IDENTIFYING DISTINCT ADAPTIVE AND PLASTIC GENE EXPRESSION PATTERNS
AND MICROBIAL INDICATORS THAT MEDIATE CORAL DISEASE RESISTANCE

by

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DISSERTATION

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Abstract

Infectious diseases are an increasing threat to coral reefs, resulting in altered community structure and hindering the functional contributions of disease susceptible species. While forecasting disease outbreaks based on environmental factors has progressed, we still lack a comparative understanding of susceptibility among coral species that would help predict disease impacts on coral communities. This dissertation compared the phenotypic, microbial, and coral host gene expression responses of seven diverse Caribbean coral species after exposure to white plague disease. Disease incidence and lesion progression rates were evaluated over a seven-day exposure. Coral microbiomes and RNA were sampled after lesion appearance or at the end of the experiment if no disease signs appeared. A spectrum of disease susceptibility was observed among the coral species that corresponded to microbial dysbiosis. This experimental exposure also determined gene expression processes involved in (i) lesion progression, (ii) within species gene expression plasticity and (iii) expression-level adaptation among species that lead to differences in disease risk. Finally, phylosymbiotic bacteria, hypothesized to provide stabilizing and probiotic contributions to the host were identified and associated with community-level microbial dysbiosis, an emerging hypothesis in coral disease etiology. Collectively, this dissertation offers insight into the adaptive constraints and plasticity of coral host gene expression patterns and microbial indicators involved in disease lesion progression and within and between species dynamics that lead to differences in disease risk that is evident on current Caribbean reefs.

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Dedication

The five years to develop this dissertation came from the exceptional contributions and sacrifice of my family and friends who helped to build this research more than they know. First of course are my parents, James MacKnight and Beth Marquis-Davis, who afforded me opportunities beyond my comfort, taught me to never squander a moment, and pushed me to grow into a person where I now find myself comfortably pursuing challenging circumstances beyond my expectations. My fiancé, Dr. Amy Yarnall, was of immense support throughout a five-year long-distance relationship. The time to now simply be together is well-earned. My older brother Jon was a source of unmatched excitement amongst nature which filled our childhood with science experiments we should have not been performing. My younger sister Brooke has been unconditionally supportive and empathetic to my distance from home. I have been fortunate to benefit from the wisdom of my Grandparents for many years and beyond the fun days with them I have also gained their insight on spiritual mentality and candid objectivity in the face of life's challenges. I have also been fortunate to learn from Gary Davis, who exemplifies a routine work ethic on doing what needs to be done, and the humility in the benefits that come from this consistency. I am grateful for the friends I have managed to maintain from kindergarten to the distant places I have lived, whose company has been exactly what I needed at the end of the day.

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Chapter 1 | Introduction

At low prevalence, disease acts as a natural selective pressure on species and has the capacity to shape species' evolution, and positively affect ecology of an environment over time (D. Harvell et al. 2009; Wood and Johnson 2015). However, infectious disease outbreaks have also been observed to reduce biodiversity at a global scale (C. Drew Harvell et al. 2002; Keesing et al. 2010), resulting in the sudden extirpation of species (Pounds et al. 2006), and fundamentally change ecological services and productivity (C. D. Harvell et al. 2019a; Hewson et al. 2014; K. M. Miller et al. 2014). Marine ecosystems are experiencing an increase in these disease outbreaks as a result of climate change and globalization (C. D. Harvell et al. 1999; Maynard et al. 2015). Marine infectious diseases are unlike terrestrial diseases, as the ocean environment is suitable for diverse microbial growth, promotes transmission through the water, and the pathogens cannot be practically removed or isolated. Therefore, disease outbreaks have become a primary threat to marine ecosystems. By understanding host susceptibility, disease scale, and pathogen virulence we can learn from these events and work towards understanding the ecology of future marine ecosystems in a changing environment.

The rise in infectious diseases emphasizes immunity and other disease tolerance and resistance mechanisms as an increasingly selective force in ecology. Although our understanding of coral innate immunity has increased, especially in naturally infected corals and those exposed to immune stimulators and bacteria within laboratory studies, we currently lack a sufficient understanding of how immune defenses and other cellular mechanisms vary among species. There is an urgent need to understand the difference between inducible immune responses to an active infection and the constitutive, lineage–specific resistance mechanisms that prevent some species from developing disease lesions. As in the example of white plague, *Montastraea*

cavernosa, *Porites porites*, and *Porites astreoides* are typically more disease-resistant, as demonstrated in the field and in this dissertation work which showed that these same species had significantly reduced relative risk of white plague disease when exposed to diseased corals (MacKnight et al. 2021). These species' resistance, however, may differ after exposure to other marine diseases, such as SCTLD, indicating different diseases stimulate different host responses, including the host immune system (Meiling et al. 2021).

Coral reefs provide a unique opportunity to understand the ecology of disease dynamics including the spatial and temporal scale of disease (E. M. Muller et al. 2020). Coral reefs are an ecologically and economically invaluable resource that have experienced gradual community biodiversity loss alongside increasingly frequent and severe disease outbreaks (Brander, Van Beukering, and Cesar 2007; Burge et al. 2014; E. M. Muller et al. 2020; Pascal et al. 2016). Coral diseases are a global threat with increased prevalence and disease outbreaks reported in nearly all major ocean basins including the Caribbean, Red Sea, Indian Ocean, Indo-Pacific, and Great Barrier Reef (Mumby, Hastings, and Edwards 2007). An example is the historical tissue-loss disease, white plague, that has gripped the Caribbean since the 1970's and is still pervasive, perhaps due to its ability to infect multiple coral-hosts (MacKnight et al. 2021). WPD is characterized by lesions originating at the base of the coral colony and expanding rapidly, resulting in significant partial and total mortality to affected colonies[17]. Newly emerging diseases such as stony coral tissue loss disease (SCTLD) are devastating what remains of Caribbean reefs by affecting multiple species, including several species previously considered disease tolerant (Meiling et al. 2021). Collectively, disease outbreaks are shifting the seascape towards more disease-tolerant coral species which changes the functionality and ecological services of coral reefs.

Previous studies on coral disease and immunity of the coral host have successfully identified genes induced by disease that contribute to biological processes such as apoptosis, autophagy, extracellular matrix maintenance, lipid metabolism, and protein trafficking (Avila-Magaña et al. 2021; Fuess et al. 2017a; Levy et al. 2021; Traylor-Knowles et al. 2021; Wright et al. 2015). However, comparing immune responses between coral species that differ in disease resistance or susceptibility, linking specific disease phenotypes to gene expression, and determining adaptive or plastic disease-resistance-associated expression patterns is understudied. By leveraging the outcome of the experimental exposure of seven coral species to white plague disease, we can identify lineage-specific expression adaptation and highly plastic genes that are linked to tangible disease phenotypes associated with coral species that are disease resistant or susceptible. Recent laboratory experimentation has confirmed lineage-specific susceptibility in response to exposure to WPD19, however, it is unknown what is driving these species differences in susceptibility. Traits that could influence disease susceptibility include immune capacity, life-history strategies, and coral-associated microbial communities20. This dissertation leverages the seven species scale of this study to broaden our investigation to challenge emerging hypothesis beyond the host gene expression to include host phylogeny and the coral microbiome.

Corals represent an excellent model system to study microbial stability and the subsequent effects on host biology. Corals experience potentially divergent species-specific bacterial transmission from parent to offspring and diverse transmission from the surrounding environment. This results in complex microbial communities which influence multiple aspects of host biology, including nutrient cycling, temperature tolerance, and disease resilience. In particular, the role of the microbiome in coral immunology has received considerable attention as coral diseases represent a critical threat to Caribbean reefs(Mera and Bourne 2018; Randall and

Van Woesik 2015; Sutherland, Porter, and Torres 2004). However, microbial profiling from diseased corals typically fails to identify etiological agents consistently between investigations. This has generated the widely held hypothesis that most documented coral diseases are poly-microbial in origin, or the causative agents are not taxonomically consistent, but are rather functionally redundant. The lack of singular etiological agents makes pathogens costly to identify through metagenomics. Moreover, broad microbial community shifts commonly observed in coral disease research have led to an increasingly popular hypothesis that many coral diseases are states of microbial imbalance, or dysbiosis, which is a stress-induced shift away from a functional, healthy community equilibrium(Egan and Gardiner 2016; MacKnight et al. 2021; Peterson et al. 2012; M. J. Sweet and Bulling 2017). The relevance of dysbiosis and need for community-level analyses has increased in coral research, in part because community imbalance is the leading hypothesized cause for the stony coral tissue loss disease (SCTLD)(Meyer et al. 2019a). SCTLD emerged from Miami, Florida in 2014 and has devastatingly swept across the Caribbean affecting coral species previously considered relatively resistant to disease(Muller et al. 2020). The first chapter of this dissertation repurposed a similarity percentage (SIMPER) analysis, a widely used analysis in coral microbial ecology, to report overall community dissimilarity. This integrated a novel method to address emerging hypothesis around dysbiosis that is also familiar enough for coral microbial ecologists to adopt in future investigations. By measuring microbial dysbiosis, we can evaluate thresholds of microbial change before host and microbiome symbiosis break down into a diseased state, consider coral disease etiology beyond the singular pathogen hypothesis, monitor the compounding effects of multiple stress events, and predict coral species survival likelihood.

To identify the microbial candidates that may contribute to stability or dysbiosis, the final chapter of this dissertation identified phylosymbiotic bacteria across divers coral species as a quantifiable indicator for micorbiome stability. Phylosymbiosis is the identification of a symbiotic organism (e.g., bateria species), whose relative abundance summarizes the phylogenetic divergence of the host (e.g., coral species) (*sensu* Fig. 1 First, that for the bacteria to have withstood historical environmental change, there are likely naturally mechanisms between microbe and host to retain that bacterial symbiont, providing stability in its abundance. Second, for this selective mechanism to have evolved, there are likely probiotic contributions between host and microbe. Investigations of phylosymbiotic microbes are meaningful because they identify probiotic candidates which have beneficial interactions with the host, provide stability within the microbiome, and assist the host in maintaining homeostasis within an increasingly changing environment (Kohl 2020).

This dissertation research is rooted in a collaborative disease transmission study where seven primary reef building Caribbean coral species were exposed to white plague disease for seven days. From this work, signatures of disease development on the coral, host gene expression, microbial ecology, antioxidant, and antibiotic metrics were collected. The first chapter presents a spectrum of disease resistance among the seven coral species which is reflected by the overall community dissimilarity. The novelty of this work lies in the scale, successful water born transmission of a marine disease, presentation of a method to measure dysbiosis, and its association with host phenotypic resistance. The second and third chapter integrate a recently developed expression variance and evolution (EVE) model to mitigate a biological caveat in multi species comparisons. The differences between species cannot be exclusively assumed to be differences associated with the species disease resistance. We observe

that phylogeny was a primary influence on host gene expression profiles, and that the genes associated with these lineage-specific differences needed to be isolated from gene expression profiles associated with the coral's response to disease exposure, a tertiary influence. This allowed our analysis to identify genes and their broader biological process that are candidates for lineage specific expression level adaptation which may explain adaptive constraints in a species ability to adapt to the rapid environmental change. This also allowed for the identification of genes and biological processes that are highly variable among these diverse coral species that elucidate immune strategies exclusive to resistant or susceptible responses to disease. The third chapter also used the EVE model to identify bacteria whose abundance demonstrate lineage-specific and highly variable patterns. However, I leverage the EVE model to identify the bacteria whose abundance is dependent on host phylogeny, which indicate phylosymbiotic bacteria. Collectively, this dissertation offers insight into the adaptive constraints and plasticity of coral host gene expression patterns and microbial indicators involved in disease lesion progression and within and between species dynamics that lead to differences in disease risk that is evident on current Caribbean reefs.

Chapter 2

Microbial dysbiosis reflects disease resistance in diverse coral species

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ABSTRACT

Disease outbreaks have caused significant declines of keystone coral species. While forecasting disease outbreaks based on environmental factors has progressed, we still lack a comparative understanding of susceptibility among coral species that would help predict disease impacts on coral communities. The present study compared the phenotypic and microbial responses of seven Caribbean coral species with diverse life-history strategies after exposure to white plague disease. Disease incidence and lesion progression rates were evaluated over a seven-day exposure. Coral microbiomes were sampled after lesion appearance or at the end of the experiment if no disease signs appeared. A spectrum of disease susceptibility was observed among the coral species that corresponded to microbial dysbiosis. This dysbiosis promotes greater disease susceptibility in coral perhaps through different tolerant thresholds for change in the microbiome. The different disease susceptibility can affect coral's ecological function and ultimately shape reef ecosystems.

INTRODUCTION

Disease is a natural force in ecosystems and at low prevalence will shape species evolution over time (D. Harvell et al. 2009; Wood and Johnson 2015). In recent decades, stressors on ecosystems driven by climate change, habitat loss and alteration, and globalization have increased disease prevalence, in some cases leading to devastating outbreak events in wild populations (Bruno et al. 2007; Pollock et al. 2014; Randall and Van Woesik 2015). These outbreaks have reshaped entire ecosystems, both terrestrial and marine (C. Drew Harvell et al. 2002). Marine disease outbreaks have driven foundational species to endangerment, including California abalone, West coast sea star, and elkhorn and staghorn corals in the Caribbean (Aronson and Precht 2001; Lafferty and Kuris 1993; Miner et al. 2018; Patterson et al. 2002).

Disease affects populations and communities globally and quantifying the impact among species will provide predictive insight into the changing functional ecology of these ecosystems.

White plague disease (WPD) is one of the most destructive diseases in the Caribbean (E. Muller et al. 2009; Weil, Croquer, and Urreiztieta 2009) affecting a large number of coral species and reducing the biodiversity and function of reef ecosystems (Descombes et al. 2015; D. O. B. Jones et al. 2014; G. P. Jones et al. 2004; Weil, Smith, and Gil-Agudelo 2006). WPD has been described affecting Caribbean corals since the 1970s and is characterized by lesions originating at the base of the colony and expanding rapidly, resulting in significant partial and total mortality to affected colonies (Sutherland, Porter, and Torres 2004). WPD is a suspected bacterial infection (Sutherland, Porter, and Torres 2004), though there has been considerable debate as to whether WPD represents one or more etiologies (Pollock et al. 2011). Recent laboratory experimentation has confirmed species-specific susceptibility in response to exposure to WPD (Williams et al. 2020), however, it is unknown what is driving these species differences in susceptibility. Traits that could influence disease susceptibility include immune capacity, life-history strategies, and coral-associated microbial communities (Velthuis et al. 2007).

As with many coral diseases, the causative agent of WPD remains unknown. Evidence among studies point to individual bacteria (Richardson et al. 1998), possible polymicrobial origins (Frias-Lopez et al. 2004), and even viral pathogens (Soffer et al. 2014), all of which question the traditional view of a singular pathogenic etiology (M. Sweet et al. 2019; M. J. Sweet and Bulling 2017). In human disease studies, there is growing literature on microbiome imbalance or dysbiosis that is responsible for disease etiology (Egan and Gardiner 2016). In coral diseases, microbiome shifts or dysbiosis also may be more appropriate than the one-pathogen-one disease concept (Cárdenas et al. 2012; Ezzat et al. 2019, 2020; Lima et al. 2020;

Meyer et al. 2016, 2019a; M. Sweet et al. 2019; M. J. Sweet and Bulling 2017; Thurber et al.

2020). By measuring microbial dysbiosis, we can evaluate thresholds of microbial change before

host and microbiome symbiosis break down into a diseased state, consider coral disease etiology

beyond the singular pathogen hypothesis, monitor the compounding effects of multiple stress

events, and predict coral species survival likelihood (Fan et al. 2013; Glasl et al. 2019; M. J.

Sweet, Burian, and Bulling 2020; Zaneveld, McMinds, and Thurber 2017).

To investigate the relationship between disease susceptibility and microbial community

responses to WPD exposure, the present study simultaneously characterized the phenotypic and

microbial responses of seven Caribbean coral species when exposed to WPD in a controlled

laboratory experiment. The seven species represented diverse life-history strategies and roles

within Caribbean reef ecosystems (Darling et al. 2012). We identified differences among coral

species not only in their phenotypic responses to WPD exposure, but in their microbial responses

as well. We then referred to the literature for any known functional roles and relevance in coral

studies of individual bacteria for their potential role as disease-associated or disease-preventing

bacteria based on their abundances among treatments and the treatment outcomes. Understanding

the link between microbial shifts and disease susceptibility can help identify the potential

mechanisms driving disease resistance, which will assist in predicting future coral assemblages.

RESULTS
Disease Prevalence

Disease prevalence significantly differed among species (Figure 1A) (Fisher's Exact

Test: p = 0.0074), and ranged from 0 to 100% (Figure 2a). Six of the seven species tested had

fragments that exhibited progressive lesions indicative of WPD. *O. faveolata* was identified as

the most susceptible species with 100% affected. Only *M. cavernosa* did not show signs of lesion

development in any fragment exposed to WPD. No lesions formed under control conditions in any species. Pairwise comparisons identified significantly different disease prevalence between species pairs (Supplementary Table 1).

Disease Severity

Disease progression rates were significantly different among species (df=5, X^2=28.627, p<0.0001). *O. annularis,* which had the fastest lesion progression rate had a significantly different lesion progression rate than *C. natans* and *S. siderea* (Supplementary Table 4). Corals in the genus *Orbicella* had the highest relative lesion progression rate (Figure 2b), with *O. annularis* and *O. faveolata* exhibiting the first and second fastest average progression rates (15.75 $cm^2 day^{-1}$ and 13.79 $cm^2 day^{-1}$, respectively). *C. natans* and *S. siderea* showed slower progression rates (3.85 $cm^2 day^{-1}$ and 2.60 $cm^2 day^{-1}$, respectively), while the only *P. astreoides* and *P. porites* colonies to exhibit disease lesions had progression rates that were considered intermediate (9.37 $cm^2 day^{-1}$) or slow (2.03 $cm^2 day^{-1}$), respectively.

Disease Incidence

Throughout the seven-day disease exposure, *O. faveolata* fragments and *O. annularis* fragments presented lesions characteristic of WPD between day 5 and 6 of exposure (Figure 3a). *C. natans* showed disease signs between 2 to 6 days of exposure, and *S. siderea* showed disease signs between days 4 and 5 of exposure (Figure 3b). Both *P. porites* and *P. astreoides* had disease incidence on day 6 of exposure. None of the *M. cavernosa* fragments showed signs of WPD over the course of the experiment (Figure 3c).

Relative Risk

The relative risk of developing WPD signs after being exposed to a WPD coral differed among species (Figure 4). *O. faveolata*, *O. annularis*, and *C. natans* all had significant risks of infection (Figure 4). Median risk of these three species was around a value of 9, indicating the

likelihood of developing WPD was 9 times greater after exposure. *S. siderea*, *P. porites*, and *P. astreoides* did not exhibit significant increased risk of disease after exposure to WPD, although their median risk values were still elevated above 1 (ranging from 3 – 8) suggesting a higher overall risk to WPD after exposure. *M. cavernosa* had an estimated relative risk close to 1.0, indicating no elevated disease risk from exposure to WPD.

Microbiome
Bacterial Community Dissimilarity and Dysbiosis

Microbiomes significantly differed among the seven (7) coral species (PERMANOVA: F = 3.91, p<0.001) (Figure 5) and were also significantly different between control and disease treatments (F=3.3381, p=0.0045). When fragments were grouped by treatment outcomes (i.e., control, disease-exposed, disease-infected), significant differences were also detected in the microbial communities (F = 1.97, p=0.0195), but pairwise comparisons showed a significant difference at p<0.1, but not at the Bonferonni corrected p-value of p=0.017 (Supplementary Table 5). These results indicate shifts in the relative abundances of the microbes once an individual was exposed to WPD or became diseased (Table 2). No interaction was detected between species and treatment (F = 0.94, p = 0.5714), or between species and treatment outcome (F=1.1026, p=0.235576) suggesting consistent differences among coral species. To look further into how each coral species' microbial community was changing based on treatment outcome, the overall dissimilarity was compared within species relative to the healthy control state (Table 1). Bacterial community dissimilarity was consistently higher in disease-infected fragments than in disease-exposed fragments relative to their paired controls (Table 1). Coral species that remained disease-exposed and did not contract the disease were more dissimilar to each other relative to the control condition dissimilarity (Table 2). While inversely, overall bacterial

community similarity was greater in coral that became disease-infected. Notably, *M. cavernosa*, the only species to not exhibit disease signs, did not show overlap with any other species in the NMDS ordination (Figure 5). All other species showed some overlap with the others regardless of treatment outcome.

Treatment Outcome-Specific Bacterial Communities

Of the 7,225 unique OTUs, 1,243 OTUs had greater than 97% sequence similarity to reference sequences allowing for species level classification of 1,243 unique bacterial species identified in this study, 29 bacteria represented 70% of the microbiome across all samples and were identified by being in 3% abundance or higher in either control, disease-exposed, or disease-infected states. Five of these 29 bacteria significantly differed in their relative abundance between treatment outcomes. Comparisons between the control, diseased-exposed, and disease-infected among all 7 coral species combined showed large overall differences of these five bacteria (Figure 6a). *Nautella italica*, *Pseudoalteromonas sp.*, and *Thalassobius mediterraneus* displayed low relative abundance in the control treatment, but were significantly higher in disease-infected fragments (Figure 6c). Conversely, *Endozoicomonas spp.* and *Burkholderia ubonesis* showed the highest abundance in disease-exposed treatments and low or no abundance in the disease-infected (Figure 6b).

Species-Specific Commensal or Beneficial Bacteria

Certain microbes were consistently present among fragments within a coral species, but absent or in low abundance in other species suggesting an identifiable species-specific microbiome (Figure 7). The bacterial family *Hahellaceae*, consisting of *Endozoicomonas sp.*, was notably dominant in *P. astreoides,* but not detectable in any great abundance in the other coral species tested. Burkholderiaceae and Spirochaetaceae were also highly abundant in *M. cavernosa* and *O. faveolata*, respectively, and not in other species. Micrococcacaea dominated

the microbiomes of *O. annularis* and *M. cavernosa* (*Anthrobacter* species; Supplementary Figure 1). Within this family specifically, *Anthrobacter ramosus* composed a large proportion of the Micrococcacaea identified (20% - 92% in *M. cavernosa* and 49% - 80% in *O. annularis*).

Microbial Diversity and Richness

Alpha and beta diversity of the bacterial community significantly differed among species and species x treatment outcome, but not between treatments, or among treatment outcome (Table 3, Figure 8). While alpha diversity (Figure 8a) showed no trends related to species susceptibility, beta diversity (Figure 8b) in disease-exposed fragments was significantly different among species and was reduced in the highly affected coral species (*O. annularis*, *C. natans*) and intermediately affected species (*S. siderea*), while no change in beta diversity was observed between control and disease-exposed fragments in low susceptible species (Figure 8c,8d, Table 3).

DISCUSSION

The present study delineated surface microbial responses of seven Caribbean reef-building coral species exposed to WPD. We found a gradient of disease susceptibility that was reflective of microbial community responses. Based on their phenotypic responses to disease exposure, the seven species fell into three groups: 1) highly susceptible (*O. faveolata* and *O. annularis*) characterized by high disease prevalence and fast lesion progression rates, 2) intermediate susceptible (*C. natans* and *S. siderea*) characterized by high disease prevalence and slow lesion progression rates, and 3) low susceptible or tolerant (*P. astreoides*, *P. porites*, and *M. cavernosa*) characterized by low to no disease prevalence and slow lesion progression rates.

As seen in field (Calnan et al. 2008; Weil, Croquer, and Urreiztieta 2009) and experimental studies (Williams et al. 2020)*, Orbicella* species displayed the highest disease

susceptibility. As major structural reef builders, the high susceptibility of orbicellids has the potential to shift the physical growth and function of coral reefs, as has already been seen in many Caribbean locales (E. Muller et al. 2009; Okazaki et al. 2017; Perry et al. 2015). Not only were individuals of both orbicellid species highly susceptible to disease in this study, but they also showed the highest severity of the disease. *P. astreoides*, *P. porites*, and *M. cavernosa* were relatively resistant to WPD. Each of these species has historically been documented as stress tolerant or weedy (Darling et al. 2012) and their relative abundances are currently increasing as overall coral cover declines (Green, Edmunds, and Carpenter 2008). As coral disease outbreaks become more common and severe because of continued degrading local conditions and the exacerbating effects of climate change, disease resistant species will likely dominate Caribbean reefs.

