**Mussismilia braziliensis** White Plague Disease Is Characterized by an Affected Coral Immune System and Dysbiosis

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**Abstract**

Infectious diseases are one of the major drivers of coral reef decline worldwide. White plague-like disease (WPL) is a widespread disease with a complex etiology that infects several coral species, including the Brazilian endemic species **Mussismilia braziliensis**. Gene expression profiles of healthy and WPL-affected *M. braziliensis* were analyzed in winter and summer seasons. The de novo assembly of the *M. braziliensis* transcriptome from healthy and white plague samples produced a reference transcriptome containing 119,088 transcripts. WPL-diseased samples were characterized by repression of immune system and cellular defense processes. Autophagy and cellular adhesion transcripts were also repressed in WPL samples, suggesting exhaustion of the coral host defenses. Seasonal variation leads to plasticity in transcription with upregulation of intracellular signal transduction, apoptosis regulation, and oocyte development in the summer. Analysis of the active bacterial rRNA indicated that *Pantoea* bacteria were more abundant in WPL corals, while *Tistlia*, *Fulvivirga*, and *Gammaproteobacteria* Ga0077536 were more abundant in healthy samples. Cyanobacteria proliferation was also observed in WPL, mostly in the winter. These results indicate a scenario of dysbiosis in WPL-affected *M. braziliensis*, with the loss of potentially symbiotic bacteria and proliferation of opportunistic microbes after the start of the infection process.

**Keywords** Coral reefs · **Mussismilia braziliensis** · White plague · Metatranscriptomic analysis · Dysbiosis

**Introduction**

A worldwide decline in reef-building coral cover has been observed in recent decades due to a combination of local and global changes [1]. Coral infectious diseases have also intensified, triggered by, for example, local pollution and/or elevated sea temperature [2, 3]. The prevalence of coral diseases is expected to increase further in future scenarios of global climate change [4–6]. White plague (WPL) disease is the most prevalent coral disease in the Atlantic and is known to infect more than 40 coral species [4, 5, 7]. WPL is characterized by a thin line of coral tissue loss, resulting in exposed skeletons adjacent to healthy tissue, with varying disease progression rates [4].

Culture-independent approaches have revealed a diverse microbial consortium in coral colonies with WPL, indicating a complex etiology of this disease [8–12]. Bacteria, eukaryotes, and viruses may be involved in tissue loss diseases in different coral species [13–17], but the best-known etiologic agents of tissue loss disease belong to Proteobacteria. *Vibrio coralliilyticus* causes white syndrome in Indo-Pacific corals [18]; *Aurantimonas coralicida* is the etiological agent of tissue loss disease in *Dichocoenia stokesi* in the Caribbean [19], and *Thalassomonas loyana* has been proposed to cause tissue loss disease in *Favia favus* in the Red Sea [20].
WPL is widespread in the Abrolhos, the main reef bank in the Southwestern Atlantic Ocean (SAO), affecting the endemic reef-building coral Mussismilia braziliensis [5, 21, 22]. The etiology of WPL in Abrolhos is not completely understood, and previous studies indicated a dysbiosis scenario, with the proliferation of opportunistic infections and different potential etiologic agents [21–25]. Dysbiosis and the consequently impaired microbiome might cause negative impacts on the coral host, impoverishing the host health condition and making it more vulnerable to infections [26, 27]. In these conditions, identification of WPL etiologic agents is elusive, demanding careful manipulative experiments [28, 29]. WPL-affected M. braziliensis are enriched with Vibrio, Alteromonadales, Bacteroidetes, and sulfate-reducing bacteria [21–25]. qPCR analysis showed an increased activity of total Vibrio spp. and Vibrio coralliilyticus in WPL-affected M. braziliensis [30]. It is not clear if these microbes represent primary infectious agents or are simply opportunistic or saprophitic, taking advantage of necrotic coral tissue as a nutrition source. Despite the high prevalence of WPL diseases, M. braziliensis physiological and cellular responses to WPL are not known.

Pathogen infections cause modulation of the coral host immune system, including induction of the innate immune system, associated cellular signaling pathways, and mobile phagocytic cells, the amoebocytes [31–33]. Humoral response includes the induction of cellular responses such as phagocytosis, apoptosis, and autophagy and activation of lysosome enzymes [31, 32, 34, 35]. Melanization, production of reactive oxygen species, and phenoloxidase proteolytic activity provide a physicochemical barrier to infections [31–33, 35, 36]. Coral mucus, gastroderm, and other tissues harbor diverse symbiotic bacterial and viral communities, which can provide both physical and biological barriers to infections [32, 37]. These barriers might function as an earlier mechanism to prevent disease infection in corals, as suggested by the lack of correlation between immune responses and survival after Vibrio spp. infections in O. faveolata [12].

