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Parasitic '*Candidatus* Aquarickettsia rohweri' is a marker of disease susceptibility in *Acropora cervicornis* but is lost during thermal stress

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Summary

Holobiont phenotype results from a combination of host and symbiont genotypes as well as from prevailing environmental conditions that alter the relationships among symbiotic members. Corals exemplify this concept, where shifts in the algal symbiont community can lead to some corals becoming more or less thermally tolerant. Despite linkage between coral bleaching and disease, the roles of symbiotic bacteria in holobiont resistance and susceptibility to disease remains less well understood. This study thus characterizes the microbiome of disease-resistant and -susceptible *Acropora cervicornis* coral genotypes (hereafter referred to simply as 'genotypes') before and after high temperature-mediated bleaching. We found that the intracellular bacterial parasite '*Ca.* Aquarickettsia rohweri' was strikingly abundant in disease-susceptible genotypes. Disease-resistant genotypes, however, had notably more diverse and even communities, with correspondingly low abundances of '*Ca.* Aquarickettsia'. Bleaching caused a dramatic reduction of '*Ca.* Aquarickettsia' within disease-susceptible corals and led to an increase in bacterial community dispersion, as well as the proliferation of opportunists. Our data support the hypothesis that '*Ca.* Aquarickettsia' species increase coral disease risk through two mechanisms: (i) the creation of host nutritional deficiencies leading to a compromised host-symbiont state and (ii) the opening of niche space for potential pathogens during thermal stress.

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Introduction

Role of the microbiome in resistance and resilience to disturbance

New studies in diverse host systems reveal the profound influence of the microbiome on animal host fitness and survival after disturbance. Beneficial microbes contribute to increased or expanded host metabolism and pathogen defence, while also occluding niche space otherwise available to opportunists (Mao-Jones *et al*., 2010; Ritchie, 2011). Therefore, a diverse microbiome containing beneficial organisms may allow for optimal host response to changing environmental conditions (Zilber-Rosenberg and Rosenberg, 2008; West *et al*., 2019). Rapid changes in microbial community composition in response to stressors may reflect the flexibility of the microbiome, allowing rapid adaptation to stress (Ziegler *et al*., 2019). In contrast, imbalances in microbiomes characterized by the loss of commensal species and an increase in opportunists are often associated with negative health consequences across host systems and are referred to as dysbiosis (Gilbert *et al*., 2016; Apprill, 2017). These changes in microbiome ecological state (e.g, community composition or variability) may be driven by and exacerbate stress from environmental change (Lewis *et al*., 2015; Zaneveld *et al*., 2017). It is suggested, therefore, that microbial dysbiosis may contribute to problems plaguing threatened species such as scleractinian corals and should be considered when developing strategies to preserve these foundational species (Lesser *et al*., 2007; West *et al*., 2019; Ware *et al*., 2020).

Disease is a major disturbance within Caribbean Acroporid corals

Infectious disease is a significant disturbance for corals in general, but especially for species within the Caribbean region including the staghorn coral *Acropora cervicornis*. Indeed, regional losses of Caribbean coral cover by up to

80% (Gardiner *et al*., 2003) are primarily attributed to the loss of these important reef-building species because of white band disease (Aronson and Precht, 2006). Acroporids are now listed as protected under the US Endangered Species Act and critically endangered under the International Union of Conservation of Nature's (IUCN) Red List (Aronson and Precht, 2001; *Acropora* Biological Review Team, 2005). The continued occurrence of disease, which may be limiting population recovery (Rogers and Muller, 2012; Miller *et al*., 2014), has led to significant focus on disease dynamics with Caribbean *Acropora* spp. and the mechanisms that may lead to disease resistance.

Influence of coral genotype on disease susceptibility

The severity of stress responses that lead to disease differs with coral genotype (Drury *et al*., 2017; Muller *et al*., 2018) suggesting that the genetic composition of the host may influence disease susceptibility and resistance. Naturally disease-resistant genotypes, observed throughout the Caribbean, may harbour potential pathogens yet do not succumb to disease-related health reductions or mortality. This trait seems to be heritable as increases in local levels of *Acropora* coral population recovery have been documented (Rogers and Muller, 2012; Edmunds, 2014; Croquer *et al*., 2016). Potential for population recovery, combined with the continued outbreaks of disease events in these species, should theoretically lead to additional increases in community level disease resistance through repeated natural selection events. In fact, prevalence of disease-resistant *Acropora* genotypes within the Western Atlantic (e.g. *Florida*) is notably higher (26% resistant to white band disease; Muller *et al*., 2018) compared with those in other parts of the Caribbean (5%–8% resistant to white band disease; Vollmer and Kline, 2008; Libro and Vollmer, 2016). Increasing frequency of thermal stress events, however, may reduce disease resistance: only two genotypes, or 13% of those tested by Muller *et al*. (2018), maintained a disease-resistant phenotype after bleaching.

Influence of the microbiome on disease susceptibility

Along with heritable traits of the host, microbiomes contribute to disease susceptibility in diverse host backgrounds ranging from the human intestine (Honda and Littman, 2012) to marine mammals (Nelson *et al*., 2015) to shrimp (Holt *et al*., 2020). In Acroporid corals, the persistence of *Endozoicomonas* bacteria within disease-resistant *A. cervicornis* populations in Panama suggests that the presence of this taxon may increase coral disease resistance (Chu and Vollmer, 2016). Histological samples of *A. cervicornis* from the Florida reef tract also showed that rates of disease-associated tissue loss were correlated with the relative abundance of Rickettsiales-like organisms (Di Lauro, 2015). Members of the Alphaproteobacterial order Rickettsiales are obligate intracellular parasites, often associated with invertebrate vectors and sometimes pathogenic to humans (Gillespie *et al*., 2012; Montagna *et al*., 2013). We previously showed that a coral-associated Rickettsiales species, '*Ca*. Aquarickettsia rohweri', possesses the genomic capacity to parasitize the coral holobiont for amino acids and ATP (Klinges *et al*., 2019). We also showed that under nutrient-enriched conditions, these parasites proliferate and reduce Acroporid coral growth (Shaver *et al*., 2017) with increasing abundances positively associated with increased disease prevalence and tissue loss in other coral species (Zaneveld *et al*., 2016). Based on their parasitic lifestyles and capabilities to flourish in various environmental conditions, we hypothesize that the mechanism by which '*Ca*. Aquarickettsia' may influence disease susceptibility is through the overconsumption of host and symbiont nutritional and energy resources (Klinges *et al*., 2019). We also suspect that under environmental conditions that are optimal for coral growth, these parasites do not generate disease phenotypes. In certain genotypic backgrounds, however, and under detrimental environmental conditions (e.g., bleaching), the dynamics of these coral parasites may influence a host genotype's disease susceptibility. But how these parasites are distributed across different host genotype backgrounds is unknown.

Influence of the environment on disease susceptibility

Host genotypes and host microbiomes alone cannot always predict disease resistance as the mechanisms that generate disease-susceptible states are highly varied and often require environmentally mediated changes in host physiology and/or pathogen virulence (Lesser *et al*., 2007; Mao-Jones *et al*., 2010). White band disease has been described as the result of the co-occurrence of a permissible host genotype, a microbial community containing single or multiple pathogens, and environmental conditions conducive to infection (Bruno, 2015). Microbiome dysbiosis has been repeatedly found to occur in response to known coral stressors, such as thermal stress including bleaching, or nutrient pollution, that result in an increased disease prevalence (Thurber *et al*., 2009; Zaneveld *et al*., 2016; Ahmed *et al*., 2019). Therefore, dysbiosis generated by environmental triggers may shift a genotype's disease susceptibility state. But as different genotypes tend to host distinct microbial communities (Glasl *et al*., 2019), it has proven difficult to determine causality of this effect. One reason for this is that the propensity for microbial dysbiosis has not been well

characterized across different genotypes, but these data are key to quantifying the relative contributions of host genetics and the microbiome to host disease susceptibility, especially in critically endangered species undergoing restoration efforts.

