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Antimicrobial and stress responses to increased temperature and bacterial pathogen challenge in the holobiont of a reef-building coral

Running title: Antimicrobial responses in coral holobiont

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Abstract

Global increases in coral disease prevalence have been linked to ocean warming through changes in coral-associated bacterial communities, pathogen virulence and immune system function. However, the interactive effects of temperature and

pathogens on the coral holobiont are poorly understood. Here, we assessed three compartments of the holobiont (host, *Symbiodinium*, bacterial community) of the coral *Montipora aequituberculata* challenged with the pathogen *Vibrio coralliilyticus* and the commensal bacterium Oceanospirillales sp. under ambient (27°C) and elevated (29.5°C and 32°C) seawater temperatures. Few visual signs of bleaching and disease development were apparent in any of the treatments, but responses were detected in the holobiont compartments. *V. coralliilyticus* acted synergistically and negatively impacted the photochemical efficiency of *Symbiodinium* at 32°C, while Oceanospirillales had no significant effect on photosynthetic efficiency. The coral, however, exhibited a minor response to the bacterial challenges, with the response towards *V. coralliilyticus* being significantly more pronounced, and involving the prophenoloxidase system and multiple immune system related genes. Elevated seawater temperatures did not induce shifts in the coral-associated bacterial community, but caused significant gene expression modulation in both *Symbiodinium* and the coral host. While *Symbiodinium* exhibited an anti-viral response and upregulated stress response genes, *M. aequituberculata* showed regulation of genes involved in stress and innate immune response processes, including immune and cytokine receptor signalling, the complement system, immune cell activation and phagocytosis, as well as molecular chaperones. These observations show that *M. aequituberculata* is capable of maintaining a stable bacterial community under elevated seawater temperatures, and thereby contributes to preventing disease development.

Introduction

The prevalence of coral disease epizootics is on the rise worldwide (Harvell et al., 2007; Sokolow, 2009), largely as a consequence of increasing anthropogenic disturbances, including ocean warming (Sokolow, 2009). The coral holobiont (*sensu* (Rohwer, Seguritan, Azam, & Knowlton, 2002) comprises inter-kingdom symbioses among the coral host and a range of microbial symbionts, including the endosymbiotic dinoflagellate, *Symbiodinium* spp., and bacteria (Bourne, Morrow, & Webster, 2016). *Symbiodinium* fixes carbon through photosynthesis and provides the coral host with carbon- and sulphur-based nutrients, while the coral-associated bacteria are believed to contribute to holobiont health through nitrogen fixation (Lema, Willis, & Bourne, 2012), sulphur-cycling (Raina, Tapiolas, Willis, & Bourne, 2009), production of antimicrobial compounds (Kvennefors et al., 2012; Nissimov,

Rosenberg, & Munn, 2009; Shnit-Orland & Kushmaro, 2009), and the exclusion of harmful bacteria through occupation of available microbial niches (Rohwer, Seguritan, Azam, & Knowlton, 2002).

Environmental disturbances can have significant impacts on the dynamic microbial communities that govern coral holobiont health. For example, elevated seawater temperatures sometimes cause shifts in coral-associated bacterial communities towards potentially more pathogenic taxa (Bourne, Iida, Uthicke, & Smith-Keune, 2008; Littman, Willis, & Bourne, 2011; Ritchie, 2006). When coupled with an increase in pathogen virulence at elevated temperatures (Sussman, Willis, Victor, & Bourne, 2008; Vidal-Dupiol et al., 2011), these altered microbiomes may enhance the probability of disease development. The higher prevalence of many coral diseases in summer when seawater temperatures are above average or during warm temperature anomalies (Bruno et al., 2007; Maynard et al., 2015; Willis, Page, & Dinsdale, 2004) is consistent with this notion. However, whether this is caused by changes in the bacterial communities, increased pathogen virulence or diminished coral host resistance has not been demonstrated for most coral diseases. The gram-negative bacterium *Vibrio coralliilyticus* has been implicated as a causative agent of a group of coral diseases known as white syndromes, (Sussman, et al., 2008; Ushijima et al., 2014). The virulence of this bacterium is temperature-dependent (Ben-Haim, Zicherman-Keren, & Rosenberg, 2003; Kimes et al., 2012) and its virulence factors attack both the coral and *Symbiodinium* (Sussman et al., 2009). Such interactions among host, its microbial symbionts and the environment highlight the challenge in understanding the drivers of disease.

Corals possess a range of innate immune and stress response mechanisms for defence against biotic and abiotic disturbances. Genomic studies have discovered Toll-like receptors (TLR) and their downstream signalling molecules in a number of coral species (Miller et al., 2007; Shinzato et al., 2011), and functional studies have revealed that this pathway is involved in both the response to wounding (van de Water et al., 2015) and bacteria (Vidal-Dupiol et al., 2014). TLR signalling is crucial for the initiation of a pro-inflammatory response, as well as the regulation and maintenance of healthy associated bacterial communities via anti-microbial peptides (AMP) in the cnidarian *Hydra* (Franzenburg et al., 2012; Fraune & Bosch, 2007). Whether AMPs have a similar function in corals remains to be determined, but they have been

implicated in the immune response of the coral *Pocillopora damicornis* to *V. coralliilyticus* (Vidal-Dupiol et al., 2011).

Other response mechanisms present in corals include the lectin-complement system and the prophenoloxidase (proPO)-activating system. The lectin-complement system is involved in the immune response against bacteria (Brown, Bourne, & Rodriguez-Lanetty, 2013), the wounding response (van de Water, et al., 2015) and potentially in the maintenance of the coral-*Symbiodinium* symbiosis (Kvennefors, Leggat, Hoegh-Guldberg, Degnan, & Barnes, 2008; Kvennefors et al., 2010). The proPO-activating system is induced in response to immune elicitors (Palmer et al., 2011), pathogens (Mydlarz, Holthouse, Peters, & Harvell, 2008) and injury (van de Water, et al., 2015; van de Water, Lamb, van Oppen, Willis, & Bourne, 2015). To prevent damage to coral tissues, corals primarily use antioxidant enzymes (Palmer, et al., 2011) for the neutralisation of reactive oxygen and nitrogen species, which are produced by various anti-microbial immune mechanisms, such as PO and the oxidative burst. The oxidative burst is induced following the phagocytosis of microbes or cellular debris by immune cells, which are activated upon pathogen exposure and physical damage. Such a mechanism is part of the response of *Acropora cervicornis* to white band disease (Libro, Kaluziak, & Vollmer, 2013).