WPL incidence is higher on summer, as a possible effect of microbial proliferation induced by higher temperatures [05]. However, seasonal variation might also affect the cnidarian immune system, as high temperatures and coral bleaching modulate coral immune pathways [32, 38]. Recent studies have shown that the establishment and maintenance of cnidarian-Symbiodiniaceae symbiosis lead to modulation of the host’s immune system, with the induced expression of TGF-B and repression of nitric oxide production and the NF-kappaB signaling pathway [39, 40]. In this sense, coral mucus and the cnidarian immune system are also responsible for the recognition and control of the diverse symbiotic interactions in the coral holobiont, making them important not only for ecological maintenance [41, 42] but also for the evolution of coral lineages [43].

Despite some advances in understanding host-microbe interactions in health and disease transitions, little is known about holobiont gene expression during WPL in the SAO endemic Mussismilia corals. Our aim was to obtain the metatranscriptomic profiles of both healthy and diseased M. braziliensis corals. This is the first attempt to shed light on the holobiont metatranscriptome to identify possible cellular processes in the coral host and associated changes in microbial communities in WPL-affected M. braziliensis.

Materials and Methods

Sampling

Coral fragments (approx. 5 cm²) were collected from shallow waters reefs (4–10 m deep) at two different locations in Abrolhos Marine National Park (Brazil, SISBIO permit 27147-2): (a) Archipelago (17° 96′ 43″ S/38° 70′ 06″ W) and (b) Parcel dos Abrolhos (17° 57′ 32.7″ S/38° 30′ 20.3″ W). Tissue fragments of asymptomatic M. braziliensis (hereafter Healthy, n = 5) and M. braziliensis visually affected with white plague-like disease (WPL, n = 9) were collected during scuba diving with a hammer and a chisel [24]. Samples were collected in the austral winter (August, 2011: two Healthy; four WPL) and summer (Feb., 2012: three Healthy; five WPL). Mean seawater surface temperature (SST) was 25.0 °C in Aug., 2011 and 27.2 °C in Feb., 2012, slightly above the maximum monthly mean temperature for the Abrolhos reef (26.9 °C, Fig. S1). Although higher SST probably occurred in shallow reefs, mass bleaching was not reported for the Abrolhos in this period. In both seasons, diseased samples presented the same morphology, with no visual signs of bleaching, and samples were collected at the tissue necrosis band. Diseased and healthy samples were collected in separate short dives. Coral fragments were collected on polypropylene tubes underwater, and transferred to RNAlater-filled tubes and stored in liquid nitrogen upon returning to the boat.

RNA Extraction

Each individual tissue fragment (100 mg) was grounded in liquid nitrogen using a mortar and pestle. Total RNA was extracted using a modified TRIZol (Invitrogen™, Carlsbad, CA, USA) and chloroform protocol [44]. TRIZol reagent (Invitrogen™, Carlsbad, CA, USA) was added to the samples and mixed by vortexing for 2 s. The tubes were kept at room temperature for 5 min, after which 200 μL of chloroform was added, and the solution was mixed for 15 s by hand shaking. The tubes were incubated at room temperature for 3 min and centrifuged at 2000 rpm for 15 min. RNA purification was performed with the RNAs paritcular kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. RNA
integrity was checked in a 1% agarose gel, and the RNA concentration quality and purity were verified in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was stored at −80 °C until processing.

RNA was treated with DNase (50 μl RNA in DEPC water to 50 μl 10× DNase buffer and 2 μl DNase I were incubated at 37 °C for 1 h; 1 μl of 0.5 M EDTA was added and heated at 75 °C for 10 min to inactivate). Precipitation was performed as follow (0.1 volume of 5 M NH₄OA₅ + 5 μg glycogen + 3 volume 100% ethanol were added and kept at −20 °C overnight. RNA was centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was discarded, and 1 ml ice-cold 70% ethanol was added and mixed by vortex. A new centrifugation was performed at 12,000 × g for 10 min at 4 °C. The supernatant was carefully discarded, and RNA was resuspended in 30 μl DEPC water. The purification for large-scale transcription reactions was performed by using the MEGAclean™ Transcription Clean-Up Kit (Ambion) according to the manufacturer’s instructions. At the end, RNA was resuspended in 30 μl of TE buffer. Depletion of potential mammal RNA contamination (rRNA and mRNA from human, mouse, or rat origin) was performed by RNA hybridization with MICROBEnrich™ Kit (Ambion), according to the manufacturer’s instructions. Depletion of bacterial rRNA (16S and 23S) was performed by MICROBExpress™ Kit (Ambion) and mRNA-ONLY™ Prokaryotic mRNA isolation kit (Ecogen), according to the manufacturer’s instructions.