To capture the expected disease incidence when WPD was present, the relative risk of infection was determined for each species in this study. From this, *O. faveolata*, *O. annularis*, and *C. natans* each showed an elevated significant risk of contracting WPD if exposed. This pattern may have been related to the phylogenetically similar *O. franksi* serving as the source coral for the experiments, thereby more easily transmitting to the other orbicellids. However, *M. cavernosa* was the least susceptible to WPD exposure yet is closest in phylogeny to the orbicellids (Pinzón C et al. 2014). In addition, the susceptibility patterns we observed in our transmission experiment corresponded with field data that show these species (*O. faveolata*, *O. annularis*, and *C. natans)* clustered together because of high disease levels following the 2005 and 2010 bleaching that resulted in significant mortality (Smith et al. 2013). This independent relative risk calculation reiterates what we have observed from other coral diseases and that our

laboratory-based experiments are consistent with field observations, making them an essential tool for predicting species assemblages in the future.

The origins of many coral diseases, including WPD, are still unknown and there are several hypotheses regarding how and why corals become diseased. For instance, coral disease can originate from a foreign individual pathogen introduction and spread from coral to coral (Jolles et al. 2002; Shore and Caldwell 2019) or from microbiome changes that cause dysbiosis in the commensal surface microbial community (Glasl, Herndl, and Frade 2016). Disease phenotypes may also be the result of a secondary infection following an extreme stress event or increased bacterial virulence or pathogenicity following some disturbance (Lesser et al. 2007; E. Muller et al. 2009; E. M. Muller and Van Woesik 2012). Our experiment and dataset provide a unique opportunity to explore some of these hypotheses in seven species of coral to determine consistencies and identify what is unique in susceptible and resistant species.

The bacterial community in diseased fragments consistently were more similar among species relative to any other treatment outcome (as seen in Table 2). This response suggests diseased fragments had similar microbial constituents while in the diseased state, and this could reflect a community of pathogenic or opportunistic bacteria. This microbial convergence is likely the result of disease exposure rather than tank effects because diseased fragments started showing signs of tissue loss at different time points in the study and were removed from tanks based on differing fragment lesion development. In addition, the microbiomes of fragments that were exposed to disease, but stayed apparently healthy became more dissimilar than in the controlled state. To further explore the convergent microbiome in disease-infected fragments, bacteria increasing in relative abundance in this disease-infected state were identified.

N. italica, *T. mediterraneus*, and *Pseudoalteromonas sp.*, showed a significant increase in abundance in fragments that displayed an active disease lesion. Interestingly, two of the species (*N. italica* and *T. meditteraneus*) belong to the family Rhodobacteraceae. Members of this family have diverse metabolic and physiological properties in marine systems, play important roles in the formation of marine biofilms (Dang and Lovell 2000; Fernandes et al. 2011; Vandecandelaere et al. 2009) and are likely early colonizers at the first sign of deteriorating coral tissue (Kviatkovski and Minz 2015). The order of Rhodobacterales also has been highlighted in disease lesions of corals affected by the highly virulent stony coral tissue loss disease (SCTLD) (Rosales et al. 2020). SCTLD was not present on corals in the US Virgin Islands at the time of our study, and so the abundance of members of this order in our study may indicate that Rhodobacterales are consistently present as opportunistic colonizers in the landscape of microbial dysbiosis.

N. italica is known to induce bleaching in a red alga (Campbell et al. 2011), and this response is temperature sensitive (Campbell et al. 2011; Kumar et al. 2016) leading to compromised host chemical defenses (Fernandes et al. 2011). Similarly, WPD increases in prevalence in conjunction with or following thermal stress and coral bleaching events (Brandt et al. 2013; Brandt and Mcmanus 2009; E. Muller et al. 2009; Smith et al. 2013). In the present study, *N. italica* had a progressively higher relative abundance in disease-exposed and even more so in disease-infected fragments among all coral species.

T. mediterraneus is a Roseobacter, and is also directly implicated in Australian white syndrome of coral (Godwin et al. 2012) and in diseases of lobsters (Ranson et al. 2019). *T. mediterraneus* has been observed in other Caribbean coral diseases, including black band disease (A. W. Miller and Richardson 2012), and is responsive to coral antibacterial activity with a

similar resistance as some *Vibrio* species (Geffen, Ron, and Rosenberg 2009). In this study, *T. mediterraneus* had significantly higher abundance in disease-infected fragments among all coral species.

Pseudoalteromonas sp., the third bacterium detected at highest abundance in diseasedfragments also has a history of disease-associated properties and has been observed in noticeable abundance in other coral disease studies (Apprill et al. 2012; Beurmann et al. 2017). This bacterium is known for its antimicrobial and bacteriolytic activity in the mucus of coral and is considered to have a protective role to the coral host (Shnit-Orland, Sivan, and Kushmaro 2012). Interestingly, the high abundance of a defensive bacteria may indicate that there was bacterial antagonism occurring. In chronic *Montipora* white syndrome induced by *Vibrio coralliilyticus* the presence of *Pseudoalteromonas piratica* accelerated the disease because the resistance of *Vibrio* bacteria to the antibacterial activity of *Pseudoalteromonas sp.* (Beurmann et al. 2017) allowed undefended spread of the pathogen. This type of polymicrobial synergy for pseudolateromonas is altering the microbiome and allows for Rhodobacteraceae, like *N. italica* and *T. mediterraneus*, to become opportunistic pathogens (Kviatkovski and Minz 2015; Vandecandelaere et al. 2009) may also be at play in WPD.(Kviatkovski and Minz 2015; Vandecandelaere et al. 2009)(Kviatkovski and Minz 2015; Vandecandelaere et al. 2009)(Kviatkovski and Minz 2015; Vandecandelaere et al. 2009)

Considerations of microbial dysbiosis as a cause for marine disease has been overshadowed by a focus on identifying singular or multiple pathogens as etiological agents. Because coral disease etiological agents are so elusive, this unique dataset can be leveraged to explore the broader microbial community dysbiosis, a process which may allow bacteria to become opportunistically pathogenic (Thurber et al. 2020). Microbial dysbiosis is a microbial

community shift that has a negative impact on the host and has the capacity to induce disease phenotypes (Egan and Gardiner 2016). Dysbiosis appears as significant microbiome shifts commonly reported in coral disease studies and may actually underlie many coral diseases (Meyer et al. 2019a; Sunagawa et al. 2009). Microbial dysbiosis has also been reported from thermal anomalies to parrotfish bites, indicating dysbiosis as a compounding effect from everyday scenarios on the reef (Bettarel et al. 2018; Ezzat et al. 2020).

In this study, the overall dissimilarity between treatment outcomes was compared within each species and was used to characterize dysbiosis. In orbicellids, the microbiome changed less in disease-infected fragments relative to control states compared with other species, possibly because orbicellids have a lower threshold for change before showing disease phenotypes, or etiological disease agents became pathogenic without statistically changing the microbiome assemblage. Microbial imbalance resulting from abiotic stressors could be a possible mechanism for increased susceptibility between stress events. Orbicellids are more susceptible to disease following thermal stress events (Brandt et al. 2013; Brandt and Mcmanus 2009; Smith et al. 2013) and our results suggest that this pattern may be due to this lower threshold for microbiome dysbiosis. Contrary to this, the disease resistant *P. astreoides* showed a much higher threshold for dysbiosis in our study; their microbiome changes were significant even in fragments that were exposed to disease but did not develop lesions. In field studies, *Porites spp.* are known to be tolerant to both thermal stress and subsequent disease (Smith et al. 2013), suggesting this species may tolerate a significant shift in their microbiome before holobiont break-down. This dichotomy emphasizes how a higher dysbiosis threshold at a time of compounding environmental stressors may be an informative measurement of understanding varying stress response outcomes among coral species (Ezzat et al. 2020; Meyer et al. 2019).

During disease exposure, stabilizing bacteria can inhibit or slow the colonization of pathogenic bacteria (Ritchie 2006), while also preventing microbial dysbiosis. In this study, *Endozoicomonas sp.* were highly abundant in fragments of *P. astreoides*, a species that remained resistant to disease, while this bacterium was notably absent in the one fragment of this species that became diseased. Additionally, the microbiome of *M. cavernosa* was dominated by *Burkholderia ubonensis* and *Arthrobacter* spp. within the order actinomycetales. Interestingly, both *Endozoicomonas* sp. and *B. ubonensis* increased in abundance in disease-exposed fragments, but were absent in diseased fragments. *Endozoicomonas sp.* has been largely regarded as part of the core microbiome of corals (Bayer et al. 2013; Lesser and Jarett 2014; Morrow, Muller, and Lesser 2018) and to be in phylosymbiosis with *P. astreoides* due to the coral's vertical transmission of bacteria through broadcast spawning (Pollock et al. 2018a). In fact, the protective features of *B. ubonensis* are well known and used as a biocontrol treatment for the tropical infectious disease, meliodidosis in humans (Price et al. 2013, 2017) and potentially play this role in *M. cavernosa*, as none of the fragments became diseased upon exposure to WPD. The dominance of these bacteria in most disease-resistant fragments suggests they may play a role in the functional stabilization of the microbiome, or their commensal presence is enough to inhibit pathogenic colonization.

The increasing prevalence and severity of diseases affect coral species differently. Because the functional contributions of these species define a reef, it is integral to understand variability in disease susceptibility among coral species to predict how disease will shape coral reefs of the future. This study characterized disease susceptibility among seven coral species that represent a diversity of life history strategies, and which have historically contributed to Caribbean coral reef assemblages. Less microbial change was observed in disease-susceptible

coral species, suggesting low microbial dysbiosis thresholds should be further investigated as a possible coral disease etiology. Furthermore, bacteria with protective properties were more prevalent in coral species tolerant to WPD. As disease increases, disease-susceptible *Orbicella* species that are primary contributors to reef structure will become less abundant, negatively affecting the physical protection that reefs provide. This lost real estate within the reef may be colonized by more disease resistant but less efficient reef-building species, making disease susceptibility an important predictor of the changing ecological function of Caribbean reefs.

METHODS

Statistics and Reproducibility

This study applied a coral disease transmission methodology recently developed and reported by Williams et al. (2020). Five parental colonies from each of seven Caribbean coral species, *Orbicella faveolata*, *Colpophyllia natans, Siderastrea siderea*, *Porites astreoides*, *Porites porites*, and *Montastraea cavernosa*, were collected from Brewers Bay (18.34403, -64.98435), St Thomas, U.S. Virgin Islands on 13 June 2017 (Figure 1A). Colony collection targeted either whole colonies (for *S. siderea, P. astreoides*, and *P. porites*) or fragments of colonies (for *O. faveolata*, *C. natans,* and *M. cavernosa*) that were between 20 and 30 cm maximum diameter. Although five parental colonies of *Orbicella annularis* were collected, one experienced mortality and was not used in experiments. Colonies were held in running seawater tables at the University of the Virgin Islands where they were fragmented into small pieces (average size 17.74 cm^2 ± 1.03 SEM) using a sterilized table saw, acclimated for nine days (allowing for tissue on fragmented edges to heal completely), and then placed in experimental conditions. Diseased (n=3) and healthy (n=5) *O. franksi* were collected by separate divers on two dives and kept isolated from each other and from fragments of the tested coral species until the

commencement of the experiment. WPD-affected colonies of *O. franksi* were identified as displaying the characteristic signs of this disease, namely large (> 5 cm wide) lesions that appeared to originate from the base or edge of a colony where no signs of predation or predators were found. Diseased *O. franksi* were targeted as the source for disease in this transmission experiment because this species is known to be consistently affected by WPD throughout the year and was also used as the source species for previous experiments (Williams et al. 2020). Diseased corals were fragmented and monitored for indications of active disease (lesion enlargement > 0.2 cm^2d^{-1}, consistent with WPD) for 24 hours. Only fragments showing active lesion progression were used in disease treatments.

Upon commencement of the experiment (June 2017), coral fragments were distributed among 5 treatment and 5 control 17-L sterilized containers (17-L), each equipped with individual aerators. Containers were placed among three outdoor shaded running seawater tables that served as water baths. Containers received water changes daily and their locations were also randomized each day over the course of the 7-day experimental period. Each treatment container consisted of a randomly assigned healthy fragment of each of the seven tested species that were placed equal distances (approximately 7-8 cm) from a central diseased *O. franksi* fragment. Control containers were identically arranged, except that healthy *O. franksi* were used as the central corals (Figure 1B,1C). During the daily water changes, the locations of the fragments within each container were randomized relative to each other, while keeping the same equal distances from the central fragment. When a disease lesion appeared on a disease-exposed coral that was previously healthy it was monitored until 30% tissue loss. If the lesion enlarged over this time period, the coral and its paired control fragment were photographed, removed, flash frozen and stored at -80˚C until further 16S rRNA analysis.

Phenotype Analysis

Metrics that encompassed a susceptibility "phenotype" were calculated for each species, including: 1) total disease prevalence (% corals that became diseased) within treatment containers during the experimental period, 2) average days until lesion appearance, and 3) average lesion progression rate (cm^2 lost/day). Coral fragments exposed to disease that did not show lesion appearance by the end of the transmission study were classified as "disease-exposed". While coral fragments that were exposed to the disease and developed expanding lesions were grouped as "disease-infected".

Disease prevalence among species was compared using a Fisher's exact test in R (Supplementary Table 1). A photograph and timestamp was captured upon appearance of lesions and then immediately before each fragment was culled around 30% tissue loss. Disease severity was measured by calculating the rate of lesion progression across the coral fragment as the amount of tissue lost between the appearance of the lesion to the time it was culled divided by that time period. Time to infection for a fragment was measured as the number of days from experiment start to the first appearance of lesions and visualized with a survival plot through a Kaplan-Meier estimate of the survivorship by using the *survfit* function in the R package *survival* (Therneau 2015) (Supplementary Table 2). The relative risk of each species was also calculated as:

$$\text{Relative risk (RR)} = \text{Risk in exposed} / \text{Risk in non-exposed}$$

where the *risk in exposed* individuals was calculated as the prevalence (diseased/total population) of those exposed to disease and *risk in non-exposed* individuals was calculated as the prevalence (diseased/total population) of those not exposed to disease. To obtain an estimate of relative risk, Markov Chain Monte Carlo simulations were used with Gibbs sampling in OpenBUGS (MRC Biostatistics Unit, Cambridge, UK). Ninety-five percent credible intervals were calculated for each

estimate of relative risk. Credible intervals that did not include a value of one were considered significant, with a credible interval above one signifying a higher risk of disease because of exposure to the diseased coral fragment. A credible interval below one signified a lower risk of disease from exposure.

Microbiome Extraction and Sequencing

DNA from the coral samples were extracted at the University of Texas at Arlington (UTA) using the DNeasy Powersoil Isolation kits (MO BIO Laboratories, Carlsbad, CA). Roughly 0.25g of tissue was removed from each of the coral fragments using a sterilized bone cutter (Supplementary Table 3). Tissue from healthy-state fragments ("control") was extracted from the center of the fragment. Tissue was extracted in a similar manner from fragments exposed to WPD that did not display lesions by the end of the experiment ("disease-exposed"). For fragments that developed a lesion(s) ("disease-infected"), tissue was extracted approximately 2 to 3mm horizontally from the lesion margin in the apparently healthy tissue and collected parallel to the lesion margin.

Tissue samples were sent to MR DNA Molecular Research LP (Shallowater, TX) for 16S rRNA gene amplification using 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) primers for the V4 region and DNA libraries were prepared follwing MR DNA protocols. Samples were sequenced on an Illumina MiSeq 2x250bp PE reads and resulting sequences were bioinformatically processed through the MR DNA pipeline (MR DNA, Shallowater, TX, USA) utilizing the QIIME analysis. Barcodes, primers, and ambiguous calls are removed from sequences as well as short sequences <150 bp. Operational taxonomic units (OTUs) are clustered at 97% similarity and taxonomically classified using BLASTn against an RDPII and NCBI database (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu) and organized into each taxonomic level as counts and percentage files based on industry standards of

homology of sequences to the NCBI reference database (Supplementary Table 6). Counts are the number of sequences read while percentage is the relative proportion of reads within each sample for each taxonomic classification.

Microbial Community Analysis

Differences in bacterial communities between coral species and treatment outcome levels (control, disease-exposed, disease-infected) were assessed with a two-way permutational multivariate analysis of variance (PERMANOVA) using the R package "vegan" (Oksanen et al. 2015). Microbial community differences were visualized using non-metric multidimensional scaling (NMDS). Bacterial abundances that were most dissimilar between species and treatment outcome were identified using a similarities percentage analysis (SIMPER) (Oksanen et al. 2015). These analyses were based on a Bray-Curtis dissimilarity matrix. One-hundred percent stacked bar graphs were created using raw abundance percentages of bacterial families (> 3% relative abundance) to visualize shifts in microbial composition of each individual coral and among species. Analysis of variance (ANOVA) and Tukey HSD post-hoc analyses identified differentially expressed bacteria between treatment outcomes. However, a non-paramatric Kruskal-Wallis test was applied to *Endozoicomonas spp*.

The Shannon diversity index and beta diversity of the bacterial community were calculated for each sample using the R package "betapart" (Andres Baselga, David Orme, Sebastien Villeger 2018). Diversity data were non-normal even with transformation, therefore differences among treatments were tested using non-parametric Kruskal-Wallis tests and relationships between diversity indices and lesion progression rates were investigated using non-parametric Spearman rank tests.

Acknowledgements

Data Availability

Source data used to create all figures including 16s sequences are stored at NCBI through BioProject accession PRJNA667272 and additionally made publicly available through the BCO-DMO project page: https://www.bco-dmo.org/project/727496.

Code Availability

Analysis for the publication was conducted in R version 3.6.2 (2019-12-12). The R scripts for the analysis are made publicly available through Github: DOI: 10.5281/zenodo.4635319

Author Contributions

EMM,LDM,MB were responsible for compiling the intellectual merit behind the research objectives, design and acquiring funding for this original research. All authors, NJM, KC, DL, ACF, AG, BD, JA, LF, CR, CB, EMM, LDM, MB, contributed to the experimental disease exposure. MB recorded phenotypic metrics, designed phenotypic analysis, and created Figure

1A. KC assisted in 16s rRNA extraction and created Supplementary Figure 1. EMM designed

and performed the relative risk analysis. NJM designed and performed microbial analysis,

performed phenotype analysis, wrote the manuscript and created all remaining figures. MB and

LDM had considerable editorial input during manuscript assembly with additional revisions from

all authors, NJM, KC, DL, ACF, AG, BD, JA, LF, CR, CB, EMM, LDM, MB.

Competing interests

The authors declare no competing interests.