Elevated seawater temperatures affect many physiological processes in corals, including several metabolic functions, calcification, fluorescence, apoptosis, antioxidant response, and the immune system (DeSalvo, Sunagawa, Voolstra, & Medina, 2010; DeSalvo et al., 2008; Leggat et al., 2011; Rodriguez-Lanetty, Harii, & Hoegh-Guldberg, 2009; Roth & Deheyn, 2013; van de Water, et al., 2015; Voolstra et al., 2009). Corals respond to environmental stress by increasing the expression of multiple immune and stress response genes (Barshis et al., 2013; Chow, Beraud, Tang, Ferrier-Pagès, & Brown, 2012; Davies, Marchetti, Ries, & Castillo, 2016; Leggat, et al., 2011; Pinzón et al., 2015; Rodriguez-Lanetty, et al., 2009), which also play a role in the immune response to pathogens in corals (Brown, et al., 2013) and other marine invertebrates (Baruah, Ranjan, Sorgeloos, MacRae, & Bossier, 2011; Sung, Pineda, MacRae, Sorgeloos, & Bossier, 2008). Heat and light stress cause reductions in phytopigments (Strychar & Sammarco, 2012) and damage to photosystems of *Symbiodinium,* resulting in the generation of cell-damaging reactive oxygen species (ROS). Prolonged heat stress can cause coral bleaching, the loss of the *Symbiodinium* cells from coral tissues, which may ultimately result in colony

mortality (reviewed in (Weis, 2008). Clearly, the health of corals depends on the efficient functioning of all partners within the coral holobiont.

In this study, we examined the responses of three components of the holobiont of the coral *Montipora aequituberculata* to challenges by the coral pathogen *V. coralliilyticus* under elevated seawater temperatures. Specifically, it entailed an assessment of the transcriptomic response of the coral and its endosymbiont *Symbiodinium*, the photosynthetic capacity of *Symbiodinium*, as well as several well-characterised host immune parameters and the composition of coral-associated bacterial assemblages.

Material & Methods

Experimental design

Fragments of the scleractinian coral *Montipora aequituberculata*, sourced from 15 different colonies (Nelly Bay, Magnetic Island, Australia) in September 2012, were placed in experimental aquaria (n=21 per aquarium; AIMS, Townsville) in ultra-filtered seawater and allowed to acclimate for 14 days at 27°C. The ultra-filtered seawater was generated using hollow fibre membranes with a nominal pore size of 0.04 μm and absolute pore size of 0.1 μm. The 27 aquaria were randomly assigned to 9 treatments, comprising all combinations of 3 temperature treatments (27°C, 29.5°C, 32°C) and 2 bacterial treatments (the coral commensal *Oceanospirillales* S47, the putative coral pathogen *Vibrio coralliilyticus* strain P1) plus a control treatment without bacterial addition (Suppl. Fig. S1). *V. coralliilyticus* was chosen as it had previously been isolated from the lesion of a white syndrome-affected *M. aequituberculata* colony (Sussman, et al., 2008), and Oceanospirillales S47 was selected as this bacterium was isolated from a coral within the same taxonomic family as *M. aequituberculata* collected in Nelly Bay. Seawater temperatures were maintained at 27°C (ambient) or gradually increased by 0.5°C every 24 hours until target seawater temperatures were reached and further maintained until conclusion of the experiment (Day 22): 29.5°C (medium heat stress) or 32°C (high heat stress). Corals were inoculated with bacteria every 3 days to maintain bacterial challenge stress levels throughout the experiment (final concentrations: $1x10^5$ per ml on Day 0, 3, 6, 9 and 12). Higher bacterial inoculation concentrations (final concentration $1x10^6$

per ml) were used to increase the chance of disease development on Day 15, 18 and 21, because of an unexpected absence of higher disease prevalence in the *V. coralliilyticus*-treatments, in contrast to previous observations (Sussman, et al., 2008). Three coral fragments were sampled from each tank 24 hours following bacterial exposure. Additional details can be found in Supplementary File S1.

Coral health parameters

Maximum (F_v/F_m) and effective ($\Delta F/Fm$) quantum yields of photosystem II of *Symbiodinium* were measured using pulse amplitude modulation (PAM) fluorometry on 3 coral fragments per experimental aquarium.

Phenoloxidase (PO) and total potential phenoloxidase (tpPO) activities, and GFP-like protein expression were analysed for three replicates per tank according to protocols described in (van de Water, et al., 2015). In short, protein concentrations in coral tissue lysates were determined using the BIO-RAD DC protein assay (BIO-RAD, USA). To quantify PO activity, the rate of dopamine hydrochloride (Sigma-Aldrich, USA) oxidation by 20 µl of coral tissue lysate was determined by measuring the absorbance at 490 nm at 5-minute intervals for 45 min. For tpPO activity quantification, 0.1 mg/ml trypsin was added to allow activation of prophenoloxidase (PPO) into PO 20 min prior to dopamine hydrochloride addition. Expression of chromoprotein was analysed by measuring the absorbance at 588 nm in 20 µl of coral tissue lysate. Fluorescence spectra were analysed by measuring the emission wavelengths between 400 and 700 nm, with a 5 nm resolution, upon excitation of fluorescent proteins at 280 nm. All data were independently obtained in triplicate and standardized to total protein content and expression levels were calculated using methods previously described (van de Water, et al., 2015). Additional details can be found in Supplementary File S1.