cDNA Library Preparation and Sequencing

Synthesis of cDNA was performed with the SeqPlex RNA amplification kit (Sigma-Aldrich) with the following amplification program: 18 °C for 10 min, 25 °C for 10 min, 37 °C for 30 min, 42 °C for 10 min, 70 °C for 20 min, and a 4 °C hold. cDNA samples were sheared in a bioruptor according to the instructions of the Quick Buitting Kit (New England Biolabs). A clean-up step with a PCR cleanup kit was performed, and the samples were eluted in a volume of 32 μl. An A′ overhang was added by using the Klenow fragment (3′-5′ exonuclease negative) according to the NEB#M0212 protocol, and sample-specific adapters were ligated using a ligation kit (NEB# M2200). After running the samples on an agarose gel electrophoresis (2%), prepared with TAE, cDNA bands at the range of 350–500 bp were excised. The extracted cDNA was enriched via qPCR, monitoring the cDNA copy numbers, and the obtained cDNA was cleaned and eluted in 30 μl of Buffer EB. Finally, the library size distribution was checked using a 2100 Bioanalyzer system (Agilent Inc., Waltham, MA) and a High Sensitivity DNA Kit (Agilent Inc., Waltham, MA). cDNA libraries were quantified in a 7500 Real-Time PCR system (Applied Biosystems) using KAPA Library Quantification Kit (Kapa Biosystems). PhiX sequencing control v3 (Illumina Inc., San Diego, CA, USA) was added at 1%, and paired-end sequencing (2 × 250 bp) was performed on the MiSeq platform (Illumina Inc., San Diego, CA, USA) at Parsons Lab, Massachusetts Institute of Technology.

Data Preprocessing and De Novo Assembly

Paired-end cDNA fastq sequences from coral were demultiplexed into separate files for each sample by barcodes using the script FASTQ/A Barcode Splitter from FASTX-toolkit version 0.0.14 [45], with a maximum barcode mismatch score of 2. Adapters were removed from both the 3 and 5′ ends using cutadapt [46]. Quality control was performed with prinseq lite version 0.20.4 [47] by trimming poly-A/T tails of more than 5 bp, filtering reads with an average quality of less than 30 (Q > 30), removing duplicates and ambiguous bases, and filtering reads by length ranging from 30 to 230 bp. Sequence quality was checked with FastQC version 0.11.3 [48] before and after quality control. High-quality overlapping paired-end reads were collapsed to form a longer sequence using pear [49].

Ribosomal RNA depletion is often incomplete [50], and remaining ribosomal sequences were filtered out, mapping the high-quality reads to small (16S/18S SSU) and large (23S/28S LSU) rRNA sequences, retrieved from the SILVA database project, release 119 [51]. Read mapping was performed with version 2.2.3 using the -very-sensitive-local parameter [52].

After quality control and rRNA removal, two healthy (MBH2, MBH5) and one diseased sample (WP8) presented a low number of reads (< 150,000) and were not used for the transcriptomic analysis. Collapsed and uncollapsed paired reads from the remaining 11 samples were pooled in a cross-assembly with Trinity version 2.0.6 [53] for de novo assembly using the –single end option. Assembly was performed with all reads across all samples combined as the input. The assembled transcriptome and the associated sequenced RNA libraries were deposited in the NCBI transcriptome repository under BioProject accession no PRJNA543846.

Transcriptomic Analysis

The assembled transcripts were annotated via Blastx comparisons with the NCBI-nr and the Uniprot (TrEMBL and SwissProt) databases (downloaded in June 2015), with an e value of 10e−5. Gene ontology annotations were determined by mapping Uniprot IDs to the UniProt-GOA database [54].

