralWatch Coral Health colorimetric chart developed by Siebeck et al. (2006) was used to determine the bleaching status of colonies. Healthy, non-thermally stressed colonies ranged in color between 3.5 and 4.5 on this chart (**Supplementary Figure 1**). Colonies with scores between 1.5 and 3.5 were considered pale, while colonies with scores < 1.5 were considered bleached.

In response to hyperthermal events in 2014 and 2015, patterns of bleaching and recovery differed between sites as well as between years. During the first bleaching event on the FRT (August–September 2014), all *D. cylindrus* colonies, including at Pickles, Coffins, and Marker 32 sites were severely bleached (**Figure 5** and **Supplementary Table 4**). Colonies at Marker 32 were the most severely bleached, followed by Coffins colonies and finally Pickles (two-way ANOVA; $p < 0.01$). Pickles and Coffins colonies regained normal coloration by December 2014 and January 2015. From January to April 2015, colonies at these two sites paled (two-way ANOVA, $p < 0.01$). Marker 32 colonies recovered slowly, remaining pale through April 2015 (**Figure 5** and **Supplementary Table 4**).

During the second hyperthermal event in August-September 2015, site-specific differences in bleaching and recovery were observed. Colonies at Coffins and Marker 32 again bleached, although Coffins colonies were less severely bleached than September 2014, indicated by higher coloration scores (two-way ANOVA, $p < 0.01$; **Figure 5** and **Supplementary Table 4**). However, unlike these two sites, Pickles colonies were more resistant to bleaching in 2015, with no significant change in colony coloration from April to September 2015 (two-way ANOVA, $p = 0.82$). While there was no bleaching observed in these colonies during the hyperthermal event, there was an increase in colony coloration from September to October ($p = 0.01$) which remained constant through January 2016 (**Figure 5** and **Supplementary Table 4**). Coffins colonies also recovered normal coloration by January 2016. Both Pickles and Coffins colonies again paled during the 2016 winter-spring transition ($p = 0.01$). Although many colonies at Marker 32 remined pale after September 2015, they appeared to recover slightly when compared with the previous year, indicated by near-normal coloration scores in November 2015 and January 2016 (mean scores: 3.46 ± 0.33 and 3.76 ± 0.33; two-way ANOVA, $p < 0.01$). Colonies at Marker 32 were considered pale again in April 2016 (mean score: 3.01 ± 1.13; one-way ANOVA $p = 0.06$; **Figure 5** and **Supplementary Table 4**).

Breviolum Species Switched Dominance Associated With Hyperthermal Events

The Symbiodiniaceae community in *D. cylindrus* was dynamic in response to hyperthermal bleaching and the subsequent recovery processes following two consecutive bleaching events. Relative abundance of the endosymbionts varied between three representative sites, especially the two species within *Breviolum* described above (**Figure 6**). At the Pickles site (Upper Keys) and associated with the August-September 2014 hyperthermal event, previously cryptic *B. meandrinium* (<10% relative abundance) became the dominant endosymbiont species in October 2014 and persisted through April 2015 while *B. dendrogyrum* declined in relative abundance through December 2014 to 20.60% but then slowly increased to 33.35% through April 2015 (**Figure 6A** and **Supplementary Table 3A**), but not to pre-bleaching abundance of 75.68% in April 2014. At the Coffins site (Middle Keys), *B. dendrogyrum* remained dominant through bleaching and recovery in 2014. *Breviolum meandrinium* remained at low