As reefs decline and coral populations dwindle towards extinction, the persistence of stress-resistant genotypes is critical to fuel population recovery. Identifying mechanisms resulting in genotypic disease resistance, and how increasing water temperatures influence that trait, will provide insight into how corals may acclimatize and adapt to ever-increasing threats. To uncover microbial signatures of disease resistance, we characterized the bacterial community of *Acropora cervicornis* genotypes that exhibited different disease susceptibility phenotypes (Muller *et al*., 2018). In this previous study, different genotypes exhibited variable responses to exposure to white-band disease homogenate after a natural bleaching event. Using the same samples, we further examined correlations between microbiome community structure and disease resistance prior to and after bleaching, as bleaching and disease susceptibility are positively correlated (Muller *et al*., 2008; Brandt and McManus, 2009; Muller *et al*., 2018). The objectives of the present study were to determine (i) whether disease-resistant genotypes possessed a significantly different microbiome compared with disease-susceptible genotypes, (ii) how high temperature-induced bleaching shifted the microbiome of disease-susceptible or resistant genotypes, and (iii) assess the contribution of microbial community members including '*Ca*. Aquarickettsia' to signatures of disease susceptibility and resistance.

Results

In the present study, we evaluated differences in the microbiomes of disease-susceptible and disease-resistant *A. cervicornis* genotypes before and after bleaching. Disease susceptibility was previously assessed for apparently healthy and bleached *A. cervicornis* genotypes in the study by Muller *et al*. (2018) using experimental exposure to homogenate of white band diseased tissue. For this study, only genotypes that had zero probability of disease after bleaching were classified as disease-resistant, while all other genotypes were classified as susceptible (see Experimental Details).

*Disease-susceptible genotypes contained more '*Ca*. Aquarickettsia' than disease-resistant genotypes*

The microbiomes of apparently healthy *A. cervicornis* disease-susceptible and -resistant genotypes were differentiated by the abundance of the bacterial parasite '*Ca*. Aquarickettsia' (Fig. 1). The genus '*Ca*. Aquarickettsia' dominated the disease-susceptible genotypes, with a mean relative abundance of 89.7% ± 2.1% (Supporting Information Figures S1 and S2). In contrast, the mean relative abundance of '*Ca*. Aquarickettsia' within resistant genotypes was only 2.5% ± 0.8%. In fact, differential abundance analysis showed that '*Ca*. Aquarickettsia' was significantly more abundant in susceptible genotypes (ANCOM, W = 25; Fig. 2A, Supporting Information Table S1). The dominance of '*Ca*. Aquarickettsia' in the disease-susceptible genotypes led to an uneven community that contained only a few additional rare taxa including *Corynebacterium* (1.0% ± 0.5%), *Alteromonas* (0.7% ± 0.3%), and *Cellulomonas* (0.7% ± 0.5%). Microbiomes of resistant genotypes were more even, with the most abundant genera, *Corynebacterium*, *Exiguobacterium*, and *Staphylococcus*, having mean relative abundances of 10.6% ± 7.3%, 10.3% ± 3.0%, and 9.0% ± 4.1%, respectively (Fig. 1, Supporting Information Fig. S1). Notably, *Corynebacterium* has been detected in sequenced negative controls (Salter *et al*., 2014; Rosales *et al*., 2019), but this genus was not detectable in our PCR negatives.

*'*Ca*. Aquarickettsia' was lost and opportunists increased during bleaching*

In contrast to apparently healthy corals, bleached corals showed a marked reduction in the relative abundance of '*Ca*. Aquarickettsia' regardless of disease resistance. Overall the mean relative abundance of '*Ca*. Aquarickettsia' was only 7.0% ± 2.3% in the bleached corals. However, as with apparently healthy corals, the relative abundance of '*Ca*. Aquarickettsia' during bleaching remained higher for disease-susceptible genotypes (7.9% ± 2.6%) than disease-resistant genotypes (1.0% ± 0.05%) although the difference was not significant. Instead, the dominant taxon in bleached samples was *Alteromonas* for both susceptible and resistant genotypes (19.0% ± 2.1% and 15.2% ± 4.9% respectively) (Fig. 1). As this taxon was only 0.7% ± 0.2% in apparently healthy individuals, bleaching thus resulted in a 26-fold increase in this opportunist compared to apparently healthy corals in both disease categories (18.5% ± 1.9%). Other dominant taxa in bleached samples included several potential opportunists such as: *Vibrio* (6.1% ± 1.6%), *Corynebacterium* (4.4% ± 1.1%), *Cloacibacterium* (3.7% ± 0.9%), and *Austauriibacter* (3.6% ± 0.4%) (Fig. 1).

Interestingly, neither '*Ca*. Aquarickettsia' nor any other taxa were significantly different in abundance between bleached-susceptible and -resistant genotypes based on differential abundance analysis with ANCOM (Fig. 2B, Supporting Information Table S1). Instead, opportunistic taxa were most variable between apparently healthy