High-quality mRNA reads were mapped back to the assembled transcriptome (bowtie2), and RSEM [55] was used to estimate sample-specific transcript abundance. Transcripts with low read support (< 15 reads) were filtered out, and mRNA libraries were normalized to account for library size.
and composition (TMM) [56]. A classic MDS was constructed with the 500 transcripts with the largest mean leading pairwise fold changes, evaluating the effect of disease category (healthy or WPL diseased) and sampling season (winter or summer) among samples [56]. Enrichment of gene ontology (GO) terms was evaluated using GO_MWU, a rank-based analysis measuring whether transcripts in a GO term are consistently upregulated or downregulated [57]. GO_MWU was conducted with signed log($p$ values) with a false discovery rate of 0.1.

Additionally, traditional GO enrichment analysis was conducted on the set of differentially expressed transcripts. Differentially expressed (DE) transcripts associated with the two categories (disease and season) were evaluated with edgeR glm likelihood ratio test, called at a 0.05 significance level and adjusted to a 0.05 false discovery rate (Benjamini-Hochberg correction [56]). Enriched GO categories were checked with Fisher’s exact test at a 0.05 significance level with the “weight01” algorithm to account for the GO topology, using the Bioconductor package topGO [58].

**Symbiodiniaceae, Bacterial, and Viral Community Structure**

Symbiodiniaceae community composition in *M. braziliensis* samples was assessed by Blastn mapping of high-quality RNA reads to a local Symbiodiniaceae ITS2 database, comprised with the dereplicated database described in [59], combined with the Symbiodiniaceae sequences previously observed in *M. braziliensis* [60]. Symbiodiniaceae ITS2 sequences were retrieved with a minimum alignment length of 150 bp and 95% identity, allowing the presence of partial ITS2 matches. A $t$ test was used to compare differences in the relative abundance of total Symbiodiniaceae reads between disease conditions and between sampling seasons. A logistic regression was used to assess the effect of hosting *Symbiodinium* A4 (the non-dominant symbiont) on disease outcome with the package glm in R [61].

Bacterial genera potentially indicative of disease conditions or sampling season were assessed with the remaining ribosomal RNA in the sequenced cDNA libraries. In contrast to high-throughput 16s metabarcoding sequencing, this analysis focused on finding bacterial biomarkers on the most abundant representatives of the active bacterial community [62]. Ribosomal RNA reads were aligned (blastn) to the SILVA SSU (16S/18S) database, release 132 [51]. rRNA reads with at least 95% identity over 200 bp were retrieved for abundance analysis, and sequences with either mitochondrial or chloroplast origin were removed from the analysis. Relative abundances of each bacterial genus were used to evaluate whether a microorganism is consistently associated with one of the conditions, based on LEfSe analysis [63]. A heatmap was constructed with the 30 most abundant bacterial genera, based on the average of the geometric mean among Healthy and WPL samples. Relative abundances were power-transformed, and bacterial genera were clustered based on Pearson correlation among samples.

The assembled transcriptome was used to assess the composition of the viral community associated with *M. braziliensis*, through tBlastx searches against the NCBI viral genomic RefSeq database (November, 2016), with an e value threshold of 10e−15. For each transcript, tBlastx best hit was retrieved for taxonomic analysis, and sequences were assigned to viral families by parsing the NCBI taxonomic tree with a custom shell script. The distribution of viral families was quantified in the total transcriptome ($n = 119,088$) and in the sets of exclusively healthy (transcripts supported by reads in healthy libraries and no read support in any diseased samples, $n = 12,788$) or WPL transcripts (transcripts supported by disease with no read support in healthy samples, $n = 28,395$). Similarly, the distribution of viral families was quantified in the winter ($n = 14,630$) and summer transcripts ($n = 17,876$). The occurrence of over- or under-represented viral families in each condition was evaluated by a hypergeometric test at the 0.05 significance level after Bonferroni correction of false positives [64].

**Results**

Sequencing of all 14 samples resulted in a total of 10,406,652 paired-end reads. A total of 8,004,107 paired-end reads were retained (average length of 270 bp) after quality control. After the removal of ribosomal RNA, a total of 6,415,051 reads were retained (average length of 270 bp) after quality control. Of the read sequences, 527 bp, containing 119,088 transcripts. A total of 38,040 transcripts (32.9%) were annotated against the UniProt protein database (Table S2).