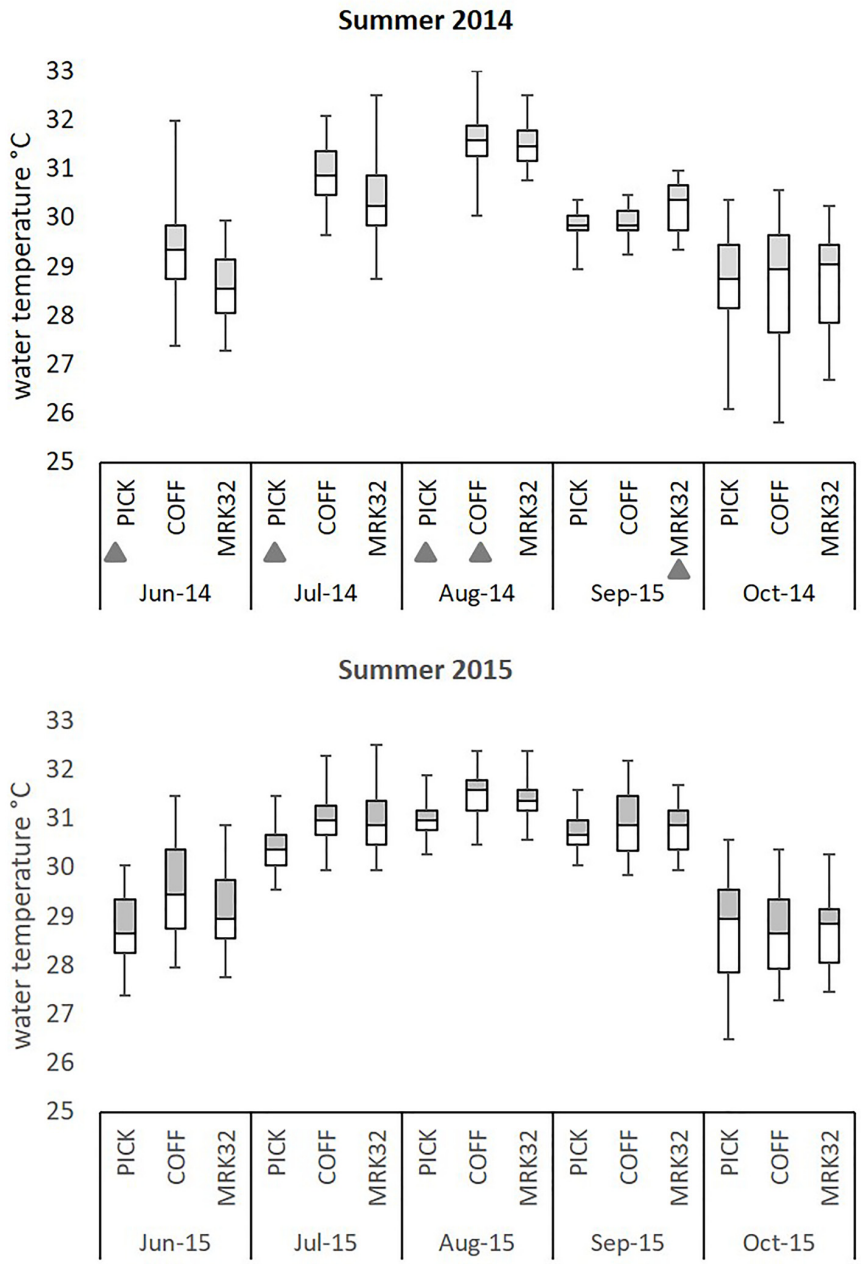

FIGURE 4 | Summary of water temperature profiles at three sites on the Florida Reef Tract during summer 2014 and 2015. Five-number summary of temperature profiles at three sites (Pickles: Upper, Coffins: Middle, Marker 32: Lower) along the Florida Reef Tract. Hourly water temperatures from pendant data loggers were used to create temperature profiles from June through October 2014 and 2015. Box plots indicate interquartile temperature ranges (Q2–Q3) and median temperatures. 'Whiskers' show maximum and minimum water temperature during this time period. Gray triangles indicate incomplete temperature profiles due to lost data loggers.

abundance but showed a slight but significant increase from October to December 2014 (two-way ANOVA $p = 0.01$), reaching maximum 20.15% relative abundance in April 2015 (**Figure 6B** and **Supplementary Table 3B**). Similar to the Coffins site, *B. dendrogyrum* remained the dominant species at Marker 32 (Lower Keys; **Figure 6C** and **Supplementary Table 3C**).

In response to the second hyperthermal event in August-September 2015, the two most abundant *Breviolum* species were again dynamic and site-specific. At the Pickles site, fluctuation in dominance was again detected in which *B. dendrogyrum* briefly increased in abundance and re-established dominance by September 2015 (53.41%; two-way ANOVA, $p = 0.02$) however, this was short-lived. Relative abundance of *B. meandrinium* slowly declined through September 2015 to 30.28% but then quickly increased during the recovery months to persist as the dominant endosymbiont through April 2016 (51.55%).

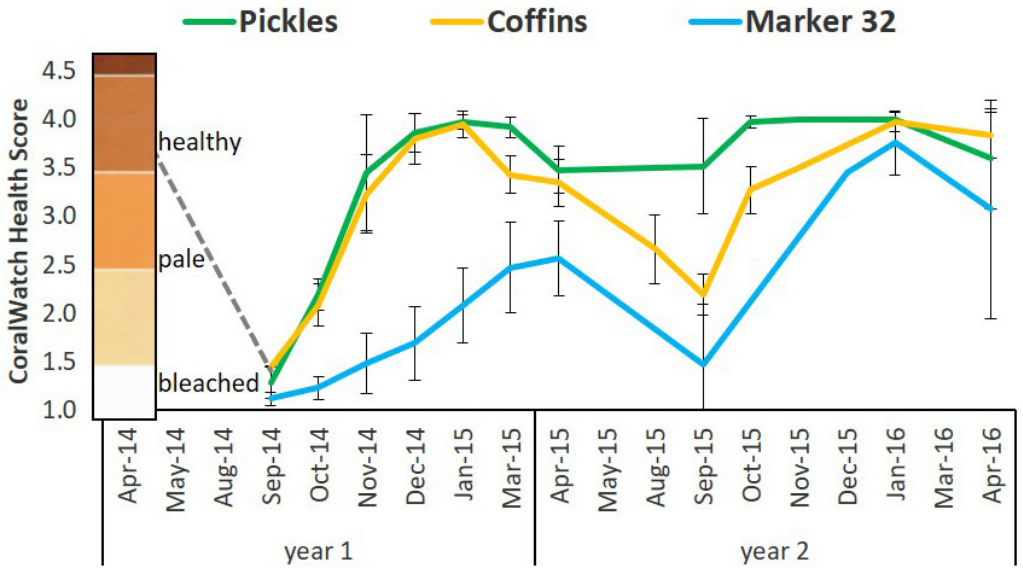

FIGURE 5 | Mean bleaching index based on coloration scores of *D. cylindrus* colonies at three sites on the FRT between 2014 and 2016. The coloration scores were determined using the CoralWatch Coral Health Chart for colorimetric reference (version: Project Aware https://coralwatch.org; Siebeck et al., 2006). Coloration ranges for *D. cylindrus* based on triannual assessments of 168 colonies: healthy 3.5–4.0; pale: 2.0–3.0; bleached: 1.0–1.5. Dashed gray line represents trajectory of estimated colony scores from April 2014. Pickles (n = 12) Upper Keys; Coffins (n = 12) Middle Keys; Marker 32 (n = 11) Lower Keys. Error bars ± 1 standard deviation.

From September 2015 to April 2016, *B. dendrogyrum* slowly declined to 29.97% (**Figure 6A** and **Supplementary Table 3A**). At the Coffins site, *B. dendrogyrum* remained the dominant species throughout the second bleaching and recovery (2015) and returned to similar abundance observed in April 2014 baseline (**Figure 6B** and **Supplementary Table 3B**). *Breviolum meandrinium* reached a maximum abundance of 20.39% in September 2015 at this site and declined to 0.12% in January 2016, remaining unchanged through April 2016 (two-way ANOVA, $p < 0.01$). And finally, at the Marker 32 site, relative abundance of *B. dendrogyrum* and *B. meandrinium* did not change from April to September 2015 or thereafter, and *B. meandrinium* remained cryptic (<0.1% abundance) through April 2016 (**Figure 6C** and **Supplementary Table 3C**).