Figures

A

O. franksi **O. faveolata** **O. annularis**

C. natans **S. siderea** **P. astreoides**

P. porites **M. cavernosa**

B

Orbicella annularis

Siderastrea siderea

Porites porites

Montastraea cavernosa

Orbicella franksi

Orbicella faveolata

Colpophyllia natans

Porites astreoides

Control Treatment
June 22nd 2017
Day 1, 12:03 pm

C

Orbicella faveolata *Porites porites* *Orbicella annularis*

Orbicella franksi *Siderastrea siderea*

Colpophyllia natans *Porites astreoides* *Montastraea cavernosa*

Disease Treatment
June 22nd 2017
Day 1

Figure 1. Coral Species and Experimental Design. (a) *Orbicella faveolata*, *Orbicella annularis*, *Orbicella franksi*, *Colpophyllia natans*, *Montastraea cavernosa*, *Porites astreoides*, *Porites porites*, *Siderastrea siderea*. All photos by M. Brandt except *M. cavernosa* by T.B. Smith. Disease transmission experimental design for (b) control and (c) disease treatment. Note the healthy *O. franksi* in the center of the control and the white plague infected *O. franksi* in the center of the disease treatment. There were five aquaria assigned as control treatments and five aquaria assigned as disease treatments. Every tank included one fragment from each of the seven coral species. When a coral colony was collected, it was split in two, with one fragment designated for control treatment and the other paired fragment going to a disease exposure treatment.
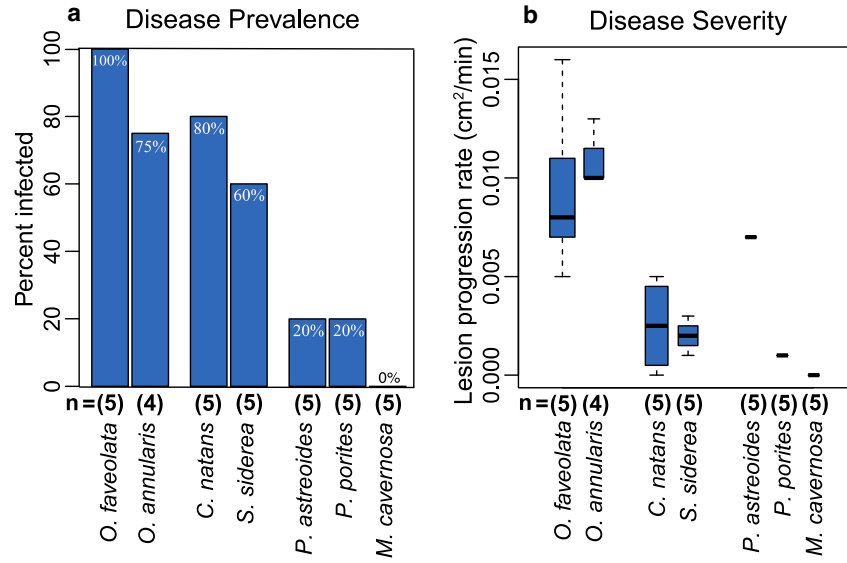
Figure 2. Disease Prevalence and Severity (a) Disease prevalence (percentage of replicates that contracted the disease) for each species. (b) Disease severity (rate at which the disease lesion progressed across the infected coral after contracting white plague disease); only the corals that contracted the disease had their lesion progression rates graphed.
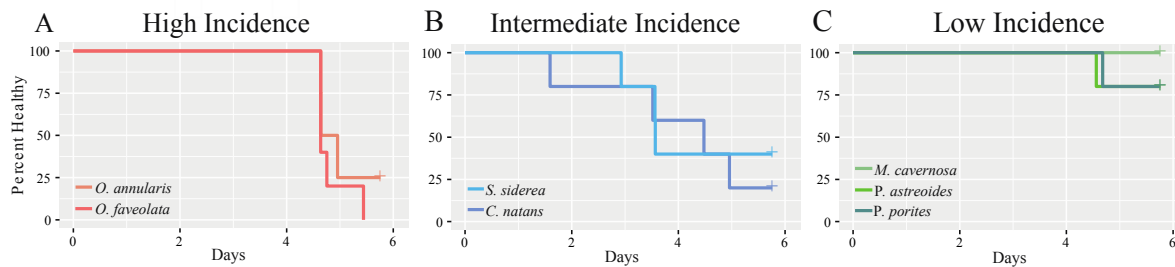
Figure 3. Disease Incidence. Survival curves showing the proportion of corals that remained healthy (i.e., did not develop lesions) over the course of the experiment for species that showed high (a), intermediate (b), and low (c) incidence of disease. Shading around survival curves represents 95% confidence intervals.
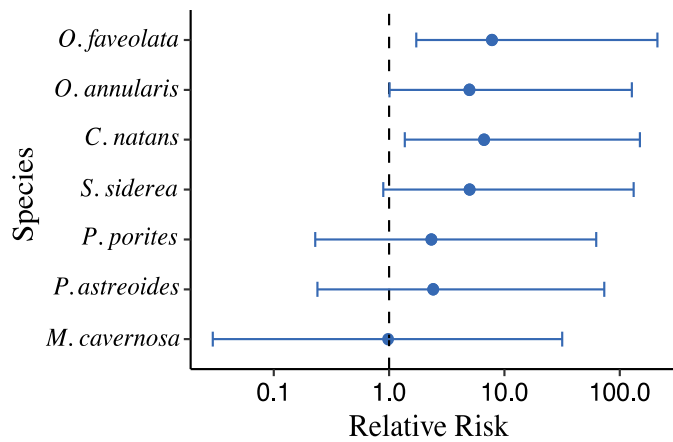
Figure 4. Relative Risk. The median relative risk the species will contract white plague disease with 95% credible intervals.
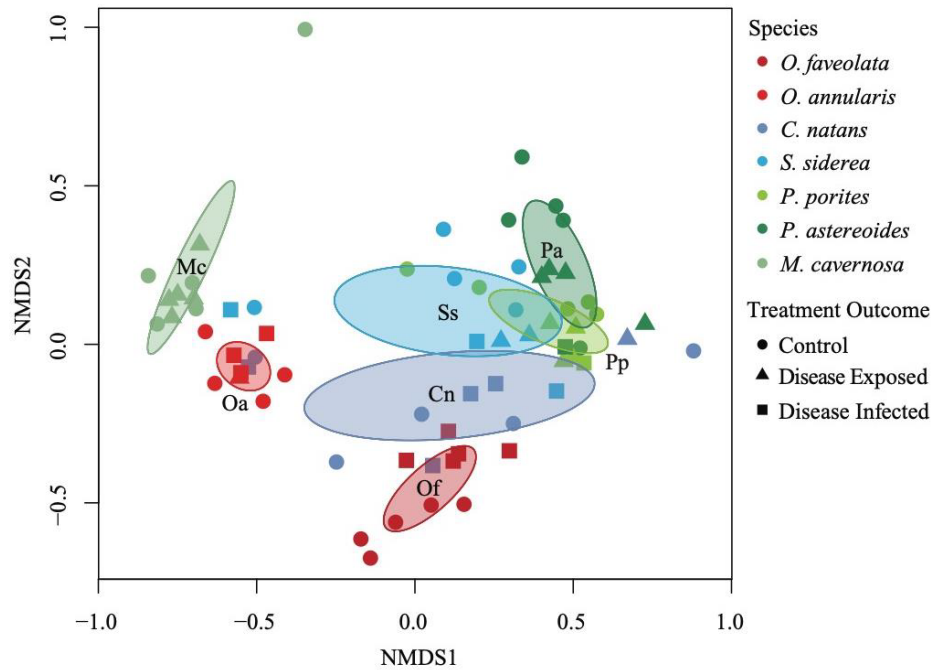
Figure 5. Non-metric multidimensional scaling (NMDS) (Stress = 0.13) of bacterial communities from the tissue of the seven species tested organized by species and treatment outcome (control, disease-exposed, disease-infected). Ovals represent 95% confidence intervals.
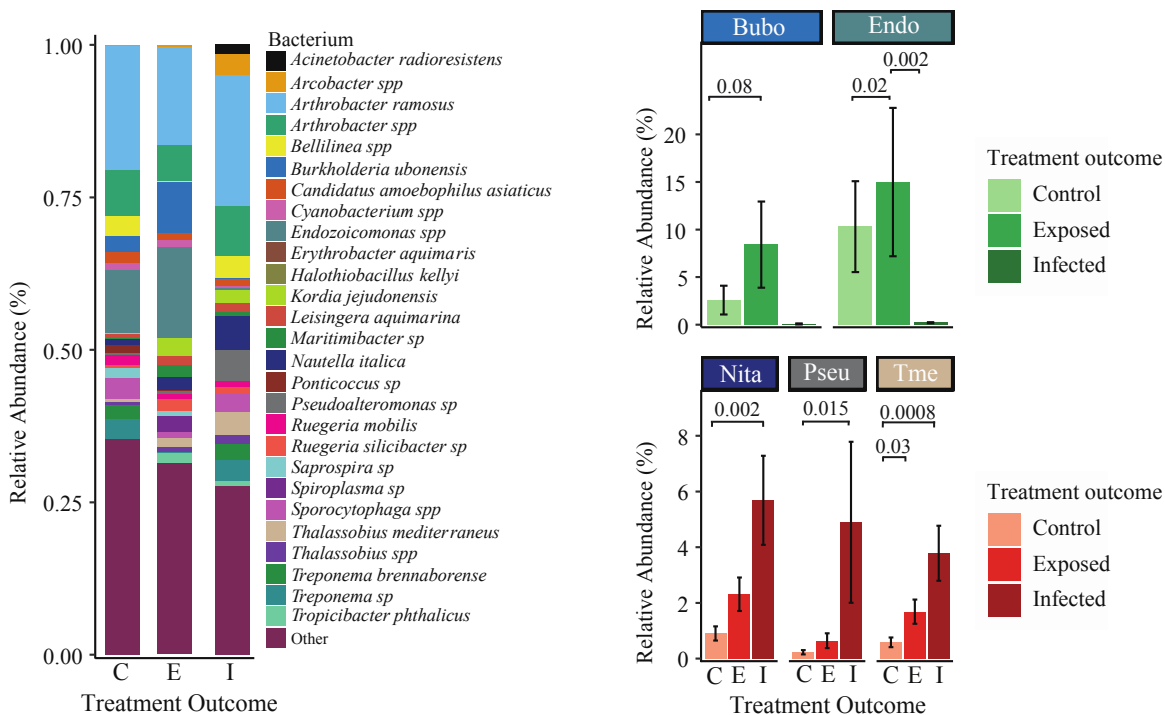
Figure 6. Treatment outcome-specific bacterial community. (a) Bacteria of 3% relative abundance or higher in any treatment outcome. (b) Two bacteria that increased in disease-exposed fragments, (c) Three bacteria that significantly increased in disease-infected fragments. Abbreviations correspond to the following bacterial species: "Bubo" = *Burkholderia ubonensis*, "Endo"= *Endozoicomonas spp.*, "Nita" = *Nautella italica*, "Pseu" = *Pseudoalteromonas sp.*, "Tme" = *Thalassobius mediterraneus*. Bar plot annotations are significant p-values from the Tukey Post hoc test, and the Dunn test for *Endozoicomonas spp.*, omitted p-value annotations are none significant comparisons. Individual data points are represented by light grey dots and error bars represent standard error.
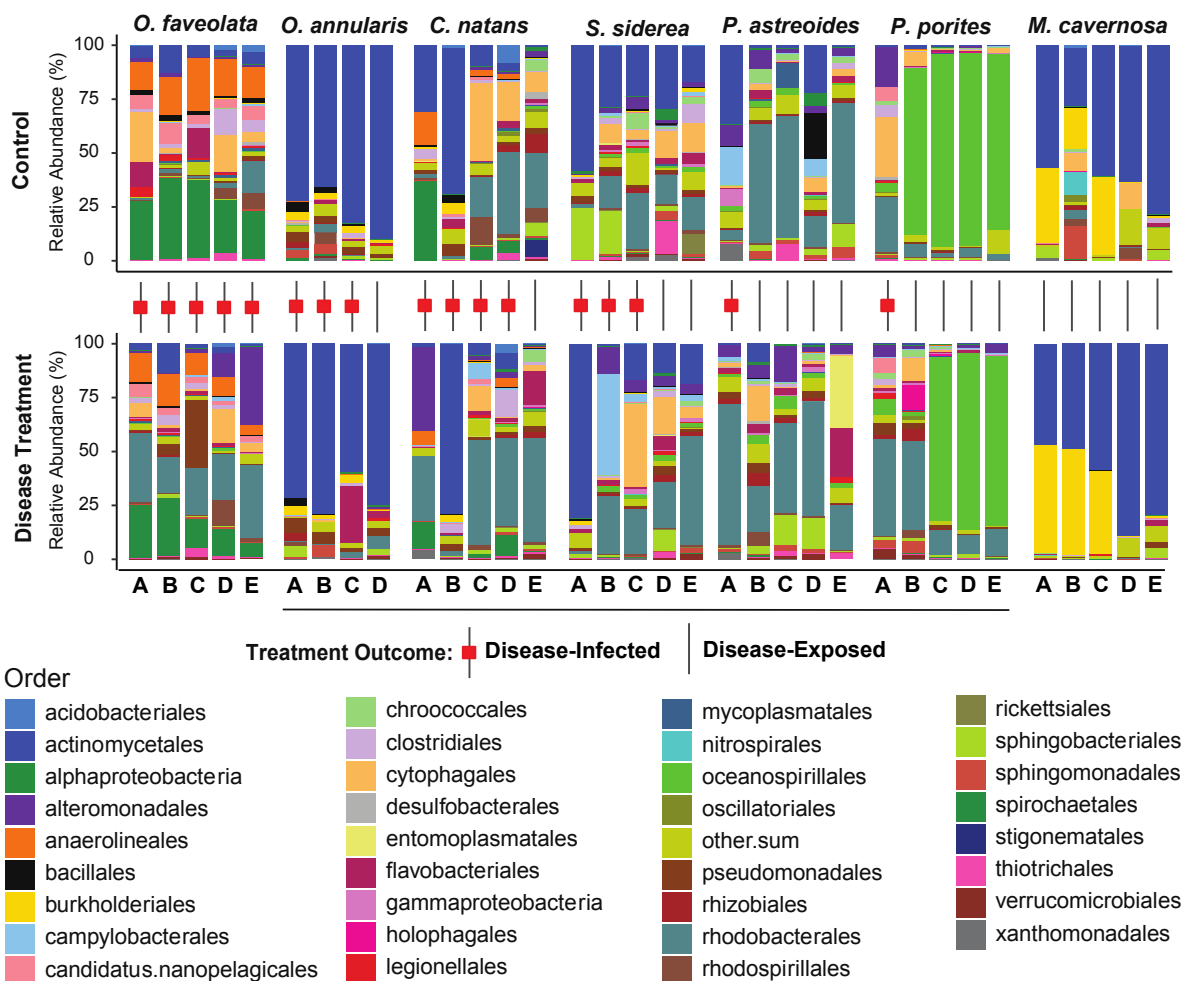
Figure 7. Species-specific bacterial community. Relative abundances of bacterial orders for each coral fragment exposed to either healthy *O. franksi* (i.e., control), or a white plague-infected *O. franksi* (i.e., disease treatment). Only bacterial order with relative abundances greater than 3% were colored individually. All other bacterial orders were grouped into the category "other.sum". Paired fragments that are genotypically identical that came from the same colony are oriented vertically to visualize how the microbiome changes from control to disease treatment exposure. The red square between vertical columns represents the genotype pair that displayed an active white plague lesion (disease-infected) by the end of the experiment.
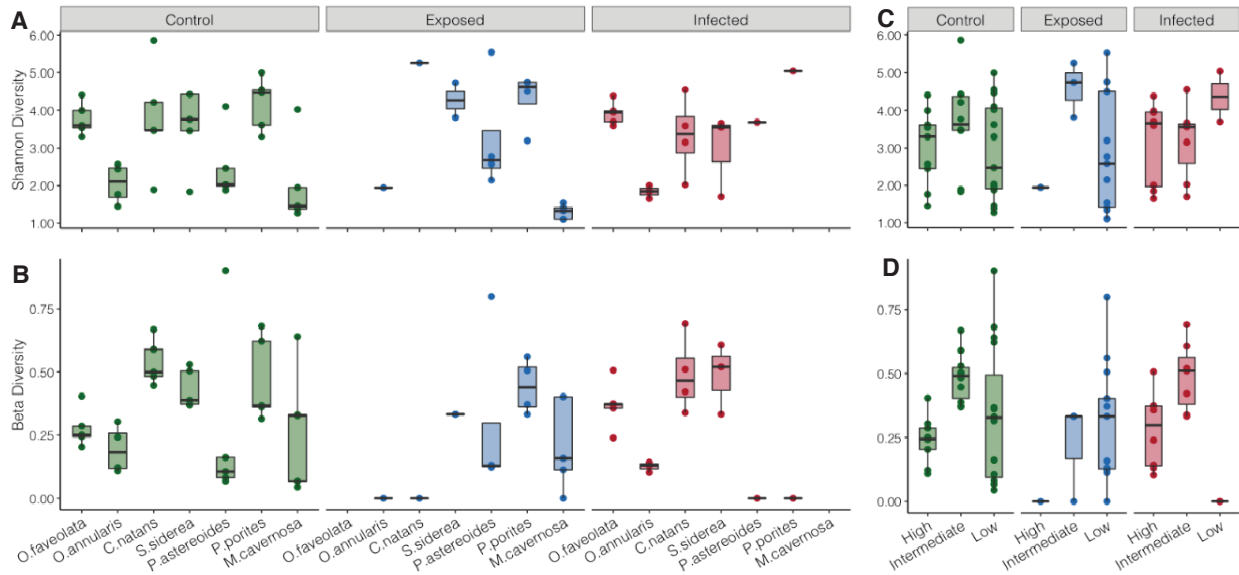
Figure 8. Microbiome Diversity. (**A**) Alpha diversity (Shannon diversity) and (**B**) Beta diversity (local diversity) per species and ordered by species disease susceptibility from left to right; and grouped by treatment outcome: control, exposed, and infected. (**C**, **D**) Alpha and Beta diversity grouped by species susceptibility (*O. faveolata*, *O. annularis* are high susceptibility, *C. natans* and *S. siderea* are intermediate susceptibility, *P. astreoides*, *P. porites*, and *M. cavernosa* are in low susceptibility).

Tables

Table 1. Dysbiosis. The values are the overall dissimilarity of microbial communities calculated by SIMPER analysis for fragments between treatment outcome within each species. Values closer to 1 indicate higher microbial dissimilarity, while values closer to 0 represent similar microbial communities.

Species	Disease-Exposed v Control	Disease-Infected v Control
O. faveolata	-	0.59
O. annularis	0.188	0.302
C. natans	0.73	0.762
S. siderea	0.701	0.746
P. porites	0.762	0.912
P. astreoides	0.393	0.716
M. cavernosa	0.381	-

Table 2. Convergent Microbiome. The values represent the overall dissimilarity from a SIMPER analysis performed on a pairwise comparison within treatment outcome (control, disease-exposed, disease-infected) for all species. Black numbers in the control table represent the origin microbial overall dissimilarity between species. Red values indicate a microbial community that is diverging and more dissimilar than in the control conditions. Green values indicate a microbial community that is converging to be more similar than in the control conditions.

Control

	O. faveolata	O. annularis	C. natans	S. siderea	P. porites	P. astreoides	M. cavernosa
O. faveolata	-						
O. annularis	0.915	-					
C. natans	0.791	0.757	-				
S. siderea	0.892	0.665	0.790	-			
P. porites	0.933	0.890	0.878	0.806	-		
P. astreoides	0.955	0.984	0.940	0.926	0.910	-	
M. cavernosa	0.936	0.436	0.808	0.707	0.907	0.987	-

Disease Exposed

	O. faveolata	O. annularis	C. natans	S. siderea	P. porites	P. astreoides	M. cavernosa
O. faveolata	-						
O. annularis	-	-					
C. natans	-	0.924	-				
S. siderea	-	0.806	0.707	-			
P. porites	-	0.916	0.670	0.688	-		
P. astreoides	-	0.964	0.800	0.853	0.834	-	
M. cavernosa	-	0.381	0.992	0.875	0.977	0.993	-

Disease Infected

	O. faveolata	O. annularis	C. natans	S. siderea	P. porites	P. astreoides	M. cavernosa
O. faveolata	-						
O. annularis	0.928	-					
C. natans	0.710	0.710	-				
S. siderea	0.840	0.673	0.780	-			
P. porites	0.797	0.975	0.802	0.820	-		
P. astreoides	0.826	0.983	0.838	0.844	0.638	-	
M. cavernosa	-	-	-	-	-	-	-

Kruskal Wallis	Species			Species:Outcome			Treatment			Outcome			Group			Spearmann to LPR	
Index	x^2	df	p	x^2	df	p	x^2	df	p	x^2	df	p	x^2	df	p	R	p
Shannon	34.8	6	<1e-5	40.7	18	0.0016	0.048	1	0.83	0.24	2	0.89	2.97	2	0.23	-0.14	0.59
Pielou	35.1	6	<1e-5	41.2	18	0.0013	0.003	1	0.95	0.48	2	0.78	4.38	2	0.11	-0.1	0.69
Simpson	35.1	6	<1e-5	41.3	18	0.0013	0.007	1	0.93	0.26	2	0.88	2.87	2	0.24	-0.06	0.82
Beta	21.7	6	0.0013	35.8	18	0.0074	0.471	1	0.49	1.37	2	0.50	12.4	2	0.002	-0.21	0.42

Table 3. Microbial Diversity. A Kruskal Wallis test was performed on the diversity indices.

"X^2" indicates Chi-square test, "p" indicates the p-value.

Chapter 3

Disease Resistance in Coral is Mediated by Distinct Adaptive and Plastic Gene Expression Profiles

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In revision

ABSTRACT

Infectious diseases are an increasing threat to coral reefs, resulting in altered community structure and hindering the functional contributions of disease susceptible species. We exposed seven reef-building coral species from the Caribbean to white plague disease and determined processes involved in (i) lesion progression, (ii) within species gene expression plasticity and (iii) expression-level adaptation among species that lead to differences in disease risk. Gene expression networks enriched in immune genes and cytoskeletal arrangement processes were correlated to lesion progression rates. Whether or not a coral developed a lesion was mediated by plasticity in genes involved in extracellular matrix maintenance, autophagy, and apoptosis. While resistant coral species had constitutively higher expression of intracellular protein trafficking. This study offers insight into the process involved in lesion progression and within and between species dynamics that lead to differences in disease risk that is evident on current Caribbean reefs.

INTRODUCTION

At low prevalence, disease acts as a natural selective pressure on species and has the capacity to shape species' evolution, and positively affect ecology of an environment over time (D. Harvell et al. 2009; Wood and Johnson 2015). However, infectious disease outbreaks have also been observed to reduce biodiversity at a global scale (C. Drew Harvell et al. 2002; Keesing et al. 2010), resulting in the sudden extirpation of species (Pounds et al. 2006), and fundamentally change ecological services and productivity (C. D. Harvell et al. 2019a; Hewson et al. 2014; K. M. Miller et al. 2014). Marine ecosystems are experiencing an increase in these disease outbreaks as a result of climate change and globalization (C. D. Harvell et al. 1999;

Maynard et al. 2015). Marine infectious diseases are unlike terrestrial diseases, as the ocean environment is suitable for diverse microbial growth, promotes transmission through the water, and the pathogens cannot be practically removed or isolated. Therefore, disease outbreaks have become a primary threat to marine ecosystems. By understanding host susceptibility, disease scale, and pathogen virulence we can learn from these events and work towards understanding the ecology of future marine ecosystems in a changing environment.

Coral reefs are an ecologically and economically invaluable resource that have experienced gradual community biodiversity loss alongside increasingly frequent and severe disease outbreaks (Brander, Van Beukering, and Cesar 2007; Burge et al. 2014; E. M. Muller et al. 2020; Pascal et al. 2016). Coral diseases are a global threat with increased prevalence and disease outbreaks reported in nearly all major ocean basins including the Caribbean, Red Sea, Indian Ocean, Indo-Pacific, and Great Barrier Reef (Mumby, Hastings, and Edwards 2007). Coral reefs provide a unique opportunity to understand the ecology of disease dynamics including the spatial and temporal scale of disease (E. M. Muller et al. 2020). An example is the historical tissue-loss disease, white plague, that has gripped the Caribbean since the 1970's and is still pervasive, perhaps due to its ability to infect multiple coral-hosts (MacKnight et al. 2021). Newly emerging diseases such as stony coral tissue loss disease (SCTLD) are devastating what remains of Caribbean reefs by affecting multiple species, including several species previously considered disease tolerant (Meiling et al. 2021). Collectively, disease outbreaks are shifting the seascape towards more disease-tolerant coral species which changes the functionality and ecological services of coral reefs.

The rise in infectious diseases emphasizes coral immunity and other disease tolerance and resistance mechanisms as an increasingly selective force in ecology. Although our understanding of immunity has increased, especially in naturally infected corals and those exposed to immune stimulators and bacteria within laboratory studies, we currently lack a sufficient understanding of how immune defenses and other cellular mechanisms vary among species. There is an urgent need to understand the difference between inducible immune responses to an active infection and the constitutive, species–specific resistance mechanisms that prevent some species from developing disease lesions. As in the example of white plague, *Montastraea cavernosa*, *Porites porites*, and *Porites astreoides* are typically more disease-resistant, as demonstrated in the field and our previous study which showed that these same species had significantly reduced relative risk of white plague disease when exposed to diseased corals (MacKnight et al. 2021). These species' resistance, however, may differ after exposure to other marine diseases, such as SCTLD, indicating different diseases stimulate different host responses, including the host immune system (Meiling et al. 2021).

Previous studies on coral disease and immunity have successfully identified genes induced by disease that contribute to biological processes such as apoptosis, autophagy, extracellular matrix maintenance, lipid metabolism, and protein trafficking (Avila-Magaña et al. 2021; Fuess et al. 2017a; Levy et al. 2021; Traylor-Knowles et al. 2021; Wright et al. 2015). However, comparing immune responses between coral species that differ in disease resistance or susceptibility, linking specific disease phenotypes to gene expression, and determining adaptive or plastic disease-resistance-associated expression patterns is understudied. By leveraging the outcome of the experimental exposure of seven coral species to white plague disease, we can

identify lineage-specific expression adaptation and highly plastic genes that are linked to tangible disease phenotypes associated with coral species that are disease resistant or susceptible.

METHODS

Experimental Design and sample preparation

The phenotypic response from the disease exposure experiment was originally reported by MacKnight et al. (2021) (MacKnight et al. 2021). Briefly, five apparently healthy parental colonies from each of seven Caribbean coral species, *Orbicella faveolata*, *Colpophyllia natans*, *Siderastrea siderea*, *Porites astreoides*, *Porites porites*, and *Montastraea cavernosa*, were collected from Brewers Bay (18.34403˚, -64.98435˚), St Thomas, U.S. Virgin Islands on 13 June 2017. Diseased *O. franksi* was targeted as the source for disease in this transmission experiment because this species is known to be consistently affected by white plague disease throughout the year and was also used as the source species for previous experiments (Williams et al. 2020). Both *O. franksi* colonies showing signs of tissue loss consistent with white plague disease (n=3) and apparently healthy colonies (n=5) were collected by separate divers and kept in isolation until the commencement of the experiment. All colonies were held in running seawater tables at the University of the Virgin Islands where they were fragmented using a sterilized table saw, acclimated for nine days, and then placed in experimental conditions. Although five healthy parental colonies of *Orbicella annularis* was collected, one experienced mortality soon after collection and was not used in experiments. Diseased *O. franksi* corals were fragmented and monitored for lesion enlargement for 24 hours in isolation. Only fragments showing active lesion progression were used in disease treatments.

Upon commencement of the experiment (22 June 2017), coral fragments were distributed among five disease treatment and five control sterilized containers (17-L), each equipped with individual aerators. Containers were filled with filtered seawater and placed among three outdoor shaded running seawater tables that served as water baths. Containers received water changes daily and their locations were also randomized each day over the course of the 7-day experimental period. Each treatment container consisted of a randomly assigned healthy fragment of each of the seven tested species that were placed equal distances (approximately 7-8 cm) from a central diseased *O. franksi* fragment. Control containers were identically arranged, except that apparently healthy *O. franksi* were used as the central corals (Figure 1A). When a lesion appeared on a disease-exposed coral that was previously healthy it was monitored until 30% of the tissue was lost. If the lesion enlarged over this time period, the coral and its paired control fragment were photographed, removed, flash frozen and stored at -80°C. Coral fragments were classified by their treatment outcome as either, "controls", "disease-exposed", or "disease-infected". Coral fragments exposed to apparently healthy *O. franksi* were classified as "controls". Coral fragments exposed to disease, but did not develop lesions by the end of the transmission experiment were classified as "disease-exposed" and considered disease resistant individuals. Coral fragments that were exposed to a diseased *O. franksi* and developed lesions that expanded through time were grouped as "disease-infected" and considered disease susceptible individuals. *O. faveolata* and *O. annularis* were classified as highly susceptible, *C. natans* and *S. siderea* were classified as intermediate susceptibility and *P. porites*, *P. astreoides* and *M. cavernosa* were classified as resistant based on lesion progression rates, and relative risk of disease incidence (Data S1) (MacKnight et al. 2021). The relative risk is a species-level

summary statistic that represents the disease prevalence for that species while the lesion progression rate is an individual-level statistic.

Tissue for total RNA extraction was collected from frozen coral fragments with a sterilized bone cutter and extracted with the RNaqueous kit (Invitrogen). To enhance RNA integrity and yield, b-mercaptoethanol (7ul) was added to the lysis stage and samples were lysed with a refrigerated Qiagen Tissuelyser II at 30 oscillations/sec for 30 seconds. Elution was performed as a 2-step elution (30ul, then 30ul) to improve RNA concentration. DNA was removed with DNAse I (Qiagen) following the manufacturer's instructions. RNA integrity was checked with an Agilent Bioanalyzer and shipped for library prep and sequencing if the RNA integrity was above 7 with greater than a 20 ng/ul concentration. RNA was Bioanalyzed again by Novogene prior to sequencing. Eukaryotic transcriptomic libraries were prepared through poly-A tail enrichment from total RNA at Novogene. Samples were sequenced on an Illumina Hiseq 4000 at 150 PE total RNA sequencing, averaging 16.4 million reads per sample (Table S1). While extraction optimization greatly improved RNA integrity and yield, not all fragments yielded sufficient RNA after multiple extraction attempts and were not sequenced as a result.