**Differential Expression and Gene Ontology Enrichment Analysis**

Transcript expression analysis indicates an effect of both the disease categories and sampling season on the similarity between samples (Fig. 1). Rank-based GO_MWU analysis indicated that WPL samples were characterized by the downregulation of immune response, cellular defense response, autophagy, and DNA/RNA metabolic processes (Fig. 2). Photosynthetic dark reactions and carbohydrate and peptide biosynthetic processes were upregulated in samples from diseased corals (Fig. 2). Coral samples obtained in the summer were downregulated in DNA integration, and upregulated in positive regulation of gene expression and carbohydrate bio-synthetic process.
A total of 212 and 251 transcripts were differentially expressed (DE) on disease and season, respectively (Tables S3 and S4). Only two transcripts were differentially expressed in both disease and season: an unannotated transcript downregulated in WPL and summer samples and a transcript coding for “Eukaryotic initiation factor 4A-I,” downregulated in WPL and upregulated in summer samples. DE transcripts in the disease were associated to biosynthetic processes, protein transport and methylation, and macroautophagy and endosome functioning (Table S5). Most of the DE transcripts in the disease were downregulated transcripts (202 of 212). DE transcripts in the season contrast were associated to regulation of gene expression, intracellular signal transduction (including multiple signaling pathways as Toll, TNF, JNK, NF-kappaB), regulation of apoptosis, and oocyte development (Tables 1, S5, and S6). In total, 177 transcripts were upregulated in the summer.

Symbiodiniaceae, Bacterial and Viral Community Structure

*Mussismilia braziliensis* preferentially hosts Symbiodiniaceae ITS2 types C3/C1 and A4 (Fig. 3), and most of the retrieved ITS2 (437 of 514) reads aligned to sequences previously observed in *Mussismilia* spp. corals [58]. Our results indicate that *Cladocopium* C3/C1 is the primary symbiont of *M. braziliensis*, but the relative abundance of *Symbiodinium* A4 can reach up to 50%. There were no differences in the relative abundance of total Symbiodiniaceae ITS2 reads, either between disease conditions (t = −1.12, p value = 0.294) or sampling seasons (t = −0.46; p value = 0.655). Also, there was no association between the relative abundance of *Symbiodinium* A4 and disease outcome (p = 0.274).

**Blastn** analysis identified 13,955 bacterial rRNA reads, after the exclusion of mitochondrial and chloroplast rRNA. As inferred from the abundance of bacterial rRNA reads, the most abundant groups in the active *M. braziliensis* bacterial community were *Vibrio*, *Leptospira*, Candidatus *Amoebophilus*, *Trichodesmium*, and an unclassified *Gammaproteobacteria* GA0077536 (Fig. 4). **LEfSe** analysis indicated that the bacterial genus *Pantoea* (*Gammaproteobacteria: Erwiniae*) was associated with WPL samples, while *Tistlia* (*Alphaproteobacteria: Rhodospirillaceae*), *Fulvivirga* (*Bacteroidia: Flammeovirgaceae*), and *Gammaproteobacteria* GA0077536 were associated with healthy colonies (Fig. 3). Considering the season contrast, *Stenotrophonomas* (*Gammaproteobacteria*:

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**Fig. 1** MDS ordination of the samples, based on the mean log2-fold change of the 500 most diverging transcripts

**Fig. 2** GO enrichment test-based ranks of the signed p value of differential expression among all transcripts (GO MWU). For the Disease contrast, repressed (red) and induced (blue) GO terms in WPL samples (a). For the Season contrast, repressed (red) and induced (blue) GO terms in Summer samples (b). p values according to the common key for each GO term.
Xanthomonadaceae) and the cyanobacteria *Lyngbia* and *Schizotrix* were more abundant on winter samples, while *Francisella* (Gammaproteobacteria: Francisellaceae) was more abundant on summer samples.

A total of 1646 viral transcripts were identified via *tBlastx* analysis. Overall, the viral transcripts were dominated by *Phycodnaviridae* (21.9%) and *Mimiviridae* (14.1%), viral families associated with Symbiodiniaceae (Fig. 5) [65, 66]. The bacteriophage families *Myoviridae* and *Siphoviridae* were also represented, as were families associated with metazoan hosts including *Polydnaviridae*, *Poxviridae*, *Iridoviridae*, *Baculoviridae*, and *Herpesviridae* (Fig. 5). A total of 114 and 262 viral transcripts were observed in the Healthy and WPL corals, respectively. *Myoviridae* (*p* = 0.0005) and *Siphoviridae* (*p* = 0.0003) were enriched in WPL, while *Herpesviridae* was enriched in healthy transcripts.