Bleaching Resistance and Resilience

The magnitude of change in relative abundance of dominant *Breviolum dendrogyrum* and cryptic *B. meandrinium* during two consecutive hyperthermal bleaching events was site specific and closely associated with differential resistance and resilience to bleaching and recovery. Colonies at the Pickles site (Upper Keys) bleached severely during August-September 2014 and then recovered by December 2014 (**Figure 5** and **Supplementary Table 4**). Concurrently, the relative abundance of dominant endosymbiont *B. dendrogyrum* declined while cryptic *B. meandrinium* increased to become the dominant symbiont. Relative abundance of these two species did not return to pre-bleaching levels (**Figure 6A** and **Supplementary Table 3A**). During the second hyperthermal event in August-September 2015, while *B. dendrogyrum* briefly regained dominance by September, *B. meandrinium* once again became the dominant species, persisting through recovery until April 2016. This change was closely associated with the observation that colonies at Pickles did not bleach during the second hyperthermal event (**Figure 5** and **Supplementary Table 4**), indicating acquired resistance to annual thermal stress (water temperatures exceeding 30.5°C, **Figures 3**, **4**). This resistance was strongly associated with a fluctuation in Symbiodiniaceae species, specifically an increase and persistence in abundance of *B. meandrinium* as it became the dominant species in *D. cylindrus* colonies at the Pickles site (MANOVA $p < 0.001$).

Colonies at the Coffins site (Middle Keys) also bleached severely in August-September 2014 (**Figure 5** and **Supplementary Table 4**). As normal colony coloration returned in December 2014, cryptic *B. meandrinium* slightly increased in abundance, reaching 20.15% by April 2015 (**Figure 6B** and **Supplementary Table 3B**). Although *B. meandrinium* never became the dominant *Breviolum* species at Coffins, colonies did not bleach as severely the second year and recovered quickly to normal coloration by January 2016 (**Figure 5** and **Supplementary Table 4**). This strongly suggests at least partial resistance to bleaching associated with an increased relative abundance in cryptic *B. meandrinium* (MANOVA $p = 0.068$). Colonies at Marker 32 (Lower Keys) bleached severely both in 2014 and 2015 (**Figure 5** and **Supplementary Table 4**) while *B. meandrinium* remained at cryptic low levels throughout, reaching a maximum abundance of only 2.60% in September

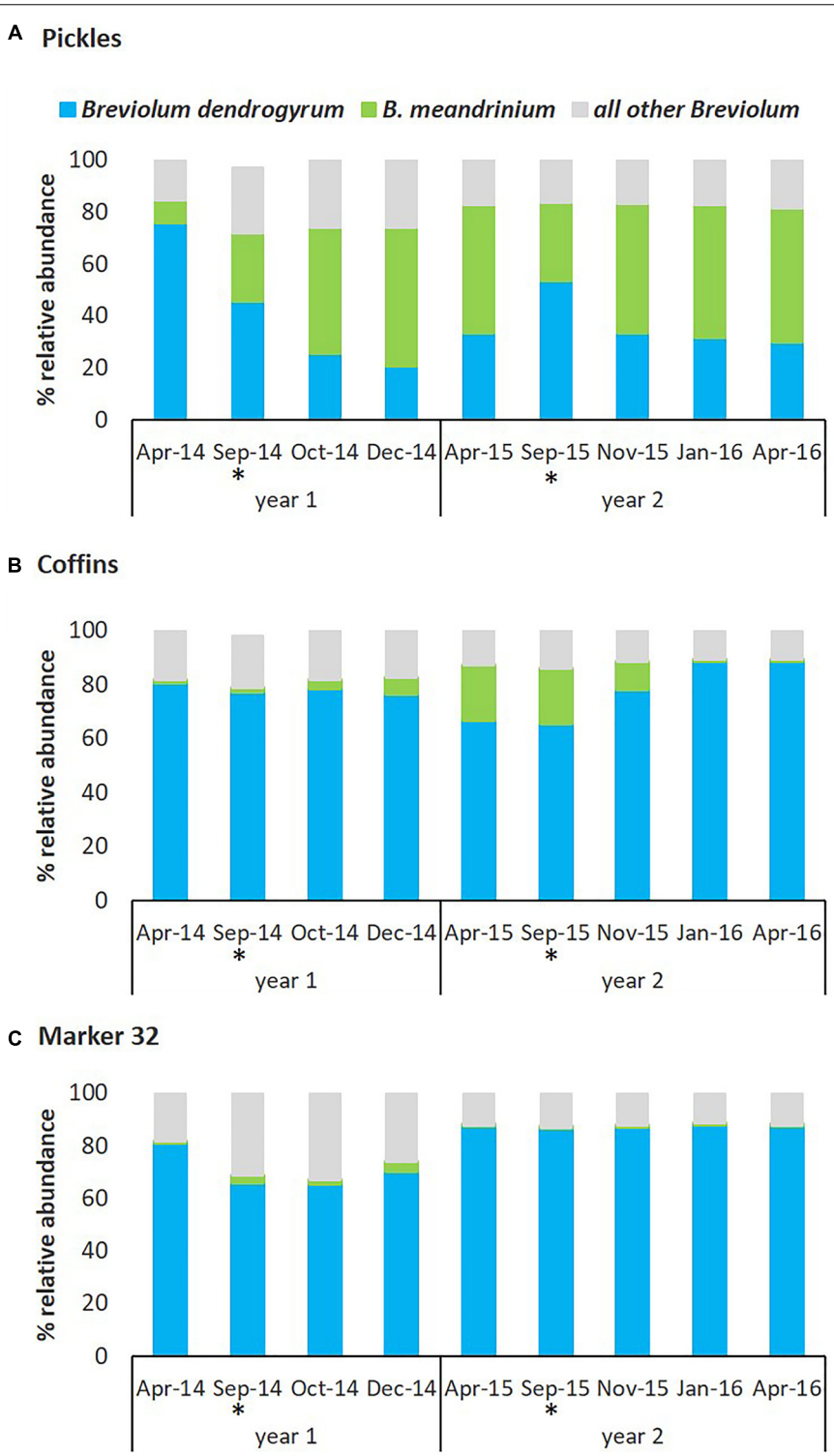

FIGURE 6 | Relative abundance of Symbiodiniaceae symbionts from the genus *Breviolum* associated with *D. cylindrus* on the Florida Reef Tract between 2014 and 2016. The dynamics of *Breviolum dendrogyrum* (blue) and *B. meandrinium* (green) between three sites and between years, by percent relative abundance of *Breviolum* spp. OTUs. *B. meandrinium* increased in relative abundance during bleaching and recovery, becoming the dominant symbiont species at the Pickles site in 2014 and 2015. **(A)** Pickles (n = 6) Upper Keys, **(B)** Coffins (n = 6) Middle Keys, **(C)** Marker 32 (n = 6) Lower Keys. 'All other *Breviolum*' (blue-gray) represents 250 OTUs combined. Asterisk (*) indicates bleaching months.