Transcriptome Assembly and Annotation

From the sequencer, raw reads were moved to the Mydlarz Lab's Texas-system high performance computing server. Trimmomatic v0.36 (Bolger, Lohse, and Usadel 2014) removed reads using these parameters which performed the following: remove adapters (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10), remove leading and trailing low quality bases (LEADING:3, TRAILING:3), scan the read with a 4-base wide sliding window, cutting when the average quality per base drops below 15 (SLIDINGWINDOW:4:15), remove reads below a 36

base pair minimum length (MINLEN:36). Trinity v2.5.1 assembled the metatranscriptomes on the Texas Advanced Computing Center (TACC)'s Lonestar 1TB RAM server and then moved back to the Mydlarz lab's server (Texas Advanced Computing Center (TACC)). To curate coral only transcriptomes, metatranscriptomes were made alignable through bowtie2 v2.3.4 and then mapped with tophat against reference transcriptomes for *O. faveolata*, *P. astreoides*, and *P. porites* were sourced from Fuess et al. 2017 (Fuess et al. 2017) (generated for internal Mydlarz lab use), while *S. siderea* and *C. natans* created de novo transcriptomes. *O. annularis* was mapped successfully to the Fuess et al. 2017 *O. faveolata* reference. The *M. cavernosa* transcriptome was created through a genome guided assembly from the Matz weebly 2018 *M. cavernosa* genome (Montastrea cavernosa Genome 2018). Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis was used to determine the completeness of each transcriptome (Simão et al. 2015). Assembled sequences were annotated using Uniprot reviewed annotations which assigned a universal gene entry i.d. to transcripts through BLASTX (Blast+ v2.2.27) (Bateman et al. 2017). All gene functions discussed were sourced from the Uniprot database (Bateman et al. 2017). Transcripts that were annotated with an evalue greater than e^{-5} were removed. Transcripts from each replicate was aligned to their respective species' transcriptome using tophat v2.1.1 (Kim et al. 2013) and implemented through the tuxedo suite. A final count matrix was curated through HTseq v0.9.1 (Anders, Pyl, and Huber 2015).

Normalization and Differential Gene Expression in Response to White Plague Disease

EDAseq was implemented to normalize gene counts by gene length using the R package EDAseq v2.24 (Risso et al. 2011). Gene length normalized expression count data from EDAseq was regularized log (rlog) normalized independently for each species and then significantly

differentially expressed genes were identified by comparing control and disease-treatments using DEseq2 v1.30 (Figure 1B) (Love, Huber, and Anders 2014). Similar differential expression responses were identified in response to disease treatment among three subset comparisons: "susceptible species" *O. annularis* and *O. faveolata*, "resistant species" *P. porites* and *M. cavernosa*, and "phylogenetically similar and distinct susceptibility" *O. faveolata* and *M. cavernosa*. To combine genes into a subset, first significant DEGs (p<0.05) shared between both species were identified. Raw expression of these annotated transcripts were concatenated into a subset and were EDAseq and rlog normalized together using Deseq2 v1.30. The log fold change of each transcript was calculated relative to the base mean expression which allowed for the identification of genes that responded similarly between species to disease treatment.

Co-expression Gene Networks Associated with Lesion Progression Rate

Through a signed WGCNA v1.69 (weighted correlation network analysis), genes that had similar expression patterns were grouped as co-expression networks to produce modules (Langfelder and Horvath 2008). These co-expression gene network modules were assembled with a power of 18 and a minimum module size of 100 genes. The summarized expression of these modules was then correlated to lesion progression rate as a continuous variable for each species independently. Six species (*O. faveolata, O. annularis, C. natans, S. siderea, P. porites, and P. astreoides*) had lesion progression rates but only five species (*O. faveolata, O. annularis, C. natans, S. siderea,* and *P. porites*) had modules significantly (p<0.1) correlated to lesion progression rate (n=16 coral fragments) (Figure 1C). Genes that were correlated to lesion progression rate for each species were pooled which included 8 modules consisting of 8,804 unique genes positively and 13 modules consisting of 8,438 unique genes negatively correlated

to lesion progression rate. The broader biological process Gene Ontology (GO) enrichments were constructed using the ClueGO v2.5.7 plugin in Cytoscape v3.8.1 using Uniprot entry identifiers. Genes that were uniquely positively or negatively correlated to lesion progression rates were also processed for their biological enrichment.

Multi-Species Comparison through an Expression Variance and Evolution Model

To create a comparable list of expressed transcripts for multispecies analysis, all transcripts with a matching Uniprot ID were identified which created a list of 446 shared genes (Figure 1D, Data S2). A species tree was generated with Species Tree from All Genes (STAG) as implemented within Orthofinder2 using predicted peptides from generated transcripts with Transdecoder where lengths of the species tree represent substitutions per site (Emms and Kelly 2018b, 2018a; Haas et al. 2013; Rohlfs, Gronvold, and Mendoza 2020). The list of 446 shared genes were rlog-normalized by species with DESeq2. This list of genes and generated species tree were input into the EVE model using the R package "evemodel" v0.0.0.9005 and "ape" v5.4.1 (Paradis and Schliep 2018; Rohlfs, Gronvold, and Mendoza 2020). EVE models a quantitative trait, such as the coral host's gene expression, to the coral species phylogenetic position in the tree. This formally determines if a gene expression pattern is being mediated by potentially evolved differences between species or mediated by white plague disease exposure. The EVE model can be used for purposes such as identifying genes with high expression divergence between species as candidates for lineage-specific expression level adaptation, and genes with high expression diversity within species as candidates for expression level plasticity (Avila-Magaña et al. 2021; Bernal et al. 2020). The beta shared test, (i.e., phylogenetic ANOVA), detected genes with increased or decreased ratios of expression divergence to

diversity, represented as the beta parameter. If there is stabilizing selection or no selection on the expression of a gene, then beta will remain constant. This works by using an Ornstein-Ulbeack process of optimization to identify an ancestrally optimal expression value for each gene where variance from this optimum is represented by beta. Significant deviations of beta from the optimal expression value are determined through the log likelihood ratio test statistic which follows a chi-squared distribution with one degree of freedom and multiple testing was corrected using a false discovery rate <0.05. If within species gene expression variation is greater than between species expression, there is diversity of expression (high beta), which represent candidates for expression plasticity. Gene candidates for lineage-specific expression level adaptation are identified when within species variation is minimal and between species expression is divergent (low beta). To determine which lineage-specific expression level adaptation genes are associated with disease resistance, a Pearson correlation test was used to determine which lineage-specific genes had correlated expression to the species relative risk. Highly plastic genes were grouped by treatment outcome and a Tukey post hoc test was applied to determine significant differences in gene expression between disease outcomes. Finally plastic and lineage-specific genes were identified in co-expression networks significantly correlated to lesion progression rates.

RESULTS

Transcriptome assembly

Sequencing yielded different numbers of average paired end reads per sample per species (Data S3). Raw sequencing reads are available for download on NCBI (SRA Accession PRJNA716052). Alignment of these reads to their respective species transcriptomes and filtering

resulted in contigs expressed only in corals. A de novo transcriptome was assembled for

Colpophyllia natans. Annotation of the final transcriptomes with UniProtKB/Swiss-Prot

database yielded unique annotations for 10,150 (approximately 7%) of *O. faveolata* transcripts,

22,126 (approximately 9%) of *O. annularis* transcripts, 34,828 (approximately 8.3%) of *C.

natans* transcripts, 17,021 (approximately 10.1%) of *S. siderea* transcripts, 20,553

(approximately 15%) of *P. porites* transcripts, 20,546 (approximately 15%) of *P. astreoides*

transcripts, 15,214 (approximately 7%) of *M. cavernosa* transcripts.

Differential Expression in Response to Disease Treatment

Thousands of annotated genes were significantly differentially expressed (padj<0.05) in

response to white plague exposure within each Caribbean coral species tested (Supplemental

Figure 2, Data S4). The number of differentially expressed genes (DEGs) between treatments

varied with the highest in *O. faveolata* at 865 DEGs, to *C. natans* with 0 DEGs. *O. annularis* had

181 DEGs, *S. siderea* had 53 DEGs, *P. porites* with 85 DEGs, *P. astreoides* with 47 DEGs, and

M. cavernosa had 787 DEGs. No DEGs were shared across all seven species, however shared

DEGs were identified between subsets of corals chosen due to similar or divergent phylogenic

and disease susceptibly comparisons (Figure 2). "Susceptible species" *O. faveolata* and *O.

annularis* shared 50 DEGs. "Phylogenetically similar and distinct susceptibility" species, *O.

faveolata* and *M. cavernosa*, had 27 shared DEGs. "Resistant species" *P. porites* and *M.

cavernosa* shared 13 DEGs (Figure 2, Data S5). Across all three subset comparisons, genes that

contributed to extracellular matrix maintenance and immunity were differentially expressed in

response to disease exposure. Susceptible species had similar patterns of differential expression

in response to disease in genes that contribute to extracellular matrix maintenance (galaxin

(GXN), collagen-alpha 2 chain (CO4A2) , mucin-like protein (MUCL), SH3 and PX domain-containing protein (SPD2A)), and immunity (Coiled-coil domain-containing protein 88B (CCDC88B), apoptosis-inducing factor 4 (AIFM4), 4-hydroxyphenylpyruvate dioxygenase (HPPD), cartilage intermediate layer proteins (CILP)), along with other biological processes. Phylogenetically similar and disease resistant species had similar differential expression of genes in response to disease exposure that contribute to extracellular matrix maintenance (matrix metalloproteinase-25 (MMP25, matrix metalloproteinase inhibitors (TIMP), galaxin (GXN)), and immunity (interferon-induced helicase C (IFIH1), cartilage intermediate layer protein 1 (CILP), cAMP regulatory subunit type 1-alpha (PRKAR1A), and universal stress protein (USP)). Resistant species had similar differential expression of genes in response to disease exposure that contribute to extracellular matrix maintenance (matrix metalloproteinase-24 (MMP24), and immunity (cAMP-responsive element modular (CREM)(Sassone-Corsi 2003), counting factor 50 (CF50), and tribbles 2 (TRIB2)) (Figure 2).

Coral Co-expression Gene Networks Associated with Lesion Progression Rate

WGCNA assigned rlog-normalized genes into modules of co-expression gene networks that were then correlated to lesion progression rate. From the six species (*O. faveolata, O. annularis, C. natans, S. siderea, P. porites, and P. astreoides*) that displayed lesion progression rates, five species (*O. faveolata, O. annularis, C. natans, S. siderea,* and *P. porites*) had co-expression gene networks significantly (p<0.1) correlated to lesion progression rate (n=16). Significant module correlation to lesion progression rate totaled 8 modules consisting of 8,804 unique genes positively correlated and 13 modules consisting of 8,438 unique genes negatively correlated to lesion progression rate (Figure 3A, Supplemental Figure 2. Species-specific

WGCNA Summary, Data S6). Module correlation to lesion progression rate was variable among species. *O. faveolata* had one module positively (p = 0.1, r = 0.8, 1468 genes) and two modules negatively (p = .02, r = -0.76, 1228 genes; p = 0.05, r = -0.68, 563 genes) correlated to lesion progression rate. *O. annularis* had two modules positively (p = 0.1, r = 0.59, 770 genes; p = 0.07, r = 0.67, 381 genes) and five modules negatively (p = 0.06, r = -0.69, 1033 genes; p = 0.08, r = -0.65, 479 genes; p=0.04, r = -0.72, 132 genes; p = .1, r = -0.58, 111 genes; p = 0.01, r = -0.81, 37 genes) correlated to lesion progression rate. *S. siderea* had two modules positively (p = 0.09, r = 0.6, 1215 genes; p = 0.1, r = 0.58, 157 genes) and three modules negatively (p = .1, r = -0.53, 580 genes; p = 0.1, r = -0.53, 357 genes; p = 0.01, r = -0.76, 266 genes) correlated to lesion progression rate. *C. natans* had one module positively (p = 1e^{-4}, r = 0.95, 208 genes) and no modules negatively correlated to lesion progression rate. *P. porites* had two modules positively (p = 0.02, r = 0.7, 2568 genes; p = 0.1, r = 0.51, 315 genes) and three modules negatively (p = 0.0006, r = -0.8, 1787 genes; p = 0.07, r = -0.59, 244 genes; p = .09, r = -0.56, 156 genes) correlated to lesion progression rate. One fragment of *P. astreoides* did display a lesion but the species did not have any co-expression gene network modules significantly correlated to lesion progression rate. *M. cavernosa* did not develop lesions. Therefore, *P. astreoides* and *M. cavernosa* are omitted from this co-expression network analysis to lesion progression rate. Enrichment of terms that were uniquely positively correlated to lesion progression rate included protein modification processes and cytoskeleton arrangement related processes (Figure 3B). Overall, biological process enrichment that was negatively correlated to lesion progression rate included processes associated with the regulation of the immune system (Figure 3B). Lymphocyte mediated immunity was significantly negatively correlated to lesion progression rate and within this process were the child processes of activation of NF-kappa B kinase and

regulation of B cell mediated immunity (Figure 3C). Four parental immune-related biological processes positively correlated to lesion progression rate had enrichment of child processes including activation of NF-kappa B kinase, regulation of tau-protein kinase, activation of protein kinase A, cysteine-type endopeptidase inhibitor activity involved in apoptotic processes (Figure3D).

Identifying Lineage-Specific and Highly Plastic Gene Expression Patterns

The clustering of samples that group by species (Figure 4A) shows that gene expression patterns are driven by coral species. To delineate phylogenetic influence compared with the influence of white plague exposure on gene expression patterns, the phylogeny of species (Figure S3) was considered in an expression variance and evolution (EVE) model. From the EVE model, 79 genes were significantly classified as either lineage-specific or highly plastic in their expression and considered "EVE genes" (Figure 4B, Data S7). Within the 79 EVE genes, 29 genes had significant lineage-specific expression relative to other species which represent gene candidates that may have contributed to species evolution (Figure 4C). The other 50 genes were identified as highly plastic in their expression and may have expression patterns related to their exposure to disease and the response to this exposure (Figure 4D). All 79 EVE genes are delineated by their lineage-specific expression level adaptation or plastic expression pattern, organized by their parental gene ontology where their log expression is visualized by color (Figure 5).

EVE gene expression is significantly correlated to treatment outcome and relative risk

While 50 genes were significantly highly diverse in their expression among all species, 10 of these genes were significantly different among treatment outcomes (i.e., control, disease-exposed, disease-infected) (Figure 6A). This indicates that the expression of these plastic genes is mediated by the treatment conditions more strongly than by species. These 10 genes were relevant in various aspects of immunity or metabolism. NF-kB suppression related interferon regulator (Figure 6A) and lipid metabolism through arachidonate 8-lipoxygenase (Figure 6A) increased in expression in disease-infected fragments. Genes that significantly increased in log expression in disease-exposed coral included inflammation related genes, tyrosine kinase receptor Tie-1, diphtheria toxin, and glycosyl hydrolase ecdE. The remaining genes had a pattern of expression which declined in expression from control to disease-infected outcomes and included, MAPK signaling (Ephrin-B2a), WNT signaling (Nephrocystin-3), lysosomal activity (Cathepsin L), and non-bacterial reactions from possible fungal presence (Echinocandin biosynthesis).

We further explored lineage-specific expression patterns to determine their association with the species relative risk of contracting white plague disease if the species is exposed. The expression patterns of two lineage-specific genes were significantly ($p < 0.1$) correlated to the relative risk of disease if exposed to white plague (Figure 6B). The lipid metabolism-related, serine incorporator gene was a constitutively expressed gene that had increased expression in species with a higher relative risk of disease incidence. Spectrin alpha chain expression which is involved in intracellular protein transport was associated with a lower relative risk of disease incidence.

Lineage-specific and highly plastic gene expression contribute to disease resilience

These 79 EVE genes were then identified in expression networks significantly correlated to lesion progression rate, a proxy for disease resilience (Figure 4S, Data S8). Thirty-seven (37) out of 50 plastic genes, and 23 out of 29 lineage specific genes were in one or more species' expression network that was correlated to lesion progression rate. These results indicate that both constitutive and highly plastic gene expression is associated with lesion progression rate in coral. We further explored this to see that highly plastic genes that are commonly correlated to disease resilience in most species have a functional role that contribute to autophagy, TLR-to-NF-kb signaling, immune suppression, and lipid metabolism (Data S8). Additionally, lineage specific genes that were constitutively expressed in gene networks correlated to lesion progression rate were commonly related to managing cytoskeleton integrity and protein translation (Figure S4, Data S8).

DISCUSSION

Marine diseases are increasing in scale and severity and have the capacity to reshape ecosystems (C. D. Harvell et al. 2019a; MacKnight et al. 2021; Meiling et al. 2021; E. M. Muller et al. 2020). By examining how disease affects coral species, we can understand the gene expression patterns that contribute to disease resistance or susceptibility and predict how disease will affect the survival, and subsequent ecological contributions of a population in a changing environment. By exposing seven coral species of diverse disease susceptibility to white plague disease, the present study links lineage-specific expression level adaptation and plasticity patterns to tangible disease phenotypes: lesion progression rate, relative risk of disease incidence, and treatment outcome. Through a combination of identifying genes with differential expression in response to disease exposure (DEGs), association with lesion progression (WGCNA), and

distinction between phylogenetically or white plague exposure mediated gene expression (EVE), we can begin to weigh the gene expression patterns that consistently lead to either survival or lesion development during disease exposure. Our study illustrates three consistent patterns. First, in corals that developed disease lesions, immunity and cytoskeletal arrangement processes were enriched and correlated to lesion progression rate. Second, whether or not a coral developed lesions was mediated by plasticity in genes involved in extracellular matrix maintenance, autophagy, and apoptosis. Third, resistant species had higher levels of intracellular protein trafficking, and these processes have a lineage-specific adaptive basis to disease resistance. Together, these patterns demonstrate that the plasticity of genes that are associated with disease resistance may be evolutionarily constrained by expression level adaptation processes.

Lesion Progression is Mediated by Immune Signaling, Cytoskeletal and Protein Modification

Genes involved in the coral innate immune system were highly correlated with lesion progression rate. Coral fragments across the five species that developed lesions and had measurable lesion progression rates, had higher enrichment of immunity-associated biological processes, driven by classical immune signaling proteins including B-cell lymphoma 3 protein (BCL3), tumor necrosis factor receptor associated factor 2 (TRAF2), NACHT, LRR and PYD domains-containing protein 3 (NLRP3), and toll-like receptor 6 (TLR6). These proteins form core components of the coral innate immune system which functions to detect pathogens and initiate immune responses (Mydlarz et al. 1995). The correlation of these immune proteins with lesion progression rate indicates that as the disease progresses through coral tissue there is activation of the immune system when the coral tissue is trying to fight infection.

Genes that were negatively correlated with lesion progression rate demonstrate a pattern of damage mitigation and slow the spread of the disease lesion. Slower lesion progression rates were mediated by genes that function in cytoskeletal organization and protein modification including spectrin alpha chain (SPTAN1), cytoplasmic dynein 1 heavy chain 1 (DYNC1H1), proteasome subunit alpha type-7 (PSMA7), B-cell receptor-associated protein 31 (BCAP), serine/threonine-protein kinase mTOR (MTOR), cathepsin B (CTSB). While not considered a classical component of innate immunity the regulation of cell structures including the cytoskeleton is an important process that promotes the cells ability to respond and slow the progression of disease by mediating vesicle-organelle transport, extracellular matrix interactions, and cell adhesion and motility (Daniels et al. 2015; Kelley et al. 2021; Young et al. 2020). These genes comprise the glandular and secretory type cells which we are now showing in this experimental work are critical at preventing lesions from killing the organism (Levy et al. 2021). Importantly, it also expands the scope of what is important and contributes to slowing the lesion progression.

Processes associated with lesion progression rate were overwhelmingly associated with signaling the immune system, rather than detection of downstream classical immune effectors such as antimicrobial peptides, reactive oxygen molecules, and antioxidant activities (Parisi et al. 2020). These immune effectors are often post-translationally regulated proteins that would not appear in transcriptomic data sets, or alternatively the sampling location or timing was not resolved enough to observe them (Christgen, Place, and Kanneganti 2020). Despite this, our study clearly shows that canonical immune signaling is involved when a disease lesion is spreading on a coral primarily in susceptible species. As these same pathways were not

significantly associated with lesion progression in species that had slower lesion progression rates and were more disease resistant, such as *S. siderea.*

Plasticity of Autophagy, Apoptosis and Extracellular Matrix Genes are Associated with Disease Outcome

Gene expression plasticity in cell fate processes including the recovery pathway of autophagy or the terminal pathway of apoptosis are relevant to disease outcomes at the individual-level. Namely, genes that contribute to autophagy are more highly expressed in corals fragments that were exposed to white plague but remained healthy, while the expression of genes that contribute to apoptosis is increased in fragments that developed lesions. Previous work has supported that this axis of cell fate is regulated differently in disease resistant versus disease susceptible corals (Fuess et al. 2017a; Mariño et al. 2014). Our current work shows this is significant within species that show variability based on disease outcome. Specifically, upregulation of lysosomal genes that promote autophagy was consistent within corals across all species that were exposed to white plague but did not develop disease lesions. CGMP-AMP synthase which activates autophagy was also upregulated in these disease resistant individuals. Further autophagic activity is presented by lysosomal vacuole assembly (Ras-like GTP-binding protein YPT1), protein unfolding (gamma-interferon-inducible lysosomal thiol reductase (GILT)) and protein degradation genes (cathepsin L (CTSL), LRP2-binding proteins, and glycosyl hydrolase ecdE). Interestingly, the expression of these genes were lower in the coral fragments among all species that developed disease lesions.

Conversely, genes associated with apoptosis including caspase recruitment domain 15 (NOD2), interferon development regulator (IRF1), allene oxide synthase-lipoxygenase (LOC), and the proteasome subunit alpha 4 (PSMA4) demonstrate higher expression in fragments that developed lesions than those which remained healthy. Interferons (IFIH1) may also play a role in cytoplasmic detection of viruses and signal downstream type I interferons and proinflammatory cytokines and act as an immune regulator. Allene oxide converts arachidonic acid into oxygenated eicosanoids that act as mediators in cell stress and inflammation and results from lipid metabolic shifts (Lõhelaid, Teder, and Samel 2014; Teder, Samel, and Lõhelaid 2019). These metabolic shifts to digest lipids have been observed during disease and bleaching while apparently healthy coral tend to reduce lipid digestion in exchange for lipid storage (Libro, Kaluziak, and Vollmer 2013; Quinn et al. 2016; Santoro et al. 2021; Williams et al. 2020; Wright et al. 2019). Excessive levels of immune activation and inflammation can lead to apoptosis which is further supported through the increased expression of caspase recruitment domains in disease-infected coral (Fuess et al. 2017b). Caspases are the effector proteins of apoptosis which are initiated through interactions with the caspase recruitment domain containing proteins (Mohamed et al. 2017; Shrestha et al. 2020). All of these genes that contribute to apoptosis represent patterns of highly plastic expression which indicate that immune-activation and inflammation could culminate in apoptosis for coral infected with white plague disease as seen in *Acropora* white syndrome (Ainsworth et al. 2007). Overall, we demonstrate that the genes involved in the autophagy-apoptosis axis (Fuess et al. 2017a; Mariño et al. 2014) show an inducible and plastic response that consistently defines resistance or lesion development across these seven diverse coral species. This advances our knowledge of cell fate decisions as a key modulator of how corals fight disease.

