Vol. 137: 217–237, 2020
https://doi.org/10.3354/dao03441

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published online January 30

Ecology, histopathology, and microbial ecology of a white-band disease outbreak in the threatened staghorn coral *Acropora cervicornis*

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ABSTRACT: This study is a multi-pronged description of a temperature-induced outbreak of white-band disease (WBD) that occurred in *Acropora cervicornis* off northern Miami Beach, Florida (USA), from July to October 2014. We describe the ecology of the disease and examine diseased corals using both histopathology and next-generation bacterial 16S gene sequencing, making it possible to better understand the effect this disease has on the coral holobiont, and to address some of the seeming contradictions among previous studies of WBD that employed either a purely histological or molecular approach. The outbreak began in July 2014, as sea surface temperatures reached 29°C, and peaked in mid-September, a month after the sea surface temperature maximum. The microscopic anatomy of apparently healthy portions of colonies displaying active disease signs appeared normal except for some tissue atrophy and dissociation of mesenterial filaments deep within the branch. Structural changes were more pronounced in visibly diseased fragments, with atrophy, necrosis, and lysing of surface and basal body wall and polyp structures at the tissue-loss margin. The only bacteria evident microscopically in both diseased and apparently healthy tissues with Giemsa staining was a *Rickettsiales*-like organism (RLO) occupying mucocytes. Sequencing also identified bacteria belonging to the order *Rickettsiales* in all fragments. When compared to apparently healthy fragments, diseased fragments had more diverse bacterial communities made up of many previously suggested potential primary pathogens and secondary (opportunistic) colonizers. Interactions between elevated seawater temperatures, the coral host, and pathogenic members of the diseased microbiome all contribute to the coral displaying signs of WBD.

KEY WORDS: Coral diseases · 16S sequencing · *Acropora cervicornis* · White-band disease · Climate change · Microbial ecology · Histopathology · *Rickettsiales*-like organism · Southeast Florida

1. INTRODUCTION

Tropical coral reefs are among the most important ecosystems on the planet because of both their intrinsic ecological value as well as the services they provide to humans (Costanza et al. 1997). Coral reefs are now degrading at an alarming rate (Spalding & Brown 2015, Hughes et al. 2018), jeopardizing these ecosystem services (Aronson & Precht 2001, Hughes et al. 2003, Hoegh-Guldberg et al. 2007, Wild et al. 2011) and threatening the extinction of a large part of Earth's biodiversity (Carpenter et al. 2008, Veron et

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al. 2009, Glynn 2011). During the summer of 2014, numerous bleaching alerts were issued in response to anomalously warm sea surface temperatures (SSTs) in Florida, USA (NOAA 2014a,b). This followed an unusually warm winter and spring (Manzello 2015), and by the early fall, many stony corals, zoanthids, and octocorals off southeast Florida had bleached, resulting in the worst regional bleaching episode since 1997–1998 (Manzello 2015). In addition to extensive coral bleaching (Manzello 2015), there were numerous reports of coral disease throughout the region, including black-band disease (Lewis et al. 2017) and the recently described outbreaks of white plague or stony coral tissue loss disease (Precht et al. 2016, Walton et al. 2018). During this time, an outbreak of white-band disease (WBD) was observed on a community of *Acropora cervicornis* being monitored off northern Miami Beach in Miami-Dade County, Florida. To document this disease outbreak and compare it to other instances of WBD, we monitored the progression of the disease and examined diseased corals using both histopathology and next-generation bacterial 16S gene sequencing. This multi-pronged approach allowed us to not only describe this outbreak and link it to observed thermal anomalies, but also explore some of the inconsistencies found in previous studies of WBD.

WBD was first described in 1976 in St. Croix by Dr. William Gladfelter (Gladfelter et al. 1977), who noted a rapidly-spreading sloughing of *A. palmata* tissue (Gladfelter 1982). Signs of WBD are sloughing coral tissue, which leaves single or multifocal bands of exposed white skeleton on otherwise healthy-looking *Acropora* spp. (*A. palmata, A. cervicornis*, and their hybrid *A. prolifera*) branches (Gladfelter et al. 1977, Peters et al. 1983). The tissue loss progresses up to a few mm per day from base to tip, leaving white skeletons that are quickly overgrown by turf algae. After a decade of epizootics, populations of acroporids have undergone catastrophic declines across the entire Caribbean and Western Atlantic (Bak & Criens 1981, Knowlton et al. 1981, Van Duyl 1985, Jaap et al. 1988, Bythell & Sheppard 1993, Sheppard 1993, Precht & Aronson 1997, Williams & Bunkley-Williams 2000, Bythell et al. 2004, Aronson & Precht 2016).

Recent reports have described at least 4 types of 'white' diseases in *A. cervicornis*, including shut-down reaction (Antonius 1977), WBD type I (Peters et al. 1983), WBD type II (Ritchie & Smith 1998), and rapid tissue loss (Miller et al. 2014), further confounding the terminology (Rogers 2010, Sheridan et al. 2013). In all cases, the visible white band is due to tissue death and exfoliation, not bleaching. Because the distinctions among these diseased states in field surveys are highly ambiguous, we have used the general term WBD. It has become evident during the last 4 decades that WBD epizootics have been the primary cause of *Acropora* spp. mortality in the Caribbean and western Atlantic (Aronson & Precht 2001, 2016, Bythell et al. 2004, Rogers 2009, Randall & van Woesik 2015), including Florida (Shinn & Wicklund 1989, ABRT 2005). This disease is partially responsible for the listing of the Caribbean acroporids as threatened on the US Endangered Species List and as Critically Endangered on the IUCN Red List (Antonius 1994a,b, Green & Bruckner 2000, Precht et al. 2002, 2004, Carpenter et al. 2008, Aronson et al. 2008a,b). This is not to say that acroporid corals have been spared the effects of local anthropogenic stressors, nor does it rule out the involvement of WBD in compound or complex mortality processes. However, WBD has been observed under a wider variety of conditions than those in which acute local impacts can be implicated as the primary cause of acroporid decline (Aronson & Precht 2001).

Transmission of WBD signs has been demonstrated numerous times, suggesting that the disease can be caused by a biotic infectious agent. Demonstrated modes of transmission include direct contact (Vollmer & Kline 2008), through the water column (Gignoux-Wolfsohn et al. 2012, Precht & Vollmer 2013), and through contact with suspected biological vectors (Williams & Miller 2005, Gignoux-Wolfsohn et al. 2012, Certner et al. 2017). However, WBD signs are not always transmissible (e.g. Smith & Thomas 2008), and spread does not follow contagious disease models (Muller & van Woesik 2012).

Infectious agents across several bacterial taxa have been suggested as potential WBD pathogens, including large bacterial aggregates, *Vibrio charchariae/ V. harveyi,* (Ritchie & Smith 1998, Gil-Agudelo et al. 2006), *Bacillus* sp., *Lactobacillus suebicus* (Sweet et al. 2014), and a *Rickettsiales*-like organism (RLO) (Casas et al. 2004). Recent studies using next-generation sequencing to characterize WBD-associated bacterial communities have suggested that the disease may be caused by multiple pathogens, either a group of related infectious or noninfectious microbial pathogens (Gignoux-Wolfsohn et al. 2017) or a loose consortium that varies with host and environmental circumstances (Gignoux-Wolfsohn & Vollmer 2015, Peters 2015), likely regulated by quorum sensing (Certner & Vollmer 2018). This suggests that WBD

could belong to a class of coral ailments that share a derangement of healthy coral microbiomes (Sweet & Bulling 2017, Zaneveld et al. 2017). In addition, histophagous ciliates have been associated with WBD, although their role in tissue loss remains unclear (Sweet et al. 2014).

Sokolow (2009) suggested that numerous environmental factors associated with global climate change may also play an increasing role in coral disease outbreaks. These climate variables likely alter coral microbiome structure and epidemiology by influencing pathogenic microorganism growth rates, transmission, virulence, and host susceptibility (Harvell et al. 2007, Palmer et al. 2010, Reed et al. 2010, Libro et al. 2013, Randall et al. 2014). Randall & van Woesik (2015) showed a clear linkage between ocean warming and contemporary WBD outbreaks. Accordingly, coral reefs will likely continue to experience disease outbreaks (Smith et al. 2006, Harvell et al. 2007), with multiple compounding stressors propelling some species like the acroporids towards extinction (Carpenter et al. 2008, Veron et al. 2009, Glynn 2011, Birkeland et al. 2013).

Given the complex biotic and abiotic factors influencing WBD, it is critical that we employ a transdisciplinary approach to studying this disease. By combining field surveys that can link disease signs to environmental factors, molecular microbial studies that can identify changes in the coral microbiome associated with macroscopic tissue loss (Ritchie & Smith 1998, Gil-Agudelo et al. 2006, Polson 2007, Sweet et al. 2014), and histopathology that can reveal visible biotic pathogens present on the surface or in the tissue and their impacts (Luna et al. 2010, Work et al. 2012, Sweet et al. 2013, Pollock et al. 2014a, Bruckner 2015), we can begin to understand the linkages among pathogens and processes (e.g. Richardson et al. 2001, Work et al. 2008, Work & Aeby 2011, Work & Meteyer 2014, Burge et al. 2016).

In this study, we used this multi-pronged approach to assess the impact of a WBD outbreak on an *A. cervicornis* community off northern Miami Beach in Miami-Dade County, Florida. The objectives were to (1) quantify the prevalence of WBD at our study site; (2) examine the potential influence of temperature on timing of the outbreak and potential links between the outbreak and coral bleaching; (3) characterize and draw links between the microbiomes and tissue condition of corals with and without disease signs; and (4) identify bacteria shared between these corals and other disease outbreaks.