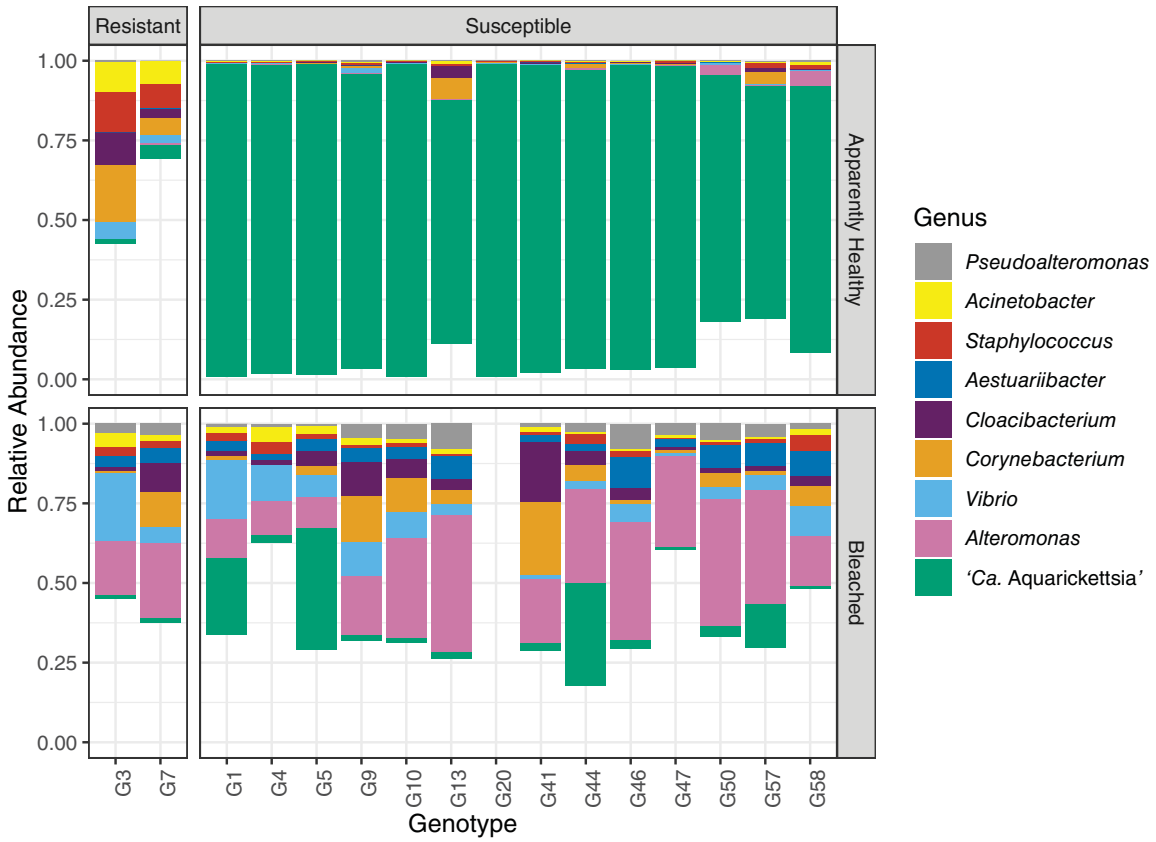

Fig. 1. Relative abundance of the most abundant genera across different resistant and susceptible genotypes in pre-bleached and bleached samples. Taxa are included in the plot if they had a relative abundance greater than 1% across the entire dataset. Each bar represents two replicates. Genotype 20 was not sampled during bleaching. [Color figure can be viewed at wileyonlinelibrary.com]

susceptible and bleached susceptible genotypes. Of the 32 observed genera included in the ANCOM analysis, '*Ca*. Aquarickettsia' (W = 31), *Aestuariibacter* (W = 31), *Exiguobacterium* (W = 30), *Marivita* (W = 29), *Roseobacter* (W = 27), *Pseudoalteromonas* (W = 26), *Alteromonas* (W = 21), and *Staphylococcus* (W = 19) showed a significant difference ($P < 0.05$) in abundance between apparently healthy susceptible and bleached susceptible genotypes (Fig. 2C). In contrast, bleaching did not significantly affect the abundance of bacterial taxa in microbiomes from resistant genotypes (Fig. 2D) indicating high stability and resistance to disturbance within the disease resistant genotypes. ANCOM results for all taxa included in the analysis ($n = 32$) can be found in the Supporting Information Figure S3.

Bleaching does not alter the structure of 'Ca. Aquarickettsia' populations

To determine whether different '*Ca*. Aquarickettsia' variants dominated different disease genotypes we examined associations of '*Ca*. Aquarickettsia' amplicon sequence variants (ASVs) with different genotypes and assessed the phylogenetic relationship of the variants present. We identified a total of 12 ASVs within the genus '*Ca*. Aquarickettsia' that shared species-level homology with '*Ca*. A. rohweri' (ranging from 98.97% to 99.66% identity). However, strain-level variation did not demonstrate clear partitioning patterns by host genotype (Fig. 3), though there were clear phylogenetic differences between the most abundant variants (Fig. 3 and Supporting Information Fig. S4). The most abundant of these ASVs (ASV 1, Fig. 3) constituted a mean 19.18% ± 2.00% of all '*Ca*. A. rohweri' sequences among apparently healthy disease-susceptible samples, while the second most abundant (ASV 2) averaged 14.61% ± 2.09% in these samples. Although these ASV abundances were considerably lower in disease-resistant compared to disease-susceptible genotypes, ASV 1 was still the most abundant of all '*Ca*. Aquarickettsia' ASVs found in disease-resistant samples (56.38% ± 37.11%). After bleaching, these two ASVs increased slightly in their relative proportion of the '*Ca*. A. rohweri' community in disease-susceptible genotypes, constituting an average of 23.56% ± 12.41% and 17.66% ± 9.44% of '*Ca*.

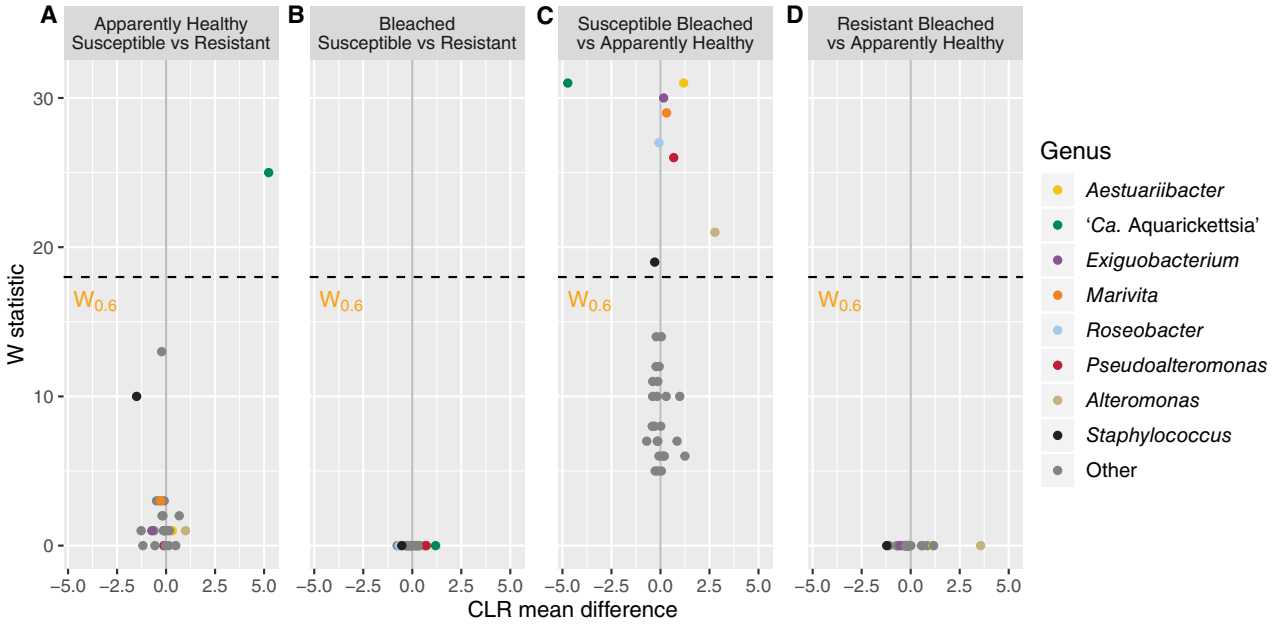

Fig. 2. Volcano plot of results from differential abundance analysis using ANCOM. The genus-level ASV table filtered to include taxa with a total count of 10 in at least 20% of samples (# taxa = 32) was used. ANCOM tests the null hypothesis that the average abundance of a given species in a group is equal to that in the other group. The W statistic represents the strength of the test for the 32 tested species and is the number of times the null-hypothesis was rejected for a given species. Taxa above the dashed line are significant with the null-hypothesis rejected 60% of the time. Non-significant taxa in any contrast are coloured grey. The x-axis value presents the effect size as the clr (centered log ratio) transformed mean difference in abundance of a given species between the two groups being compared. For the first panel, a positive x-axis value means the genus is abundant in apparently healthy susceptible samples compared to apparently healthy resistant samples or vice versa for a negative x-axis value. [Color figure can be viewed at wileyonlinelibrary.com]

A. rohweri' reads, respectively, although total relative abundance of '*Ca*. A. rohweri' declined dramatically in response to bleaching. All other ASVs each averaged less than 10% of the '*Ca*. Aquarickettsia' community before and after bleaching.