2014 (**Figure 6C** and **Supplementary Table 3C**; MANOVA, $p = 0.981$).

Symbiodinium, *Cladocopium*, and *Durusdinium* Persisted at Cryptic Low Abundance

Although *Breviolum* (formerly clade B) remained the dominant genus in *D. cylindrus* through two consecutive bleaching and recovery events, cryptic *Symbiodinium, Cladocopium,* and *Durusdinium* species (formerly clades A, C, and D, respectively) were also detected and often persisted through recovery (**Supplementary Table 3**). *Symbiodinium* spp. were detected in cryptic low abundance (<0.01–5.38%) from April 2014 through December 2014 and then not thereafter at Pickles site (Upper Keys). At the Coffins site (Middle Keys), *Symbiodinium* spp. were detected intermittently through both bleaching and recovery events. At Marker 32 (Lower Keys), *Symbiodinium* spp. were detected in April 2014 and persisted at cryptic low abundance though April 2016 (≤0.2%).

Cladocopium spp. were only detected from April to October 2014 and then not after at the Pickles site (**Supplementary Table 3**). *Cladocopium* spp. were detected at Coffins after the first bleaching in September 2014 and persisted intermittently during recovery until November 2015. At Marker 32, *Cladocopium* spp. were detected at cryptic levels in the April 2014 baseline sampling (<0.001%) and persisted through April 2016 (0.21% abundance).

A single OTU, classified as *Durusdinium* sp. and identified by BLAST-search as type D1a (putatively *D. trenchii*, formerly *S. trenchii*), was first detected in cryptic low abundance at Pickles and Coffins sites in October 2014 after the 2014 bleaching event and then intermittently through the second bleaching and recovery period until January 2016 (**Supplementary Table 3**). At Marker 32, *Durusdinium* sp. was detected pre-bleaching in April 2014 and then intermittently throughout both bleaching and recovery periods through April 2016.

DISCUSSION

Research over the last two decades has documented the influence of the symbiont composition and the dynamic processes of symbiont repopulation during the coral response to single episodes of hyperthermal stress events, followed by years of normal environmental conditions (Edmunds et al., 2014; Kemp et al., 2014). Our study characterized changes in symbiont species within some colonies of *Dendrogyra cylindrus* in response to a consecutive hyperthermal event. This study contributes to further understanding how coral-algal mutualisms may respond through shifts in partnerships under long-predicted environmental scenarios (annual bleaching) (Hoegh-Guldberg, 1999; Grottoli et al., 2014), which coral reefs worldwide are now experiencing (Hughes et al., 2017). Our findings show unexpectedly diverse assemblages of Symbiodiniaceae associated with *Dendrogyra cylindrus*, and that one low abundant host-compatible species was not a transient associate but persistent and ecologically relevant symbionts that plays a role during thermal stress. Furthermore, site-specific shifts in Symbiodiniaceae species dominance were associated with greater bleaching resistance during consecutive hyperthermal events.

Symbiodiniaceae Community Dominated Mainly by a Single Symbiont Along With a Diverse Assemblage of Cryptic Associates

By revealing a Symbiodiniaceae community represented by 266 OTUs, with the majority in very low relative abundances (<0.1%), our findings indicate that past studies have considerably underestimated the actual diversity of endosymbionts associated with *D. cylindrus,* and likely many other coral species, although at least some of these rare and cryptic OTUs may in fact be sequencing artifacts (Arif et al., 2014). Most symbiont types detected in our study (>99%) belonged to the genus *Breviolum* spp. (formerly clade B). Three other genera, *Symbiodinium*, *Cladocopium*, and *Durusdinium* (formerly clades A, C, and D, respectively) were detected at extremely low levels (0.001–5.38%) in *D. cylindrus*. Discovery of this tremendous sequence diversity within the genus *Breviolum* associated with *D. cylindrus* opens new questions regarding whether these symbiont types are the reflection of population variability within species or are indeed independent evolutionary lineages (i.e., species). Symbiont assemblages across sites and within individual *D. cylindrus* colonies were dominated by a single *Breviolum* species of endosymbiont. Using single copy microsatellite markers (see Materials and Methods), it was confirmed that this symbiont corresponded to the recently described *Breviolum dendrogyrum* (Lewis et al., 2018). Furthermore, low concentrations of *Breviolum meandrinium*, which was also identified and confirmed with the use of diagnostic microsatellites, increased in relative abundance as a function of environmental stress. *Breviolum meandrinium* is common to corals in the family Meandrinidae from shallow habitats (1-10 m) across the Greater Caribbean. *Dendrogyra cylindrus* is one notable exception of the family in that it harbors a unique host-specialist, which appears adapted to associating only with this host. These findings highlight how a host-generalist, *B. meandrinium,* normally rare in *D. cylindrus,* can proliferate in colonies subjected to severe thermal stress.