2. MATERIALS AND METHODS

2.1. Study site

This study was undertaken on the innermost (1.5–2 km offshore) of a series of submerged (6–11 m deep), shore-parallel fossil reef terraces off Miami Beach (Walker 2012) — the remains of a 150 km long barrier-reef system dominated by *Acropora* spp. during the Holocene (Lighty et al. 1978, Precht & Aronson 2004) (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d137p217_supp.pdf). The terrace now consists of patches of *A. cervicornis* with an average density of about 0.05 colonies m^{-2} (DERM 2008). In recent decades, there have been localized increases in *A. cervicornis* populations in this area (Vargas-Ángel et al. 2003, Precht & Aronson 2004, Wirt et al. 2013, D'Antonio et al. 2016, S. Blair pers. comm.), which is believed to be the current northern range limit of this species (Walker 2017). While thickets are extremely transient (Walker et al. 2012, D'Antonio et al. 2016), this new growth may be a northern range expansion due to warming SSTs (Precht & Aronson 2004, Precht et al. 2014, Kuffner et al. 2015).

2.2. Seawater temperatures

We used SST data from the NOAA National Data Buoy Center, Fowey Rock Station (NOAA 2015), located within Biscayne National Park, 28 km away from the survey site. Timing of temperature changes was compared to timing of coral bleaching and disease prevalence data recorded at our monitoring site.

2.3. Ecological field data collection

To collect data on coral condition, we used timed swim surveys along the inner-reef tract (Fig. S1). In total, 6 surveys were performed between October 2013 and October 2014 (see Table 1). The diver entered the water at a fixed geographic coordinate and swam in a roughly rectangular pattern approximately 1 m above the substrate, stopping occasionally to take notes and photographs, returning to the start within 30 min. Water depth varied between 6 and 8 m, and approximately 600 m^2 of reef benthos were sampled during each survey. We were unable to place permanent transects at this location due to permitting restrictions associated with the ad hoc nature of our surveys.

The diver recorded the total number and condition of *A. cervicornis* colonies seen, with individual colonies defined as 'connected skeletal branches with a common basal attachment to the substrate' (Huntington & Miller 2014). Various signs of coral stress, recent mortality, and recent partial mortality were noted using a standard set of diagnostic criteria developed by W. F. Precht (unpubl.). Signs of previous stress include WBD (evenly distributed tissue loss from base to tip, sensu Gladfelter 1982), feeding scars from the corallivorous gastropod *Coralliophila abbreviata* (presence of snail, uneven tissue loss up one side; Ott & Lewis 1972), the presence of the fireworm *Hermodice carunculata* (tissue loss on tips of branches only, Ott & Lewis 1972), territories and spot-biting of the threespot damselfish *Stegastes planifrons* (spots of tissue loss colonized by algae; Precht et al. 2010), and coral bleaching (loss of pigmentation but intact tissue; Glynn 1993) (Fig. 1). On many colonies, more than 1 condition or combination of conditions were noted. Recent mortality was classified as white skeleton with minimal algal turf. Corals observed during the surveys were photographed using a Sealife DC600 underwater camera and housing. A follow-up timed-swim survey was undertaken 1 yr after the initial outbreak on 20 September 2015.

2.4. Sample collection for laboratory analysis

In total, divers collected 20 branch fragments (2 cm) from 5 living colonies of *A. cervicornis* on 1 October 2014. When the outbreak was first documented, permits were requested, but delays in the process meant that sampling could only occur in October. The permits only allowed sampling of colonies already displaying disease signs to protect unaffected colonies. Each of the 5 colonies had multiple branches with active disease as well as multiple branches that appeared healthy (no outward signs of disease, bleaching, or predation). Two (2 cm) branch tips that appeared to be healthy and 2 that had tissue loss (including the disease margin, 60% tissue, and 40% recently exposed skeleton; Fig. 1) were taken from each parent colony using garden clippers. Because the tissue-loss margins were removed from these colonies, the apparently healthy tissue remained intact when observed 1 mo after sampling.

On returning to the boat, 5 apparently healthy and 5 diseased samples (H1–5 and D1–5, respectively; 1 pair from each parent colony) were placed in individual pre-labeled sample vials containing 1 ml of guanidine thiocyanate DNA buffer (Fukami et al. 2004) for 16S sequencing (see Section 2.5 for details). The apparently healthy and diseased branch tips for histopathology were transferred to 50 ml plastic centrifuge tubes containing a formaldehyde-based fixative solution made of 1 part Z-Fix Concentrate (Anatech) diluted with 4 parts 35 ppt seawater (marine salts diluted with deionized water) and tightly sealed (see Section 2.6 for further details on histopathology).

2.5. 16S sequencing

2.5.1. Library preparation and sequencing

DNA was extracted from the 10 samples and a blank control using the Agencourt DNAdvance bead

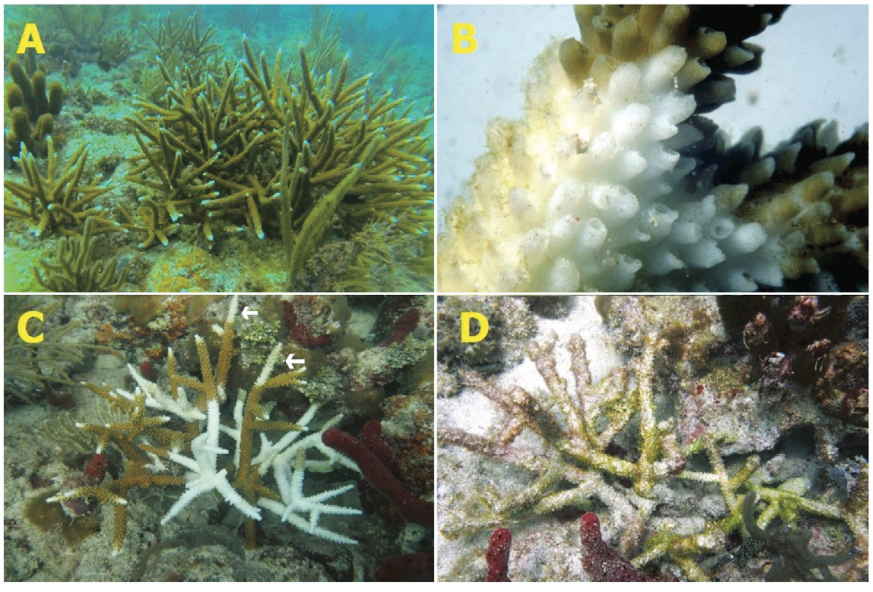

Fig. 1. (A) Apparently healthy thickets of *Acropora cervicornis* at the northern Miami Beach monitoring site, 16 October 2013. (B) Close-up white band disease (WBD) progression; note colonization by turf algae on recently dead, exposed skeleton. (C) Colony with both WBD and fireworm predation scars (indicated by arrows), 8 July 2014. (D) Same colony as in (C), with 100% mortality approximately 2 mo later, on 17 September 2014

extraction kit (Agencourt Bioscience). The V6 hypervariable region of the 16S gene was chosen for its high sensitivity to diversity in a short region (Barriuso et al. 2011, Caporaso et al. 2012). The region was amplified in individual PCR reactions with custom barcoded primers as described by Gignoux-Wolfsohn & Vollmer (2015). The concentration of PCR products was determined using a Qubit 2.0 fluorometer (Thermo Fisher Scientific), and products were pooled to normalize concentration across samples. To remove primer-dimers and dNTPs, final PCR products were cleaned with DNAmpure beads (Agencourt); the Agilent 2100 Bioanalyzer was then used to check concentration and length of products. PCR products were sequenced using paired-end 150 bp sequencing on the Illumina Hiseq 2000.

2.5.2. Bioinformatics

Overlapping of paired reads was performed using FLASH (Mago & Salzberg 2011). Sequences were then demultiplexed, and primers were trimmed and quality filtered (ends with phred score <20 were removed and resulting sequences <60 bp were thrown out) using a custom Python script (https://github.com/sagw/Python_scripts/blob/master/SD1/SD1_demultiplex.py).

Using Qiime 1.9.0, 97% operational taxonomic units (OTUs) were picked using the pick open reference OTUs method (http://qiime.org/scripts/pick_open_reference_otus.html), and taxonomy was assigned using BLAST against the SILVA database (Quast et al. 2013). Chimeras were detected and removed with UCHIME (Edgar et al. 2011). Details of bioinformatics and analyses can be found at https://github.com/sagw/Miami_WBD/tree/master/Notebooks.

2.5.3. Statistical analyses

Normalized counts of OTUs were calculated using a modified version of the 'sizeFactors' function in the R package DESeq2, where counts are normalized by the geometric mean of each sample (Love et al. 2014, McMurdie & Holmes 2014). Non-metric multidimensional scaling (nMDS) to visualize Bray-Curtis dissimilarities was performed using the metaMDS function in the R package 'vegan.' The significance of the community-level effect of disease state was tested by PERMANOVA of Bray-Curtis dissimilarities using the 'Adonis' function in 'vegan.' Diversity metrics were calculated using the 'diversity' and 'betadisper' functions in 'vegan' (Oksanen et al. 2013), and phylogenetic diversity was calculated using the 'pd' function in 'picante.' Significant differences in individual OTU abundance were identified with negative binomial generalized linear models (GLMs) run for each OTU, using the R package DESeq2. The data were fit to a negative binomial distribution, and the likelihood ratio test for significance of GLM terms was used to identify OTUs that differed significantly between diseased and apparently healthy samples (p-value adjusted by FDR < 0.05; Love et al. 2014). Bar charts and bubble charts were created using ggplot2 (Wickham 2009); bubble charts were made for orders that made up at least 5% of the microbiome of at least 1 coral.