Disease susceptibility classification and bleaching state alter patterns in microbiome alpha diversity

Overall, disease-resistant *A. cervicornis* genotypes had a significantly greater Simpson's diversity (0.98 ± 0.002) than disease-susceptible genotypes (0.94 ± 0.005; $P < 0.05$, $\chi^2 = 5.313$, Supporting Information Table S2). Bleaching also significantly increased Simpson's diversity (0.98 ± 0.001) compared with apparently healthy corals (0.92 ± 0.005; $P < 0.001$, $\chi^2 = 36.923$). The interaction between resistance groupings and bleaching status was also significant ($P < 0.001$, $\chi^2 = 44.271$) and pairwise comparisons showed that apparently healthy disease-susceptible genotypes were significantly less diverse than all other groups ($P < 0.001$, Fig. 4A).

To evaluate the effect of the dominant taxon '*Ca*. Aquarickettsia' on alpha diversity dynamics, we repeated the analyses with ASVs from this taxon removed from the community. After removal, the Simpson's diversity index of disease-resistant genotypes was not significantly different from that of disease-susceptible genotypes ($P = 0.171$, $\chi^2 = 1.877$), although bleaching still significantly increased Simpson's diversity ($P < 0.001$, $\chi^2 = 15.292$, Supporting Information Table S2). The interaction between resistance groupings and bleaching status remained significant ($P < 0.001$, $\chi^2 = 17.582$), however, apparently healthy susceptible genotypes were only different from bleached susceptible genotypes ($P < 0.001$). After *in silico* removal of '*Ca*. Aquarickettsia', the Simpson's diversity index of apparently healthy susceptible genotypes was no longer different from apparently healthy resistant genotypes (Fig. 4B).

Bleaching homogenized resistant and susceptible microbiomes

Apparently healthy disease-susceptible microbiomes were less variable than, and compositionally distinct from, all other microbiomes. Beta diversity analysis showed that disease-susceptibility classification (PERMANOVA; $P < 0.001$, $R^2 = 0.079$), bleaching condition ($P < 0.001$, $R^2 = 0.391$), and their interaction ($P < 0.001$, $R^2 = 0.055$) produced distinct communities of microbes (Fig. 4C, Supporting Information Table S3). Pairwise comparisons showed that all combinations were significantly different from each other ($P < 0.05$), except for bleached disease-susceptible and bleached disease-resistant genotypes ($P = 0.628$).

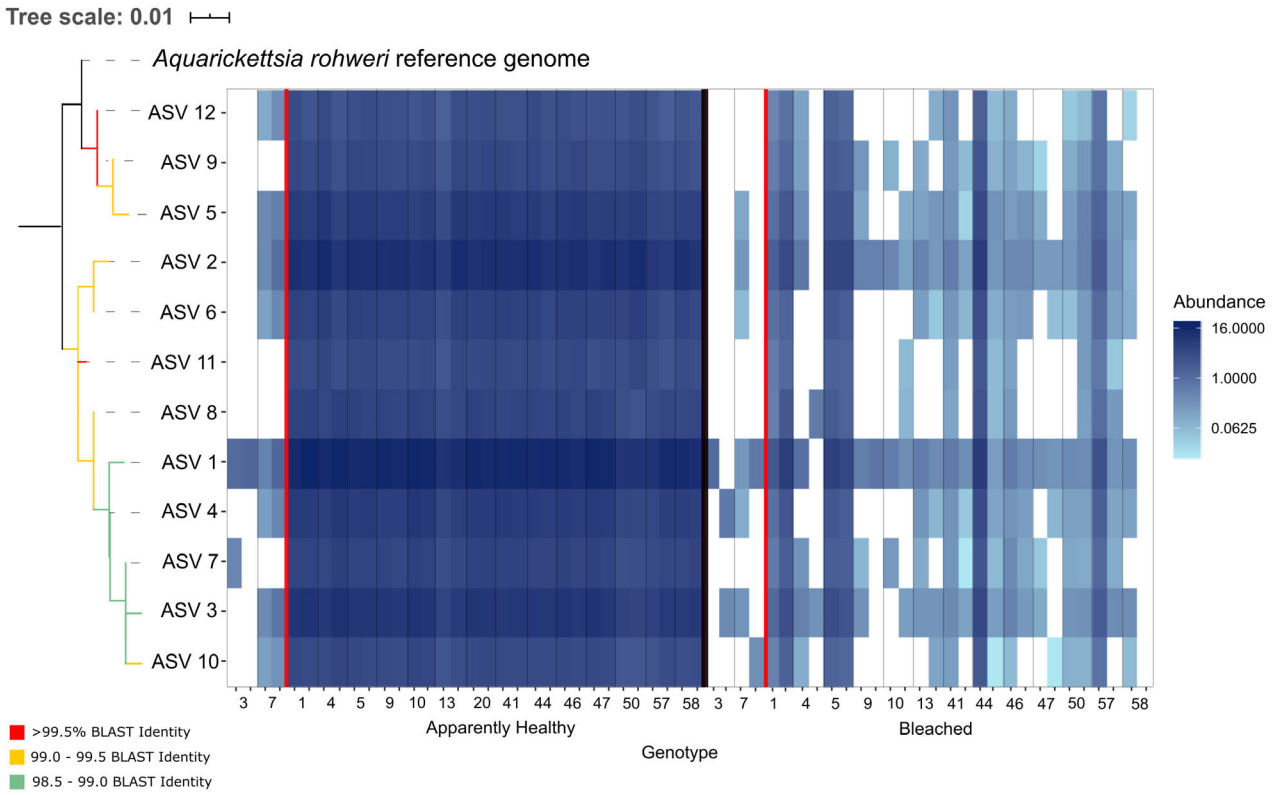

Fig. 3. Relative abundance of ASVs from the genus '*Ca*. Aquarickettsia' across all genotypes in August (apparently healthy state) and September 2015 (bleached state). ASVs are numbered according to their overall abundance in the dataset, and phylogenetic relationships are plotted on the left. Red line separates disease-resistant and susceptible genotypes. BLASTn percent identity to the 16S rRNA sequence of '*Ca*. Aquarickettsia rohweri' (NCBI accession number MT544612.1) is plotted as coloured branches on the phylogenetic tree. [Color figure can be viewed at wileyonlinelibrary.com]

Tests for differences in group dispersion or sample-to-sample variation showed that while disease susceptibility did not affect dispersion (PERMDISP; $P = 0.287$, $F = 1.153$), dispersion significantly increased when corals were bleached ($P < 0.001$, $F = 22.143$, Supporting Information Table S4). The interaction between disease susceptibility groups and bleaching status was a significant predictor of group dispersions ($P < 0.001$, $F = 73.187$) with pairwise comparisons showing that apparently healthy disease-susceptible genotypes were less variable than all other genotypes ($P < 0.01$) (Fig. 4D). As unbalanced designs are known to bias PERMANOVA results in the presence of heterogeneity of dispersion (Anderson and Walsh 2013), we verified these results with 1000 permutations of down-sampling to a balanced design (Supporting Information Tables S3 and S4).