### Table 1 Most abundant enriched biological process GO terms observed in either the Disease or Season contrasts

| GO.ID      | GO.Term                              | Total_transcripts | DE_transcripts | Up  | Down | *P* value |
|------------|--------------------------------------|-------------------|----------------|-----|------|-----------|
| Disease    |                                      |                   |                |     |      |           |
| GO:0009058 | Biosynthetic process                 | 10,197            | 24             | 3   | 21   | 0.0286    |
| GO:0015031 | Protein transport                    | 3443              | 12             | 1   | 11   | 0.0264    |
| GO:1901575 | Organic substance catabolic process  | 4736              | 11             | 1   | 10   | 0.0857    |
| GO:0045927 | Positive regulation of growth        | 468               | 5              | 0   | 5    | 0.0785    |
| GO:0007584 | Response to nutrient                 | 298               | 4              | 0   | 4    | 0.0429    |
| GO:0002183 | Cytoplasmic translational initiation | 132               | 3              | 1   | 2    | 0.0070    |
| GO:0006479 | Protein methylation                  | 263               | 3              | 0   | 3    | 0.0378    |
| GO:0007568 | Aging                                | 428               | 3              | 0   | 3    | 0.0499    |
| GO:0016236 | Macrophagophagy                      | 735               | 3              | 0   | 3    | 0.0730    |
| Season     |                                      |                   |                |     |      |           |
| GO:0010468 | Regulation of gene expression        | 5992              | 29             | 25  | 4    | 0.072     |
| GO:0035556 | Intracellular signal transduction    | 4718              | 21             | 19  | 2    | 0.011     |
| GO:0006468 | Protein phosphorylation              | 2796              | 16             | 14  | 2    | 0.032     |
| GO:0006357 | Regulation of transcription by RNA polymerase II | 2321 | 16 | 14 | 2 | 0.053 |
| GO:0042981 | Regulation of apoptotic process      | 2069              | 13             | 13  | 0    | 0.002     |
| GO:0009887 | Animal organ morphogenesis           | 2137              | 10             | 8   | 2    | 0.044     |
| GO:0008104 | Protein localization                 | 5183              | 9              | 8   | 1    | 0.005     |
| GO:0008285 | Negative regulation of cell proliferation | 752          | 9              | 8   | 1    | 0.006     |
| GO:0007409 | Axonogenesis                         | 1089              | 7              | 4   | 3    | 0.074     |
| GO:0045930 | Negative regulation of mitotic cell cycle | 434        | 6              | 6   | 0    | 0.000     |
| GO:0009408 | Response to heat                     | 330               | 6              | 5   | 1    | 0.000     |
| GO:0033209 | Tumor necrosis factor-mediated signaling pathway | 194 | 6 | 6 | 0 | 0.000 |
| GO:0048599 | Oocyte development                   | 139               | 6              | 5   | 1    | 0.014     |

Total: number of transcripts observed in the total *M. braziliensis* metatranscriptome, DE transcripts: number of differentially expressed transcripts in either Disease or Season contrasts, Up: number of induced transcripts in WPL (or summer) samples, Down: number of repressed transcripts in WPL (or Summer) samples, *p* value: Fisher’s exact test

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Fig. 3 Relative abundance of Symbiodiniaceae ITS2 reads among samples. Underline separate the contrasts (Disease and Season) in sample legend: H healthy, WP WPL samples, W winter, S summer

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Considering the season contrast, 109 and 165 transcripts were observed in the winter and summer samples, respectively. Myoviridae was enriched in winter samples ($p = 0.0003$).

**Discussion**

The metatranscriptomic analysis indicates that disease condition and seasonal environmental variation have diverging effects on the *M. braziliensis* meta-transcriptomic response. Results of WPL-affected samples suggest a widespread down-regulation of transcription, with the exhaustion of the cnidian immune system and cellular defense responses (Fig. 2, Table 1). Contrastingly, seasonal environmental variation leads to a coordinated regulation, indicating plasticity in the *M. braziliensis* transcriptional response (Fig. 2, Table 1). Moreover, the etiology of WPL in *M. braziliensis* involves proliferation of opportunistic microbes and loss of potentially symbiotic bacteria.