2.6. Histopathology

Each sample was photographed, then cut into ~2 cm long portions with a diamond-coated tile-cutting blade fitted on a Dremel tool. To capture organisms or material on denuded skeletal surfaces, subsamples with a tissue-loss margin were enrobed in 1.5% agarose. All subsamples were decalcified with 10% disodium EDTA at pH 7, changing the solution every 48 h, then rinsed with tap water for about 30 min. Subsamples were trimmed into 2–3 mm slices, placed in tissue cassettes, and embedded in Paraplast Xtra® (Miller et al. 2014, Peters 2015). Sections (5 µm thick) were cut with a microtome, mounted on glass microscope slides, stained with Harris's hematoxylin and eosin and Giemsa (Noguchi 1926), and a coverslip was attached with Permount™ mounting medium. The sections were examined with a Zeiss Student 16 compound microscope, and photomicrographs were taken using an Olympus BX43 microscope fitted with an Olympus DP-72 camera.

Semi-quantitative data (Jagoe 1996) were collected about each sample based on condition at the time of fixation (tissue architecture, cellular integrity, Symbiodiniaceae abundance, pathological changes; 0 = excellent, 1 = very good, 2 = good, 3 = fair, 4 = poor, 5 = very poor) and the severity of tissue changes, ranging from normal to severe (0 = within normal limits, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe; see Table S1 in Miller et al. 2014). Symbiodiniaceae condition and abundance were also scored, as well as 6 parameters of polyp health (both cell and tissue), bacterial aggregates, and RLOs (from the Giemsa-stained sections). Presence/absence was noted for the following phenomena: hypertrophied calicodermis foci, necrotic cell spherules, apicomplexans, and ciliates. When possible, the stage of gonad

development was noted (Szmant 1986). Mean scores for each sample (across sections created from tissue blocks) were calculated. Descriptive statistics were calculated for the scored parameters in the apparently healthy and diseased groups, frequency distributions were examined, and comparisons were made using Student's *t*-tests, applying the sequential Bonferroni correction for p-values (Rice, 1989) and Mann-Whitney *U*-tests (Rice 1989).

3. RESULTS

3.1. Disease occurrence and prevalence

The first sign of a WBD outbreak (prevalence >5%) was noted on 8 July 2014, when the disease prevalence was measured at 15% (15 of 101 corals surveyed). The maximum prevalence of the disease outbreak was recorded as 63% (69 out of 110 corals surveyed) in September 2014. The maximum number of recently dead corals (28 out of 95) was recorded in October following the maximum disease prevalence (Table 1, Figs. 1 & 2).

3.2. Links between temperature, bleaching, and disease

The beginning of the disease outbreak coincided with regional SSTs of ~29°C (Fig. 2). By 17 September, 1 mo after the peak recorded temperature, 63% of the colonies surveyed (69 out of 110) showed signs of WBD (Figs. 1 & 2). Following regional cooling of SSTs in early October, the prevalence dropped quickly and by 22 October, was measured at 6% (5 out of 90 colonies surveyed).

Peak coral bleaching occurred on 17 September 2014, when 30% (33 of 110 *Acropora cervicornis* corals surveyed that day) showed signs of bleaching (Fig. 2). Soon after the temperatures decreased, bleached corals began showing signs of recovery, with most *A. cervicornis* colonies regaining their color within a few weeks (Table 1).

3.3. Difference between diseased and apparently healthy coral bacterial communities

Ten samples were sequenced, yielding 684 925 reads, which clustered into 6605 OTUs. The bacterial communities of apparently healthy coral tissue were significantly different from those of diseased coral tissue (PERMANOVA df = 1, 9, p = 0.049, R^2 = 0.23). This result was corroborated by the nMDS plot, where all diseased coral tissue samples clustered together except for sample D5 (Fig. 3). Diseased coral tissue exhibited a characteristic increase in bacterial diversity as measured by Shannon's index (healthy = 2.65, diseased = 4.95, *t*-test p = 0.015) and Faith's *D* phylogenetic diversity (Faith 1992) (healthy = 36.93 ± 4.59, diseased = 90.42 ± 33.39, *t*-test p = 0.022) but

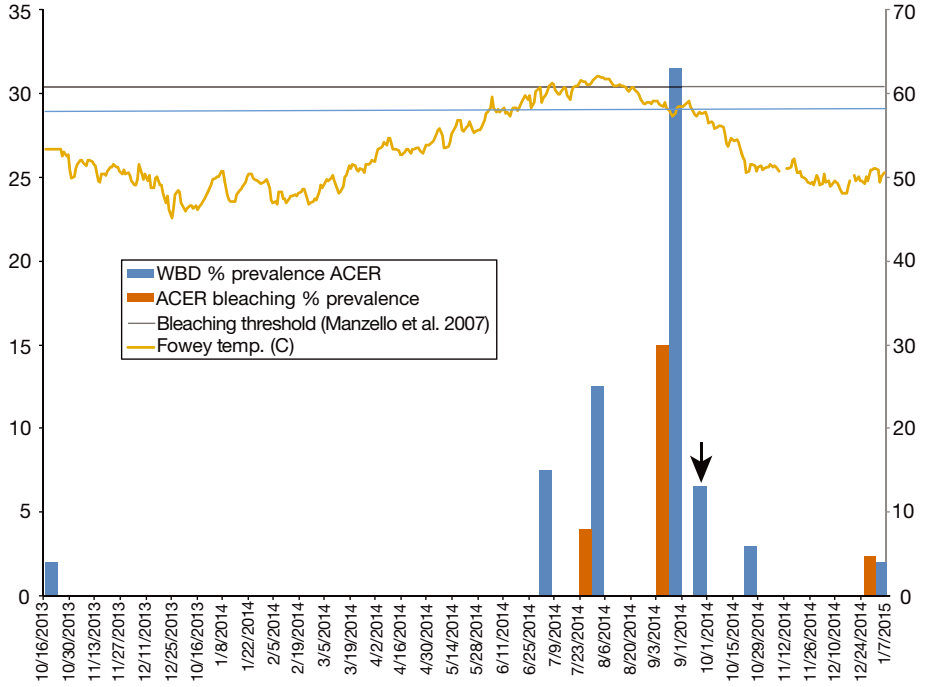

Fig. 2. Relationship between sea surface temperature and prevalence of coral bleaching and white band disease (WBD) on *Acropora cervicornis* (ACER) colonies monitored during timed-swim surveys. Temperature data were downloaded from NOAA National Data Buoy Center, Fowey Rock Station (FWYF1), east of Soldier Key within Biscayne National Park. Grey horizontal line at 30.4°C represents regional coral bleaching threshold (from Manzello et al. 2007). Light-blue line at 29.0°C represents the approximate turn-on and turn-off threshold for WBD in this study. Coral bleaching prevalence is the proportion of paled and bleached *A. cervicornis* colonies recorded during the timed-swim surveys. WBD prevalence is the proportion of actively diseased *A. cervicornis* recorded during these same surveys. Arrow denotes timepoint when samples were taken for histology and sequencing

Table 1. Combined data for all *Acropora cervicornis* colonies observed during the timed-swim survey sites. 'Apparently healthy' column represents colonies that had no outward stress indicator(s) or partial mortality during the survey. On many colonies, more than 1 condition or combination of conditions were observed (including feeding scars from three-spot damselfish, snails, and fireworm) to have been responsible for causing recent partial mortality. Colonies with exposed white skeleton, with minimal algal turf, and no visible living coral tissue were deemed 'recently dead'. Because not all colonies were surveyed at every time point, each time point should be considered independently; total numbers of apparently healthy and dead corals cannot be calculated from these data

Date (mo/d/yr)	Total colonies	Apparently healthy	Apparently unhealthy (some corals had multiple lesion types)						
			Fish	Snails	Fireworm	Disease (prevalence)	Bleached (prevalence)	Partial new mortality	Recently dead
10/16/2013	109	89 (82%)	6	4	12	5 (4%)	0	20	0
7/8/2014	101	69 (68%)	8	8	15	15 (15%)	0	42	2
8/6/2014	97	43 (44%)	6	5	7	24 (25%)	8 (8%)	48	4
9/17/2014	110	12 (11%)	8	6	8	69 (63%)	33 (30%)	83	15
10/1/2014	95	40 (42%)	7	10	11	12 (13%)	0	27	28
10/22/2014	90	46 (51%)	7	5	7	5 (6%)	0	21	25
9/20/2015	82	60 (73%)	6	8	10	3 (4%)	4 (5%)	19	3

dispersion was not significantly higher (healthy = 0.36, diseased = 0.48, ANOVA p = 0.15). Apparently healthy coral tissues were dominated by *Rickettsiales* and *Campylobacterales* (Fig. 4). In contrast, diseased coral tissue had lower proportions of *Rickettsiales* and *Campylobacterales* and were instead dominated by *Alteromonadales*, *Flavobacteriales, Sphingobacteriales*, and *Oceanospirillales*. The outlier sample D5 contained a microbiome more similar to the apparently healthy corals, dominated by *Rickettsiales*.