Apparently healthy disease-susceptible and disease-resistant microbiomes were also more similar in sample-to-sample variability and composition when '*Ca*. Aquarickettsia' was removed from the analysis. Overall, disease-susceptible and disease-resistant genotypes were no longer different after removal of '*Ca*. Aquarickettsia' sequences (PERMANOVA; $P = 0.314$, $R^2 = 0.015$, Supporting Information Table S3). And although bleaching still produced distinct communities ($P < 0.001$, $R^2 = 0.132$), the interaction between bleaching and disease-susceptibility was no longer significant ($P = 0.514$, $R^2 = 0.014$) (Fig. 4E). Pairwise comparisons showed that apparently healthy disease-susceptible and disease resistant were not different for each other ($P = 0.336$) in addition to bleached disease-susceptible and disease-resistant genotypes ($P = 0.550$). Likewise, when '*Ca*. Aquarickettsia' was removed, disease-susceptibility had no effect on dispersion (PERMDISP; $P = 0.627$, $F = 0.239$), while bleaching significantly reduced group dispersions ($P < 0.001$, $F = 32.916$, Supporting Information Table S4). The interaction between disease-susceptible groups and bleaching status was a significant predictor of dispersion ($P < 0.001$, $F = 10.194$), and pairwise comparisons showed that for disease-susceptible genotypes, bleached samples were less variable than apparently healthy samples when '*Ca*. Aquarickettsia' was removed from the data (Fig. 4F).

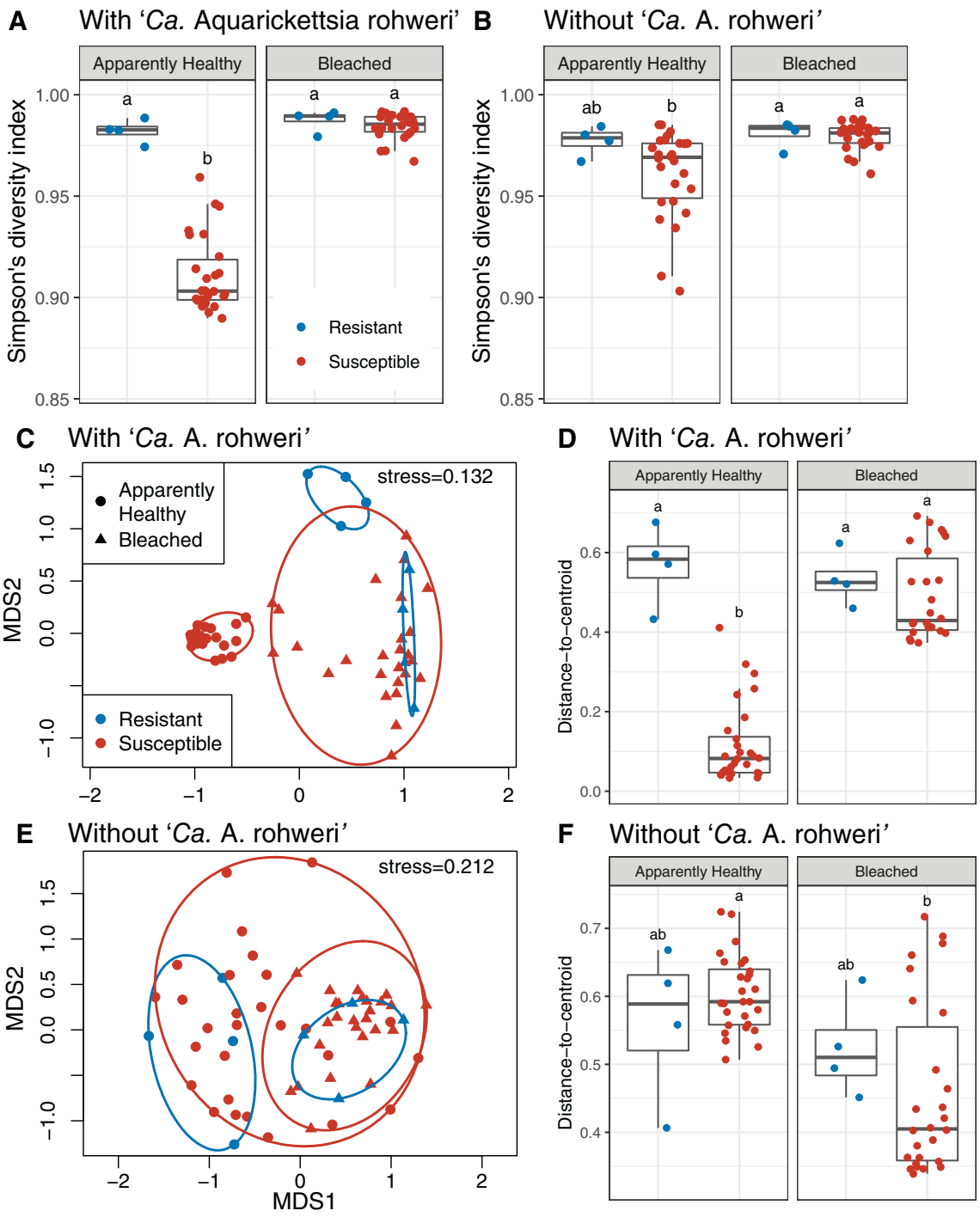

Fig. 4. Community diversity metrics by genotype (resistant vs susceptible) and health state (apparently healthy vs bleaching) for microbiomes with and without the dominant taxon '*Ca*. Aquarickettsia rohweri'.
A and B. Shannon's diversity index.
C and E. NMDS ordinations of samples using Bray–Curtis distance.
D and F. Distance-to-centroid measures. Note that detection of significant differences between disease-susceptible and disease-resistant genotypes may be influenced by the difference in replication ($n = 4$ versus $n = 26$, respectively) causing disparate dispersion between the two groups (Anderson and Walsh, 2013). See Supporting Information Tables S3 and S4. Boxes sharing a letter are not significantly different from each other using an FDR corrected significance level of $P < 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

Discussion

Coral reef ecosystems continue to be significantly altered by disease epizootics, but why some host populations or even individuals remain resistant while others succumb to outbreaks remains unknown. Previously, Muller *et al*. (2008) showed that corals susceptible to temperature-induced bleaching were more likely to suffer from disease-related mortality. More recently, however, Muller *et al*. (2018) documented that even after bleaching, some genotypes of *A. cervicornis* maintained high levels of disease resistance. This variation in disease susceptibility even after bleaching may have resulted from differences in coral host immune responses, holobiont structure, or a combination of the two factors. To explore the effect of holobiont structure, we examined differences in microbial community composition across 15 genotypes of *A. cervicornis* both before and after a 2015 natural thermal stress event that induced bleaching. To account for unbalanced distribution of samples across disease response phenotypes due to the rarity of disease resistance, we verified statistical analyses by down-sampling to a balanced design. We found that coral genotypes phenotypically characterized as disease-susceptible had a distinctive microbial community signature characterized by low diversity, low evenness, and a strikingly high abundance of the genus '*Ca*. Aquarickettsia'. This parasitic genus was roughly 36-fold more relatively abundant in disease-susceptible genotypes than in resistant genotypes. After bleaching, however, the microbiomes of these disease-susceptible genotypes were no longer distinct from resistant genotypes as the microbiomes of all corals became similarly heterogeneous in composition as measured by beta diversity. Importantly, however, in disease-susceptible genotypes, these shifts were characterized by a decrease in '*Ca*. Aquarickettsia' and an increase in the relative abundance of a variety of opportunists. Our findings suggest then that '*Ca*. Aquarickettsia' plays a critical role in the susceptibility of *A. cervicornis* to white band disease, but that disease resistance is complex and may be influenced both by microbiome history and host factors external to the microbiome, such as genetic capacity to combat disease and the role the host may play on shaping the microbiome.