Disease resistance was also characterized by the induced expression of genes associated with extracellular matrix stability. Corals exposed to disease but did not develop lesions consistently downregulated degradation of the extracellular matrix through a metalloproteinase (ADAMTS). While those coral that developed lesions downregulated extracellular matrix stabilizing genes (Alpha L-Fucosidase (FUCA) and FAM92A). Degradation proteins of the extracellular matrix are frequently upregulated in disease-infected coral, such as astacin and gelatinase (Wright et al. 2015). Pathogens such as *Vibrio corralliilyticus* have been shown to significantly upregulate zinc metalloproteinases to better infect coral hosts within minutes of detecting stressed coral mucus (Gao et al. 2021). The coral mucus layer is a first-line, barrier defense held together by the extracellular matrix that is integral for preventing pathogen penetration and directing immune responses such as cytokine activity and wound healing (G. Y. Chen and Nuñez 2010; Midwood and Piccinini 2010). The coral mucus layer also serves the maintenance of beneficial coral-associated microbial communities (Ritchie 2006) and as a means to discriminate beneficial microorganisms from pathogens (Boilard et al. 2020). In our previous study, we demonstrate that white plague resistant species such as *M. cavernosa* and *Porites spp.* show a tolerance for microbial change (MacKnight et al. 2021) and now we show that these species also induced plastic expression of extracellular matrix stabilizing genes. This furthers our understanding of how host-microbiome associations can contribute to host resistance.

Extracellular matrix stability through possible mechanisms of collagen alpha chain, protocadherin, and hemicentin have been associated with disease resistant individuals (Traylor-Knowles et al. 2021; Young et al. 2020). DMBT-1 is a putative mucosal immunity gene involved in coral microbial pattern recognition and signaling processes suspected to maintain mucosal

immunity and microbial homeostasis (Fuess et al. 2018; Huang et al. 2017; Wright et al. 2017). DMBT-1 was significantly upregulated in disease resistant *M. cavernosa*, but significantly downregulated in disease susceptible *O. annularis*, further demonstrating the relevance in extracellular matrix maintenance as a plastic expression associated with disease susceptibility across species. Processes like extracellular matrix stability are proving to be very important in not only the disease response but resistance to disease demonstrating the valuable contributions of other aspects of coral physiology that complement or bolster the classic immune response.

Protein Trafficking Delineates Disease Resistance among Species

Constitutive lineage-specific expression patterns were dominated by genes that contribute to intracellular protein trafficking, suggesting these genes are candidates for disease adaptation. Genes responsible for protein and vesicular transport had on average higher constitutive expression in resistant species such as *M. cavernosa, P. astreoides*, and *P. porites*, than species with intermediate *C. natans*, *S. siderea* or high-risk *O. faveolata*, *O. annularis* of contracting white plague. Protein trafficking is critical for mediating immune processes (Benado, Nasagi-Atiya, and Sagi-Eisenberg 2009) such as the transport of immune vesicles, antimicrobials or sequestration of damaged organelles (M. C. Chen et al. 2003; Geffen, Ron, and Rosenberg 2009) and expressed higher in resistant species in this study. Namely genes that contribute to cytoplasmic scaffolding (ISCU), cytoplasm to mitochondria transporters (Phosphate carrier protein (SLC25A3)), cytoskeletal movement (spectrin alpha chain (SPTAN1)), cytoskeletal motility (cytoplasmic dynein 1 heavy chain 1 (DYNC1H1)), exocytosis (Ras-related protein Rab-3), and protein folding stability (AN1 Zinc Finger (ZFAND1)) were more highly constitutively expressed in the resistant species. Protein trafficking has demonstrated significant

differential expression in response to several cellular dysfunctions such as coral disease and bleaching (DA et al. 2016; Daniels et al. 2015; Kenkel, Meyer, and Matz 2013). Recent single cell gene expression work in *Stylophora pistillata* show that coral immune cells have upregulated expression of vesicular trafficking, protein stability and lysomal genes supporting the notion that these processes go hand-in-hand (Levy et al. 2021). Our study shows that protein turnover and trafficking are expressed in a lineage-specific pattern that prevents corals from getting white plague disease.

While our data show consistent patterns that more trafficking is associated with survival, spectrin alpha chain in particular is significantly correlated to the coral host's relative risk of getting disease (70,71). Spectrin alpha chains interact with calmodulin and aids in calcium-dependent movement of the cytoskeleton to the membrane and is involved in secretion(Simonovic et al. 2006). This increased vesicular transport and protein trafficking in disease resistant species such as *Porites spp.* and *M. cavernosa* may indicate better preparation to respond and fight off potential infections before lesion development occurs through inflammatory or apoptotic events. Therefore, preventing any tissue destruction through inflammatory or apoptotic events. The lower expression of genes that contribute to protein trafficking in susceptible species suggests there is an adaptive constraint which limits the susceptible species ability to mitigate a changing environment, while demonstrating a process that allows resistant corals to tolerate change. These lineage-specific expression level disease adaptation candidates also relate to the apoptosis-autophagy axis as autophagy requires the sequestration and transport of damaged cellular components to lysosomes for turnover. A key regulator of intracellular transport that initiates autophagy is ras-like GTP binding YPT1 (Lipatova et al. 2012) which is facilitating homeostatic expression of autophagy that is

interrupted in a disease-infected coral (Kraft, Reggiori, and Peter 2009). This initiator of autophagy is also a master regulator of intracellular protein transport which is more highly constitutively expressed in resistant coral that demonstrates why autophagy, rather than apoptosis, is successfully employed in resistant coral. These resistant species have higher lineage-specific adaptive expression of the protein transport mechanisms that support this autophagic protein recycling pathway.

CONCLUDING REMARKS

This study provides a novel framework to identify coral-wide disease resistance traits. By leveraging a disease transmission experiment with seven coral species, we weigh the variable immune strategies that consistently lead to either a susceptible or resistant disease exposure outcome that is both considerate and independent of phylogeny. The integration of disease phenotypes (disease outcome, lesion progression rate, relative risk), into our analyses also identified processes directly involved in lesion. Considering these phenotypes, phylogeny, and the gene expression broadens our understanding on what processes are relevant at mediating the holobiont's innate immune system across coral species (MacKnight et al. 2021). Faster lesion progression is widely dominated by immune signaling while lesion arrest is promoted by the coral's modification of cytoskeletal arrangement and ability to traffic vesicles and organelles. Maintaining coral health when exposed to disease is also associated with intracellular protein trafficking mechanisms to fulfill pro-survival autophagic processes over apoptotic ones. These analyses offer insight into the evolutionary constraints of species to mitigate disease and present predictive gene-level markers and broader biological processes consistent across coral species that will shape coral reef populations in this changing environment.

Acknowledgements

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Author Contributions

Conceptualization: EMM, LDM, MB

Methodology: NJM, BAD, LDM

Experimental Disease Exposure: NJM, BAD, EMM, MB, LDM

Extraction and Bioinformatics: NJM, BAD

Statistical Analysis: NJM

Visualization: NJM

Supervision: LDM

Writing—original draft: NJM, BAD, LDM

Writing—review & editing: NJM, BAD, KB, EMM, MB, LDM

Competing Interest

The authors declare no competing interests.

Data and Materials Availability

Source data used to create all figures including RNA sequences are stored at NCBI though

BioProject accession PRJN and additionally made publicly available through the BCO-DMO

project page https://www.bco-dmo.org/project/727496. Analysis for the publication was

conducted in R version 3.6.2 (2019-12-12). The R scripts for the analysis are made publicly

available through Github:

Figures
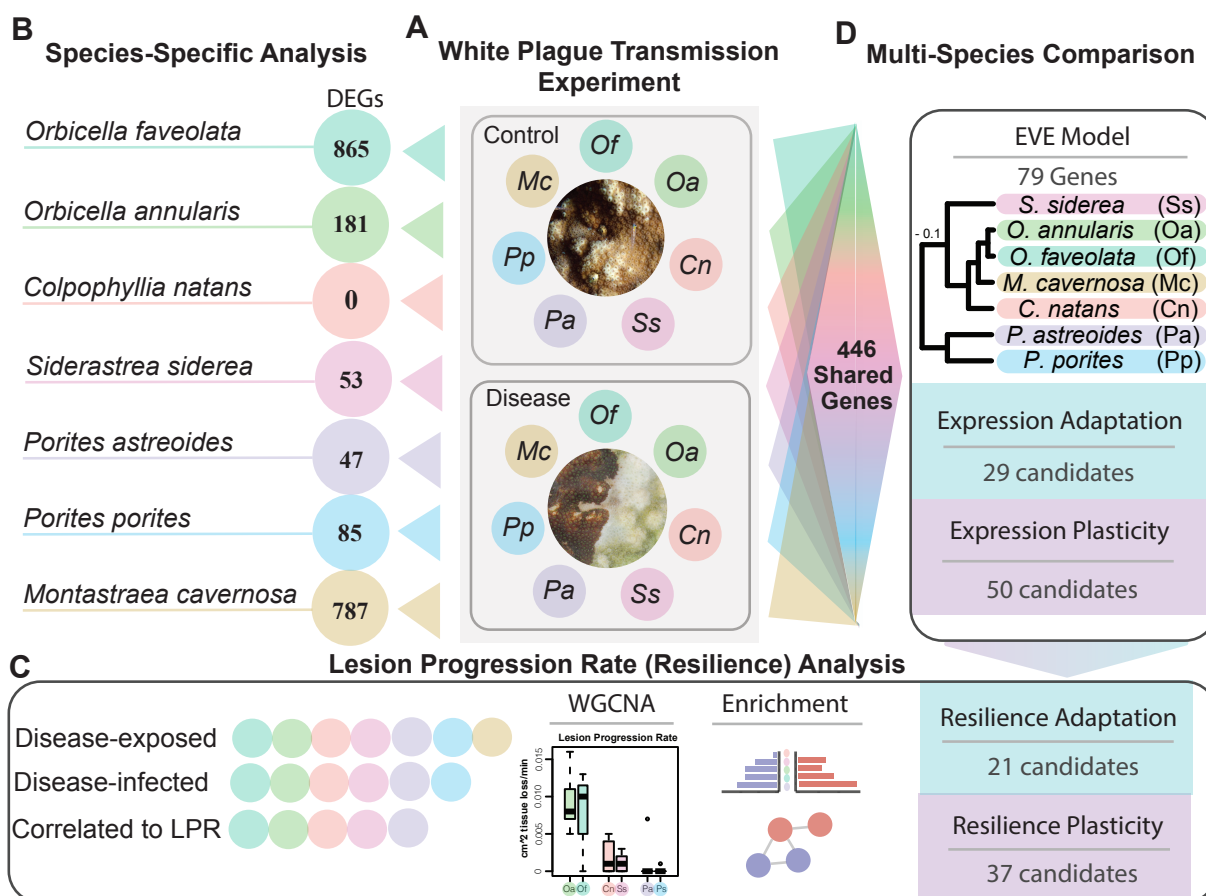
Figure 1. Experimental Design and Statistical Analysis Overview. (**A**) White plague disease transmission involved apparently healthy (control) and white plague-infected *O. franksi* (disease) exposure to seven coral species. Each treatment had five replicates and coral species were genotypically paired between treatments. (**B**) RNAseq was performed on all coral fragments (Table S1) and significantly differentially expressed genes (DEGs) were identified between the control and disease treatments within each species (Figure S1). Shared DEGs between relevant species comparisons was then identified (Figure 2). (**C**) From the seven species exposed to disease (disease-exposed), six species developed lesions, while five of those six species had gene co-expression networks correlated to lesion progression rate (LPR). Gene modules correlated to LPR had enrichment of biological process determined within each species (Figure S2), and also

among all species (Figure 3). (**D**) Pooling all annotated genes among the seven species identified 446 genes shared across all coral species. The expression of these genes and the phylogenetic divergence (Figure S3) of the coral species was integrated into the Expression Variance and Evolution (EVE) model (Figure 4). This delineated when a gene had an expression pattern that was lineage-specific and a candidate for expression-level adaptation, or when a gene's expression was highly plastic, and not mediated by phylogenetic differences, but likely by the disease exposure. Distinct lineage-specific and highly plastic candidates were then correlated to either relative risk, treatment outcome, or presence in modules correlated to LPR to determine their relevance with disease resistance or susceptibility (Figure 5, Figure S4).
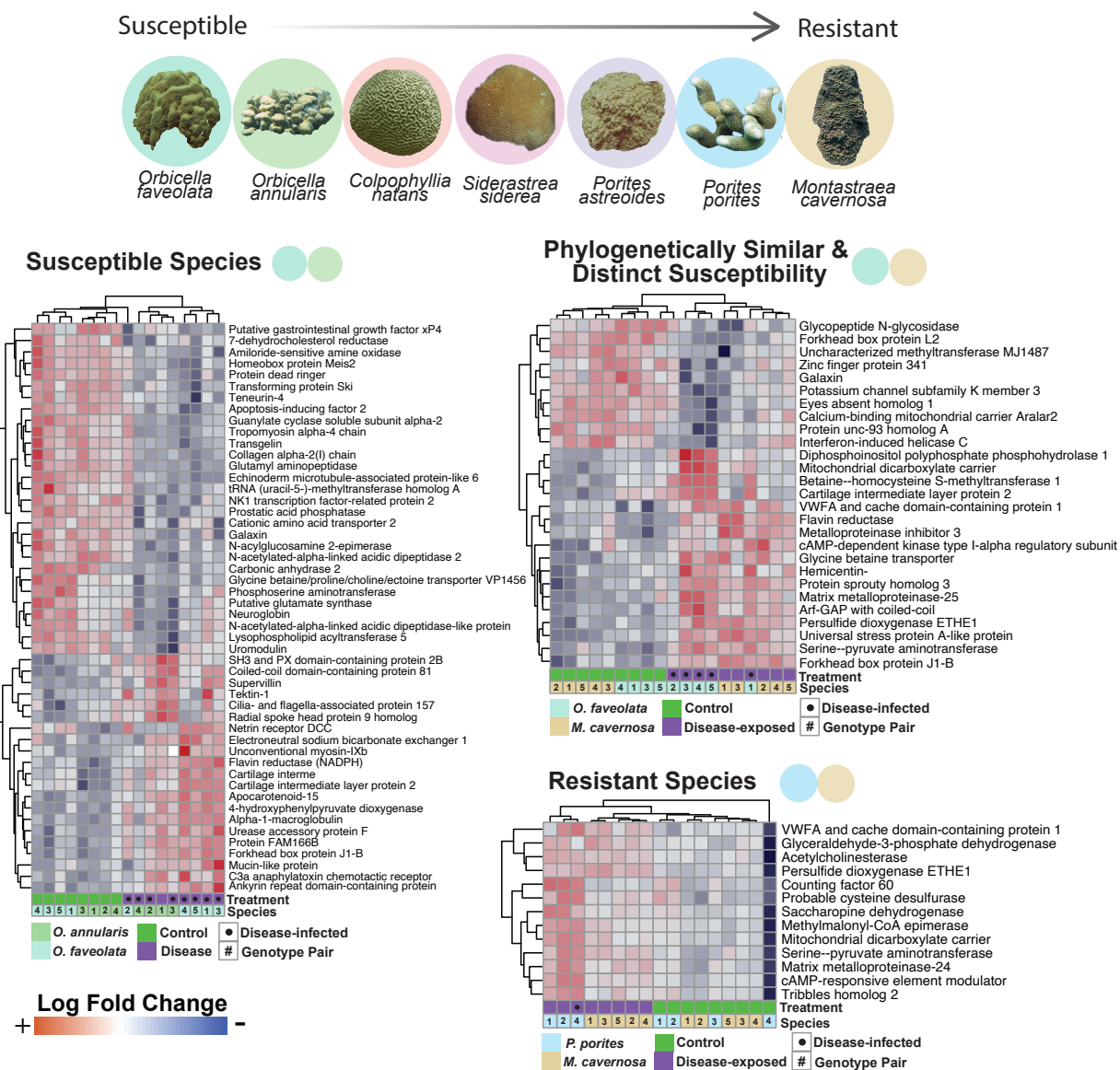
Figure 2. Relevant Comparisons of Significantly Differentially Expressed Genes. Heatmaps represent the log fold change expression of shared DEGs to demonstrate the genes that are being similarly expressed in response to treatment among relevant species comparisons.

A Genes Negatively Correlated to Lesion Progression Rate

Genes Positively Correlated to Lesion Progression Rate

C. natans
S. siderea
O. annularis
O. faveolata
P. porites

Negative Modules Enrichment

Positive Modules Enrichment

B

protein modification process
cellular protein modification
ion transport
protein phosphopantetheinylation
protein catalytic activity
cellular component organization
cytoskeleton organization
protein conjugation or removal
cellular component biogenesis
small protein conjugation
protein ubiquitination
cellular protein catabolic process
regulation of cell cycle process
cytoskeleton organization
nucleoside-triphosphatase activity
GTPase regulator activity
protein complex localization
receptor regulator activity
receptor activator activity
receptor ligand activity
axoneme assembly
lymphocyte mediated immunity

phosphate-containing compound metabolic process
regulation of transcription by RNA polymerase II
response to other organism
positive regulation of immune system process
regulation of protein catabolic process
regulation of immune response
nuclease activity
cilium movement
positive regulation of immune response
mitotic spindle organization
activation of immune response
cilium-dependent cell motility
cilium or flagellum-dependent cell motility
extracellular fluid cilium movement

P-value
0.01
0.02
0.03
0.04

Number of Genes

C Negative Enrichment of Immune Processes

Activation of NF-kappa B kinase

MALT1   TRAF2

regulation of acute inflammatory response to antigenic stimulus

hypersensitivity

C3

postive regulatino of myeloid leukocyte mediate immunity

Regulation of B cell mediated immunity

BTK

positive regulation of B cell mediated immunity

RIF1

TP53BP1

regulation of immunoglobulin mediated immune response

SUPT6H

negative regulation of double-stranded break repair via homologous recombination

D Positve Enrichment of Immune Processes

regulation of cAMP-dependent protein kinase activity

ADRB2   PRKAR1A

cAMP-dependent protein kinase activity

cAMP-dependent protein kinase inhibitor activity

PRKAR1B

ADCY5

cyclic nucleotide-dependent protein kinase activity

Activtation of protein kinase A

NLRP3   SERPINB9

SOS1   RB1

TRAF2   TRAF6   MALT1

TLR6

Cysteine-type endopeptidase inhibitor activity involved in apoptotic process

Regulation of tau-protein kinase activity

Activation of NF-kappa B kinase

Figure 3. Lesion Progression Rate Among Coral Species. (**A**) WGCNA identified gene co-expression modules correlated to lesion progression rate for five of the seven species exposed to white plague disease. The genes in WGCNA modules that were positively correlated to lesion progression rate were pooled among species and then the genes in WGCNA modules that were negatively correlated to lesion progression rate were pooled among species. (**B**) Enrichment of biological processes was separately determined for the pooled genes that were positively and negatively correlated to lesion progression rate. (**C**) Child terms of the parental Immune-related biological processes was identified and the genes that contributed to that enrichment was identified. Larger circles represent the enriched biological process and smaller circles represent the genes that contribute to that enrichment.
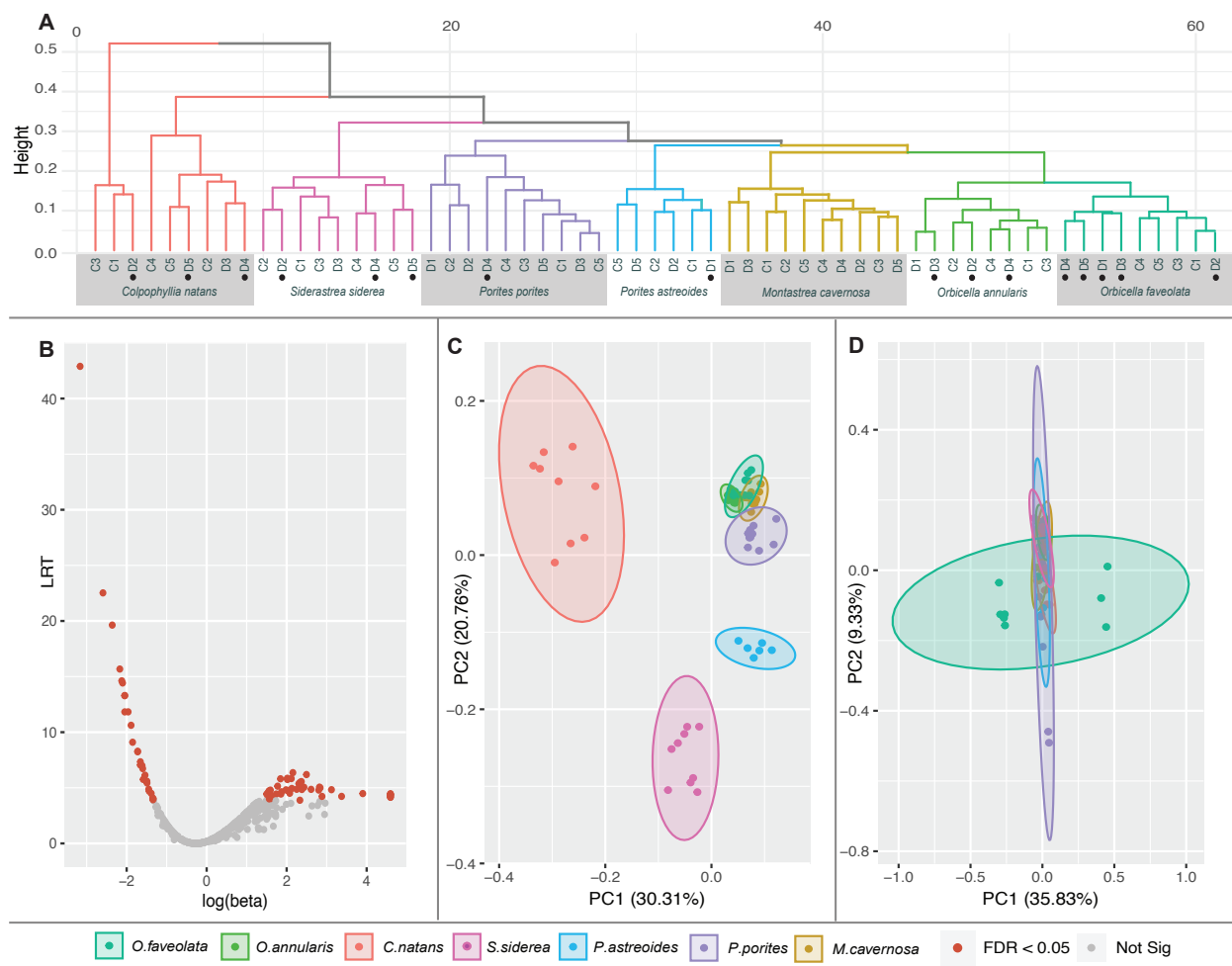
Figure 4. Expression Variance and Evolution (EVE) Model. (**A**) From the gene expression of 446 shared genes, this hierarchical tree demonstrates that samples (i) separate by host species, (ii) organized by phylogenetic divergence among host species, (iii) tend to cluster by genotypic pair, regardless of treatment, and then (iv) cluster by treatment outcome if the response was influential enough (i.e. *Orbicella faveolata*). Because response to disease is seemingly the fourth in hierarchical influence on multi-species gene expression comparisons, this warrants the need for an EVE model to isolate phylogenetic (i-iii) influential factors from the response to disease we seek to explore (iv). (**B**) Gene expression of 446 shared genes and the phylogenetic divergence of the coral host species was integrated into the EVE model. (**C**) This identified 29