**M. braziliensis Processes Associated with WPL: Immune System, Cellular Defense Response, and Autophagy**

The results of this study indicate a vulnerable *M. braziliensis*, evidenced by the repression of immune system, cellular defense response, and autophagy in WPL samples (Fig. 2, Tables 1, S3, and S5). Selective autophagy is the main route for degradation of invading pathogens in eukaryotic cells, and its inhibition in WPL samples is supported by the repression of macroautophagy, the autophagy regulator *Beclin 1*, of E3-ubiquitin ligase, and endosome/lysosome transcripts (*FIVE, Huntigtin, mvb2, snx4*; Fig. 2, Tables 1 and S3) [67, 68].

Inhibition of autophagy was also observed in disease-susceptible *O. faveolata*, but not in the resistant *Porites astreoides*, when exposed to bacterial lipopolysaccharides (LPS) [35]. Induction of cell adhesion and phagocytosis transcription were observed in the *Eunicea* sp. octocoral with black band disease [35].

Lower activity of vesicle transport in WPL *M. braziliensis* is evidenced by the repression of transcripts related to
cytoskeleton and intracellular motor activity (dynein, tubulin-B, unconventional myosins 1a and 5a, WASP2C, Table S3) [69]. Impacts on the cellular cytoskeleton and tissue organization are also indicated by the repression of transcripts involved in cell migration, tissue development, and the maintenance of extracellular matrix (GO: basolateral plasma membrane, Table S5; ADGRE3, EPHA4, FMCA1, F-actin, mucin, pcd1, Table S3) [70, 71]. More specifically, reduced calcification is indicated by the inhibition of carbonic anhydrase, an extracellular calcium pump (FMCA1) and transcripts associated with the organic matrix of the aragonitic skeleton (mucin, pcd1) [72, 73]. Repression of cellular adhesion transcripts was also observed in WPL-affected O. faveolata [14]. These processes may be related to necrosis of the coral tissue. Necrosis can also be caused by bacterial enzymatic processes. Metalloproteases from the coral pathogen *Vibrio coralliilyticus* are known to cause tissue necrosis in *Pocillopora damicornis* and other Pacific corals [18, 74, 75]. In this model, pathogen infection induces a temperature-dependent innate immune response and cytokine production in *P. damicornis* [75, 76].

Upregulated in WPL samples, photosynthesis, carbohydrate, and organonitrogen biosynthesis processes are putatively associated with Symbiodiniaceae (Fig. 2). The repression of TGF-B and nitric oxide signaling (NOS) regulator nostrin suggests a breakdown of the symbiosis, with a possible transition to free-living lifestyle in Symbiodiniaceae (Table S3) [39]. *Deleted in malignant brain tumors 1 (dmbt1)*, a pattern recognition protein associated with symbiosis maintenance [12], was also repressed in WPL (Table S3). To a lesser extent, carbon fixation and photosynthesis transcripts were also upregulated in summer samples (Fig. 2, Table S4).

**Seasonal Effects on *M. braziliensis* Metatranscriptomic Response**

Positive regulation of gene expression in summer samples was associated with upregulation of signal transduction pathways and protein phosphorylation, suggesting a coordinated metatranscriptomic response to environmental variation in *M. braziliensis*. This response shows a remarkable similarity with the proposed cnidarian environmental stress response (ESR) [77], with the upregulation of general stress response transcripts (*dnaJ* and *hsp1*), the apoptotic regulator Bcl-2, and TNF factors (Tables S4, S6). These results support the hypothesis that seasonal variation selects for plasticity of ESR in *M. braziliensis* transcription. Seasonal plasticity was also observed on *Porites astreoides* [78], while a constitutive uploading of the ESR genes was observed in *Acropora hyacinthus* subjected to acute daily environmental variation [77].

It is striking to note that immune system and stress-related biological processes were affected differently in the disease and season contrasts. Regulation of apoptosis was repressed in WPL samples, while the apoptotic regulator Bcl-2 and TNF factors were induced in the summer (Fig. 2, Tables S4 and S5). Similarly, cell adhesion and migration were repressed in WPL, but induced in summer samples (Fig. 2, Table S3). Given the mild summer conditions in 2012 and the positive regulation of gene expression, these results suggest that the observed seasonal biological processes reflect *M. braziliensis* physiological response. Interestingly, *M. braziliensis* spawning period begins in the summer [79], when induction of oocyte development transcripts was observed (Tables S4 and S6).