'Ca. Aquarickettsia' sp. dominate the microbiome of disease susceptible A. cervicornis *genotypes prior to bleaching*

Based on its genomic composition and cosmopolitan distribution, we previously showed that '*Ca*. A. rohweri' is a ubiquitous parasite of diverse coral hosts that can translocate both amino acids and ATP from the holobiont (Klinges *et al*., 2019; Fig. 5A). We also found that members of this genus proliferate during nutrient enrichment and their abundance is strongly correlated with reduced coral growth (Shaver *et al*., 2017) and increased tissue loss and mortality (Zaneveld *et al*., 2016). Although '*Ca*. A. rohweri' is highly abundant in disease-susceptible *A. cervicornis* genotypes, the presence of this organism does not appear to be an early symptom of disease, as only corals exposed to a white band disease homogenate developed symptoms while controls remained visually healthy (Muller *et al*., 2018). Furthermore, this species has been found globally in samples of healthy coral spanning diverse genera (Klinges *et al*., 2019). The considerable dominance of a few '*Ca*. A. rohweri' variants is apparent as samples of disease-susceptible genotypes were markedly less diverse than samples of resistant genotypes. There was no apparent association of specific variants of '*Ca*. A. rohweri' with particular genotypes, but rather a few variants dominated across all samples. The presence of abundant Rickettsiales in apparently healthy *A. cervicornis* from Florida has been observed in multiple previous studies (Di Lauro, 2015; Rosales *et al*., 2019, Gignoux-Wolfsohn *et al*., 2020), but interestingly, samples of *A. cervicornis* from Panama are instead dominated by Endozoicomonadaceae (Gignoux-Wolfsohn *et al*., 2017). The absence of Endozoicomonadaceae in Floridian individuals in this study and others (Rosales *et al*., 2019; Gignoux-Wolfsohn *et al*., 2020) may be indicative of a compromised health state of these corals compared to Panamanian populations, as *Endozoicomonas* are proposed to be commensal or beneficial symbionts of corals (Neave *et al*., 2016).

*'*Ca*. Aquarickettsia' populations decline in response to bleaching but predispose corals to disease*

As '*Ca*. Aquarickettsia' species lack the ability to produce most essential amino acids and thus rely heavily on the coral host, Symbiodiniaceae, or both, we predicted that '*Ca*. Aquarickettsia' would be negatively affected by elevated ocean temperatures as host and symbiont metabolic activity are dramatically reduced during bleaching (Weis, 2008, Fig. 5). '*Ca*. Aquarickettsia' mean abundances indeed declined dramatically in disease-susceptible genotypes during bleaching to the extent that they no longer differed in relative abundance compared to the disease-resistant genotypes (Fig. 1). The loss of microbiome distinctiveness between disease-susceptibility groups after bleaching suggests that bleaching eliminates the microbiome signatures of disease-resistance by homogenizing the microbiomes of these two groups. Bleaching did, however, increase community dispersion, upholding results from previous studies which suggested that stressors induce microbiome instability (Zaneveld

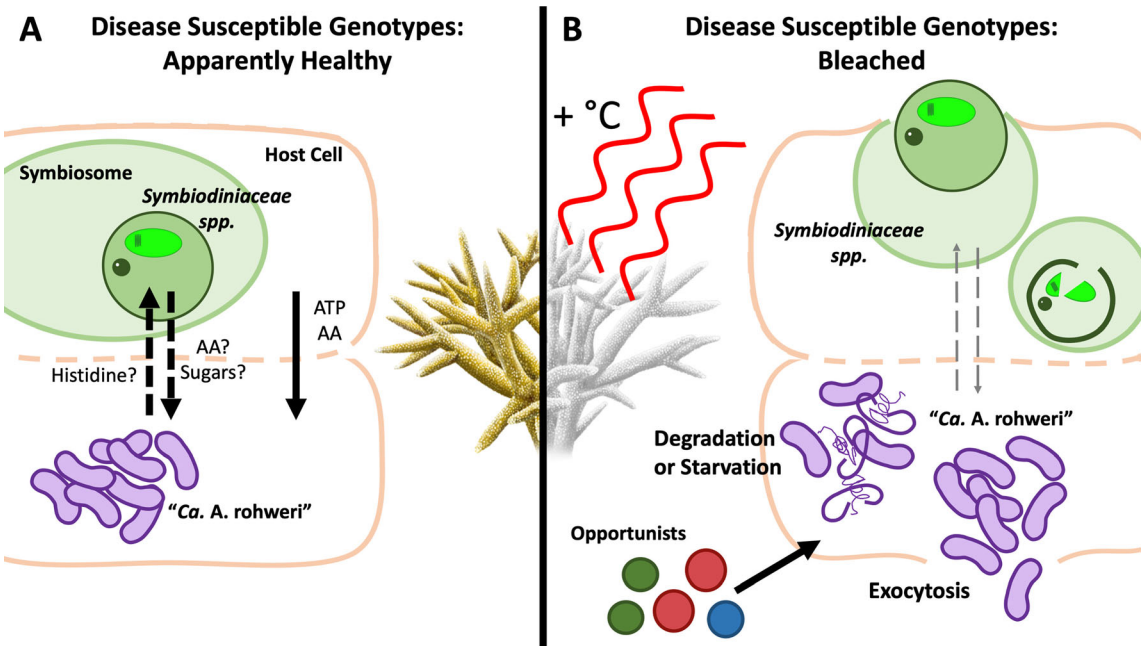

Fig. 5. Model of predicted impacts of heat stress on Symbiodiniaceae and '*Ca*. Aquarickettsia rohweri' interactions in *Acropora cervicornis*. Intracellular '*Ca*. Aquarickettsia' sp. acquire amino acids (AA) and ATP either from Symbiodiniaceae or from the coral host, or both. Decreased metabolic activity of the coral and algal symbiont during bleaching could lead to the starvation of '*Ca*. Aquarickettsia' populations, as there are fewer available resources to parasitize and the bacterium possesses sparse metabolic capabilities of its own. Due to the close colocalization of '*Ca*. Aquarickettsia' with Symbiodiniaceae, these two organisms could also be lost simultaneously via host mechanisms targeted at the symbiont, inducing expulsion and degradation. AA amino acids, ATP adenosine triphosphate. [Color figure can be viewed at wileyonlinelibrary.com]

et al., 2017; Maher *et al*., 2019). However, this effect was reversed with the removal of '*Ca*. Aquarickettsia' from the dataset (Fig. 4). These results suggest that the variation in '*Ca*. Aquarickettsia' abundance among disease-susceptible genotypes, rather than variation in minor taxa, affected dispersion. Furthermore, the homogenization of the communities after bleaching suggests that in this scenario, '*Ca*. Aquarickettsia' is likely not a primary pathogen because it was no longer in higher abundances after bleaching when disease emerged in these susceptible genotypes. Nevertheless, the rapid proliferation or maintenance of large abundances of '*Ca*. Aquarickettsia' may predispose hosts to increased disease susceptibility via overconsumption of holobiont resources prior to the bleaching event, making it the trigger of the disease instead of the sole pathogen.