Maximum and effective quantum yields, and fluorescent protein expression were analysed using linear mixed effects models. Phenoloxidase activities (PO, tpPO) and chromoprotein content were analysed using linear fixed effects models. Models were compared using analysis of variance (ANOVA). Detailed descriptions of the statistical analyses can be found in Supplementary File S1.

Microbial community analyses

DNA was extracted using a modified protocol of Wayne's DNA preparation method (Wilson et al., 2002). Identity of *Symbiodinium* was determined via the nuclear ribosomal DNA internal transcribed spacer 1 (rDNA ITS1) using single strand conformation polymorphism (SSCP) analysis (van Oppen, Palstra, Piquet, & Miller, 2001). For bacterial community analysis, bacterial 16S rDNA amplicon libraries were generated using the 28F/519R primer set, followed by 454 pyrosequencing. Sequencing data was processed using the QIIME pipeline (Caporaso et al., 2010). Chimeric sequences were removed using UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011), sequences of 97% similarity were clustered in operational taxonomic units (OTU) using UCLUST (Edgar, 2010) and Greengenes taxonomy (version gg_13_5) was assigned using BLAST. OTU tables were generated and alpha diversity metrics calculated. The phyloseq package (McMurdie & Holmes, 2013) was used to graphically present 1) the microbiome composition at the class level and 2) shifts in overall microbiome composition based on a principal coordinate analysis on the Bray-Curtis dissimilarity matrix. Permutational multivariate analysis of variance (PERMANOVA) and pairwise comparisons were used to test for statistical differences in overall microbiome composition between treatments and time points. The DESeq2 package (Love, Huber, & Anders, 2014) was employed on a DESeq2-compatible OTU counts file generated by conversion of the phyloseq object to investigate which OTUs were differentially abundant among treatments and time points,. The complete dataset was deposited in the NCBI Sequence Read Archive (SRA) database with accession number SRP125476. Additional details on protocols and data analysis are presented in Supplementary File S1.

De novo transcriptome & Tag-based RNA-Seq

The procedure to generate the cDNA library for transcriptome sequencing was based on the protocol by (Meyer et al., 2009) and adapted for sequencing on Illumina platforms. Libraries were 250bp paired-end sequenced on the Illumina MiSeq. The *de novo Montipora aequituberculata* transcriptome was assembled using a Trinity platform protocol (Haas et al., 2013). After trimming of non-template sequences using Cutadapt (Martin, 2011) and quality filtering using the Fastx Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), transcriptome assembly was performed using an input of 3,521,179 sets of paired reads and 1,592,295 unpaired reads. Average read length was 195.7 bp (standard deviation = 68.6). The assembly included

180,971 contigs greater than 200 bp, with a total size of 123.5 Mb. Average contig length was 682 bp and N50 was 1012 bp. BlastX of the assembly against the Uniprot database (e-value cut-off of 10^{-20}), returned 29,292 hits. Of these, 57% covered at least 40% of the length of hit sequence. Assembled contigs were annotated based on BlastX (Altschul et al., 1997) hits against the annotated proteomes of *Nematostella vectensis* (Putnam et al., 2007), and *Acropora digitifera* (Dunlap et al., 2013). The complete dataset of trimmed reads was deposited in the NCBI SRA with accession number SRP125476. The annotated transcriptome is available from https://matzlab.weebly.com/data--code.html.

TagSeq cDNA libraries were prepared following a protocol described in (E. Meyer, Aglyamova, & Matz, 2011), with modifications for sequencing on Illumina HiSeq platforms and to remove PCR duplicates (Dixon et al., 2015). Libraries from 40 differently bar-coded samples were multiplexed and sequenced (50bp single end) on the Illumina HiSeq 2000. The complete dataset of trimmed reads was deposited in the NCBI SRA with accession number SRP125476.

The DESeq2 package (Love, et al., 2014) was used to analyse differential gene expression patterns and Wald tests were performed to identify genes uniquely differentially expressed between specific stressors. GO analysis was conducted using a rank-based methodology with adaptive clustering of GO terms (Wright, Aglyamova, Meyer, & Matz, 2015), available at https://github.com/z0on/GO_MWU. Detailed descriptions of protocols and analyses can be found in Supplementary Files S1 and S4.

Results

The majority of parameters analysed in this study did not show any interactive effect of temperature stress and bacterial treatment. From here on, when referring to: 1) the effect of a temperature treatment, the statement is made for all treatment combinations at that temperature, regardless of bacterial treatment, and 2) the effect of a bacterial treatment, the statement is made for all treatment combinations that underwent that bacterial treatment, regardless of temperature. When both a bacterial and a temperature treatment are reported together, the result addresses that specific treatment combination.

Macroscopic assessment of coral fragment health

Over the course of the experiment, most coral fragments appeared visually healthy based on the level of pigmentation and the absence of tissue lesions (Suppl. Fig. S2). Only seven fragments showed signs of disease, characterised by tissue loss and exposure of the underlying skeleton, signs that are consistent with a white syndrome, followed by secondary colonisation forming a grey-black film covering the lesion (Suppl. Fig. S3). Timing of disease development was variable, with disease found in only one *V. coralliilyticus*-challenged fragment at 27°C on Day 7. All other cases occurred on Day 15 (two non-challenged at 32°C, two *V. coralliilyticus*-challenged at 29.5°C and 32°C, respectively, and two Oceanospirillales-challenged corals at 29.5°C and 32°C, respectively). Progression was relatively slow and no fragment sustained >25% mortality by the time of sampling or the end of experiment.