3.4. Significantly different OTUs

We identified 122 OTUs that significantly differed in abundance (hereafter 'enriched') between diseased and apparently healthy corals: 1 was enriched in apparently healthy corals and 121 were enriched in diseased corals. The healthy-enriched OTU belonged to the order *Sphingobacteriales* (family *Chitinophagaceae*). OTUs belonging to many orders that have previously been associated with diseased corals were identified among the disease-enriched OTUs: *Flavobacteriales* (family: *Flavobacteriaceae*), with 36 OTUs and *Sphingobacteriales* (family *Saprospiraceae*), with 22 OTUs, both within the phylum *Bacteroidetes*; *Rhodobacterales* (family *Rhodobacteraceae*), with 10 OTUs; *Alteromonadales* (family *Alteromonadaceae*) with 3 OTUs; and *Vibrionales* (family *Vibrionaceae,* genus *Vibrio*; Fig. 5).

Several families of disease-enriched OTUs in this study were previously found to be enriched in diseased *A. cervicornis* in Panama as well as multiple species of coral with signs of stony coral tissue loss

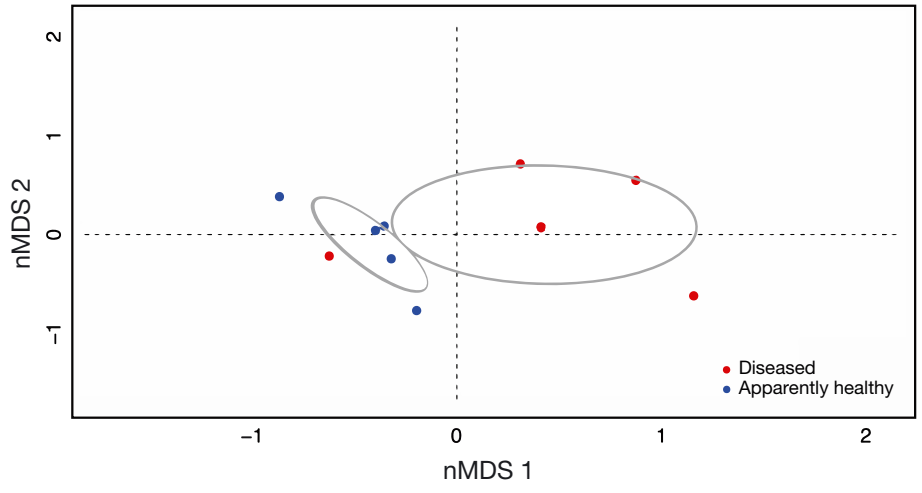

Fig. 3. nMDS of Bray-Curtis dissimilarities between diseased and apparently healthy samples, 2D stress = 0.08. Individuals are labeled according to disease state. Ellipses are 95% confidence ellipses around their respective centroids

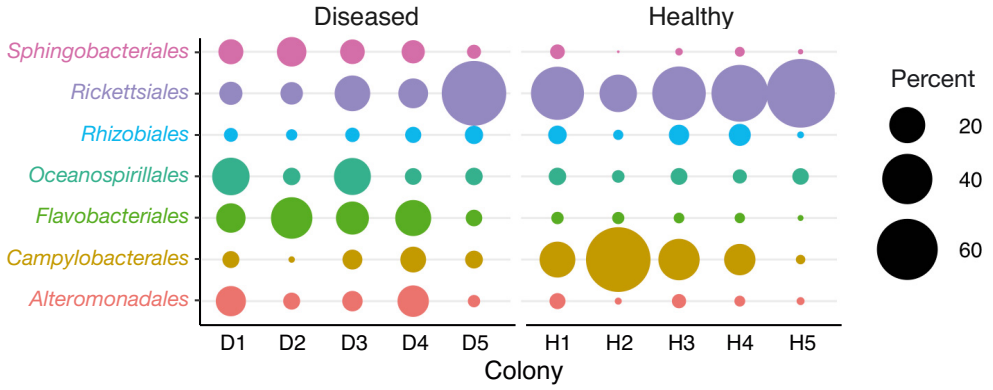

Fig. 4. Dominant bacterial orders of diseased and healthy coral microbiomes. Only orders making up ≥5% of at least one coral were included. Size of bubble corresponds to the average percent of the microbiome made up of that order for either healthy or diseased individuals

in Florida (Meyer et al. 2019). Several families, including *Vibrionaceae*, *Saprospiraceae*, *Rhodobacteraceae*, and *Flavobacteriaceae* were enriched in all species of diseased samples across all 3 studies. Other families, such as *Rhodospirillaceae*, *Enterobacteriaceae*, *Chitinophagaceae*, and *Bacteriovoraceae* were only enriched in *A. cervicornis* displaying signs of WBD in both Panama and Florida (Fig. 6).

3.5. Histopathology

The apparently healthy samples were all from growing branch tips, about 3–6 cm long, tapering from the apical polyp to about 1 cm in diameter at the cut end. Four out of 5 diseased samples came from older portions of branches, about 1.5–2 cm in diameter and 2–6 cm long, with the exception of D5, which was an apical branch tip 11 cm long and 1.3 cm in diameter at the cut end. The diseased samples had severe acute tissue (polyps and coenenchyme) loss generally consistent with signs of WBD type I, although samples D3 and D5 had patches of tissue remaining on the skeleton consistent with rapid tissue loss (Miller et al. 2014, their Table 1). D4 did not appear to have tissue loss, but had a greenish discoloration on the basal end, which presented microscopically as endolithic fine pink filaments and debris on the H&E-stained section.

Apparently healthy samples were in very good to good condition (Table 2), with cellular and tissue architecture and staining characteristics resembling normal apical tip development. All had abundant coenenchyme epidermal mucocytes with pronounced mucus secretion. Mild to moderate RLO levels were found in tentacles, oral disk, actinopharynx, cnidoglandular bands, and gastrodermal mucocytes of each polyp; fewer were seen in the apical polyp and most recently formed lateral polyps at the branch tip (Fig. 7). H4 had small primary oocytes developing in mesenteries

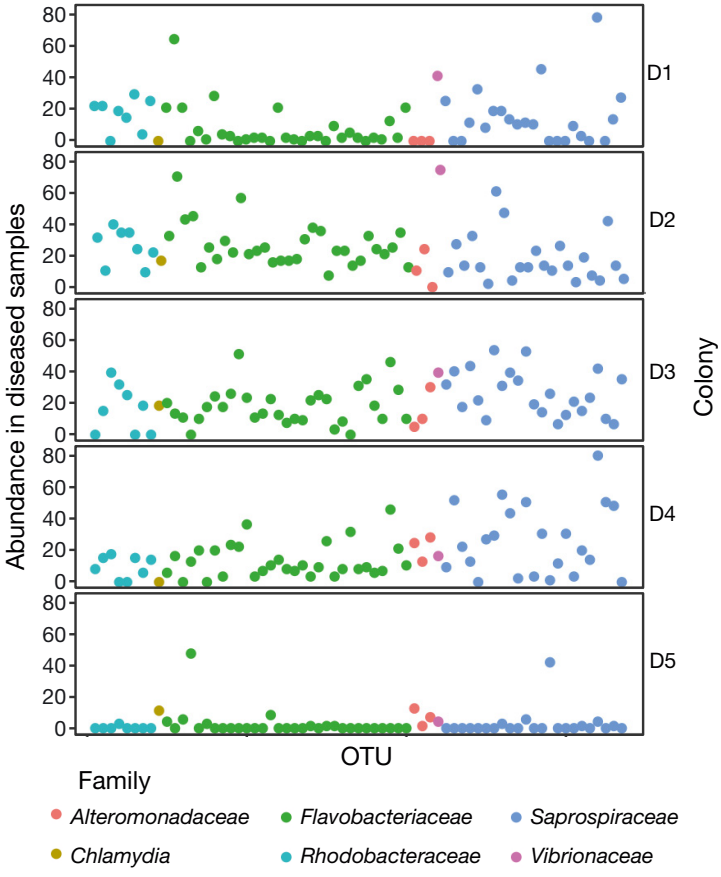

Fig. 5. Disease-associated operational taxonomic units (OTUs). The abundance of each OTU is shown for the diseased sample from each colony

Fig. 6. Families of disease-enriched bacteria found across studies. Panama white band disease (WBD) are OTUs enriched in diseased samples from Gignoux-Wolfsohn et al. (2017), Florida WBD are from this study, stony coral tissue loss disease (SCTLD) samples are from Meyer et al. (2019). Families are color-coded by phylum, and size corresponds to the number of OTUs (this study, Gignoux-Wolfsohn et al. 2017) or amplicon sequence variants (ASVs; Meyer et al. 2019). Data from Meyer et al. (2019) were adapted from their Figs. S4–S6. All disease-enriched families found in this study are shown, while only those families that overlap with one other study are shown for Gignoux-Wolfsohn et al. (2017) and Meyer et al. (2019)

in the basal subsample. H1 and H3, in contrast, had dissociation and necrosis of deep polyp cnidoglandular band epithelial cells and atrophied, lysing interior calicodermis lining deep gastrovascular canals in the basal subsamples typical of coral affected by tissue loss (Fig. 8).