Mechanisms affecting 'Ca. Aquarickettsia' abundance in apparently healthy and bleached corals

Bleaching results in a breakdown of several aspects of symbiosis in the coral holobiont. During bleaching, less carbon production and transfer occurs between the algal symbiont and the host, resulting in temporary starvation of the holobiont unless coral heterotrophy is significantly increased. The production of reactive oxygen species by the symbiont and the subsequent diffusion of these free radicals into host tissue initiates a cascade of events ultimately leading to the loss of Symbiodiniaceae (Weis, 2008; Lesser, 2011). '*Ca*. Aquarickettsia' and Symbiodiniaceae observed in fluorescent *in situ* hybridization imagery of *A. cervicornis* show colocalization of both microbes within the gastrodermis (Klinges *et al*., 2019), suggesting the possibility for the physical loss of '*Ca*. Aquarickettsia' simultaneously with Symbiodiniaceae. Alternately, as '*Ca*. Aquarickettsia' sp. are believed to be nutritionally dependent on the holobiont, coral bleaching may directly lead to the starvation and population decline of these parasites (Fig. 5).

Members of '*Ca*. Aquarickettsia' sp. possess few genes involved in carbon or nitrogen metabolism or for the synthesis of amino acids, suggesting that like other members of Rickettsiales, they are dependent on their host for nutrients and metabolic byproducts (Klinges *et al*., 2019). While their acroporid hosts are unable to synthesize a full set of amino acids, Symbiodiniaceae synthesize all essential amino acids, including mycosporine-like amino acids (Yellowlees *et al*., 2008; Rosic and Dove, 2011). We therefore hypothesize that '*Ca*. Aquarickettsia' sp. may depend on the algal symbiont either by directly or by parasitizing amino acids from *Acropora* sp. after acquisition from Symbiodiniaceae (Fig. 5A). The loss of Symbiodiniaceae during bleaching would thus negatively affect '*Ca*. Aquarickettsia' sp. and

could contribute to a significant decline in these species after a thermal event (Fig. 5B). An alternate cause for significant loss of '*Ca*. Aquarickettsia' sp. during heat stress could be the physical loss of '*Ca*. Aquarickettsia' cells due to Symbiodiniaceae expulsion and degradation (Fig. 5B). Studies in *Aiptasia pulchella* and in corals have documented both the exocytosis of Symbiodiniaceae and the detachment of necrotic host cells containing Symbiodiniaceae during bleaching (Brown *et al*., 1995; Weis, 2008). As '*Ca*. Aquarickettsia' were previously found in close proximity to Symbiodiniaceae in host tissue imagery (Klinges *et al*., 2019), they may be simultaneously eliminated in the same manner. '*Ca*. Aquarickettsia' were previously detected in water samples despite their obligate parasitic lifestyle, supporting the hypothesis that they may be released into the water column after bleaching (Klinges *et al*., 2019).

Dysbiosis in disease-susceptible genotypes after loss of 'Ca. Aquarickettsia' rohweri

While single-taxon dominance is common in coral microbiomes, as the genus *Endozoicomonas* often exceeds 50% of the coral microbial community (Bayer *et al*., 2013; Pogoreutz *et al*., 2018; Maher *et al*., 2019), low diversity and single-species dominance has been linked to disease in human systems (Kriss *et al*., 2018). Compounding this, long-term dominance of a parasite likely has additional detrimental effects on host fitness as discussed above. Loss of this dominant microbial community member observed during heat stress may allow for the invasion of opportunists or primary pathogens (Fig. 5B). In fact, heat stress increased alpha diversity, consistent with previous results (McDevitt-Irwin *et al*., 2017), but a significant change in individual community members with bleaching only occurred in disease-susceptible genotypes (Figs. 2 and 3).

It was previously proposed that coral diseases may be the result of dysbiosis in the form of unchecked growth of normally commensal bacterial species triggered by changes in the environment such as increases in water temperature (Lesser *et al*., 2007; Muller and van Woesik 2012). Heat stress led to a sudden expansion of minor community members in disease-susceptible genotypes, with members of the order Alteromonadales and other potentially opportunistic genera significantly increasing in these samples. While these genera were also found in disease-resistant samples, their change in abundance with bleaching was insignificant (Fig. 2), suggesting these genotypes may harbour opportunistic species even when in a healthy state. Although not included in the present study, control samples during DNA extraction would be helpful in verifying the significance of minor or rare community members.

Members of Alteromonadales have been found to increase in stressed or diseased coral microbiomes compared to healthy states, and at high levels may be pathogenic to corals (Sunagawa *et al*., 2009; Gignoux-Wolfsohn and Vollmer, 2015). *Exiguobacterium* and *Marivita* may also be opportunistic pathogens: *Exiguobacterium* was found in White Syndrome lesions in Pacific *Acropora* species (Krediet *et al*., 2013) and *Marivita* was found in corals exposed to heat stress (Pootakham *et al*., 2019). A similar increase in opportunists in *A. cervicornis* exposed to heat stress was observed by Gignoux-Wolfsohn *et al*. (2020), who observed lower abundance of Rickettsiales species in white band diseased tissue compared to apparently healthy tissue, and a correspondingly higher abundance of Alteromonadales species. The significant and sudden increase of previously minor microbial community members after bleaching may be an indicator of dysbiosis and may have contributed to increased disease susceptibility of these genotypes (Muller *et al*. 2018).

Diverse microbiomes may contribute to disease resistance

Samples of apparently healthy disease-resistant genotypes possessed a diverse, even microbiome with no single member exceeding 11% of the community. While high microbial diversity in itself is not necessarily an indicator of host resilience, as disease-susceptible genotypes increased in diversity after bleaching, higher diversity during non-stressful conditions may provide a greater arsenal of antimicrobial defences provided by the microbial community itself, and a coral host that has encountered many different types of bacteria may be better equipped to combat future infections (Zilber-Rosenberg and Rosenberg, 2008; West *et al*., 2019). A diverse, evenly distributed microbial community may also occupy the majority of available niches and exclude opportunistic parasites such as '*Ca*. Aquarickettsia' sp. or other pathogens involved in disease. Furthermore, considering the demand a high level of parasitic Rickettsiales infection may pose upon the immune system of its host (Gillespie *et al*., 2012), it is likely that the resistant genotypes (with low abundance of '*Ca*. A. rohweri') are better equipped to deal with the additional burden of exposure to a disease homogenate. The greater resistance of genotypes 7 and 3 to white band disease compared to other genotypes may then be due to a combination of these factors.

'Ca. Aquarickettsia' abundance as a metric of host potential for resilience

The role of coral-associated Rickettsiales as an agent of coral disease has been debated as these species were identified in corals suffering from white band disease

(type I) but are also highly prevalent community members in apparently healthy corals (Fig. 1, Casas *et al*., 2004; Miller *et al*., 2014; Gignoux-Wolfsohn *et al*., 2020). While '*Ca*. Aquarickettsia' infections are associated with reduced coral growth (Shaver *et al*., 2017), some antibiotics that effectively treated white band disease are ineffective against intracellular pathogens (Kline and Vollmer, 2011; Sweet *et al*., 2014). As species of *Vibrio* were also identified as putative white band disease pathogens, it has been suggested that disease signs may be a product of a bacterial consortium instead of a single pathogen (Kline and Vollmer, 2011; Gignoux-Wolfsohn and Vollmer, 2015; Klinges *et al*., 2019). Genomic evidence suggests '*Ca*. Aquarickettsia' sp. possess parasitic capabilities and *in vivo* data suggest that these species detrimentally affect their hosts when present at high levels (Shaver *et al*., 2017). Chronic '*Ca*. Aquarickettsia' infections in otherwise healthy corals may increase susceptibility to opportunistic infections. In bleached corals, '*Ca*. Aquarickettsia' loss may open up available niche space for the proliferation of pathogens. Regardless of bleaching status, the diverse microbiome of disease-resistant genotypes could provide protection against opportunistic pathogens in the form of antibacterial defences in addition to reducing available niche space for invaders. As disease-resistant *A. cervicornis* genotypes are rare, with only two genotypes thus far shown to maintain resistance during thermal stress, the application of robust quantitative metrics such as qPCR to quickly characterize disease resilience potential of other genotypes is essential.