Response of Symbiodinium to bacterial challenges and heat stress

Symbiodinium C• (van Oppen, 2004) was the only *Symbiodinium* type detected in all fragments, as determined by SSCP profiling of the nuclear ribosomal ITS1 region (Suppl. Fig. S4A). Both effective (Y(II) and maximum (Fv/Fm) quantum yield declined over time in all treatments (Suppl. Fig. S4B,D and Suppl. Fig. S4C,E). Generally, a consistent pattern was observed, comprising an initial decrease in Y(II) and Fv/Fm during the heating stage (Days 0 – 9), followed by a slight recovery and then a subsequent second period of decline. The rate of decline differed significantly among treatments (Suppl. Fig. S4B,C). For Fv/Fm, the slope of the models for all treatments at 32°C had a significantly larger negative coefficient compared to all treatments at 27°C and 29.5°C; however no statistically significant difference was detected between the treatments at 27°C and 29.5°C. Similar results were obtained for Y(II), with the exception that yields for Oceanospirillales-challenged corals at 27°C were higher than for all treatments at 29.5°C. Notably, effective quantum yield of corals exposed to *V. coralliilyticus* at 32°C was significantly lower than the yields of Oceanospirillales S47-challenged or unchallenged corals at 32°C. The results of statistical analyses for both effective and maximum quantum yield can be found in Supplementary File S2 – ST1.

Analyses of the *de novo* transcriptome of the *M. aequituberculata* holobiont identified 12,913 genes as *Symbiodinium* genes. Of these genes, 11,909 were also found in the RNA Seq dataset. Gene expression was significantly impacted by

temperature stress (ADONIS $p < 0.001$), but not by bacterial challenges (Fig. 1A; Suppl. File S2 - ST2). Overall, 540 genes were differentially expressed in response to elevated temperatures (Suppl. File S3), but significant overlap in the differentially expressed genes was observed between the different heat stress treatments (Fig. 1B,C). In the 32°C treatments, *Symbiodinium* upregulated 209 genes and downregulated 257 genes compared to the 27°C treatments. Gene enrichment analysis based on the Gene Ontology annotations showed that at 32°C, mostly DNA, RNA, amino acid and nitrogen metabolic processes were affected (Fig. 1D; Suppl. File S2 – ST3). However, *Symbiodinium* also showed a significant differential response in the GO category 'humoral immune response', potentially involving a range of transmembrane receptors (Fig. 1D; Suppl. File S2 – ST3). This immune response by *Symbiodinium* was also found when comparing gene expression signatures of the 29.5°C and 32°C treatments (Fig. 1E; Suppl. File S2 – ST3), suggesting it was elicited by a factor related to high heat stress. Analysis at the gene level showed that the expression of homologues of genes involved in anti-viral (*mind-bomb, CHMP5, cdc37, ZC3HAV1, sec13*) and immune responses (e.g. *ADAMTSL4, CdPK2, MIF, DUSP10, calmodulin, ANXA4*), as well as cell survival and heat shock/chaperone (*HSP90B1, calnexin, DNAJB6, STIP1, FES1*) genes were significantly modulated (Suppl. Fig. S5). In addition, the profile of 57 up- and 52 downregulated genes in the 29.5°C-32°C comparison indicated significant negative effects on photosynthesis, particularly due to impacts on the thylakoid and other chloroplast components at 32°C (Fig 1E; Suppl. File S2 – ST3).

The expression profile of *Symbiodinium* genes in corals at 29.5°C showed expression characteristics of *Symbiodinium* at both 27°C and 32°C (Fig. 1A,C). The expression of 58 genes was increased while the expression of 64 genes was decreased at 29.5°C compared to 27°C treatments, and GO analysis suggested that RNA processing was significantly reduced at mild heat stress (Suppl. File S2 – ST3). No genes were differentially expressed by *Symbiodinium* in response to bacterial challenges. Full details on differential gene expression analysis, including DESeq2 results and gene annotations can be found in Supplementary Files 3 and 4.

Gene expression response of the coral host under elevated temperatures

The *M. aequituberculata* holobiont transcriptome contained 54,196 sequences that were identified to be of coral host origin. Through TagSeq analysis, we obtained

reads for 36,454 of these sequences. Temperature was found to be the main driver of the differential gene expression patterns observed (ADONIS p = 0.001; Fig. 2A,B; Suppl. File S2 – ST2). While only 79 genes (51 down- and 28 upregulated) were differentially expressed following a 2.5°C increase in seawater temperature to 29.5°C, the coral host differentially expressed 1062 genes (527 down- and 535 upregulated) in response to a 5.5°C temperature increase (Fig. 2C; Suppl. File S5). Further, 373 DEGs (190 down- and 183 upregulated) were found between the 29.5°C and 32°C treatments (Fig. 2C; Suppl. File S5). Significant overlap in the DEGs was observed between all temperature treatments (Fig. 2C). Using differential GO category analysis, we found only limited impacts of DEGs on functional processes in the coral host: the primary cellular processes affected were vesicle-mediated transport and signalling processes (increased at elevated temperatures), and ribosome biogenesis and nucleosome assembly (decreased at elevated temperatures (Fig. 2D; Suppl. File S2 – ST3)

To investigate the stress and immune responses of *M. aequituberculata* under elevated seawater temperatures, we assessed differential expression at the gene level (Fig 2E, Suppl. Fig. S6). At elevated temperatures, the coral expressed various molecular chaperones from the Hsp90, Hsp26/42 and DNAJ families at significantly higher levels than at 27°C. Multiple genes involved in the antioxidant response were also upregulated, including peroxiredoxin and transcription factors belonging to the Maf family. In contrast, the bicarbonate transporter SLC26 was downregulated at elevated temperatures. Numerous genes with a putative role in the coral immune response (i.e., transcripts annotated with gene names associated with immune responses in other organisms) were also differentially expressed; at 32°C, 98 genes were upregulated and 63 downregulated; at 29.5°C, 1 was down-regulated and only were 8 upregulated compared with 27°C; while at 32°C 19 genes were down-regulated and 41 up-regulated compared with 29.5°C (Suppl. Fig. S6). These genes were involved in various processes of the immune response, including Toll-like receptor signalling, apoptosis, cytokine production, anti-viral responses, phagocytosis, complement system, immune cell activation, hypoxia-induced inflammation as well as in negative feedback loops that regulate the aforementioned processes (Suppl. Fig. S6; Suppl. Table S1). Details of all differentially expressed genes, including DESeq2 results and gene name annotations can be found in Supplementary Files 4 and 5.