Changes in Balance Among Host-Compatible Symbiodiniaceae During Recurrent Environmental Stress

It has been suggested that most cryptic Symbiodiniaceae are transitory and likely provide minimal ecological significance for their coral hosts (Lee et al., 2016). However, recent studies have challenged this view and suggested that rare symbionts tend to be non-random clusters of coral host-symbiont communities and may provide environmental resilience for the coral holobiont (Ziegler et al., 2015, 2017). In agreement with this, our data show a clear association between a site-specific increase and persistence in relative abundance of the low abundance background *B. meandrinium* and the overall stability of the host–symbiont community during subsequent hyperthermal stress. At the Pickles site in the Upper Keys region of the Florida

Reef Tract, *B. meandrinium* increased in relative abundance during the first hyperthermal bleaching event in 2014 and rapidly switched to become the dominant symbiont during the first 7 months of recovery (**Figure 6A** and **Supplementary Table 3**). This change in the relative abundances of two species persisted for at least 11 months under normal conditions, but by September 2015, *B. dendrogyrum* displaced *B. meandrinium* as the dominant symbiont. This reversal of endosymbiont assemblages is consistent with other studies that have monitored changes before and after non-recurrent hyperthermal events, where changes in symbionts during bleaching episodes reverted to the original state after several months or even years (Thornhill et al., 2006; Sampayo et al., 2008; LaJeunesse et al., 2009b; Grottoli et al., 2014). Nevertheless, the reversion to the normal symbiont in *D. cylindrus* was short-lived as *B. meandrinium* again become the dominant symbiont species among colonies at the Pickles site in response to the second hyperthermal event in August–September 2015. These host-symbiont combinations persisted and, at the time of the last sampling for this study in April 2016, *B. meandrinium* remained the dominant species (**Figure 6A** and **Supplemental Table 3A**). Importantly, corals at this site did not lose color during this second event (**Figure 5**); the relative abundance of *B. meandrinium* was 3–4 times higher during the onset of the 2015 hyperthermal event in comparison to pre-bleaching levels in April 2014. Under scenarios of annual hyperthermal bleaching events, impacted coral communities may not have sufficient time to fully recover their stable host-symbiont pairings.

The Adaptive Bleaching Hypothesis posits that when corals bleach, they expel less thermally tolerant endosymbionts and then acquire new, more favorable endosymbionts, allowing them to acclimatize and adapt to environmental stressors (Buddemeier and Fautin, 1993; Baker et al., 2004; Buddemeier et al., 2004). Berkelmans and Van Oppen (2006) demonstrated that thermal stress may induce changes in the dominant symbionts among experimental corals, thus providing thermal tolerance and decreased mortality to the coral animal, supporting the hypothesis that the presence of a thermally tolerant endosymbiont, even in low abundance, may impart an ecological advantage to their coral host (LaJeunesse et al., 2009b). Sampayo et al. (2009) demonstrated that shifts in dominance between closely related species in the genus *Cladocopium* can also impart differential thermal tolerance and, ultimately, differential colony survival. However, unusual host-symbiont partnerships that emerge during bleaching events revert to the original state after normal environmental conditions return (Stat et al., 2009).

It appears that during a scenario of hyperthermal stress events, followed by multiple years of less-stressful environmental conditions, new host-symbiont combinations that appear following the bleaching event are short-lived and the Symbiodiniaceae community eventually reverts back to its original state (Berkelmans and Van Oppen, 2006; Sampayo et al., 2008). This is likely as normal host-compatible symbionts are more effective at growing inside their hosts under non-stressful conditions (Jones and Berkelmans, 2010). However, under sustained disturbance events, such as annually recurring hyperthermal events, new thermally tolerant partner pairings may be longer-lived and maintain higher relative proportions within the Symbiodiniaceae community as the period of less-stressful environmental conditions between disturbances becomes shorter (Grottoli et al., 2014). Such was the case at the Pickles site where relative abundance of *B. meandrinium* remained near 50% and, while trace amounts of *Durusdinium* persisted through April 2016, *Symbiodinium* sp. and *Cladocopium* sp. disappeared altogether (**Supplementary Table 3A**). Our data did not show a shift in dominance at the other two sites (**Figures 6B,C**) and, unlike Pickles colonies, they bleached again during the second hyperthermal event in August-September 2015 (**Figure 5**). However, at the Coffins site (Middle Keys), we detected an increase in relative abundance of *B. meandrinium* to 20.39% during the second hyperthermal event (August–September 2015; **Figure 6B** and **Supplementary Table 3B**), which was associated with only paling in most colonies at this site (**Figure 5** and **Supplementary Table 4**). While *B. meandrinium* did not become the dominant Symbiodiniaceae species, perhaps some threshold abundance may also impart at least partial bleaching resistance for the coral animal (Bay et al., 2016), as was observed at the Coffins site. The Lower Keys site (Marker 32) showed minimal fluctuation in the endosymbiont community but also bleached more severely and recovered more slowly after both bleaching events.

This difference across sites seems not linked to different temperature profiles during the two hyperthermal events, since all sites experienced similar exposure to elevated temperatures above the 30.5°C bleaching threshold for the FRT (**Figures 3**, **4**). While we cannot explain why the shift of Symbiodiniaceae assemblages did not occur across all sites, it is important to note that baseline abundance of the cryptic *B. meandrinium* prior to the first hyperthermal event in April 2014 was higher in Pickles colonies than the other two sites (**Supplementary Table 3**). Perhaps some critical minimum abundance may be required for a rare symbiont to out-compete dominant symbionts when the opportunity arises, such as during bleaching and recovery. However, the relative abundance of *B. meandrinium* in Coffins colonies was higher (20.15%) prior to the second hyperthermal event and still we did not see a shift in symbiont assemblages during the second bleaching and recovery event at this site. This suggests the existence of other site-specific factors influencing the dynamics and competition processes of symbiont repopulation after environmental stressors. Alternatively, genotypic differences in the coral animal may contribute to the observed differential symbiont flexibility, but their role is thus far unknown. Recent work on genetic diversity in Florida's *D. cylindrus* population indicates that each of these three sites is represented by unique coral genotypes (Chan et al., 2018). All colonies at the Pickles site comprised one genotype, indicating a high level of clonality at this local. The same pattern of clonality was also detected for colonies at the Coffins site. The Marker 32 site had the most genetic diversity, composed of five different coral animal genotypes, and yet was the population most affected by these hyperthermal events. Continued work on the role of genetic diversity in the coral host and its symbiont requires further investigation.