genes with expression patterns that were significantly lineage-specific expression-level adaptation, and (**D**) 50 genes that were significantly highly plastic in their expression.
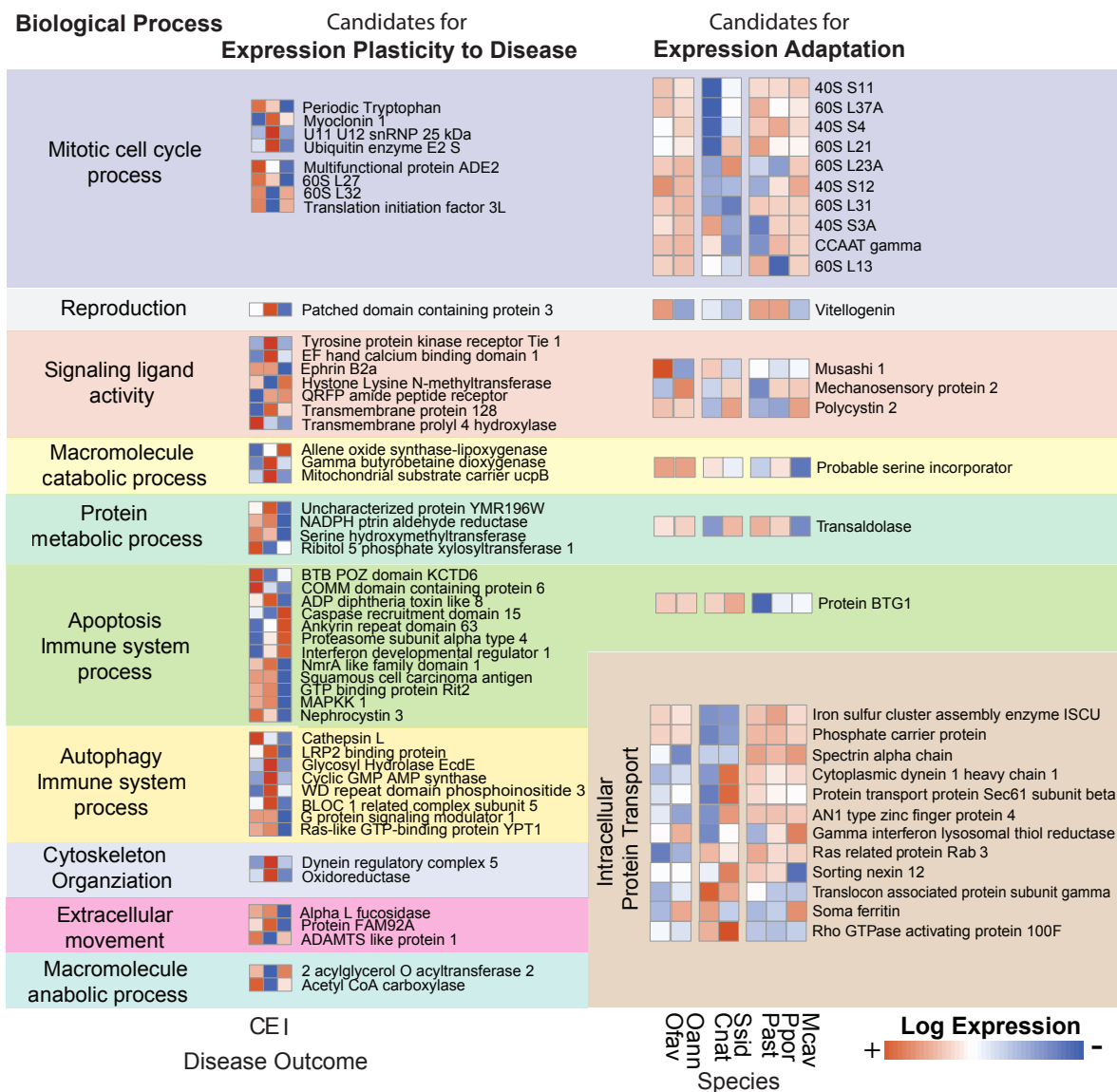
Figure 5. Biological Process Enrichment of EVE Genes. EVE genes were organized by the parental biological process that they contribute to as determined by Universal Uniprot gene ontology ID's. Log expression of each gene was color coordinated to indicate that red was higher expression, while blue was lower expression of a particular gene. Disease outcomes are represented by the control "C", disease-exposed "E", and disease-infected "I" response to disease.
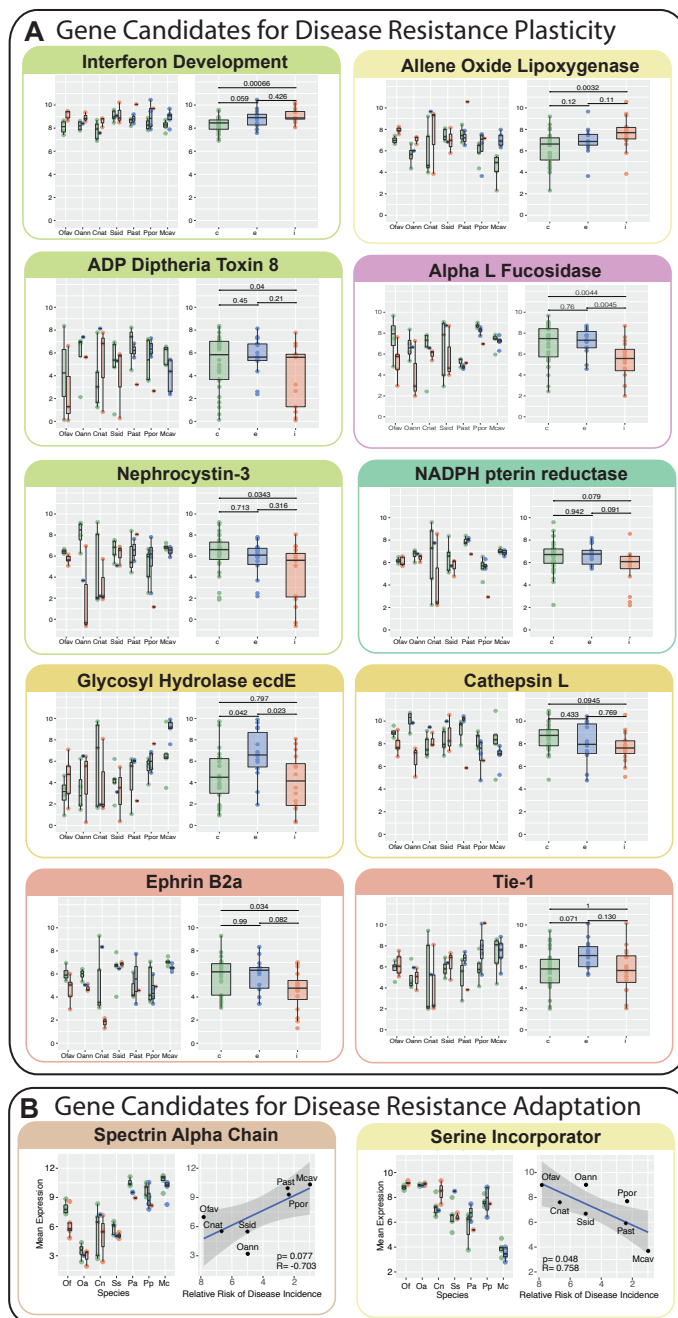
Figure 6. Disease Resistance Plasticity and Adaptation Candidates. Gene sub header colors correspond to Figure 5 biological processes. Within each gene panel, there is the log expression of the gene from each sample, and then the expression of that gene is pooled by treatment outcomes (A) or by species (B). (**A**) From the 50 highly plastic genes, 10 were significantly

different among treatment outcomes. (**B**) From the 29 lineage specific genes, 2 significantly

correlated to relative risk of disease incidence.

SUPPLEMENTAL MATERIAL

Orbicella faveolata — 1123 DEGs

Highest LFC Genes

Transcriptome Wide Gene Ontology

Biological Process
- 5/26 regulation of cell cycle
- 20/73 signal transduction
- **24/107 cell cycle process**
- *4/24 G2/M transition of mitotic cell cycle*
- 9/51 mitotic cell cycle phase transition
- 39/293 cellular nitrogen compound metabolic process
- **5/33 DNA recombination**
- **16/61 DNA metabolic process**
- **10/43 cellular response to stimulus**
- *8/34 peptide metabolic process*
- *3/47 ncRNA processing*
- *13/89 positive regulation of biological process*

Molecular Function
- 2/19 phosphotransferase, phosphate group as acceptor
- **105/822 organic cyclic compound binding**
- **6/20 peptide receptor**
- **47/291 DNA binding**
- 7/26 hydrolase, acting on carbon–nitrogen bonds
- 60/201 peptidase
- 1/21 ubiquitin–like protein–specific protease
- **63/174 molecular transducer**
- 4/41 hydrolase, acting on glycosyl bonds
- *19/43 enzyme inhibitor*
- 29/61 metallopeptidase
- 20/70 transition metal ion binding
- **9/37 iron ion binding**
- *7/41 voltage–gated channel*
- 16/68 gated channel
- **14/65 structural molecule**

Cellular Compartment
- **16/139 chromosomal part**
- 7/53 chromatin
- 3/23 chromosomal region
- 26/257 nuclear part
- 12/118 intracellular non–membrane–bounded organelle
- 4/37 kinetochore
- 5/32 neuron part
- 3/35 peptidase complex
- *16/172 catalytic complex*
- 6/68 whole membrane

Differentially Expressed Genes

Orbicella annularis — 213 DEGs

Highest LFC Genes

Transcriptome Wide Gene Ontology

Biological Process
- 7/33 ion transport
- 15/96 localization
- 4/50 organic substance transport
- 2/25 regulation of cell cycle
- 4/23 G2/M transition of mitotic cell cycle
- 4/49 mitotic cell cycle phase transition
- 11/68 signal transduction
- 6/61 regulation of biological quality
- 2/28 chromosome organization
- 8/272 cellular nitrogen metabolic process
- **1/36 DNA repair**
- **2/56 DNA metabolic process**
- 2/40 cellular response to stimulus

Molecular Function
- 7/94 exopeptidase
- 25/268 DNA binding
- 2/36 structural constituent of ribosome
- 51/747 organic cyclic compound binding
- 10/57 structural molecule
- 19/64 transition metal ion binding
- 12/32 iron ion binding
- 17/55 metallopeptidase
- 2/58 hydrolase, acting on glycosyl bonds
- 6/39 voltage–gated cation channel
- 7/62 ion gated channel

Cellular Compartment
- 0/35 nuclear chromosome part
- 7/238 nuclear part
- 3/23 chromosomal region
- 45/804 cytoplasmic part
- 1/31 neuron part
- 2/125 chromosomal part
- 0/50 chromatin
- 3/34 peptidase complex
- 11/161 catalytic complex

Differentially Expressed Genes

Siderastrea siderea — 104 DEGs

Highest LFC Genes

Transcriptome Wide Gene Ontology

Molecular Function
- 24/221 transporter
- 4/33 transferase, transferring alkyl or aryl groups
- 10/91 channel
- 22/188 molecular transducer
- 12/109 frizzled binding

p < 0.05
p < 0.1
p < 0.2

Differentially Expressed Genes

Porites astreoides — 46 DEGs

Highest LFC Genes

Transcriptome Wide Gene Ontology

MF
- 49/152 molecular transducer

CC
- 16/91 endoplasmic reticulum part
- 30/141 plasma membrane

Differentially Expressed Genes

Porites porites — 97 DEGs

Highest LFC Genes

Differentially Expressed Genes

Montastraea cavernosa — 787 DEGs

Highest LFC Genes

Transcriptome Wide Gene Ontology

Biological Process
- 5/46 movement of cell or subcellular component
- 5/52 microtubule–based process
- 8/68 establishment of organelle localization
- 14/139 response to stimulus
- 1/46 DNA repair
- 3/84 DNA metabolic process

Molecular Function
- 23/66 metallopeptidase
- 41/322 DNA binding
- 113/410 molecular transducer
- 16/79 G–protein coupled amine receptor

Differentially Expressed Genes

Colpophyllia natans — 0 DEGs

Highest LFC Genes
- 60S ribosomal protein L36
- ADP
- ATP synthase subunit a
- 60S ribosomal protein L37
- 40S ribosomal protein S2

Differentially Expressed Genes

Legend

Highest LFC Genes
2 1 0 −1 −2
Log Fold Change

Transcriptome Wide Gene Ontology
- Increased Enrichment — p < 0.001
- Decreased Enrichment — p < 0.01
- p < 0.1

Differentially Expressed Genes
- padj < 0.05
- log fold change > 1
- padj < 0.05 and log fold change > 1

Supplemental Figure 1. Species-Specific Transcriptome DEG and GOMWU Results.

Among the tens of thousands of transcripts expressed in each species, the significantly differentially expressed genes are highlighted in the volcano plot. Blue dots indicate genes with a log fold change greater than 1, while pink dots indicate genes with significant ($p<0.05$) change in gene expression between treatments, and purple dots indicate genes with both a log fold change greater than 1 and significant differential gene expression between treatments. Log fold expression of the most differentially expressed genes for each species is presented as a heatmap with red representing upregulated genes and blue representing downregulated genes. Each species' gene ontology for their transcriptome is presented as enriched biological processes, molecular function, and cellular components.
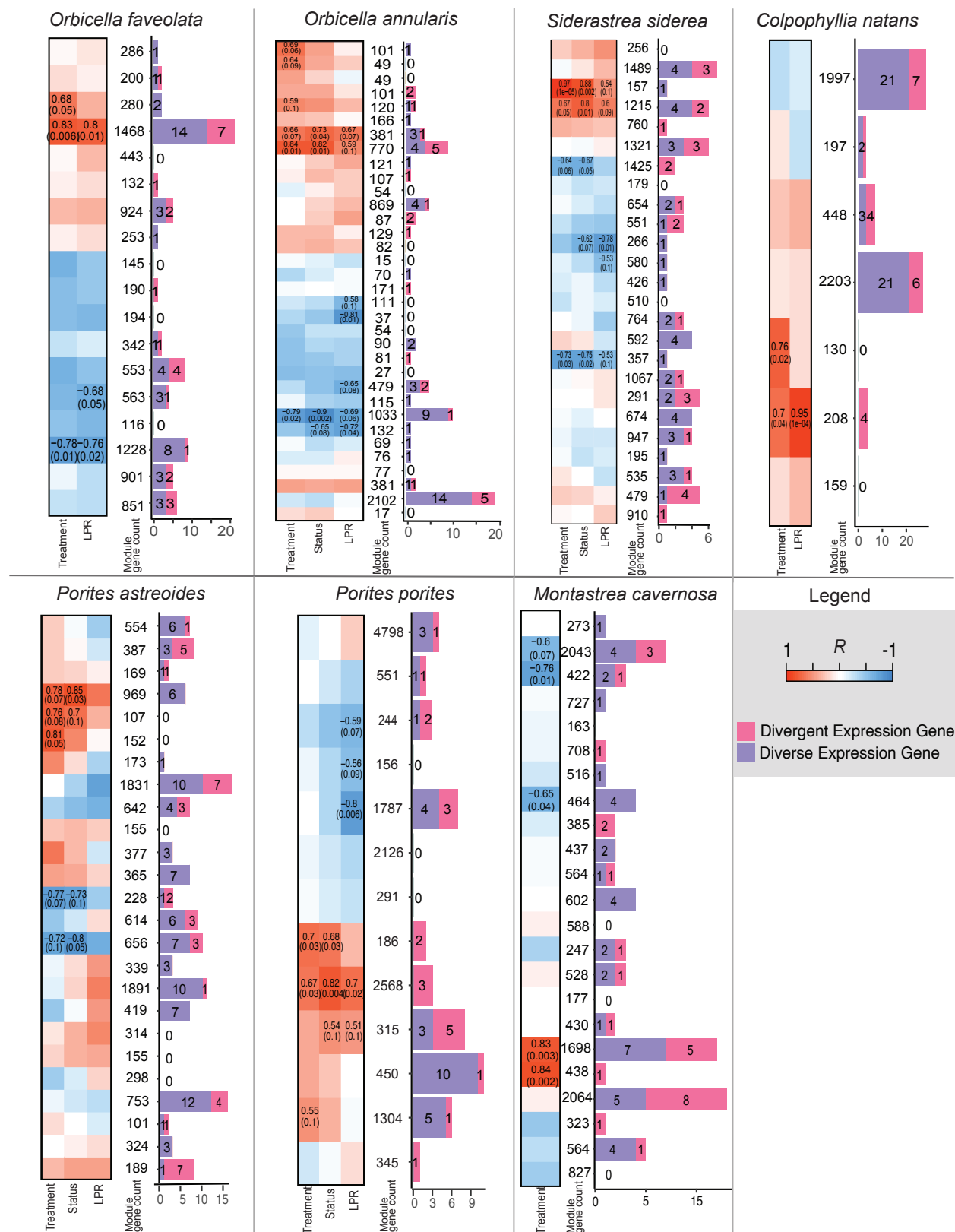
Supplemental Figure 2. Species-specific WGCNA Summary. Co-expression gene modules were correlated to disease phenotypes: Treatment (control or disease-exposed), Status (control, disease-exposed, or disease-infected), LPR (lesion progression rate). Red to blue colored cells represent positive to negative correlation, respectively. Numbers within colored cells represent correlation value (on top) and p-value (on bottom). The number of genes in each module is identified to the right of the heatmap, in between the bar plot. The bar plot represents the number of genes that are in each module that also displayed a divergent (pink) or diverse (purple) gene expression pattern from EVE.
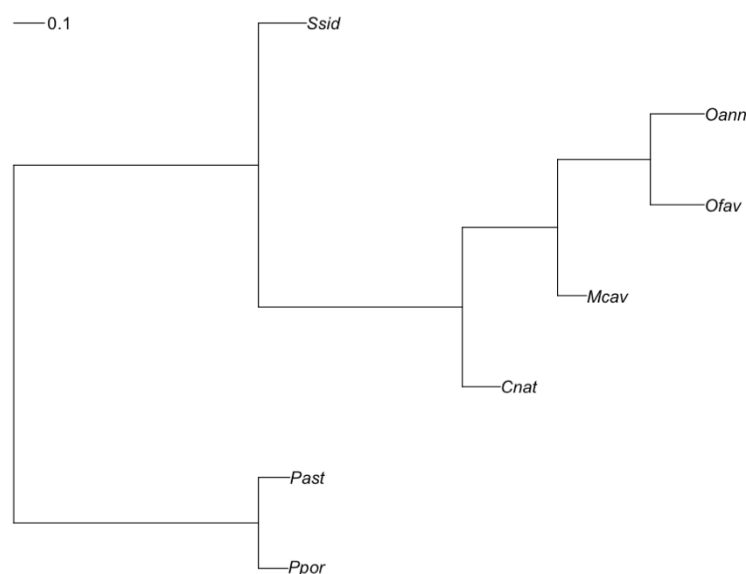
Supplemental Figure 3. Coral Species Phylogenetic Tree. Generated from Species Tree from all Genes (STAG) where lengths of the species tree represent substitutions per site.
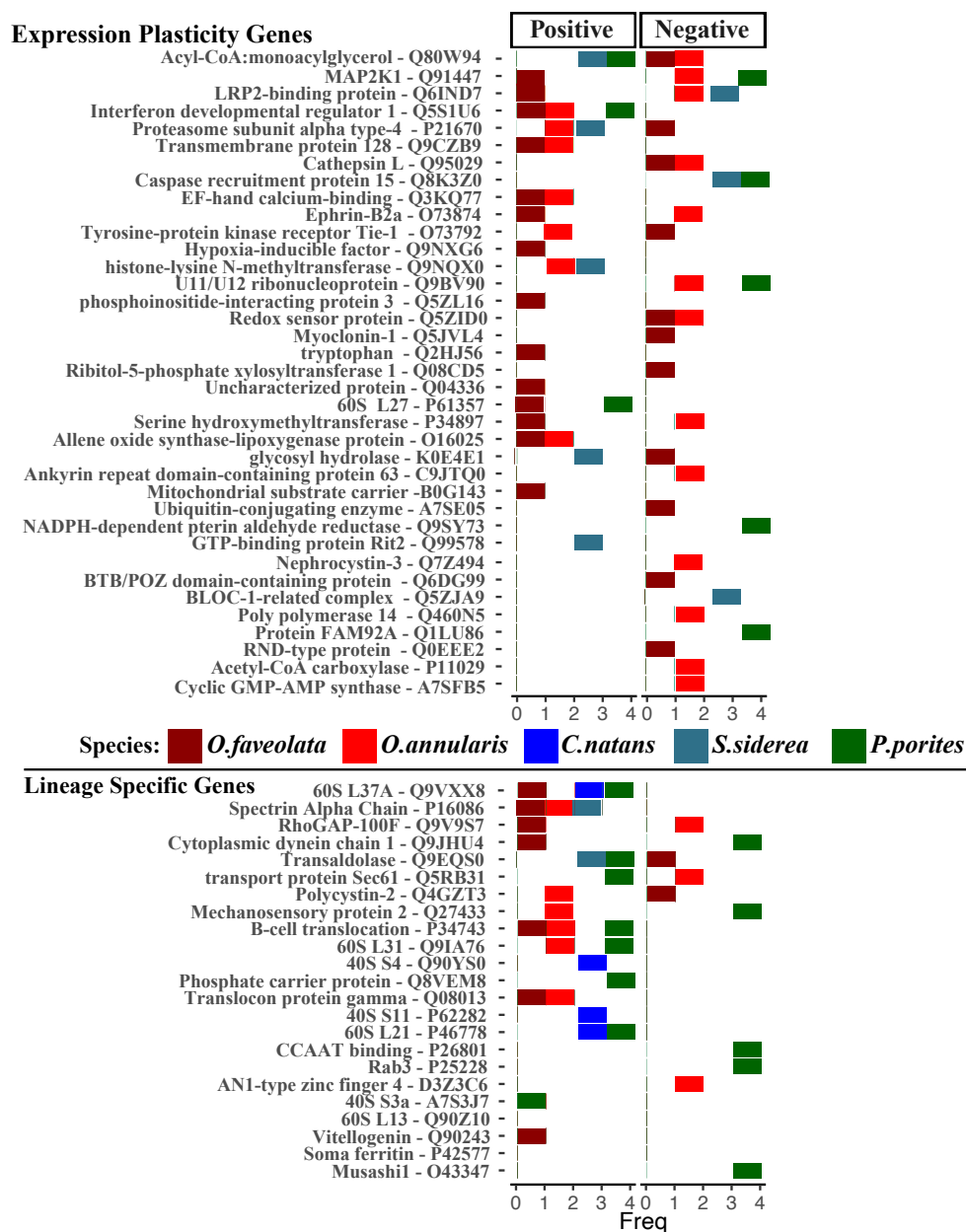
Supplemental Figure 4. Plastic and lineage-specific genes correlated to lesion progression rate. Gene names of the EVE genes that were identified to be in WGCNA modules significantly correlated to lesion progression rate (**Figure S2**). Genes are ranked by frequency, or total number of times that gene was in a species module correlated to lesion progression rate. Cells are color coded to visually separate species. Species color is based on their spectrum of disease resistance with red representing susceptible species (*O. faveolata* and *O. annularis*), blue representing

intermediately susceptible species (*C. natans* and *S. siderea*), and green representing the only relatively resistant species that developed disease lesions and had gene co-expression networks correlated to lesion progression rate (*P. porites*).

Chapter 4

Phylosymbiotic probiotics: Identifying bacteria that promote microbiome stability in coral

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In prep

Abstract

In recent decades, coral reefs around the world have experienced environmental stressors driven by climate change which have increased the incidence of infectious disease outbreaks. Coral diseases break down the host-bacteria relationship which reduces the coral's health and ecological contributions as a foundational ecosystem engineer. There is an emerging hypothesis among coral researchers that coral diseases are the result of a distinct destabilization of the coral microbiome. Identifying the bacteria associated with this microbial shift from "healthy" to "diseased" states is critical for preventing and diagnosing coral reef degradation. To identify stabilizing bacteria, we utilized a phylogenetic ANOVA, the expression variance and evolution (EVE) model, to identify bacteria that exhibit an evolved symbiotic relationship with the coral host, termed "microbial phylosymbiosis". The development of this advantageous symbiotic relationship suggests there exists mechanisms to maintain the symbiosis which promote stability, and beneficial contributions between host and microbe that have withstood environmental change on an evolutionary timescale. We experimentally challenged this hypothesis by determining if the relative abundance of putatively stabilizing phylosymbionts are correlated to microbial dysbiosis, and the relative risk of disease incidence. This investigation provides diagnostic and predictive disease susceptibility biomarker candidates, microbial species for bacteria-host interaction, and bacteria candidates that mediate microbiome community-level stability.

INTRODUCTION

Microbial Contributions to Host Health

Stony corals are ecosystem engineers that build reef foundations and offer irreplaceable ecological and economic global services (Brander, Van Beukering, and Cesar 2007; Pascal et al. 2016). To perform these ecosystem services, corals rely upon beneficial symbiotic relationships with algae, bacteria, viruses, and archaea that live within the coral tissue (van Oppen and Blackall 2019). In recent decades, climate change has destabilized these symbiotic relationships and corals' ecological productivity (Brander, Van Beukering, and Cesar 2007; C. D. Harvell et al. 1999; Pascal et al. 2016). A rising threat to coral health is the increasing frequency and severity of infectious disease outbreaks, which break down host-bacteria symbiosis and subsequently alter corals' ability to provide ecological services (K. M. Miller et al. 2014). In extreme cases, outbreaks can result in the local functional extinction of coral species (C. D. Harvell et al. 2019b; Hewson et al. 2014) and therefore reduce biodiversity on a global scale (C. Drew Harvell et al. 2002; Keesing et al. 2010). From extensive, widespread coral disease research, there is an emergingly consistent inference that disease susceptible coral experience a microbiome community-level shift, or perhaps microbial dysbiosis (MacKnight et al. 2021; Meyer et al. 2016, 2019a; M. J. Sweet and Bulling 2017). Identifying the key bacteria associated with this microbial shift from healthy to susceptible states is critical for diagnosing and preventing coral reef degradation.