Diseased samples exhibited moderate to severe pathology in all parameters, including tissue fragmentation, cell necrosis or lysing, and loss of mucocytes. Epithelia were atrophied or lysed where covering costae. Symbiodiniaceae were present in gastrodermal cells, but fewer than in apparently healthy samples, with increased degradation in the more severely affected samples. RLO levels were similar to those found in the apparently healthy samples, but decreased as mucocytes were lost, resulting in a broader range in scores (Table 2, Fig. 8). Neither apparently healthy nor diseased samples contained the bacterial aggregates first reported to be associated with WBD (Peters 1984). The diseased samples had multifocal atrophy and lysing of the calicodermis, particularly along the deep gastrovascular canals. However, 4 of the 5 diseased samples (compared to 1 of the 5 apparently healthy samples; Table 2) also had multifocal areas of hypertrophied calicoblasts secreting coral acid-rich proteins (eosinophilic apical granules, Fig. 9). One sample, D5, had a few well-degraded necrotic cell spherules in the agarose (Miller et al. 2014). Two samples (D2 and D5) contained ciliates. In sample D5, ciliates were trapped in the agarose that covered and infiltrated the skeleton well away from coral tissue. Most of these ciliates had bullet-shaped nuclei and lacked Symbiodiniaceae, but a few contained degraded algal cells in their vacuoles, and some of the ciliates were too degraded to verify presence or absence of Symbiodiniaceae. Three degraded ciliates lacking Symbiodiniaceae were also identified in sample D2 adjacent to necrotic coral tissue in the gastrovascular cavities (Fig. 10). Most tissue parameters were significantly different between apparently healthy and diseased samples (Mann-Whitney *U*-test; Fig. 11), but scores of mesenterial filament mucocytes, dissociation of mesenterial filaments, and RLO intensities in the tentacle epidermis, cnidoglandular bands, and gastrodermal mucocytes were not significantly different.

4. DISCUSSION

4.1. Links between WBD outbreak, temperature, and bleaching

The 2014 outbreak of WBD was strongly linked to increased SST. Elevated temperatures likely compromise the host and increase pathogen prevalence

Table 2. Summary statistics for histopathological observations on all apparently healthy (n = 5) and diseased (n = 5) samples collected off northern Miami Beach. RLO: *Rickettsiales*-like organism

Parameter	Apparently healthy			Diseased		
	Mean	SD	Range	Mean	SD	Range
General condition (100×)	1.5	0.3	1–1.7	3.8	0.2	3.5–4.0
Symbiodiniaceae (100×)	1.1	0.1	1–1.3	3.2	0.6	2.5–4.0
Epidermal mucocyte condition	2.0	0.3	1.5–2.3	3.9	0.3	3.3–4.3
Mesenterial filament mucocytes	2.5	0.6	1.5–3.0	4.1	0.7	3.0–5.0
Degeneration of cnidoglandular bands	1.6	0.4	1.0–2.0	3.9	0.5	3.0–4.3
Dissociation of mesenterial filaments	1.1	0.7	0.0–1.7	3.2	1.3	1.0–4.0
Costal tissue loss	0.8	0.2	0.5–1.0	3.5	1.0	2.5–4.0
Calicodermis condition	1.4	0.3	1.0–1.7	3.6	0.4	3.0–4.0
Bacterial aggregates	0.0	0.0	0.0–0.0	0.0	0.0	0.0–0.0
Epidermal RLOs	2.2	0.7	1.0–3.0	2.7	0.6	2.0–3.5
Filament RLOs	2.3	0.4	2.0–3.0	2.3	0.6	1.5–3.0
	2.1	0.6	1.0–2.5	2.1	1.1	0.5–3.7
	Percent affected (presence/absence)					
Coccidia		0			0	
Calicodermis repair		20			80	
Necrotic cell spherules		0			20	
Symbiodiniaceae-containing ciliates		0			20	
Non-Symbiodiniaceae ciliates		0			40	
Oocytes		20			0	
Spermaries		0			0	

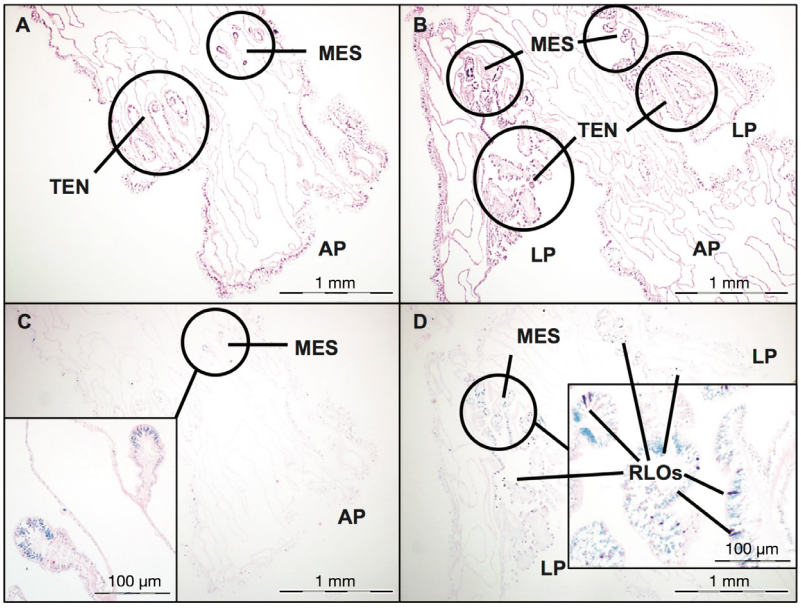

Fig. 7. (A) Photomicrograph of apical polyp (AP) on the apparently healthy (H5) branch tip above the first lateral polyps (LPs) (H&E). Cells in this region of the coral are still dividing, and the gastrodermal cells have not yet phagocytosed Symbiodiniaceae. Mesenteries (MES) and tentacles (TEN) of newly developing LPs are present in the circled areas. (B) Section through the first fully developed LPs below the AP (H&E). Tentacles, mesenteries, and filaments in the gastrovascular cavities are circled. (C) Same area of AP shown in (A) but stained with Giemsa. Inset shows higher magnification of the developing mesenteries lacking *Rickettsiales*-like organisms (RLOs) in the cnidoglandular band epithelium; scale bar = 100 µm. (D) Same area of LP shown in (B) but stained with Giemsa, showing moderate level of dark purple RLOs in sections of the tentacles, actinopharynx, and cnidoglandular bands of the mesenterial filaments. Inset shows higher magnification of the cnidoglandular bands and lower actinopharynx epithelia (on right) circled in D

and virulence. These findings contrast with a previous study of WBD in Florida that did not find a link between temperature and disease prevalence (Miller et al. 2014). Across species, evidence is increasing that corals exposed to elevated seawater temperatures are more susceptible to tissue-loss diseases than those that have not been heat stressed (Brandt & McManus 2009), with the frequency of temperature-induced disease outbreaks continuing to accelerate (Maynard et al. 2015). Recent work by Muller et al. (2018) found that *Acropora cervicornis* that had bleached due to heat stress were less disease resistant than corals that had not experienced this stress. Elevated seawater temperature and lowered salinity, which was also recorded during the 2014 outbreak (Carsey et al. 2016), are known to alter mucus production and composition as they also kill coral and algal cells (Peters & Pilson 1985, Vargas-Ángel et al. 2007, Downs et al. 2009). Whether these abiotic factors alone elicited the disease signs, compromised the coral allowing pathogens to infect, or alternatively influenced existing bacteria allowing them to become pathogenic remains somewhat unclear (Mera & Bourne 2018).

4.2. Characterization of apparently healthy colonies by histology and microbiome sequencing

Comparisons of the microbiomes of apparently healthy and diseased fragments found few OTUs that were consistently enriched in apparently healthy fragments, supporting previous work which found that the majority of coral-associated bacteria are not part of the core intransient healthy microbiome (Ainsworth et al. 2015). The only OTU more abundant in apparently healthy tissues was in the order *Sphingobacteriales* (family *Saprospiraceae*). Bacteria in this order have previously been associated with both diseased and apparently healthy *A. cervicornis* in Panama (Gignoux-Wolfsohn & Vollmer 2015), but little is known about the roles they play in coral health and disease.

The microscopic anatomy of apparently healthy fragments had minimal changes, except for RLO presence, tissue atrophy, and dissociation of mesenterial filaments deep in the branch. The degradation of the deeper aboral tissues of the polyps and gastrovascular canals is probably the result of physiological changes in the oral surface tissues (containing most of the actively photosynthesizing Symbiodiniaceae) as cells divide to maintain the polyps on the surface of the skeleton (Vaughan & Wells 1943, Barnes & Lough 1992)

4.3. Molecular and histological signatures of disease

Histological changes were more pronounced in diseased fragments, with atrophy, necrosis, and lysing of surface and basal body wall and polyp structures at the tissue-loss margin. Interestingly, diseased samples more often had foci of hypertrophied calicoblasts filled with coral acid-rich proteins (Mass et al. 2014) near the tissue-loss margins, which we hypothesize may signify an effort to form skeletal