It has been proposed that colonies exposed to moderate thermal stress are better conditioned to dealing with episodes of severe thermal stress (Oliver and Palumbi, 2011; Ainsworth et al., 2016). Thermal profiles showing a pre-bleaching spike in water temperatures, followed by a recovery period of cooler temperatures, prior to a hyperthermal event, reduced the severity of bleaching. Temperature profiles at our three study sites show a similar sub-bleaching spike in water temperatures in June 2014 and May 2015 (**Figure 3B**), however, severe bleaching occurred in August–September both years. This may be due to the recovery period being too short or the ensuing hyperthermal stress was too severe (i.e., temperatures exceeding the bleaching threshold for too many weeks, **Supplementary Table 1**), exceeding the capacity of the thermally primed corals. As predicted by Ainsworth et al. (2016), climate change leading to annual bleaching and excessive thermal stress may indeed disable this protective thermal priming scenario.

Persistent Cryptic Communities as a Source of More Thermally Tolerant Strains for Acclimatization and Adaptation

Our data support that under certain circumstances, a low abundant symbiont may emerge during thermal bleaching and/or recovery to enhance acclimatization of the coral host (Kemp et al., 2014). *Durusdinium* sp., known to be opportunistic in certain Caribbean corals exposed to stress (LaJeunesse et al., 2009b), remained at trace levels (<0.03%) during the 2014 and 2015 bleaching and recovery events on the FRT. Due to the high resolution of this study, this is the first reporting of *Durusdinium trenchii* (formerly *Symbiodinium trenchii*) in *Dendrogyra cylindrus*, suggesting an expansion of this invasive, more thermally tolerant species into another Caribbean coral host (LaJeunesse et al., 2010, 2014; Wham et al., 2011). *Durusdinium trenchii* is a stress-tolerant species within the genus *Durusdinium*, commonly found in the Indo-Pacific and known to impart thermal tolerance and bleaching resistance to its coral host (Pettay and Lajeunesse, 2009). This species is considered invasive in the Greater Caribbean and has been increasingly found in corals inhabiting marginal habitats or under high environmental stress, particularly after bleaching events (Pettay and Lajeunesse, 2009).

One of three cryptic *Symbiodinium* OTUs detected in *D. cylindrus* and identified putatively as *Symbiodinium necroappetens* (strain A13), is considered an opportunist, emerging transiently to associate with thermally stressed or diseased corals (LaJeunesse et al., 2015). Our data show that *Symbiodinium* spp. were part of the cryptic community in *D. cylindrus* even prior to the 2014–2015 thermal events (April 2014; **Supplementary Table 3**). At sites showing an increased abundance of *B. meandrinium* in response to annual thermal events (Pickles and Coffins), *Symbiodinium* and *Cladocopium* genera were not detected, and may have been displaced as *B. meandrinium* approached 20% relative abundance. Alternatively, the occurrence and persistence of cryptic symbionts in *D. cylindrus* and other Caribbean corals may also be an indicator of long-term physiological stress due to deteriorating environmental factors, e.g., water quality (Boyer et al., 1999; Boyer and Jones, 2002; Wagner et al., 2010) and pulsed thermal events (i.e., previous Florida Reef-wide bleaching events in 1987, 1990, 1997, 1998, 2005, as well as other localized bleaching episodes (Manzello et al., 2007).

CONCLUSION

While it is encouraging to substantiate that coral symbioses respond ecologically and thus "acclimatize" to a changing climate through shifts in the dominant symbiont partner, it may not be enough for their long-term survival in the Anthropocene. As annual thermal bleaching is predicted to become more prevalent on many reefs in the coming decades, the collateral damage to biological, physiological, and immunological functions of the coral holobiont (Bellantuono et al., 2012; Pinzón et al., 2015) may negate their innate ability to acclimatize. It is imperative that we address the issues of environmental stressors in the hopes that at least some reef ecosystems will be able to acclimate and survive in a changing climate. Even this glimmer of hope for survival may prove to be 'too little, too late' as alarming coral losses due to escalating disease outbreaks in warming oceans may overcome these slow-growing monarchs of the reef more quickly than they can adapt.

NOMENCLATURE

- cp23S-HV primers - utilize length variation in Domain V of large sub-unit rDNA chloroplast 23S hyper-variable gene region
- ENSO - El Niño/ La Niña Southern Oscillation is characterized by oscillating changes from expected sea surface temperatures in the eastern and central equatorial Pacific Ocean (El Niño – warm phase, and La Niña – cool phase)
- FRT – Florida Reef Tract
- Illumina Mi-Seq platform – high resolution parallel amplicon sequencing diversity assay
- Symbiodiniaceae Family (LaJeunesse et al., 2018) – photosynthetic endosymbiotic dinoflagellates
    - *Symbiodinium* spp. (living together, whirling) – formerly Clade A
    - *Breviolum* spp. (short and small) – formerly Clade B
    - *Cladocopium* spp. (branch and plenty) - formerly Clade C
    - *Durusdinium* spp. (tough and whirling) – formerly Clade D

DATA AVAILABILITY

Water temperature data at Pickles, Coffins, and Marker 32 sites: NSF RAPID project 637743 https://www.bco-dmo.org/dataset/699988. 10-year mean monthly water temperatures: National

Data Buoy Center, Molasses Reef C-MAN station MLRF1 data: https://www.ncbi.nlm.nih.gov/sra. Cp23S-Illumina Sequencing: all fastq files deposited in NCBI Sequence Read Archive – BioProject #PRJNA513329.