Gene expression response of the coral host to bacterial challenges

Although no significant effect of the bacterial challenges (ADONIS p = 0.428) or an interactive effect of elevated temperatures and bacterial challenges (ADONIS p = 0.483) on coral gene expression patterns were observed (Suppl. File S2 – ST2), *M. aequituberculata* differentially expressed a number of genes following exposure to *V. coralliilyticus* or Oceanospirillales S47 (Fig 3; Suppl. File S5). Corals exposed to either bacterium showed a differential expression of genes involved in the circadian clock: 1) an upregulation of circadian locomotor output cycles kaput (*CLOCK*) and 2) a downregulation of cryptochrome (*CRY*). In addition, a subunit of protein phosphatase-1 (*PPP1R3C/D*) was downregulated, while the hairy/ enhancer of split related with YRPW motif (*HEY*) gene was upregulated in both Oceanospirillales and *V. coralliilyticus*-challenged corals. These effects were significantly more pronounced in *V. coralliilyticus*-challenged corals than those exposed to Oceanospirillales S47. Corals exposed to this potential coral pathogen also showed an increased expression of histamine receptors and the transporter of the phenoloxidase substrate L-DOPA *SLC16A10*, while the antioxidant thioredoxin was downregulated.

Biochemical responses of the coral host

PO activity was significantly higher in *V. coralliilyticus*-challenged corals compared to controls and Oceanospirillales S47-challenged corals on Day 2, and changed over time in corals exposed to *V. coralliilyticus* at 29.5°C and those exposed to Oceanospirillales S47 at 32°C (Suppl. Fig. S7A; Suppl. File S2 – ST4). Total potential PO activity changed over time depending on the bacterium that corals were challenged with (Suppl. Fig. S7B; Suppl. File S2 – ST4). Particularly, corals that were challenged with *V. coralliilyticus* at 29.5°C or 32°C, or with Oceanospirillales S47 at 32°C all showed higher tpPO activity levels on Day 10 compared to Day 22. Oceanospirillales S47-challenged corals at 29.5°C, however, showed significant increases over time in tpPO activity. No patterns in GFP-like protein expression could be discerned (Suppl. Fig. S7C-G).

Effect of bacterial exposure and heat stress on coral-associated bacterial communities

The microbiome of *M. aequituberculata* was highly dominated by Alphaproteobacteria and to a lesser extent by Gammaproteobacteria,

Deltaproteobacteria, Clostridia and Flavobacteria (Fig. 4A). Shifts in the beta diversity of the microbiome were apparent between the sampling time points (all $p < 0.0001$) (Figure 4B; Suppl. File S2 – ST5) and time was also the explanatory factor in the observed differences in alpha diversity (Suppl. File S2 - ST6/ST7). Surprisingly, however, we did not observe any effects of temperature ($p = 0.3227$) or bacterial challenges ($p = 0.4098$) on microbiome diversity, nor any interactive effects (Suppl. File S2 – ST5/ST6/ST7). Using differential abundance analysis at the OTU level, we investigated which bacteria (out of a total of 19,915 unique OTUs) were responsible for the observed temporal shifts. While some rare OTUs increased or decreased in number over time, the temporal shifts in diversity could be largely attributed to OTUs belonging to the most abundant classes (Suppl. File S6) such as members of the Rhodobacterales (families of Rhodobacteraceae and Hyphomonadaceae), Rhizobiales (family Hyphomicrobiaceae), Flavobacterales (family Flavobacteriaceae) and Planctomycetia (orders Pirellulales and Planctomycetales) as well as various Gammaproteobacteria. Main differences observed were 1) higher relative abundances of bacteria in the genera *Rhodovolum* and *Dinoroseobacter* (Rhodobacteraceae) on Days 10 and 22 compared with Day 1, and 2) higher relative abundance of Alphaproteobacteria on Day 10 due to increases in Rhodobacteraceae, BD7-3 and Rhizobiales and lower numbers of Flavobacteria and Planctomycetia OTUs. It should, however, be noted that no other particular patterns could be discerned as OTUs belonging to the same taxonomic order/family both decreased and increased in abundance between the same time points. Surprisingly, no increases in Oceanospirillales S47 or *V. coralliilyticus* OTU abundances were observed. All *Vibrio* sp. sequences observed in our data were identified as *V. coralliilyticus* and were only present in corals challenged with this bacterium. Overall, these results indicate that the shifts were primarily caused by restructuring of the native host-associated bacterial assemblages.

Discussion

This study used a holistic approach to elucidate the responses of three components of the *Montipora aequituberculata* holobiont (coral host, *Symbiodinium* and the bacterial community) to bacterial challenges and elevated seawater temperatures. The coral host exhibited 1) a transcriptomic response at high seawater

temperatures involving many differentially expressed genes homologous to genes implicated in the immune response of other organisms, and 2) a differential gene expression response following exposure to the potentially pathogenic bacterium *V. coralliilyticus* or the commensal bacterium Oceanospirillales S47. *Symbiodinium* responded at the transcriptome level to temperature stress rather than to the pathogen challenge, despite negative impacts of the coral pathogen *V. coralliilyticus* on the effective quantum yields of *Symbiodinium* when the holobiont was exposed to high temperatures. The coral-associated bacterial community did not change with temperature stress or bacterial exposures. We hypothesize that the absence of visual signs of coral disease development over the course of our 22-day study indicates that holobiont responses were sufficient to prevent any visual signs of coral disease development over the course of our 22-day study.