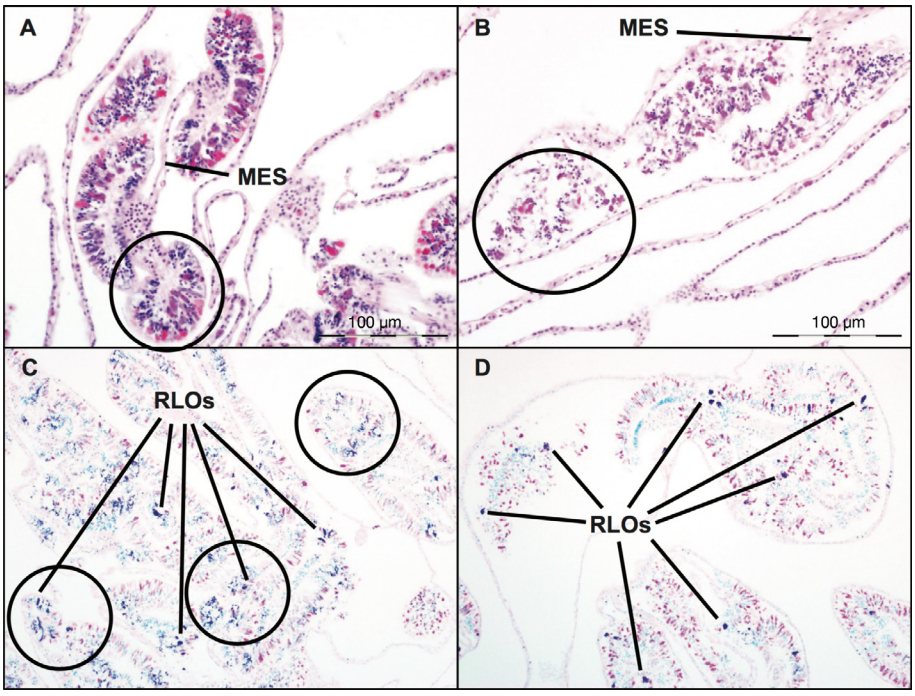

Fig. 8. (A) Intact cnidoglandular bands (one circled) and mesenteries (one visible at MES) from H3 sample near surface of branch about 3.5 mm from the branch tip (H&E). (B) Dissociated and necrotic cnidoglandular bands (one circled) and mesenteries (MES, one visible) section taken deeper (aborally) from the same sample of H3 with markedly atrophied basal body walls (H&E). (C) Area of intact cnidoglandular bands and mesenteries from H3 with moderate levels of RLOs in mucocytes present as dark blue cells (lines pointing to some of the individual infected cells); some of the mucocyte-rich areas are circled (stained with Giemsa, scale bar as in A). (D) Area of dissociated cnidoglandular bands with loss of mucocytes and release of RLOs from D3 (Giemsa, scale bar as in B)

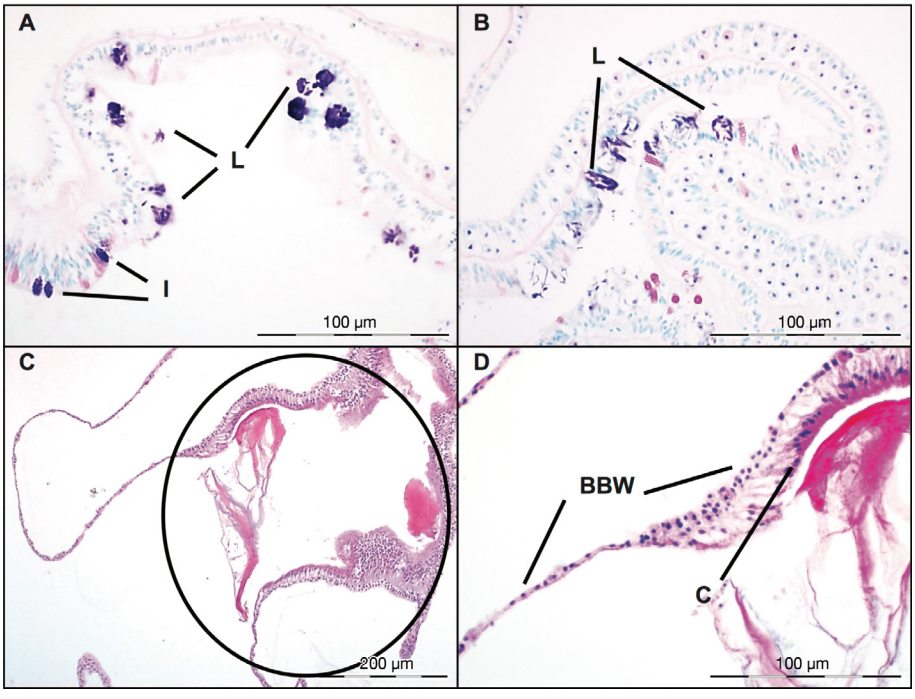

Fig. 9. (A) Section from sample H5 tentacle showing different stages of RLO-infected cells in epidermis, intact (I) and lysing (L) as multiplied larger reticulate cells disrupt the mucocyte plasmallema and are released to seawater (stained with Giemsa. (B) Section from sample D5 tentacle showing loss of mucocytes and lysing RLO-infected mucocytes (L) in epidermis (Giemsa). (C) Area of calicodermis with thin columnar calicoblasts producing abundant apical granules of coral acid-rich proteins to enhance calcification (H&E). (D) Higher magnification at junction of hypertrophied calicoblasts (C) on right with squamous, atrophied calicodermis-mesoglea-gastrodermis of basal body wall (BBW) on left (H&E)

partitions in the face of the more rapid than normal cell death to maintain polyp integrity and tissue thickness. These samples show the same microscopic changes that have been found during histological examinations of acroporid species and hybrids sampled throughout the Caribbean since 1975 (Peters et al. 1983, Peters 1984, Miller et al. 2014, Patterson 2015, E. C. Peters unpublished data). With light microscopy and histochemical staining, the only bacteria that have been clearly seen in WBD-affected corals are the bacterial aggregates (*Pseudomonas* spp.) (Peters et al. 1983, Polson 2007) and RLOs. The large bacterial aggregates were not seen in any of these Miami Beach samples, and RLO abundance did not differ with gross disease signs (see Section 4.4 for further discussion).

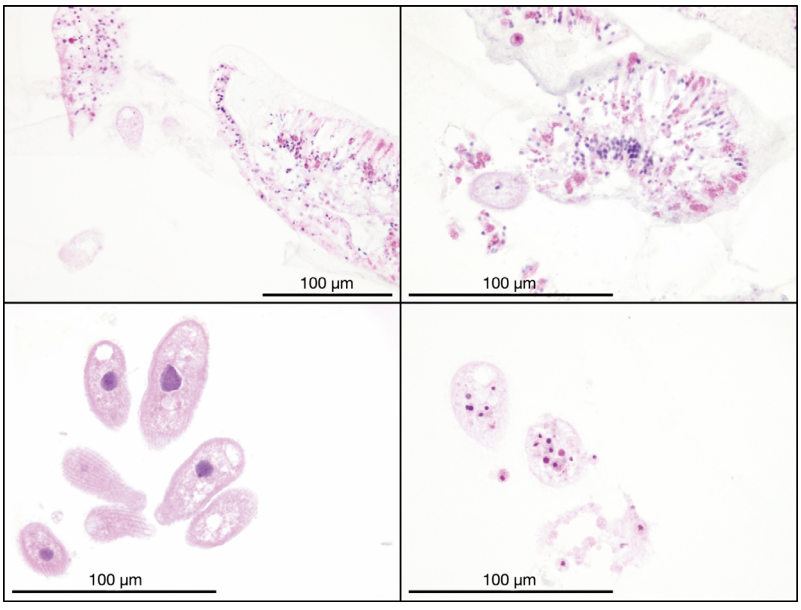

Fig. 10. Examples of the ciliates found (all stained with H&E). (A) Two of 3 ciliates seen in section from sample D2; coral cnidoglandular bands and basal body walls are necrotic and the ciliates are pale, appear to be degenerating. (B) The remaining ciliate found near A from D2. (C) Cluster of ciliates with bullet-shaped macronuclei lacking Symbiodiniaceae from sample D5. (D) Cluster of ciliates from D5 with digested dinoflagellates in vacuoles; the lower one has lysed

Ciliates have been associated with multiple coral white syndromes (Sweet & Bythell 2012), including WBD (Sweet et al. 2014). While many of these ciliates are presumably attracted to and feeding on the increased bacteria and metazoans colonizing the denuded skeleton, some appear to consume the coral tissue either directly off the coral or after it has become detached (Sweet & Séré 2016). While we observed ciliates in the agarose surrounding the skeleton of 2 out of 5 samples, only a few of the observed ciliates in 1 sample contained visible Symbiodiniaceae, suggesting that they were not actively consuming tissue (Table 2). It is possible that ciliates lacking Symbiodinaceae had consumed and already digested coral tissue, based on the appearance of debris in the digestive vacuoles of a few ciliates in sample D5. This observation does support the need for time-series sampling, since ciliate abundances may vary widely due to their mobility. Cilliates may also be lost during sample collection and processing — although the agarose-enrobing technique can reduce loss during decalcification — or they were not present in the particular sections examined. Genetic identification is required to confirm ciliate absence in the other samples.