**Dysbiosis and Changes in Microbial Communities in WPL Colonies**

Bacterial genera that are often associated with corals were among the most abundant in healthy *M. braziliensis* samples, including *Candidatus Amoebophilus*, *Vibrio* spp., and *Flavobacteriaceae* NSS Marine group (Fig. 4) [80–82]. Changes in the *M. braziliensis* microbiome composition were...
associated with the affected host immune system, with the proliferation of opportunistic species and the loss of nondominant potentially symbiotic species, suggesting a condition of dysbiosis [26, 27]. The biomarker genera of the Healthy condition (Tistilia, Fulvivirga, and the Gammaproteobacteria Ga0077536) formed a cluster with bacteria commonly observed in healthy corals, as Blatocatella and Candidatus Amoebophilus (Fig. 3) [80]. Although some of the species associated with healthy M. braziliensis might indeed be commensal bacteria [26], it is noteworthy that the single described species in Tistilia (T. consotensis) is a nitrogen-fixing bacterium [83]. Efficient nitrogen regulation is essential to the coral-Symbiodiniaceae interaction, and changes in this cycle are often observed in dysbiosis conditions, either caused by disease or environmental factors [10, 11, 84].

_Pantoea_, identified as a WPL biomarker, is a diverse genus that includes commensal species and pathogens of plants and animals [85]. _Pantoea_ was previously associated with WPD in Diploria strigosa, but not in Siderastrea siderea [86]. In the present study, _Pantoea_ clustered together with cyanobacteria which proliferated in some WPL samples, but not consistently. Proliferation of opportunistic cyanobacteria is possibly related to the colonization of the exposed skeleton in diseased colonies [5]. Cyanobacteria proliferation was more evident in the winter, when Lyngbia and Schizotrix were more abundant and the relative abundance of Trichodesmium reached 65% in the sample WP01 (Fig. 4). Changes in bacterial community composition were accompanied by enrichment of the phage families Myoviridae and Siphoviridae in WPL samples. The observed enrichment of the phage families Myoviridae in WPL-affected and winter samples (Fig. 5) was largely driven by cyanobacterial phages (Table S7). Virus-bacteria interactions in coral samples may have diverse roles, ranging from viral-facilitated infections to viruses conferring protection to bacterial and coral hosts from invading pathogens [37, 87].

_Sampling and Sequencing Strategy_

In this study, a cross-sectional comparison of healthy and diseased _M. braziliensis_ was presented. However, it is known that the effect of diseases on coral immune system varies with time [32, 33, 42]. Coral immune system and signaling pathways can be induced by pathogen infection, but decline with disease progression [42]. Physical injuries also elicit immune responses that decline over time with healing [33]. WPL-affected _O. faveolata_ showed repression of cellular adhesion transcripts, but increased transcription of the ubiquitin degradation pathway [14]. It remains to be investigated whether the observed discrepancies between WPL-affected _M. braziliensis_ and _O. faveolata_ indicate different stages of the same etiology or different etiologies for WPL disease in each coral species.

Healthy and WPL _M. braziliensis_ coral colonies were collected both in winter and summer, but the limited number of samples obtained after processing precluded a full factorial analysis of the interaction between disease and season categories. The pooling of different samples in each category (e.g., winter and summer samples in the WPL samples) tend to inflate biological variation between replicates, leading to a lower statistical power to differentiate between conditions [56, 88]. Statistical power of the study was also affected by the relatively low sequencing depth. A sequencing platform with higher throughput would improve transcript assembly and quantification in the meta-transcriptome, leading to a more precise holobiont response [88]. In this sense, it is expected that the obtained DE transcripts are dominated by the most abundant transcripts, but it is unlikely that the observed biological responses are caused by the pooling of samples. This conclusion is supported by the small overlap on DE transcripts, biological processes, and biomarkers between the disease and season categories.

**Conclusion**

This study brings new insight into the metatranscriptomic response of _Mussismilia_ corals. The exhaustion of the coral defenses on WPL disease and the lower responsiveness of the coral host to infections are evidenced by the repression of immune processes, autophagy, and cellular adhesion transcripts. Dysbiosis and proliferation of opportunists follow the onset of WPL disease. Despite the potentially synergistic effect of higher temperatures and disease infection, a different transcriptomic response was observed in the season categories, with the active regulation of ESR genes.

The macroscopic observations of tissue necrosis can be attributed to the inhibition of transcripts associated with cellular adhesion and the proliferation of potentially tissue necrotizing bacteria. The collection of induced and repressed genes identified in WPL as well as the detection of potentially symbiotic microorganisms in healthy coral colonies in the present study may serve as tools for monitoring coral health in face of escalating global changes and infectious diseases.

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