Symbiodinium response to heat stress

The algal endosymbiont *Symbiodinium* C• regulated a multitude of genes under elevated seawater temperatures, including various stress and immune response genes. Under experimental temperatures 2°C above long-term summer means at the study site, several heat shock proteins and antioxidants were upregulated, and the expression of genes involved in metabolism and photosynthesis were downregulated. Temperature stress is known to result in the generation of reactive oxygen species (ROS), triggering an antioxidant response by the algae to mitigate their cell-damaging effects, as well as misfolding of and damage to proteins; this likely explains the upregulation of various heat shock proteins and their co-chaperones. Our findings are in line with a previous study showing significant transcriptomic responses of cultured *Symbiodinium* under heat stress involving antioxidant and heat shock response genes (Gierz, Forêt, & Leggat, 2017; Levin, Voolstra, Weynberg, & van Oppen, 2017). However, other studies have found no or limited transcriptomic responses in coral-associated *Symbiodinium* (Barshis, Ladner, Oliver, & Palumbi, 2014; Leggat, et al., 2011) or in culture (Baumgarten et al., 2013) under high temperatures (up to 36°C). Differences between our work and these previous studies may be partly due to different *Symbiodinium* types regulating a different set of genes, and this may contribute to differences in bleaching susceptibility among corals harbouring different *Symbiodinium* types. However, significant transcriptomic responses to heat stress in

Symbiodinium may also be observed primarily when phenotypic effects are present, as found here and previously (Gierz, et al., 2017; Levin, et al., 2017). In addition, we may have observed an immune response by *Symbiodinium* under heat stress, which may in part be related to the general cellular stress response (CSR; described in more detail below). However, *Symbiodinium* also exhibited an anti-viral response, suggesting that these algae experienced a virus infection when seawater temperatures were elevated, which may in part be responsible for the observed reduction in photochemical efficiency. This finding is consistent with recent studies linking viruses to the thermal sensitivity of *Symbiodinium* and bleaching (Correa et al., 2016; Levin, et al., 2017). Overall, *Symbiodinium* appeared to have experienced a viral infection that may have impacted its photochemical efficiency under heat stress, causing cellular stress and resulting in corresponding immune and stress responses.

Symbiodinium C• did not show a transcriptomic response to bacterial challenges, despite a significant drop in its photochemical efficiency in the *V. coralliilyticus* exposure treatment at 32°C, but not at 29.5°C. The virulence of *V. coralliilyticus* increases at elevated seawater temperatures (Ben-Haim, et al., 2003; Kimes, et al., 2012), which is possibly driven by prophages (Weynberg, Voolstra, Neave, Buerger, & van Oppen, 2015) and results in the secretion of a zinc metalloprotease virulence factor that damages the photosystem II of the dinoflagellate (Sussman, et al., 2009). Our results indicate that *V. coralliilyticus* P1 was virulent towards the *M. aequituberculata* holobiont at temperatures where photochemical efficiency of *Symbiodinium* was also reduced due to significant heat stress. Taken together, the effect of heat stress on *Symbiodinium* (photo)physiology may have been synergistically exacerbated by heat stress-induced *V. coralliilyticus* pathogenicity and viral infections.

Coral-associated bacterial community

Elevated seawater temperatures have previously been shown to cause shifts in bacterial assemblages towards pathogenic species (Bourne, et al., 2008; Littman, et al., 2011; Ritchie, 2006), potentially resulting in increased disease prevalence. Surprisingly, temperature did not cause any shifts in the bacterial community associated with *M. aequituberculata*. Even the repeated addition of large cell numbers of Oceanospirillales S47 and *V. coralliilyticus* did not result in measurable changes in the bacterial assemblages towards these species (although *V. coralliilyticus* was

detected only in those challenged fragments). All shifts observed in our study occurred over time and appeared to be the result of an alteration in the native bacterial community as a whole and not due to changes in the abundance of a small number of species or families. The *Montipora* genus, unlike other reef-building corals, produces a wide range of active compounds with antimicrobial properties, including montiporic acids A, B, C and D which can inhibit the growth of a range of marine pathogens including *Serratia marcescens* and *Vibrio harveyi* (Fusetani, Toyoda, Asai, Matsunaga, & Maruyama, 1996; Kodani, Sato, Higuchi, Casareto, & Suzuki, 2013; Marquis, Baird, de Nys, Holmström, & Koziumi, 2005; Sato, Casareto, Suzuki, & Kodani, 2013). These molecules potentially act as a selective filter on the associated bacterial communities and might have prevented shifts towards *Vibrio*- or Oceanospirillales-dominated communities and temperature-induced changes.

Stress and immune responses by M. aequituberculata under elevated seawater temperatures

The coral host showed major stress and immune responses under elevated seawater temperatures, particularly at 32°C, involving several major innate immune response mechanisms (Table S1). We hypothesize that the immune response was initiated by the upregulated TLR signalling pathway (Figure 5(I)), which, in turn, was responsible for the production of pro-inflammatory cytokines (Figure 5(III)). Immune cells are activated through cytokine receptor signalling (Figure 5(IV)) and migrate towards the site of infection along a chemotactic gradient of cytokines (Figure 5(III)). In addition, the lectin-complement system may have been induced to tag invading microbes for phagocytosis (Figure 5(VI)). Using their ficolin/lectin receptors and scavenger receptors (Figure 5(VII)), immune cells phagocytose (Figure 5(VIII)) and subsequently eliminate the microbes (Figure 5(X)). There were also indications of an anti-viral response by *M. aequituberculata* under heat stress (Figure 5(XI). In addition, we found evidence of negative feedback mechanisms regulating the coral immune response. The exact causative agent eliciting the major immune response in this coral at 32°C, however, remains to be identified.

Under stressful conditions, organisms exhibit a cellular stress response (CSR), which is conserved throughout the taxonomic kingdoms and induced regardless of the nature of the stress. Overall, the CSR can be subdivided into 1) the unfolded protein response (UPR), 2) DNA damage response (DDR), 3) heat shock response (HSR) and

4) oxidative stress/redox regulation (Kultz, 2005), and generally coincides with increased energy metabolism, but reduced RNA metabolism and ribosome biogenesis (Gasch et al., 2000). A meta-analysis of studies investigating the environmental stress response of oysters under various stress conditions also identified a group of consistently modulated stress genes involved in immunity (lectins, MAMP-binding proteins, AMPs and complement system), cell signalling and the cytoskeleton (Anderson et al., 2015). In our study, we observed that numerous genes involved in the CSR were indeed differentially expressed and that ribosome biogenesis was also negatively impacted, indicating that the CSR was activated in *M. aequituberculata* under heat stress conditions. Besides, the differential expression of lectins and components of the complement system and the cytoskeleton, suggests that the environmental stress response in corals and oysters may be similar. Concerning the CSR, our results are also consistent with previous studies investigating the coral response to elevated seawater temperatures, showing heat shock, unfolded protein and oxidative stress responses as well as decreased ribosome biogenesis (Bay & Palumbi, 2014; Bay & Palumbi, 2015; Bellantuono, Granados-Cifuentes, Miller, Hoegh-Guldberg, & Rodriguez-Lanetty, 2012; Maor-Landaw et al., 2014; Maor-Landaw & Levy, 2016; Meyer, et al., 2011; Rodriguez-Lanetty, et al., 2009; Vidal-Dupiol, et al., 2014; Voolstra, et al., 2009).