16S sequencing allows us to identify bacteria associated with diseased fragments that do not form aggregates visible by histology. Most of the diseased

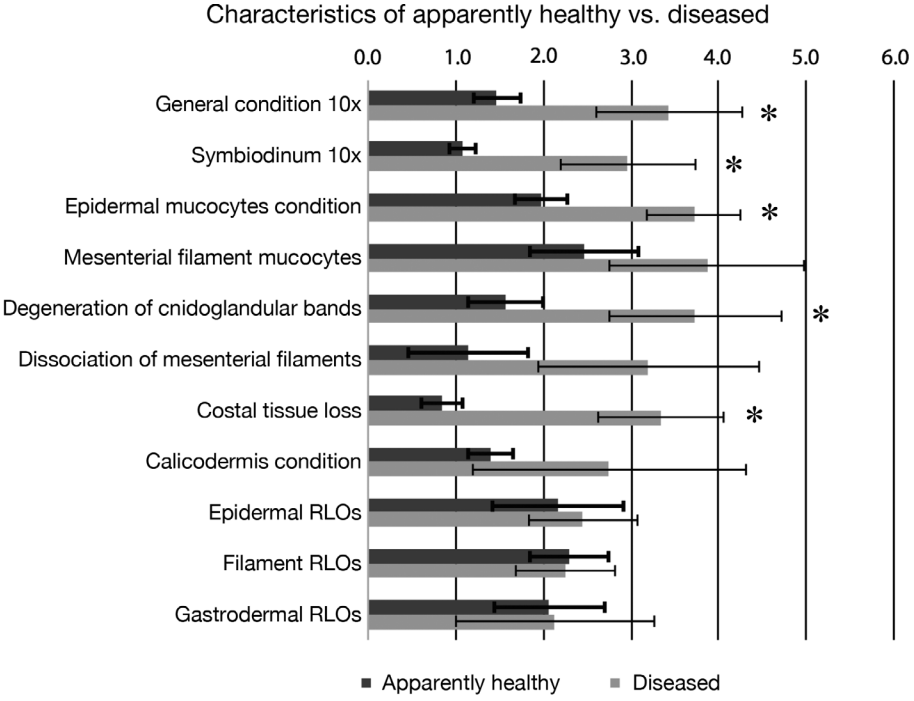

Fig. 11. Mean scores for tissue parameters examined in the apparently healthy samples compared to the diseased samples. Scales for each parameter are as follows: general condition, epidermal mucocyte condition, mesenterial filament mucocytes, calicodermis condition, 0 (excellent) to 5 (very poor); Symbiodiniaceae 0 (highest abundance) to 5 (no Symbiodiniaceae present); degeneration of cnidoglandular bands, dissociation of mesenterial filaments, costal tissue loss, 0 (none) to 5 (heavy); epidermal RLOs, filament RLOs, gastrodermal RLOs, 0 (none present) to 5 (highest abundance). ∗: significant differences between the groups based on sequential Bonferroni correction for p-values in Student's *t*-tests ($p < 0.0045$) and Mann-Whitney *U*-tests ($p < 0.05$)

fragments contained a notably more diverse bacterial community both in terms of classic diversity metrics and phylogenetic diversity than those of the apparently healthy fragments (Fig. 4), consistent with previous studies of WBD and other coral diseases (e.g. Pantos & Bythell 2006, Closek et al. 2014, Roder et al. 2014b, Gignoux-Wolfsohn & Vollmer 2015). While not significant, the diseased coral microbiomes were more dispersed around their centroid, meaning that apparently healthy microbiomes may be more similar to each other than diseased microbiomes are, as has been found previously (e.g. (Zaneveld et al. 2016). Dysbiosis, rather than the presence of a single pathogen, may therefore be the primary indication that a coral is diseased (Rosenberg et al. 2007, Bourne et al. 2009, Sunagawa et al. 2009, Zaneveld et al. 2017). Due to technical limitations, we were not able to sample corals multiple times over the course of the disease outbreak, which would have provided us with a more complete picture of how changes in the microbiome are linked to the development of disease signs. Previous research tracking changes in abundance of disease-associated bacteria through time has shown that most of the increased diversity on diseased corals is caused by opportunistic associates or secondary pathogens increasing in abundance after corals display disease signs (Gignoux-Wolfsohn et al. 2017).

Rhodobacteraceae (28 disease-associated OTUs) form the most widespread associations with diseased corals across species and disease signs, indicating that they are not disease-specific primary pathogens (Mouchka et al. 2010, Klaus et al. 2011, Cárdenas et al. 2012, Roder et al. 2014b, Ng et al. 2015, Pollock et al. 2017). *Rhodobacteraceae* are more abundant on coral-dominated reefs than on algal-dominated reefs, indicating that they may be symbionts of multiple species of coral (Haas et al. 2016). In corals displaying disease signs, *Rhodobacteraceae* may therefore be responding to the disease as opportunists or defensive symbionts. Like *Rhodobacteraceae*, *Alteromonadaceae* are commonly found in high abundances on both healthy (Cárdenas et al. 2012, Ceh et al. 2013) and diseased corals (Frias-Lopez et al. 2002, Sunagawa et al. 2009, Roder et al. 2014a, Gignoux-Wolfsohn & Vollmer 2015).

Similarly, bacteria in the family *Flavobacteraceae* have been associated with many other coral diseases (Frias-Lopez et al. 2002, Apprill et al. 2013, Roder et al. 2014b, Ng et al. 2015), but are found in higher abundance on algae-dominated than on coral-dominated reefs (Haas et al. 2016). The high variation across samples in OTU abundances is to be expected from a group of bacteria that may be secondary colonizers responding to the dying coral or secondary metabolites of the primary pathogen(s). We also found multiple OTUs belonging to *Saprospiraceae* to be disease-enriched, in keeping with previous studies (Gignoux-Wolfsohn & Vollmer 2015, Gignoux-Wolfsohn et al. 2017).

Often presumed to be the most widespread coral pathogens, *Vibrio* spp. (1 OTU) have been linked to both bleaching (e.g. Rosenberg & Ben-Haim 2002, Ben-Haim et al. 2003, Luna et al. 2010) and tissue-loss diseases (e.g. Ushijima et al. 2012, 2014) in multiple coral species. *V. carchariae* has been previously found in the bleaching margin of WBD type II, in both the Bahamas (Ritchie & Smith 1998) and Puerto Rico (Gil-Agudelo et al. 2006), suggesting that this pathogen may be involved in the loss of Symbiodiniaceae that we observed in intact tissue adjacent to tissue sloughing. Whether the WBD signs observed here are caused by the same pathogen as WBD observed in other parts of the Caribbean is unknown, and targeted genetic analysis is needed to determine if the *Vibrio* found here, which was present in all diseased samples and absent from healthy samples, is similar to the previously isolated *V. carchariae*. A new disease-associated OTU in the order *Chlamydiales* was abundant in 3 out of 5 diseased samples. This is the first time a molecular identification of *Chlamydiales* has been made on *A. cervicornis*.

While we found significant differences in both histology and microbiome composition between apparently healthy and diseased samples, the variation in both metrics among samples underscores the value of considering 'disease' and 'health' along a continuum rather than as binary states (Figs. 5 & 9). The histology revealed differences in tissue condition among apparently healthy colonies, with 2 out of the 5 displaying minor early signs of tissue loss. While these differences did not correlate with any noticeable changes in the microbial community, we found considerable variation in OTU abundance across disease-associated OTUs and samples, indicative of the complexity of this disease. Interestingly, the diseased sample from colony 5 did not have as diverse a microbiome as the other corals. While the gross appearance of this sample was that of rapid tissue loss, there were no significant differences in the histology between this colony and the other samples displaying disease signs. The lower overall diversity of sample 5 was reflected in the abundance of disease-enriched OTUs, with disease-enriched OTUs in the families *Rhodobacteraceae, Saprospiraceae*, and *Flavobacteriaceae*, and the genus *Vibrio* all displaying lower abundance in sample 5 than the other samples. It is possible that the tissue on this coral may be sloughing more rapidly,

not allowing as much time for secondary colonization on the tissue as the other diseased samples (Gignoux-Wolfsohn et al. 2017). Alternatively, this may be an example of tissue loss that is not caused by bacterial pathogens. This pattern did not hold true for all groups of disease enriched OTUs; *Alteromonadaceae* had much lower abundances in the diseased sample from colony 1, potentially indicating that these groups of bacteria may be playing different roles in the diseased-coral microbiome. The bacteria identified here may be playing multiple roles in diseased corals: pathogens causing tissue loss, digesters of cells and mesoglea at the margin, and also settlers on the denuded skeleton. Critical questions that cannot be answered by molecular microbiology analyses are (1) Exactly where are these WBD-associated microbial pathogens found in the tissues? and (2) How do they contribute (if at all) to the loss of the coral tissue from the skeleton? The next step is to use *in situ* hybridization procedures to explore the relationships of specific categories or strains of microbes and distinguish between bacterial diseases and cell necrosis, apoptosis, or lysing in tissue-loss diseases caused by other stressors (Ainsworth et al. 2007, Work & Meteyer 2014, Sweet & Séré 2016).

4.4. Rickettsial abundance is independent of gross disease signs

Likely an obligate intracellular bacterium, an RLO recently identified as *Candidatus* Aquarickettsia rohweri (Klinges et al. 2019) has been associated with both apparently healthy *A. cervicornis* and those displaying disease signs (Casas et al. 2004). RLOs were seen in all corals examined here, regardless of disease state, in keeping with previous findings that the RLOs are present in all acroporid samples examined thus far from diverse Caribbean locations collected for histological examination since 1975, (Peters 2014, Di Lauro 2015). Previous histological examination and *in situ* hybridization have shown that the RLOs occupy staghorn coral mucocytes, multiplying and growing to form reticulate bodies, which fill the mucocytes and rupture the plasmallema (Fryer et al. 1992, Sun & Wu 2004, Miller et al. 2014, Norfolk 2015). Whether the effects of RLOs on coral mucocytes influence tissue loss is unclear, but an alteration of the surface mucopolysaccharide layer produced by the mucocytes could influence the beneficial microbial community as well as production of antimicrobial compounds, resulting in a more susceptible coral (Koh 1997, Brown & Bythell 2005, Ritchie 2006, Wooldridge 2009). Klinges et al. (2019) found that *Ca.* A. rohweri lacks genes required for amino acid synthesis, suggesting that it acquires them from the coral host and algal symbionts, likely weakening the host and making it more susceptible to further infections. The corals surveyed here have a high abundance of OTUs in the order *Rickettsiales*, with the bacterial communities of all apparently healthy samples consisting of at least 20% *Rickettsiales*, and 1 microbiome made up of 76% *Rickettsiales*. There appears to be no difference in normalized *Rickettsiales* abundance between apparently healthy and diseased samples, only a difference in percent relative abundance due to an increase in other taxa. RLOs were also visible in roughly equal abundance inside the mucocytes of the polyps via histological examinations in both apparently healthy and diseased corals.