Phylosymbiosis: Symbiosis that recapitulates host evolution

Phylosymbiosis characterizes a symbiotic organism (e.g., bateria species), whose relative abundance aligns with the phylogenetic divergence of the host (e.g., coral species) (sensu Fig. 1A). For phylosymbiosis to develop, this symbiotic relationship must have withstood environmental change on an evolutionary timescale. This suggests that there are mechanisms to

maintain the symbiosis and beneficial functional contributions between host and microbe (Kohl 2020). Investigations of phylosymbiotic microbes have the potential to identify probiotic candidates which have beneficial interactions with the host, provide stability within the microbiome, and assist the host in maintaining homeostasis within an increasingly changing environment (Brooks et al. 2016; Van Opstal and Bordenstein 2019).

Corals as a Model for Phylosymbiosis

Corals represent an excellent model system to study microbial stability and the subsequent effects on host biology. Corals experience potentially divergent species-specific bacterial transmission from parent to offspring and horizontal transmission from the surrounding environment. This results in complex microbial communities which influence multiple aspects of host biology, including nutrient cycling, temperature tolerance, and disease resilience (Ceh et al., 2013; Dupont et al., 2020; Ravenscraft et al. 2019). In particular, the role of the microbiome in coral immunology has received considerable attention as coral diseases represent a critical threat to Caribbean reefs (Mera and Bourne 2018; Randall and Van Woesik 2015; Sutherland, Porter, and Torres 2004). However, microbial profiling from diseased corals typically fails to identify etiological agents consistently between investigations. The lack of singular etiological agents makes pathogens costly to identify through metagenomics. Moreover, broad microbial community shifts commonly observed in coral disease research has led to an increasingly popular hypothesis that many coral diseases are states of microbial imbalance, or dysbiosis, which is a stress-induced shift away from a functional, healthy community equilibrium (Egan and Gardiner 2016; MacKnight et al. 2021; Peterson et al. 2012; M. J. Sweet and Bulling 2017). The relevance of dysbiosis and need for community-level analyses has increased in coral

research, in part because community imbalance is the leading hypothesized cause for the stony

coral tissue loss disease (SCTLD) (Meyer et al. 2019). SCTLD emerged from Miami, Florida in

2014 and has devastatingly swept across the Caribbean affecting coral species previously

considered relatively resistant to disease (Muller et al. 2020).

Using the EVE Model to Identify Phylosymbiotic Bacteria

An increasingly popular hypothesis among coral microbial ecologists suggests that

microbial community-level imbalance is an etiological force for coral disease (Glasl, Herndl, and

Frade 2016; Meyer et al. 2019; Rosales et al. 2020; Sweet and Bulling 2017). To explore this

possibility, the present study identified phylosymbiotic bacteria across diverse coral species as a

quantifiable indicator for microbiome stability. Previous investigations have identified that the

bacteria, *Endozoicomonas sp.*, is in phylosymbiosis with the coral host, *Porites astreoides*

(O'Brien et al. 2020; Pollock et al. 2018a). The relevance of this phylosymbiotic bacterium with

disease resistance was demonstrated when the absence of this bacterium was associated only

with *P. astreoides* fragments that developed lesions during white plague exposure (MacKnight et

al. 2021). In the present study, we explored phylosymbiosis in the context of disease resistance

by integrating the host phylogeny and bacterial abundance into the expression variance and

evolution model (hereafter "EVE"). This analysis models the evolution of quantitative traits

(e.g., bacteria abundance) to the host's phylogeny through a phylogenetic ANOVA (Rohlfs and

Nielsen 2015). EVE can identify bacteria that have highly divergent abundances among host

species (i.e., are lineage-specific) and bacteria that recapitulate host phylogeny (i.e., present a

phylosymbiotic signal). Identifying lineage-specific and phylosymbiotic bacteria provides targets

for further host-microbe research and an analytical framework to address emerging questions surrounding dysbiosis as a cause for coral disease.

METHODS

Experimental Design and Sample Preparation

The disease exposure experiment data and microbial sampling was originally reported by MacKnight et al. 2021. Briefly, five parental colonies from each of seven Caribbean coral species, *Orbicella faveolata*, *Colpophyllia natans*, *Siderastrea siderea*, *Porites astreoides*, *Porites porites*, and *Montastraea cavernosa*, were collected from Brewers Bay (18.34403, -64.98435), St. Thomas, U.S. Virgin Islands on 13 June 2017. These colonies were fragmented, acclimated for nine days, and then exposed to active white plague infection on *Orbicella franksi* while an experimentally paired fragment was exposed to an apparently healthy *Orbicella franksi* as control treatments. This experimental disease transmission was conducted for seven days in running seawater tables at the University of the Virgin Islands, St. Thomas.

When a disease lesion appeared on a disease-exposed coral that was previously healthy, it was monitored until 30% of the tissue was lost. If the lesion enlarged over this time period, the coral and its paired control fragment were photographed, removed, flash frozen and stored at -80˚C. Coral fragments were classified by their treatment outcome as either, "controls", "disease-exposed", or "disease-infected". Coral fragments exposed to apparently healthy *O. franksi* were classified as "controls". Coral fragments exposed to disease, but did not show signs of tissue loss by the end of the transmission study were classified as "disease-exposed" and considered disease resistant individuals. Coral fragments that were exposed to a diseased *O. franksi* and developed tissue loss lesions that expanded through time were grouped as "disease-infected" and therefore

considered disease susceptible individuals. *O. faveolata* and *O. annularis* were classified as highly susceptible, *C. natans* and *S. siderea* were classified as having intermediate susceptibility, and *P. porites*, *P. astreoides* and *M. cavernosa* were classified as resistant based on percent infected, lesion progression rates, and relative risk of disease incidence (Supplemental Figure 1.) (MacKnight et al. 2021).

Microbial Isolation and Sequencing

DNA from the coral samples was extracted at the University of Texas at Arlington using the DNeasy Powersoil Isolation kits (MO BIO Laboratories, Carlsbad, CA). Roughly 0.25g of tissue was removed from each of the coral fragments using a sterilized bone cutter (Supplementary Table 3). Tissues from healthy-state fragments (i.e., controls) were extracted from the center of the fragment. Tissues were extracted in a similar manner from fragments exposed to white plague disease that did not display lesions by the end of the experiment (i.e., disease-exposed). For fragments that developed one or more lesions (i.e., disease-infected), tissues were extracted approximately 2-3 mm horizontally from the lesion margin in the apparently healthy tissue and collected parallel to the lesion margin. The V4 region of bacterial 16s rRNA was amplified using the 515f and 806R primers and sequenced on an Illumina MiSeq at MR DNA Molecular Research LP (Shallowater, TX). Sequences were bioinformatically processed using the QIIME analysis and then cleaned up by removing barcodes, primers, ambiguous calls, or sequences less than 150 bp. Operational taxonomic units (OTUs) were clustered by 97% similarity and taxonomically classified using BLASTn against the NCBI database (www.ncbi.nlm.nih.gov) (MacKnight et al. 2021).

Multi-Species Comparison through an Expression Variance and Evolution Model

To create a list of comparable bacteria for multispecies analysis, bacteria taxonomic names were identified among the coral hosts which created a list of 1674 bacteria with abundance data in all seven coral hosts (Figure 1D). A species tree was generated with Species Tree from All Genes (STAG) as implemented within Orthofinder2 using predicted peptides from generated transcripts with Transdecoder where lengths of the species tree represent substitutions per site (Emms and Kelly 2018b, 2018a; Haas et al. 2013; Rohlfs, Gronvold, and Mendoza 2020). The abundance of bacteria and generated species tree were input into the EVE model using the R package "evemodel" v0.0.0.9005 and "ape" v5.4.1 (Paradis and Schliep 2018; Rohlfs, Gronvold, and Mendoza 2020).

The EVE model links the expression data from the samples to the host's phylogenetic position in the tree. The beta shared test (i.e., phylogenetic ANOVA) can detect genes with increased or decreased ratios of expression divergence to diversity, represented as the beta parameter. EVE can be used for purposes such as identifying genes with high expression divergence between species as candidates for expression level adaptation, and genes with high expression diversity within species as candidates for expression level conservation and/or plasticity. This works by using an Ornstein-Ulbeack process of optimization to identify an ancestrally optimal expression values for each gene where variance from this optimum is represented by beta. The log likelihood ratio between the individual and shared beta fit indicates whether the individual beta was a better fit (i.e., the gene has an increased or decreased ratios of expression divergence to diversity). Significant deviations of beta from the optimal expression value are determined through the log likelihood ratio test statistic which follows a chi-squared distribution with one degree of freedom. Multiple comparisons were corrected using false

discovery rate. To determine phylosymbiotic candidates from the lineage-specific bacteria, a phylogenetic correlation using Pegal's Lambda was applied to determine bacteria that significantly recapitulated host phylogeny.

Pearson correlations were used to determine which lineage-specific and phylosymbiotic bacteria had correlated expression to species relative risk of disease incidence, and overall community dissimilarity between treatment outcomes. Within each coral host, a similarity percentage (SIMPER) analysis ranked bacteria that are most dissimilar between controls whose genotypic pair disease treatment outcome was disease-exposed to controls whose genotypic pair disease treatment outcome was disease-infected. Significant difference of the most dissimilar bacteria was identified through an ANOVA and Tukey post hoc test. Ultimately, *O. faveolata* and *M. cavernosa* were excluded from this analysis because their treatment outcomes were either exclusively disease-infected or disease-exposed, respectively. Bacteria that were significantly more highly abundant in control coral fragments whose genotypic pair developed disease lesions were categorized as candidates for co-evolved pathogens. By contrast, bacteria that were significantly more highly abundant in control coral fragments whose genotypic pair remained disease resistant were categorized as candidates for co-evolved probiotics.

RESULTS

Isolating phylogenetic and treatment expression patterns

To delineate phylogenetic influence compared with environmental influence in bacterial abundance, the phylogeny of coral species (Figure 1A) was considered in an expression variance and evolution (EVE) model. From EVE, 268 bacteria were classified as either (i) highly variable, (ii) lineage-specific, or (iii) phylosymbiotic in their abundance (Figure 1B, Data S1). Within the

268 bacteria, 160 bacteria were identified as highly variable in their expression and represent bacteria of potential microbiome plasticity that may have expression patterns related to their environmental exposure to disease and response (Figure 1C). The other 108 bacteria had significantly lineage-specific expression relative to other species which represent bacterial candidates that may have contributed to host evolution (Figure 1D). Within the 108 lineage-specific bacteria, a phylogenetic correlation determined that the relative abundance of 33 bacteria recapitulate coral host phylogenetic divergence, and considered phylosymbiotic candidates (Figure 1E, Data S2). All of the 33 phylosymbiotic bacteria were more highly abundant in *P. astreoides*, *P. porites*, and *S. siderea* relative to *O. faveolata*, *O. annularis*, *C. natans*, and *M. cavernosa*.

Lineage-Specific Bacteria are Associated with Microbial Dysbiosis and Relative Risk

We further explored lineage-specific abundance patterns to determine their association with the species relative risk of contracting white plague disease if the species is exposed. The abundance patterns of seven lineage specific bacteria were significantly ($p < 0.05$) correlated to the relative risk of disease incidence if exposed to white plague (Figure 2, Data S3). All seven bacteria correlated to relative risk were most highly abundant in disease-susceptible *O. faveolata*. Furthermore, the constitutive lineage-specific abundance of 17 bacteria were significantly correlated to the coral host's overall microbiome dissimilarity between control and disease-infected treatment outcomes (Data S4). From the 33 phylosymbiotic bacteria identified, 8 were also correlated to the coral host's overall microbiome dissimilarity between control and disease-infected treatment outcomes (Figure 3, Data S5).

Co-evolved Probiotic and Pathogenic Candidates

Lineage-specific bacteria contributed to a variable percentage of the total microbiome in individual coral fragments, ranging from up to 93% in *M. cavernosa* to 10% in *C. natans* (Figure 4). The total abundance of phylosymbiotic was also highly variable among coral fragments ranging from approximately 55% in a *P. astreoides* fragment to nearly 0% among several *M. cavernosa*, *O. faveolata*, and *O. annularis* fragments. Phylosymbiotic bacteria were predominantly identified in extant robust coral lineages (*P. porites*, *P. astreoides*, and *S. siderea*) which possessed 76.9% of the total abundance of microbial phylosymbionts identified. Higher abundance of phylosymbiotic bacteria in control fragments was associated with disease resistance in *P. porites*, *S. siderea*, and *C. natans*. By contrast, higher abundance of phylosymbiotic bacteria in control treatments was associated with disease susceptibility in P. astreoides (Figure 4). In total, 27 lineage-specific or phylosymbiotic bacteria were significantly different between control fragments whose paired treatment outcome was either disease-exposed or disease-infected. Eight of these 27 bacteria were significantly more highly expressed in controls whose paired genotype remained resistant to disease, relative to controls whose paired genotype developed disease lesions, and considered candidates for probiotic co-evolution (Figure 5, Data S7). In contrast, 15 bacteria are considered candidates for pathogenic co-evolution for their higher abundance in control fragments whose genotypic pair developed disease lesions, relative to the controls whose genotypic pair remained resistant to disease exposure (Figure 6, Data S7). The remaining four bacteria did not demonstrate abundance patterns that aligned with these conservative categorizations.

DISCUSSION

The study highlights three adaptive patterns of microbial symbiosis across seven Caribbean coral species in the context of disease resistance. First, phylosymbiotic bacteria were more highly abundant in extant, complex lineages of coral (*P. astreoides*, *P. porites*, *S. siderea*). These species also demonstrated a higher tolerance for microbial dysbiosis before developing disease lesions. Second, phylosymbiotic bacteria consisted of microbial candidates that demonstrated either pathogenic or probiotic patterns of abundance. Finally, further evidence of co-evolved pathogens is present in susceptible coral species which had a higher abundance of lineage-specific bacteria whose abundance is also positively correlated to the relative risk of disease incidence among all coral hosts. Collectively, this study presents microbial candidates that represent the co-evolved constraints and tolerance of microbial symbiosis associated with the coral host survival which reflects the disease susceptibility that is evident on current Caribbean reefs.

Microbial Phylosymbiosis is Present in Extant, Dysbiosis-Tolerant Coral Lineages

Phylosymbiotic bacterial candidates were consistently more abundant in "older" extant complex coral species such as *P. astreoides*, *P. porites*, and *S. siderea*, relative to *C. natans*, *M. cavernosa*, *O. faveolata*, and *O. annularis*. This pattern is consistent across all 33 phylosymbiotic bacteria identified. The consistency of this presents the possibility that not all coral species possess microbial phylosymbionts and may not have an equally evolved functional dependence between microbiome and host. Furthermore, while 108 bacteria were identified as lineage-specific, the abundance of only a select 33 bacteria were influenced by host phylogenic divergence (phylosymbiosis). While phylogeny is evidently an influential factor in the abundance of these bacteria, it also demonstrates that there are likely host physiology, or

biogeographic factors influencing this pattern. For phylosymbiosis to develop, there are likely

probiotic and stabilizing mechanisms in place to have withstood the millions of years of

historical environmental change (Kohl 2020). This suggests the robust coral species that have a

lower abundance of phylosymbiotic bacteria may be lacking in the probiotic and stabilizing

benefits that complex coral species could be benefitting from by having a higher abundance of

microbial phylosymbionts. This implication is supported in previous work, where complex coral

species demonstrated a higher disease tolerance and a lower disease prevalence in situ (Pinzón C

et al. 2014). Additionally, when mucosal, tissue, and skeletal layers of the coral were isolated,

microbial phylosymbiosis was determined to be present in the skeletal and tissue layers, and not

in the mucosal layer (Pollock et al. 2018). While our study demonstrates complex coral had a

higher abundance of microbial phylosymbiosis, it also presents a possible physiological

preference for this co-evolution to develop. A key difference between complex and robust coral

is that complex lineages typically possess a more porous skeleton while robust coral have a more

solid calcified skeleton (Romano and Palumbi 1996). The porous vacuoles that complex coral

possess may have provided the environment suitable for co-evolution to develop. A similar

relationship between life-history strategies and microbial phylosymbiosis was reported in high

microbial abundance sponges, relative to low microbial abundance sponges. Subsequently,

through metagenomics, the high microbial abundance sponges are more likely to possess

specialized biochemical functions (Pankey et al. 2022). The potential stabilizing benefits of

phylosymbiotic partners is also supported by this disease exposure experiment which

demonstrated that these extant, complex coral species have a greater tolerance for microbial

community imbalance (MacKnight et al. 2021). Collectively this suggests these microbial

phylosymbionts could be contributing to the disease tolerance of these extant, complex coral

lineages through microbe to host contributions that robust coral do not benefit from. The particular bacteria contributing to this were identified in eight phylosymbiotic bacteria that were positively correlated to the microbial community-level imbalance. The original hypothesis in this study is that microbial phylosymbiosis could have developed through probiotic and stabilizing contributions between host and microbe. This positive correlation suggests the tolerance for microbiome imbalance could be mediated by phylosymbiotic bacteria, but these microbes may also possess pathogenic properties. This expands the potential functional contributions of these phylosymbiotic bacteria to include co-evolved pathogens that lead to the hosts adaptive vulnerability and co-evolved probiotics that lead to the hosts adaptive tolerance in a changing environment. Furthermore, the variance in microbial phylosymbiosis among coral lineages and the potential functional contributions of these symbionts suggests coral may not rely on their holobiont the same across coral species to mitigate disease exposure (Pollock et al. 2018b; Pankey et al. 2022). Further exploration of holobiont dependance to mitigate environmental change could help focus predictive and diagnostic biomarker development that more accurately captures coral species vulnerability.

Patterns of Pathogenic Co-evolution

The intention to identify microbial phylosymbiosis was to identify bacteria with probiotic or stabilizing contributions to the host. While our results align with this hypothesis, the abundance patterns of phylosymbionts also suggests this approach has detected possible pathogens that have co-evolved with the coral hosts. The single *P. astreoides* fragment that developed disease lesions had a significantly higher abundance of phylosymbiotic bacteria in the control state relative to the other controls of this species whose disease treatment pair remained

resistant. Furthermore, in the disease-infected fragment, the abundance of these phylosymbiotic bacteria increased to 50% of the total microbiome. This opportunity to identify co-evolved pathogens responsible for these differences was driven by five bacteria, *Nautella italica*, *Erythrobacter citreus*, *Roseivirga sp.* and *Thalassomonas sp*. In MacKnight et al 2021, *Nautella italica* was identified as a candidate pathogen among all coral hosts and we further that conclusion by understanding this microbe has a lineage-specific relationship with coral hosts associated with disease susceptibility. Finally, lineage-specific bacteria associated with relative risk of disease incidence were more highly abundant in disease susceptible *O. faveolata* and *O. annularis*. This pattern suggests these bacteria may be co-evolved pathogens and demonstrates an adaptive constraint in the microbiome that inhibits the survival of these reef-building coral. These susceptible species also demonstrated the lowest overall microbiome change before developing disease lesions (MacKnight et al. 2021). One explanation is that a pathogenic infection may have led to lesion development without shifting the overall community in this species. However, the results of this study show that the microbiome in the control treatment already possess candidate pathogens which would result in a relatively lower overall community change before developing lesions. Further investigation on the functional contributions of these microbes is worthwhile because they suggest adaptive constraints on the microbiome of coral species associated with their relative risk of disease incidence and tolerance for dysbiosis in a changing environment.
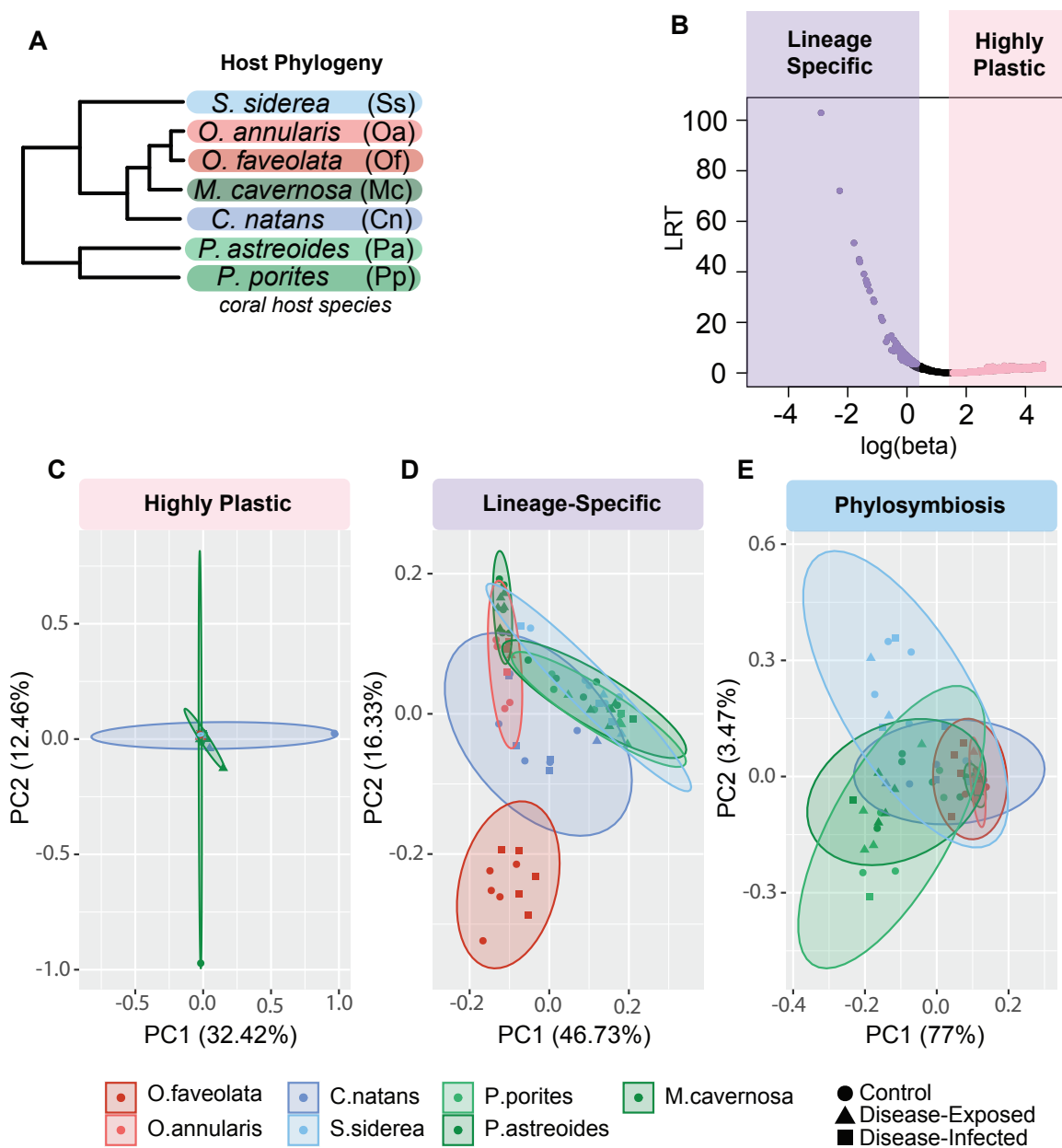
Figure 1. Microbial Abundance Dependence on Coral Host Phylogeny. (A) Coral host phylogenetic tree where colors represent host disease susceptibility red is high susceptibility, blue is intermediate susceptibility, and green is low susceptibility. (B) Volcano plot of the 160 highly variable bacteria and 108 lineage specific bacteria. (C) The relative abundance of the 160 highly variable bacteria do not separate by host lineage but share an average relative abundance.