In parallel to the conserved stress responses, organisms exhibit responses specific to the stress encountered. Here, we showed that *M. aequituberculata* exhibited a major immune response under elevated seawater temperature, upregulating a large number of genes that are putatively involved in various immune defence pathways (Table S1), while downregulating negative regulators of the immune response. In fact, immune genes represented nearly 18% of the differential transcriptome. The potential stimulatory cross talk between the CSR and the immune system in case of infection and inflammation (Muralidharan & Mandrekar, 2013), could have been implicated in boosting the immune response observed and a causative agent of microbial origin is therefore most probable.

Although the major immune response observed may have prevented a shift in the coral-associated bacterial community and the establishment of known coral pathogens under heat stress conditions, it cannot be excluded that some of the immune responses observed were elicited by non-bacterial microbes. Interestingly, we found upregulation of numerous genes involved in anti-viral responses, including detection,

signalling and effector molecules (see Table S1 for gene functions). In fact, the toll-like receptor TLR4 is known to recognize viruses, initiating the immune response via signal transduction pathways, and TNFR signalling has also been linked to anti-viral responses. Their simultaneous signalling could result in the activation of IRF1, a key regulator of the anti-viral response, and reinforcement of pro-inflammatory cytokine signalling. Increased expression of various MAMP-binding proteins that may target viruses, including scavenger receptors (e.g. DMBT1, DSCAM), ficolins and lectins, indicate involvement of the complement system and phagocytosis. Using their PtdSer-specific receptors activated immune cells, may have cleared the apoptotic cells. Although speculative, our results provide indications for active virus infections in *M. aequituberculata* under heat stress conditions. This is in accordance with recent transcriptomic studies that found increased numbers of potential viral transcripts in stressed and diseased corals. It is therefore crucial to further assess the roles viruses play in coral holobiont health and their implications in disease development. Which viruses may have been involved in this study, is currently under investigation.

In contrast to our study, downregulation of putative immune and apoptosis genes under heat stress (Rodriguez-Lanetty, et al., 2009; Vidal-Dupiol, et al., 2014) or in bleached corals (Pinzón, et al., 2015) has been previously reported. Suppression of the immune system and apoptosis could lead to a reduced capacity to respond to pathogens, which may result in increased disease incidence (Ainsworth, Kvennefors, Blackall, Fine, & Hoegh-Guldberg, 2007; Libro, et al., 2013). The significant immune response combined with the lack of disease development under heat stress conditions observed here, shows that *M. aequituberculata* is relatively tolerant to elevated temperatures and able to resist changes in its bacterial community. The population where we sourced our coral fragments from experiences relatively high seawater temperatures in summer (average 30.5C), and as such this thermal history may have provided these corals with increased stress resistance through both local adaptation and acclimatisation (Barshis, et al., 2013; Bellantuono, et al., 2012).

Host response to bacteria and bacterial community regulation in corals

By challenging *M. aequituberculata* with both a potentially pathogenic and a commensal bacterium, we were able to reveal a general mechanism employed by corals to regulate their bacterial community. First, we identified two genes involved in the circadian cycle (cryptochrome and CLOCK), which may be at the basis of this

microbiome regulatory process. These genes may play a role in the coral immune response; a function only recently described in mice and humans (Curtis, Bellet, Sassone-Corsi, & O'Neill, 2014; Narasimamurthy et al., 2012). Cryptochrome, a repressor of expression of the *CLOCK* gene, was downregulated in response to bacterial challenges, which was likely linked to the upregulation of *CLOCK*. Reduced levels of cryptochrome have previously been linked to increased expression of pro-inflammatory cytokines and inducible nitric oxide synthase, which plays a role in the anti-microbial oxidative burst following phagocytosis or encapsulation (Curtis, et al., 2014), and was recently reported in pathogen-challenged corals (Wright et al., 2017). In addition, CLOCK represses the anti-inflammatory function of the glucocorticoid receptor, enhances the activity of NF-κB and positively regulates the expression of Toll-like receptors (e.g. TLR9) and their downstream transcription factors (FOS and JUN) (Curtis, et al., 2014), (Scheiermann, Kunisaki, & Frenette, 2013). To orchestrate a proper immune response, TLR signalling is tightly regulated via the interferon-γ and Notch signalling pathways (Hu et al., 2008). Notch signalling is crucial in the development of immune cells (Radtke, Fasnacht, & MacDonald, 2010; Yuan, Kousis, Suliman, Visan, & Guidos, 2010). Our results showed an increased expression of the Notch target gene HEY, which selectively regulates expression of several interleukin cytokines (Hu, et al., 2008), in response to both bacterial challenges. Immune signalling was further regulated through the downregulation of protein phosphatase-1 (*PP1*). This protein inhibits the Erk2, MAPK p38 and NF-κB pathways (Jin, Yan, Ma, Cao, & He, 2011; Nika et al., 2004; Saxena, Williams, Tasken, & Mustelin, 1999), which play major roles in the TLR-mediated immune response, and reduced PP1 expression may therefore result in higher activity of these pathways, leading to increased immune function. Previous studies have also identified genes involved in the TLR pathway to be upregulated following pathogen challenges of corals (Vidal-Dupiol, et al., 2014). Taken together, the general mechanism to regulate the microbiome appears to primarily involve TLR-dependent immune system regulation. As TLRs are essential for anti-microbial peptide expression and maintenance of a stable microbiome in *Hydra* (Franzenburg, et al., 2012), our results provide novel insights into the potential mechanisms underlying the regulation of coral-associated microbial communities.