Conclusion: '*Ca*. Aquarickettsia' abundance as a metric of host potential for disease susceptibility

Acropora cervicornis has declined precipitously over the last 50 years and is now the primary species used for large-scale coral restoration efforts throughout the Florida reef tract. However, disturbances such as chronic disease events limit long-term population recovery (Miller *et al*., 2014; Ware *et al*., 2020). As bleaching events become increasingly frequent worldwide, the extent of coral disease may also increase as bleaching reduces disease resistance (Muller *et al*., 2018). The results of the present study, however, suggest there may be a microbial signature to identify disease-resistant genotypes (i.e., low abundances of '*Ca*. Aquarickettsia' species). The presence of these parasites leads to nutritional deficiencies in both the coral host and the algal symbiont, contributing to a compromised health state of the holobiont. Long-term parasitism by these species may have chronic effects, and based on the results of this study '*Ca*. Aquarickettsia' are associated with increased disease risk after bleaching. This potential 'biomarker' could be integrated into the decision-making framework associated with coral restoration initiatives and is a potential intervention tool to increase the resilience of restored populations.

Experimental procedures

In August 2015, two replicate fragments from 16 genotypes of *Acropora cervicornis* were collected from the Mote Marine Laboratory *in situ* coral nursery. In September, another set of two fragments from 15 of the same genotypes were collected (one genotype, G20, was not available due to high mortality rates). By September, the nursery corals had been experiencing anomalously high water temperatures reaching approximately $\sim 2^{\circ}$C above historical averages. For further details, see Supplementary Methods SI and Muller *et al*. (2018).

All samples were flash frozen in liquid nitrogen and stored in at -80°C until processing. A total of two polyps were scraped from each replicate using a sterile razor blade, and total DNA was extracted from polyp tissue using the MoBio Powersoil kit. The microbiome of each sample was assessed using 16S rRNA Illumina sequencing on the MiSeq platform. Amplification of the 16S rRNA gene was conducted using the 515F-806R primer set, which targets the V4 region of the 16S rRNA, with barcodes on the forward primer (Apprill *et al*., 2015). Polymerase chain reaction (PCR) and library preparation protocols are detailed in the Supporting Information Methods SI. A PCR negative control was included in library preparation but did not produce a viable library. Paired-end sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) using a single flow cell on a MiSeq following the manufacturer's guidelines.

Demultiplexing and barcode removal was performed using sabre (v1.0) (Copyright© Nikhil Joshi, UC Davis), during which reads with no barcode match were discarded from the initial read pool (Supporting Information Table S5). Reads were subsequently processed using DADA2 (v1.16) (Callahan *et al*., 2016) in R (v1.1.383) (R Development Core Team, 2017). Supporting Information Table S5 presents reads lost during quality control, performed using parameters detailed in the Supporting Information. After quality control, amplicon sequence variants (ASVs) were inferred from unique reads and paired-end reads were subsequently merged. Two-parent chimeras (bimeras) were removed and taxonomy was assigned at 100% sequence identity using the Silva reference database (v132) (Quast *et al*., 2012). The resulting ASV table contained 2979 unique ASVs across 62 samples and was imported into *phyloseq* (v1.30.0) (McMurdie and Holmes, 2013). ASVs that were annotated as mitochondrial or chloroplast sequences as well as rare ASVs (total counts in the bottom quartile) were

removed resulting in a total of 2133 unique ASVs (Supporting Information Table S5).

Alpha and beta diversity metrics were analysed with and without the dominant taxon, genus '*Ca*. Aquarickettsia', at rarefaction levels of 8663 and 823 reads respectively. Genotype 20 was not sampled during bleaching and was therefore not included in statistical analyses. Differences observed in Simpson's diversity index (Heip *et al*., 2001) between sample types were tested with the Kruskal-Wallis chi-squared test and the Pairwise Wilcoxon Rank Sum Test with FDR correction for multiple testing. Using Bray–Curtis distances, permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) and permutational analysis of multivariate dispersions (PERMDISP; Anderson, 2006) were conducted to test for differences in the beta diversity with the package vegan (v2.5.5) (Oksanen *et al*., 2019). Due to the potential bias of an unbalanced design with these tests (Anderson & Walsh, 2013), we performed a down-sampling procedure with 1000 permutations to verify our results (Supporting Information SI). Statistical analyses determined differences among groups including resistance to white band disease (resistant/susceptible), bleaching status (apparently healthy/bleached) and the interaction of the two groups. Metadata is included in the Supporting Information Table S6. An analysis of composition of microbiomes (ANCOM) (Mandal *et al*., 2015) was performed to identify key genera discriminating the microbiomes of susceptible and resistant *Acropora* genotypes before and during bleaching using an unrarefied ASV table summarized to the genus level and taxa with at least 10 counts in 20% of samples. A 16S rRNA phylogeny of ASVs within '*Ca*. Aquarickettsia' was produced by aligning ASV sequences with six other described members of the genus including the type species '*Ca*. A. rohweri' using cmalign (part of the INFERNAL 1.1.1 package, Nawrocki and Eddy, 2013). PhyML v3.1 (Guindon *et al*., 2010) was used for phylogenetic analysis, with parameters selected using jModelTest 2 v0.1.10 (Supporting Information Table S7) (Darriba *et al*., 2012).

Data accessibility

Raw sequence data were deposited into the NCBI Sequence Read Archive (SRA) under accession number PRJNA639601. All scripts involved in the preparation and analysis of this dataset are available at https://github.com/maherrl/Aquarickettsia_bleaching_dynamics.

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Author Contributions

E.M. designed and performed the bleaching experiment, R.L.M. and J.G.K. performed analyses, and all authors contributed to data interpretation and writing of the manuscrip

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supplementary Methods.

Figure S1 Supplementary Figures

Table S1 Results from ANCOM analysis for each of four pairwise contrasts. An unrarefied ASV table was first summarized to the genus level, then was filtered to contain only taxa with at least 10 counts in 20% of the samples. A significance level at W = 0.6 was used in which the null hypothesis for a given taxon was rejected in 60% of the tests and p-values were corrected with Benjamini-Hochberg FDR. MD3-55 and HIMB11 are the genus identifications in Silva for *Ca. Aquarickettsia* and *Roseobacter*, respectively.

Table S2 Kruskal Wallis tests for differences in Simpson's diversity with and without *Ca. Aquarickettsia rohwerii* sequences with pairwise comparisons

Table S3 Results of PERMANOVA tests for differences between groups with and without *Ca. Aquarickettsia rohwerii* sequences and with pairwise comparisons. Perm column presents the percentage of 1000 tests that produced a $p < 0.05$ after permutational down-sampling.

Table S4 Results of PERMDISP tests for differences in group dispersion with and without *Ca. Aquarickettsia rohwerii* Sequences. Perm column presents the percentage of 1000 tests that produced a $p < 0.05$ after permutational down-sampling.

Table S5 Reads and corresponding ASVs lost through quality control processing in sabre, DADA2 and Phyloseq.

Table S6 Sample metadata with sequencing depth after removal of rare ASVs and ASVs annotating to mitochondria or chloroplasts.