This observed dominance of *Rickettsia* in apparently healthy coral microbiomes contrasts with a study by Gignoux-Wolfsohn et al. (2017), who found that the microbiomes of healthy *A. cervicornis* colonies in Panama were either dominated by bacteria in the genus *Endozoicomonas* or had no dominant group of bacteria. In that study, only 1 out of 10 colonies was infected by *Rickettsiales,* although RLOs have been previously found on Panamanian reefs (Casas et al. 2004). Loss of *Endozoicomonas* has been shown to precede the development of disease signs (Gignoux-Wolfsohn et al. 2017, Certner & Vollmer 2018). Our finding that these apparently healthy *A. cervicornis* from Miami do not have microbiomes dominated by the potentially beneficial *Endozoicomonas*, but are instead dominated by *Rickettsiales*, may indicate decreased resilience and increased susceptibility to disease compared to corals from healthier reefs. The lack of *Endozoicomonas* may also be a result of increased temperature and coral bleaching, and therefore decreased disease resistance (Muller et al. 2018). Alternatively, the absence of any colonies dominated by *Endozoicomonas* may be indicative of the 'pre-disease' state of these apparently healthy tissues. We are unable to definitively rule out RLOs as the primary cause of this disease outbreak given our lack of sampling prior to the start of the outbreak. It is possible that RLO abundance had previously increased only in corals that would subsequently show disease signs when triggered by other factors, although this was not the case in Panamanian corals sampled over time (Gignoux-Wolfsohn et al. 2017). More likely, the high abundance of *Rickettsiales* seen in these corals may be due to their proximity to developed land and therefore increased nutrient runoff. Shaver et al. (2017) found that while an experimental increase in

nutrients did not affect WBD prevalence, it did increase *Rickettsiales* abundance, shifting the microbiome to *Rickettsiales* dominance.

Determining the specific interactions between the RLOs and both diseased and apparently healthy corals will involve additional research using transmission electron microscopy (TEM), which was beyond the scope of this study due to lack of resources and funding. TEM may also reveal other subcellular changes or agents (e.g. viruses) involved in tissue loss. Shifts in viral communities have been documented between apparently healthy and affected samples using both TEM and sequencing in other coral diseases including white syndrome (Pollock et al. 2014b) and white plague (Soffer et al. 2014). Future work integrating bacterial and viral morphology and location (TEM) and bacterial and viral diversity (sequencing) will help to better illuminate the roles these groups of microbes may be playing in the development of disease signs both individually and in conjunction.

4.5. Comparison of disease-enriched families to other studies of coral disease

The species-specificity of coral tissue loss diseases remains up for debate, as diagnostic criteria tend to vary with host species (Rogers 2010). Similar taxa are often enriched on diseased corals regardless of species (Roder et al. 2014a). Furthermore, the similarity (in terms of both cause and effect) of disease outbreaks separated by time and location remains understudied. The microbiomes of diseased corals have been shown to vary across site (Roder et al. 2014b, Gignoux-Wolfsohn & Vollmer 2015), and there is evidence that similar disease signs can be caused by different pathogens (Sutherland et al. 2016). To help address these issues, we compared disease-enriched OTUs to results from 2 previous studies, one conducted on diseased and healthy *A. cervicornis* in Panama (Gignoux-Wolfsohn et al. 2017), and one on stony coral tissue loss disease (SCTLD) on 3 species of coral in Florida (Meyer et al. 2019). It should be noted that the Florida and Panama *A. cervicornis* were sequenced on the same sequencing run using the same 16S region (V6 vs. V4 for SCTLD) and therefore may have similarity biases. We found many families that were enriched in all 3 species, including suggested pathogens such as *Vibrionaceae* (Ritchie & Smith 1998, Gil-Agudelo et al. 2006), as well as *Rhodobacteraceae*, which have been found associated with multiple coral diseases (Mouchka et al. 2010). The family *Flavobacteriaceae*, which has consistently been associated with WBD-affected *A. cervicornis* as colonizers before corals show disease signs (Gignoux-Wolfsohn & Vollmer 2015, Gignoux-Wolfsohn et al. 2017, Certner & Vollmer 2018), were also found in all 3 species of coral affected by SCTLD. Further work is needed to determine if these families generally colonize compromised corals or if certain species or strains cause disease in different host species. Interestingly, all families that were shared between the Florida WBD corals and SCTLD corals were also enriched in the Panama WBD corals, indicating that disease/host species is a stronger driver of the disease-enriched families than location. This finding is especially interesting in light of the observed resistance of *A. cervicornis* to SCTLD (Precht et al. 2016, Walton et al. 2018). Families not enriched in either WBD dataset should be further investigated for their role in SCTLD. Families enriched in both WBD datasets but not in corals with SCTLD were generally less-well studied as coral pathogens or colonizers, including *Victivallaceae*, *Puniceicoccaceae*, *Marinilabiaceae*, and *Chitinophagaceae*. While preliminary, this comparison of disease-enriched families across studies provides a possible list of families that may be involved specifically in WBD, rather than general coral tissue loss.

4.6. Conclusions

Clearly, WBD continues to present a significant, ongoing threat to the acroporid populations throughout the region. Our results suggest that the seasonal nature of WBD outbreaks is largely due to the frequency of thermal-stress events, and that the severity of these outbreaks may be increased by rates of coral bleaching. These findings also point to the likely increase in the impacts of coral diseases with increasing SSTs. Thus, the increasing prevalence and incidence of marine diseases and links to global climate change (Bruno et al. 2007, Brandt & McManus 2009, Randall et al. 2014, Randall & van Woesik 2015) make applied research that synthesizes genetic, histological, epidemiological, and ecological perspectives all the more imperative.

Overall, the bacteria enriched in these Miami *A. cervicornis* displaying WBD signs are similar to those enriched in previous studies of diseased *A. cervicornis* in Panama (Gignoux-Wolfsohn & Vollmer 2015), suggesting that there is an *A. cervicornis* tissue-loss disease microbiome that is consistent at multiple locations in the Caribbean. This may indicate that these disease signs have the same etiology, or that the increased abundance of multiple bacterial taxa is

a common response of *A. cervicornis* to disease or tissue loss. The impact of the observed *Rickettsiales* association with the coral host is mediated by the coral's nutritional status and exposure to other stressors. These compromised corals may be more susceptible to colonization by other pathogenic bacteria or their toxins, which may cause apoptosis or necrosis and lysing of atrophied epithelia with visually evident tissue sloughing. We now need to identify where the other bacteria identified here reside in the tissue and their roles. We also need to know what conditions control tissue loss, since WBD can stop before a colony completely dies, suggesting that factors can change to bring the coral back to a state of visually apparent health.

Under future scenarios of increasing frequency and duration of coral disease outbreaks, coral genotypes with high disease resistance and resilience will have an ecological advantage over low-resistance genotypes provided that ability to cope with disease does not require detrimental tradeoffs. To this end, understanding the coral immune system, and in particular its heritable components, and understanding the links between host genetics and beneficial microbes will be crucial in the search for the host genotypes best suited to restoration efforts with this species (Vollmer & Kline 2008, Gignoux-Wolfsohn et al. 2017, Muller et al. 2018). In addition, deciphering the specific roles of the pathogens versus the host is another missing piece of the puzzle that needs to be addressed and solved (Libro et al. 2013). It is quite possible that many or most coral disease syndromes are the result of a general dysfunction of the coral microbiome–host relationship rather than being attributable to a monolithic pathogen (Mera & Bourne 2018). Such information may potentially lead to the prevention, or at least mitigation, of future coral disease outbreaks. Understanding the full nature and function of the coral microbiome, the genomic basis for disease resistance, pathogen recognition, and both the innate and adaptive or adaptive-like immunity of reef-building corals will be essential for restoring populations of endangered corals like the *Acropora* spp. throughout their range and for fostering the health and welfare of these ecosystems for future generations to use and enjoy.

Data archive. Sequences have been deposited to the SRA (Bioproject ID: PRJNA511881, accession nos. SAMN10644736–SAMN10644745).

Acknowledgements. Thanks to Martha Robbart, Ryan Fura, and Lindsey Precht for assistance with scientific diving, data collection, and analysis. Mike Rice of Dial Cordy and Associates, Inc. prepared Fig. 2. S.G.W. thanks Felicia Aronson for assistance in packaging samples and Erik Holum for assistance with bioinformatics. E.C.P. thanks Katheryn Patterson, William Norfolk, Maryam Hosseinpour, Haleh Shariat Madari, and Jessica Havens for assisting in methods development, analyses, and discussions on the histology of the Caribbean acroporid tissue-loss diseases. W.F.P. thanks Rich Aronson, Andrew Bruckner, John Bruno, Steven Miller, Kim Ritchie, Caroline Rogers, Robert van Woesik, Steve Vollmer, and Ernesto Weil for numerous conversations over the years about the role of WBD on Caribbean reefs. We also thank Rebecca Certner and Katrina Lohan for critically commenting on an earlier version of this manuscript. Coral samples were collected under Florida Fish and Wildlife Conservation Commission Special Activity License SAL-14-1618-SRP issued to L.S.K. on 15 August 2014.

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Editorial responsibility: Stephen Feist, Weymouth, UK

Submitted: February 21, 2019 ; Accepted: November 27, 2019
Proofs received from author(s): January 24, 2020