(D) The relative abundance of the 108 lineage specific bacteria begin to separate by coral host species. (E) The relative abundance of 33 phylosymbiotic bacteria.
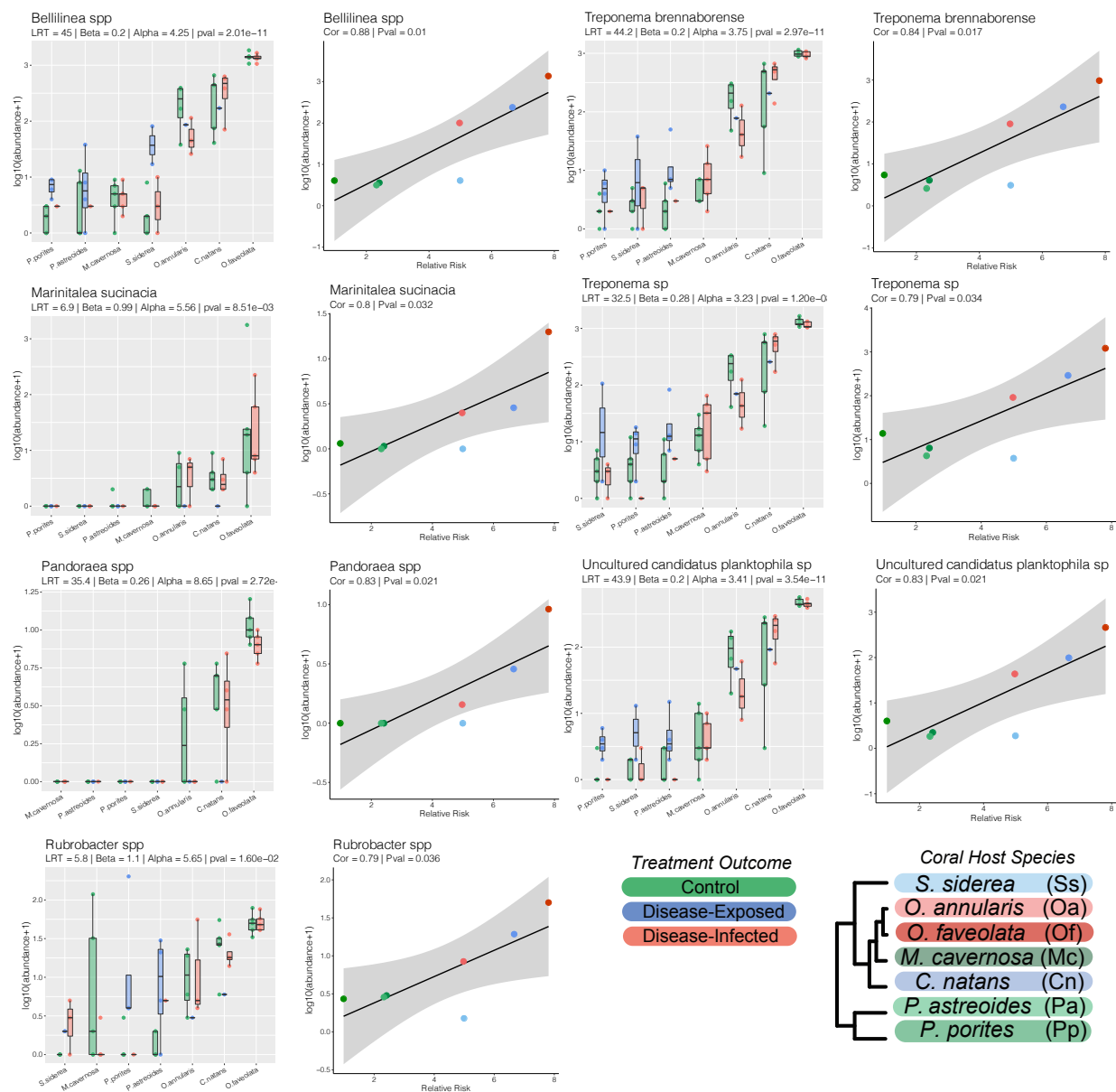
Figure 2. Lineage-Specific Bacteria Correlated to Relative Risk of Disease Incidence. Of the 108 lineage-specific bacteria, 7 were correlated to the relative risk of disease incidence, presented here.
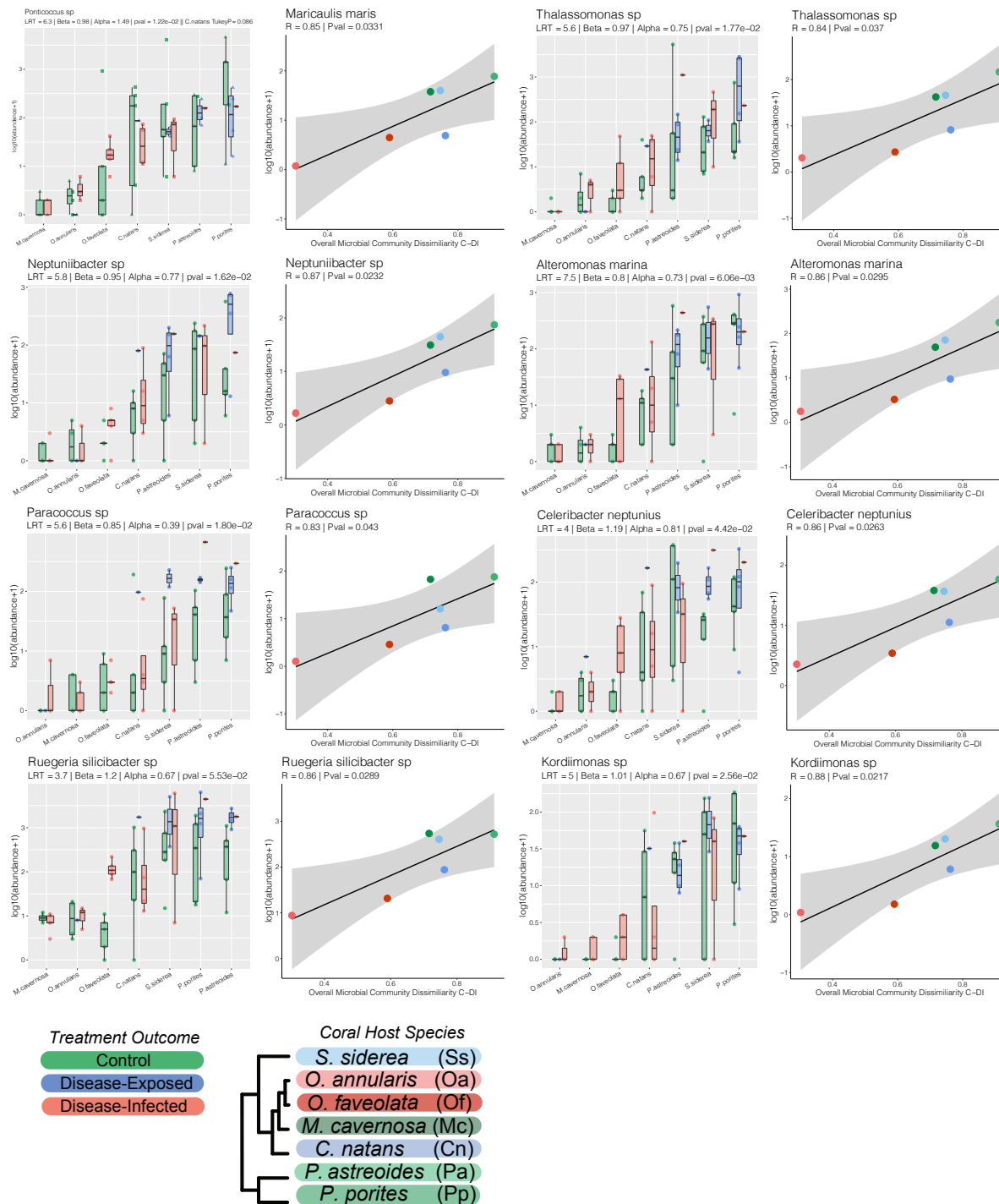
Figure 3. Phylosymbiotic Bacteria Associated with Microbial Dysbiosis. Of the 33

phylosymbiotic bacteria, 8 were correlated to Microbial Community Dissimilarity between

Control and Disease-Infected Treatment Outcomes, presented here.
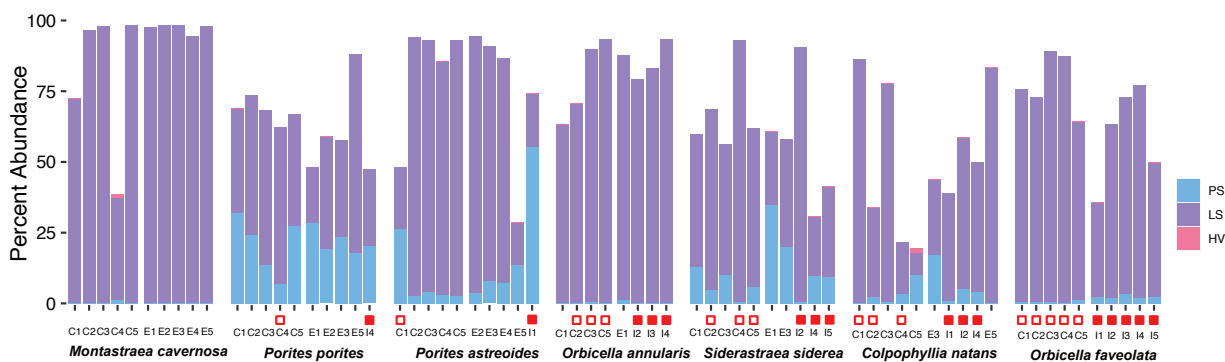
Figure 4. Percent Abundance of Co-evolved Bacteria. Bacterial abundance was pooled based on their classification as either phylosymbiotic (PS), lineage-specific (LS), or highly variable (HV) candidates to determine their abundance relative to the abundance of the entire microbiome. Red hollowed squares indicated controls, whose genotypic pair (indicated by a corresponding number 1 through 5) developed disease lesions and considered disease-infected (I).
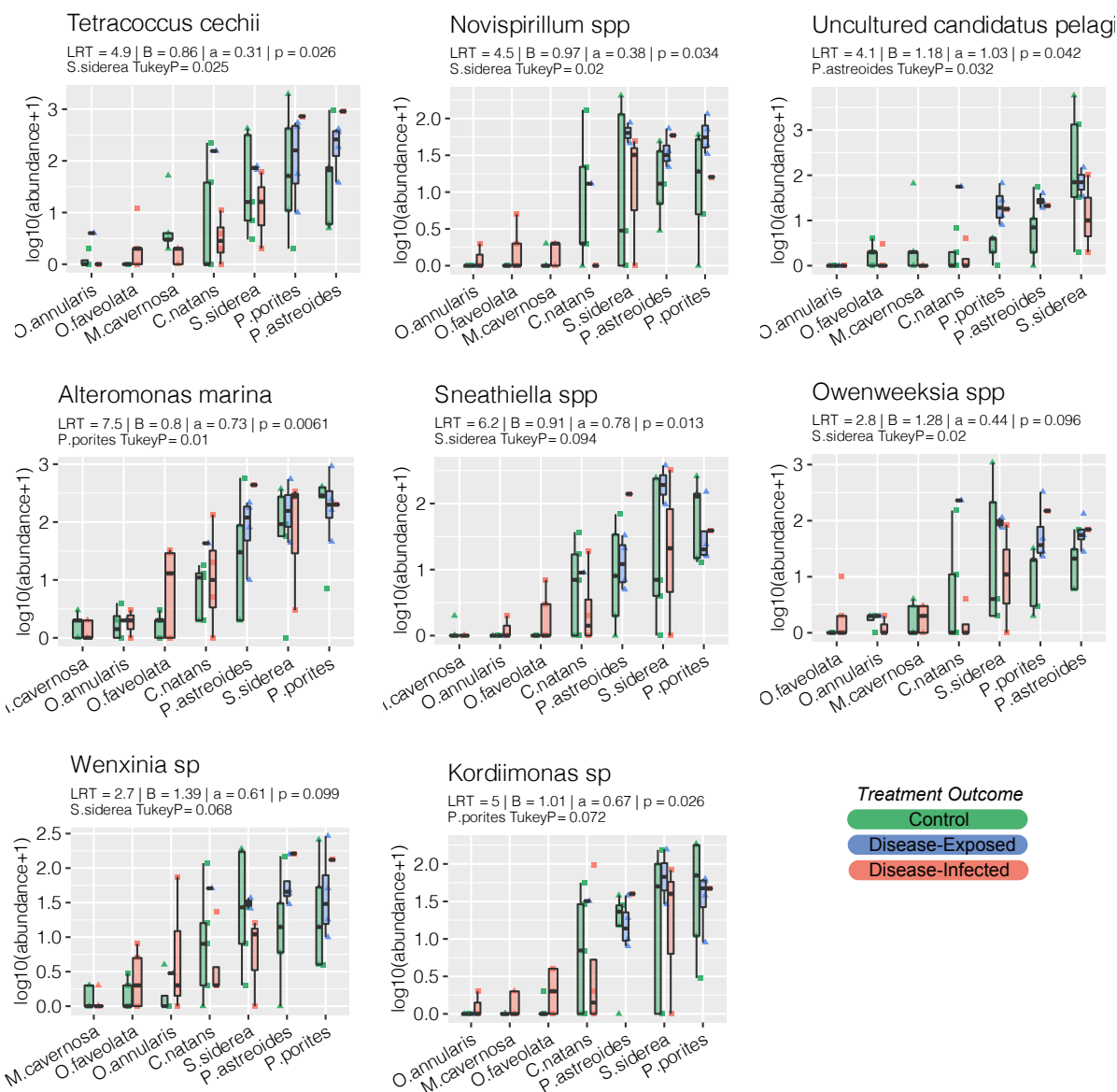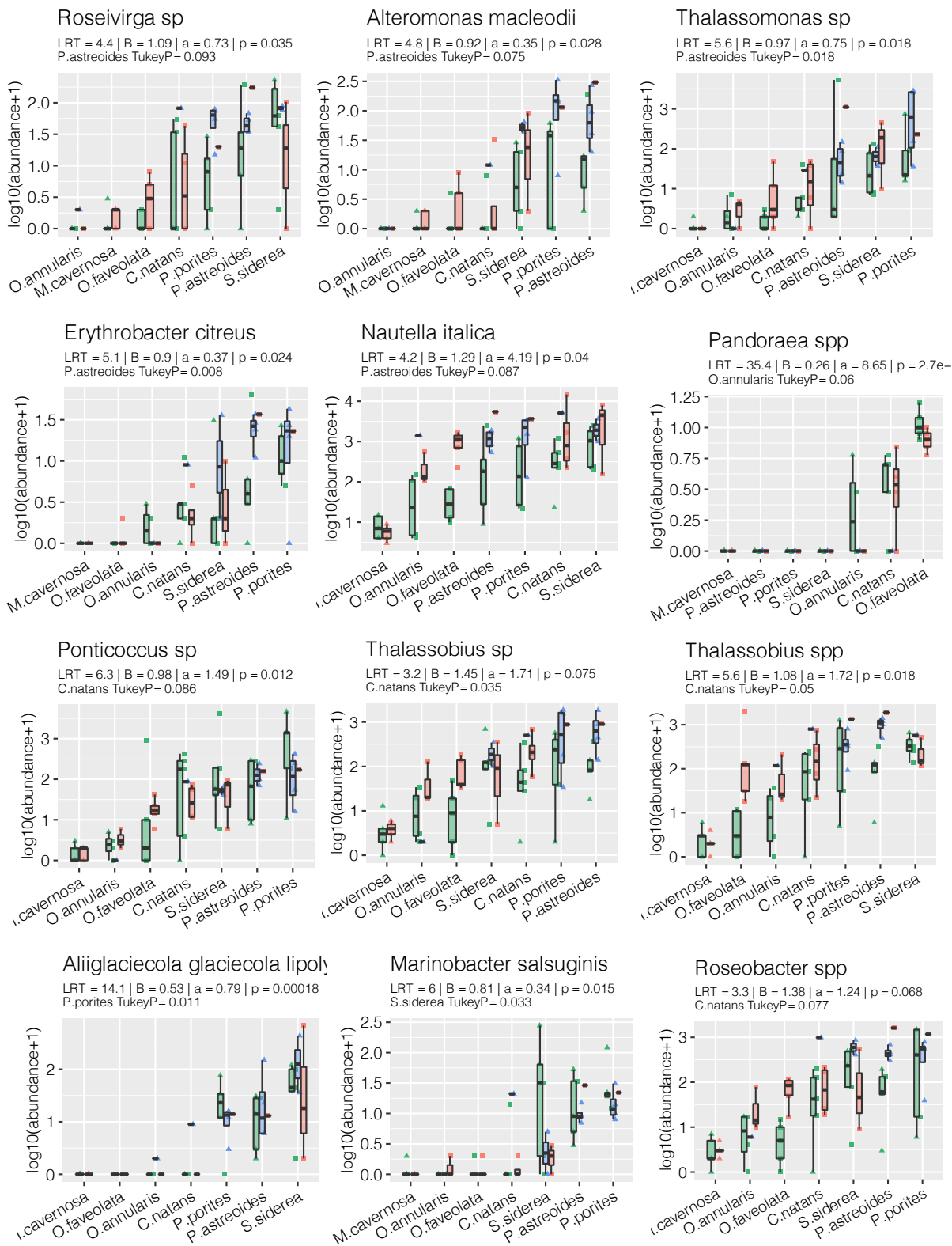
Figure 5. Co-evolved Probiotic Candidates. Significantly different abundances of lineage specific, phylosymbiotic, and highly variable bacteria was determined between controls whose genotypic pair's treatment outcome was either disease-exposed or disease-infected. From these significantly different bacteria, control fragments whose paired control was disease resistant and had significantly higher abundance in the control treatment were considered probiotic candidates.
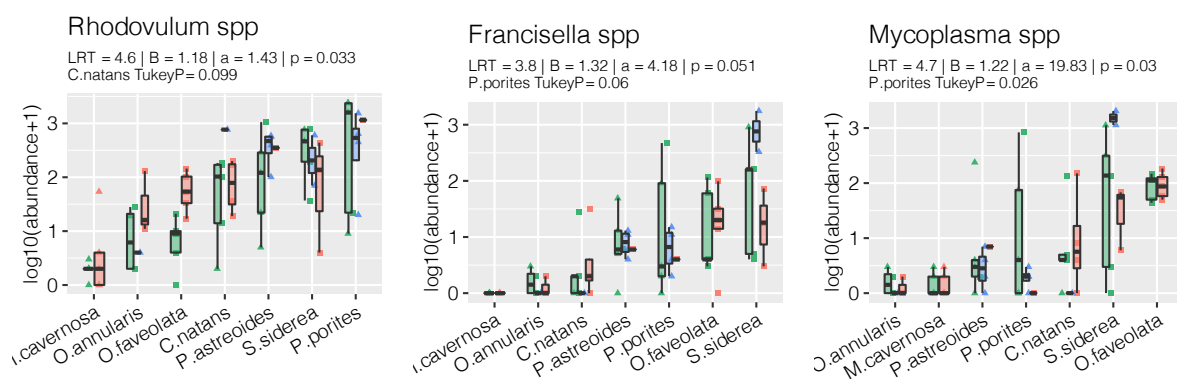
Roseivirga sp
LRT = 4.4 | B = 1.09 | a = 0.73 | p = 0.035
P.astreoides TukeyP = 0.093

Alteromonas macleodii
LRT = 4.8 | B = 0.92 | a = 0.35 | p = 0.028
P.astreoides TukeyP = 0.075

Thalassomonas sp
LRT = 5.6 | B = 0.97 | a = 0.75 | p = 0.018
P.astreoides TukeyP = 0.018

Erythrobacter citreus
LRT = 5.1 | B = 0.9 | a = 0.37 | p = 0.024
P.astreoides TukeyP = 0.008

Nautella italica
LRT = 4.2 | B = 1.29 | a = 4.19 | p = 0.04
P.astreoides TukeyP = 0.087

Pandoraea spp
LRT = 35.4 | B = 0.26 | a = 8.65 | p = 2.7e−
O.annularis TukeyP = 0.06

Ponticoccus sp
LRT = 6.3 | B = 0.98 | a = 1.49 | p = 0.012
C.natans TukeyP = 0.086

Thalassobius sp
LRT = 3.2 | B = 1.45 | a = 1.71 | p = 0.075
C.natans TukeyP = 0.035

Thalassobius spp
LRT = 5.6 | B = 1.08 | a = 1.72 | p = 0.018
C.natans TukeyP = 0.05

Aliiglaciecola glaciecola lipoly
LRT = 14.1 | B = 0.53 | a = 0.79 | p = 0.00018
P.porites TukeyP = 0.011

Marinobacter salsuginis
LRT = 6 | B = 0.81 | a = 0.34 | p = 0.015
S.siderea TukeyP = 0.033

Roseobacter spp
LRT = 3.3 | B = 1.38 | a = 1.24 | p = 0.068
C.natans TukeyP = 0.077

Figure 6. Co-evolved Pathogenic Candidates. Significantly different bacteria between controls whose paired genotypic fate was either disease-exposed or infected were identified. Bacteria that were significantly higher in control treatments whose genotypic pair became disease-infected were categorized as co-evolved pathogenic candidates.

Chapter 5 | CONCLUSIONS

The increasing prevalence and severity of diseases affect coral species differently. Because the functional contributions of these species define reef ecology, it is integral to understand variability in disease susceptibility among coral species to predict how disease will shape coral reefs of the future. This dissertation characterized disease susceptibility among seven coral species that represent a diversity of ecological contributions which have historically contributed to Caribbean coral reef assemblages. Less microbial change was observed in disease-susceptible coral species, suggesting low microbial dysbiosis thresholds should be further investigated as a possible coral disease etiology. As disease increases, disease susceptible *Orbicella* species that are primary contributors to reef structure will become less abundant, negatively affecting the physical protection that reefs provide. This lost real estate within the reef may be colonized by more disease resistant but less efficient reef-building species, making disease susceptibility an important predictor of the changing ecological function of Caribbean reefs.

This dissertation leverages a novel disease-transmission experimental design and expands upon analysis common in the field to identify coral-wide disease resistance traits. The phenotypic, genetic expression, microbial, immune protein, and antibacterial activity of seven diverse Caribbean coral species was captured in response to a seven-day exposure to a white plague disease-infected *Orbicella franksi*. While the experimental disease transmission of seven species is unprecedented in the field, the successful water transmission of white plague, and the coral disease susceptibility which aligned with field observations set a confident backbone for omics investigation. The following conclusions expands the frontier of knowledge in the field, while bringing visibility to innovatively curated methodology which appeases previous

challenges in the field head on. Subsequently, these chapters demonstrate replicable analysis to empirically investigate microbial dysbiosis, identify adaptive and plastic gene expression, and scalable identification of co-evolved microbial symbionts which can be applied even beyond the research focus of coral immunology. This innovation begins in with a repurposed similarity percentage (SIMPER) analysis that allowed for us to measure overall microbial community dissimilarity and address emerging hypothesis on microbial dysbiosis as an etiological force. This concluded that the tolerance for microbial dysbiosis reflects the coral's disease resistance. We expand the frontier of knowledge in the field by addressing this emerging hypothesis and empirically demonstrating that microbial dysbiosis is an etiological factor supported by our study, delivers candidate pathogenic and probiotic bacteria, and provides a familiar methodology for others to measure microbial dysbiosis in a globally changing environment.

Immune strategies that consistently lead to either a susceptible or resistant disease exposure outcome were identified that were either dependent or independent of host phylogeny. The integration of disease phenotypes (disease outcome, lesion progression rate, relative risk of disease incidence), into our analyses also identified gene expression processes directly involved in lesion development. Considering these phenotypes, phylogeny, and the gene expression broadens our understanding on what processes are relevant at mediating the holobionts' immune system across coral species. This built support for the non-canonical immune processes associated with adaptive innate immunity among coral species. Particularly, barrier defense through plastic maintenance of the extracellular matrix, and intracellular execution of autophagy over apoptosis was consistently associated with disease resistance among seven diverse coral species. The integration of the EVE model identified intracellular protein trafficking as a lineage-

specific process associated with autophagy which suggests an adaptive constraint which may mediate why some resistant species initiate the plastic expression of autophagy over apoptosis.

We leveraged the EVE model further to identify co-evolved (lineage-specific and phylosymbiotic) microbial symbionts of the coral host. Previous research on coral phylosymbiosis recognizes a singular bacterium, *Endozoicomonas sp.* with *Porites astreoides* by applying a phylogenetic generalized linear model to the beta diversity of microbial symbionts. The use of EVE demonstrated 108 lineage-specific bacteria, 33 of which significantly recapitulated host phylogenic divergence (phylosymbiosis) which demonstrates the powerfully scalable integration of the EVE model to identify co-evolved bacteria with animal hosts. We further this analysis by demonstrating that eight are correlated with microbial dysbiosis and candidates as high network connectance bacteria, another eight are candidates as co-evolved probiotics, and 15 are candidates as co-evolved pathogens. These dissertation chapters offer insight into the evolutionary constraints of species to mitigate disease and present predictive microbial and gene-level markers and broader biological processes consistent across coral species that will shape coral reef populations in this changing environment.

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