AUTHOR CONTRIBUTIONS

CL conceived experimental design and sampling strategy, site selection, sampling methodologies for fieldwork, sample processing and data analysis, manuscript preparation including figures and tables, and reviewed manuscript drafts. KN contributed site selection, field assessments and dive support, assisted with tables and figures, and reviewed manuscript drafts. MR-L conceived experimental design and sampling strategy, financial support, manuscript preparation, and reviewed the manuscript drafts.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00005/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

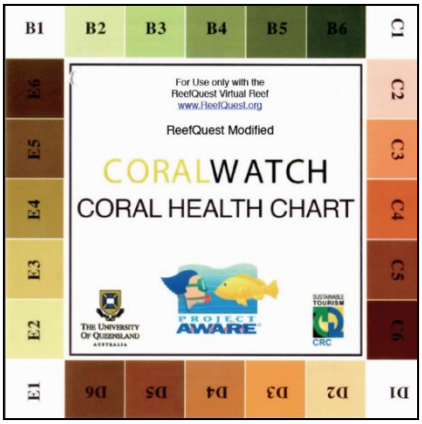
Supplemental 2

Graphical abstract:

Acquired site-specific bleaching resistance associated with a switch in dominant *Breviolum* species during two consecutive hyperthermal bleaching events. Photos represent a single colony at Pickles site during two hyperthermal bleaching and recovery events in 2014 and 2015. (photos: C Lewis)

SUPPLEMENTAL FIGURES & TABLES

Supplemental Figure 1. Coral Health Chart used to calculate the level of bleaching on *Dendrogyra cylindrus* colonies monitored between 2014 and 2016.



Coloration of live tissue was determined by comparing with the gradient of color on the Coral Health Chart (https://www.projectaware.org) *(Siebeck et al. 2006)*. Color scores were further broken down (0.5) if color appeared between two values. Percent of each color value on an individual colony was estimated visually. Chart score (column A) was multiplied by estimated percent of colony live tissue (column B) to calculate colony score for each chart score value (column C). All colony scores for each coloration value (column C) were added to determine Total Colony Coloration Score.

Calculation example:

Bleach Status	**A** **Coral Health Chart Score**	**B** **Estimated proportion of total live tissue on colony**	**C** **Colony Score** (col A x col B)
Bleached	1.0	.20	1.0 x 0.20 = 0.20
	1.5	0	0
Pale	2.0	.30	2.0 x 0.30 = 0.60
	2.5	0	0
	3.0	0	0
Healthy	3.5	.50	3.5 x 0.50 = 1.75
	4.0	0	0
Total Colony Coloration Score		**1.00**	**2.55**

Supplemental Table 1. Summary of thermal profiles from three sites on the Florida Reef Tract 2014-2016.

HOBO data loggers recorded hourly water temperatures at three sites (Pickles: Upper, Coffins: Middle, Marker 32: Lower) 2014-2016 along the Florida Reef Tract (FRT). **(A).** Maximum weekly and maximum annual temperatures were recorded at each site. Number of weeks maximum water temperatures exceeded the FRT bleaching threshold (30.5°C) were calculated. for each site. **(B).** Ten-year mean monthly maximum (MMM) water temperatures were calculated for July, August, and September from archived C-man Station data at Molasses Reef (MRLF1) 2004-2013. Degree heating weeks (DHW, °C-weeks) were calculated for each site using the mean monthly maximum temperatures

for July, August, and September and compared to NOAA Coral Reef Watch DHW. Degree heating weeks were also calculated at each site using the FRT bleaching threshold for comparison. Weeks where DHW were <1°C-weeks were not counted, as per standard protocols. ND represents insufficient data for meaningful calculations.

A. 10-yr Mean Monthly Maximum 2014-2013 at Molasses Reef (MRLF1)	
month	temperature (°C)
July	30.80
Aug	31.31
Sept	30.78
FRT bleach threshold	30.50
NOAA Coral Reef Watch 50-km Satellite Degree Heating Weeks (DHW) for FRT	
2014	2015
5°C-weeks	7°C-weeks

B.	2014			2015		
	Pickles*	Coffins	Marker 32	Pickles	Coffins	Marker 32
DHW using MMM	ND	7.48	2.8	0.00	5.21	4.07
DHW -using 30.5°C	ND	13.82	8.65	5.88	13.96	10.59
# weeks >30.5°C	ND	10	8	12	13	13
max temperature °C	30.36	33.01	32.50	31.88	32.39	32.50

*data loggers were lost at Pickles site from 4/1/2014-9/12/2014

Supplemental Table 2. Primers used for cp23S amplicon sequencing and microsatellite genotyping analysis in Symbiodiniaceae.

Primer name	**Primer Sequence**	**Reference**
cp23S hyper up (forward)	TCA GTA CAA ATA ATA TGC TG	Santos et al 2003
cp23S hyper down (reverse)	TTA TCG CCC CAA TTA AAC AGT	
B7*Sym*15 forward	CTC ACC TTG AAA TCA GTA GCC A	Pettay & LaJeunesse 2007
B7*Sym*15 reverse	CGT AGC TTC TGA AGG TAC GAC AC	

Supplemental Table 3. Mean relative abundance of Symbiodiniaceae genera in *D. cylindrus* at three sites on the Florida Reef Tract 2014-2016.

Percent mean relative abundance (± standard deviation) of operational taxonomic units ($OTU_{0.03}$). Asterisk (*) indicates bleaching months. **(A)** Pickles (n=6 colonies sampled) Upper Keys **(B)** Coffins (n=6 colonies sampled) Middle Keys **(C)** Marker 32 (n=6 colonies sampled) Lower Keys

(A) Pickles n=6 Upper Keys	**Year 1**				**Year 2**				
	Apr-14	***Sep-14**	**Oct-14**	**Dec-14**	**Apr-15**	***Sep-15**	**Nov-15**	**Jan-16**	**Apr-16**
B. dendrogyrum 1 OTU	75.68 (16.15)	45.47 (25.91)	25.35 (14.73)	20.60 (19.67)	33.35 (14.18)	53.41 (19.15)	33.29 (12.77)	31.63 (14.65)	29.97 (8.02)
B. meandrinium 1 OTU	8.75 (0.88)	29.53 (22.06)	48.74 (3.24)	53.49 (5.79)	49.37 (11.82)	30.28 (13.02)	49.60 (10.35)	51.10 (12.12)	51.55 (6.58)
all other *Breviolum* 243 OTUs	15.56 (1.79)	25.23 (9.47)	25.90 (5.11)	25.90 (4.34)	17.27 (2.38)	16.31 (6.16)	17.11 (2.44)	17.27 (2.54)	18.48 (1.59)
all *Symbiodinium* 94 OTUs	0.01 (0.01)	5.38 (13.85)	0.001 (0.004)	0.002 (0.002)	0	0	0	0	0
all *Cladocopium* 3 OTUs	0.001 (0.001)	0.001 (0.001)	0.01 (0.03)	0	0	0	0	0	0
all *Durusdinium* 1 OTU	0	0	0.001 (0.002)	0	0.001 (<0.001)	0.0003 (0.0007)	0.001 (<0.001)	0.001 (<0.001)	0

(B) Coffins n=6 Middle Keys	**Year 1**				**Year 2**				
	Apr-14	***Sep-14**	**Oct-14**	**Dec-14**	**Apr-15**	***Sep-15**	**Nov-15**	**Jan-16**	**Apr-16**
B. dendrogyrum 1 OTU	80.57 (6.54)	77.60 (5.60)	78.58 (3.68)	76.50 (5.46)	66.78 (0.66)	65.54 (0.56)	78.26 (12.71)	88.87 (0.53)	88.87 (0.17)
B. meandrinium 1 OTU	0.88 (0.12)	0.94 (0.14)	3.24 (1.92)	5.79 (5.05)	20.15 (0.14)	20.39 (0.40)	10.02 (11.42)	0.12 (0.01)	0.15 (0.02)
all other *Breviolum* 243 OTUs	18.52 (6.45)	19.34 (2.91)	18.18 (3.01)	17.71 (2.24)	13.07 (0.62)	14.04 (0.20)	11.58 (1.46)	10.77 (0.53)	10.73 (0.20)
all *Symbiodinium* 94 OTUs	0.02 (0.01)	2.12 (0.01)	<0.001	0.003 (0.01)	0	0	<0.001	0.003 (0.002)	0.003 (<0.001)
all *Cladocopium* 3 OTUs	0	0.003 (0.01)	<0.001	<0.001	0	0	0.14 (0.16)	0	0
all *Durusdinium* 1 OTU	0	0	<0.001	0	<0.001	0.03 (0.06)	0.002 (0.002)	0.004 (0.002)	0.003 (0.003)

(C) Marker 32 n=6 Lower Keys	**Year 1**				**Year 2**				
	Apr-14	***Sep-14**	**Oct-14**	**Dec-14**	**Apr-15**	***Sep-15**	**Nov-15**	**Jan-16**	**Apr-16**
B. dendrogyrum 1 OTU	81.09 (2.24)	66.26 (11.33)	65.58 (15.69)	70.33 (6.90)	*87.82 (0.44)*	86.80 (1.05)	87.43 (0.87)	88.34 (0.39)	87.60 (0.30)
B. meandrinium 1 OTU	0.76 (0.08)	2.60 (4.55)	1.44 (0.54)	3.71 (3.04)	0.08 (0.04)	0.17 (0.08)	0.11 (0.05)	0.08 (0.02)	0.06 (<0.01)
all other *Breviolum* 243 OTUs	18.07 (2.21)	31.03 (9.98)	32.76 (15.43)	25.96 (5.34)	11.80 (0.45)	12.72 (1.00)	12.23 (0.88)	11.32 (0.39)	12.12 (0.31)
all *Symbiodinium* 94 OTUs	0.02 (0.01)	0.01 (0.01)	0.20 (0.54)	0.003 (0.005)	0.002 (0.001)	0.007 (0.01	0.003 (0.001)	0.003 (0.001)	0.002 (0.002)
all *Cladocopium* 3 OTUs	0.03 (0.07)	<0.001	0	<0.001	0.29 (0.01)	0.29 (0.01)	0.22 (0.02)	0.25 (0.02)	0.21 (0.02)
all *Durusdinium* 1 OTU	0.002 (0.005)	0	0	0	0.006 (0.003)	0.009 (0.005)	0.005 (0.001)	0.005 (0.001)	0.01 (0.007)

Supplemental Table 4. Mean colony coloration scores at three sites on the Florida Reef Tract 2014-2016.

Mean colony coloration scores (±1 standard deviation) at three sites on the Florida Reef Tract April 2014 to April 2016. CoralWatch Coral Health Chart (version: Project Aware https://www.projectaware.org) was used to determine a score for each colony. See Figure 4.5 for calculating colony coloration scores. NA = no data. Colorimetric chart was not utilized during April 2014 assessments; coloration scores were later estimated by reviewing colony photographs (estimated mean coloration scores 3.75).

	Year 1							**Year 2**					
	Apr-14	**Sep-14**	**Oct-14**	**Nov-14**	**Dec-14**	**Jan-15**	**Mar-15**	**Apr-15**	**Sep-15**	**Oct-15**	**Nov-15**	**Jan-16**	**Apr-16**
Pickles													
n=10		1.29	2.19	3.45	3.87	3.98	3.93	3.48	3.52	3.98	4.00	4.00	3.60
Upper Keys	NA	(0.16)	(0.16)	(0.60)	(0.20)	(0.08)	(0.11)	(0.24)	(0.49)	(0.07)	(<0.01)	(<0.01)	(0.52)
Coffins													
n=10		1.45	2.09	3.23	3.80	3.95	3.43	3.35	2.20	3.28	3.50	3.98	3.84
Middle Keys	NA	(<0.01)	(0.22)	(0.41)	(0.14)	(0.14)	(0.19)	(0.24)	(0.21)	(0.24)	(<0.01)	(0.10)	(0.24)
Marker 32													
n=12		1.12	1.23	1.48	1.70	2.08	2.47	2.57	1.48		3.46	3.76	3.08
Lower Keys	NA	(0.07)	(0.12)	(0.31)	(0.38)	(0.39)	(0.47)	(0.39)	(0.62)	NA	(0.33)	(0.33)	(1.13)

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PUBLICATIONS AND PRESENTATIONS

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