Several immune system-related genes were also specifically upregulated in response to exposure to *V. coralliilyticus*, suggesting the coral did recognise this

potential pathogen as a significant threat. Homologues of histamine receptors, which are known to regulate immuno-stimulatory cytokine production (O'Mahony, Akdis, & Akdis, 2011) as well as a member of the solute carrier family 16 (SLC16A10) were upregulated. Increased expression of histamine receptors was recently also found in coral tissues affected by white syndrome, the disease caused by *V. coralliilyticus* (Wright, et al., 2017), indicating that this might be a specific response to this pathogen. As SLC16A10 transports the PO substrate L-DOPA (Kim et al., 2002), this suggests involvement of the melanisation cascade in the anti-*V. coralliilyticus* response. Indeed, the amount of active PO was generally higher in *V. coralliilyticus*-challenged corals thus requiring more substrate to be available to fulfil its immune function. Overall, these results corroborate a recent study showing a primary role for the melanisation cascade in the response of *Pocillopora damicornis* against *V. coralliilyticus* (Vidal-Dupiol, et al., 2014). Similarly, the disease-resistant coral *Porites astreoides* also exhibits a PO-based immune response when exposed to PAMPs (Palmer, et al., 2011). Overall, our results provide insights into the mechanistic basis of microbiome regulation in corals that facilitate maintenance of a healthy bacterial community, and the specific immune response mechanisms against bacterial pathogens.

Resistance to coral pathogens?

The general lack of disease development in our study was surprising. The *V. coralliilyticus* P1 strain had been isolated from colonies of *M. aequituberculata* exhibiting white syndrome signs and used to successfully re-infect healthy colonies of *M. aequituberculata* with the isolated strain, thereby fulfilling Koch's postulates and identifying this bacterium as the causative agent of this disease (Sussman, et al., 2008). To make sure that culturing would not affect properties of *V. coralliilyticus*, we conducted our study using the primary isolate of this strain and sourced our corals from the same population. Despite these precautions, we were unable to cause disease in healthy corals and therefore hypothesise that this *M. aequituberculata* population has developed some degree of resistance to this coral pathogen. Similarly, *Vibrio shilonii* used to be implicated in bleaching in the coral *Oculina patagonica* (Kushmaro, Loya, Fine, & Rosenberg, 1996; Kushmaro, Rosenberg, Fine, & Loya, 1997); however, following several outbreaks, infection by this pathogen has not been observed in the field and cannot be re-established experimentally (Reshef, Koren,

Loya, Zilber-Rosenberg, & Rosenberg, 2006). *Serratia marcescens* has also been reported to be incapable of infecting *Acropora palmata*, despite being associated with white pox in this species a decade earlier (Joyner et al., 2015). While the exact mechanisms of resistance to pathogens is unknown, it could be the result of the selective elimination of disease-sensitive corals from the population, holobiont adaptation or potentially immunological memory. In addition, the coral probiotic hypothesis (Reshef, et al., 2006) may also be applicable to the disease resistance observed in our and other studies, posing that adjustments in the microbiome towards bacteria capable of warding off pathogens may prevent infection and disease. Taken together, if coral populations are indeed capable of developing resistance to diseases over relatively short time frames, it could be very promising for the future of coral reefs.

In summary, we assessed three components of the holobiont of the coral *Montipora aequituberculata* exposed to elevated seawater temperatures and a potentially pathogenic or commensal bacterium. We found that, regardless of heat stress, the coral was capable of orchestrating an immune response towards the pathogenic bacterium and maintain a stable bacterial community, and identified potential conserved bacterial-response mechanisms used by corals. As we did not observe any significant impact on the coral-associated bacterial communities under elevated seawater temperatures either, the immune responses exhibited by both the coral host and *Symbiodinium* under these conditions were likely directed against both heat stress and a microbe of non-bacterial origin. Anti-viral responses in both the coral and *Symbiodinium*, suggest that viral infections may affect holobiont health during climatic stress events. Overall, however, the responses exhibited by the holobiont were sufficient to prevent the development of visual signs of disease and tissue loss.

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Data Accessibility

All sequencing data (16S rRNA gene amplicon, TagSeq and Transcriptome) has been made available through the NCBI Sequence Read Archive under BioProject number PRJNA419467 and SRA Accession number SRP125476.

Montipora aequituberculata transcriptome – assembled and annotated: http://matzlab.weebly.com/data--code.html

Author Contributions

J.vd.W., B.W., D.G.B. and M.v.O. designed the experiment. J.vd.W and M.C.D.M. conducted the experiment. J.vd.W and M.C.D.M. analysed the samples and data. G.D. developed the transcriptome. J.B.R. isolated and characterised the Oceanospirillales S47 strain. All authors contributed to the writing of the manuscript.

Supporting Information

Suppl. Figure S1 – Experimental Design

Suppl. Figure S2 – Photo Timeline Coral Fragments

Suppl. Figure S3 – Diseased Coral Fragment

Suppl. Figure S4 – Symbiodinium Genotyping and Photochemical Efficiency

Suppl. Figure S5 – Heatmap DEGs *Symbiodinium* at elevated temperatures

Suppl. Figure S6 – Heatmap DEGs *M. aequituberculata* at elevated temperatures

Suppl. Figure S7 – Biochemical Parameters (Phenoloxidase Activity and GFP-like Proteins)

Suppl. File S1 – Material & Methods (detailed)

Suppl. File S2 – Tables Statistical Analysis and Primer Sequences

Suppl. File S3 – DEGs *Symbiodinium* (DESeq2 Results)

Suppl. File S4 – Scripts and Input Data

Suppl. File S5 – DEGs *M. aequituberculata* (DESeq2 Results)

Suppl. File S6 – Differentially Abundant OTUs (DESeq2 Results)

Suppl. Table S1 – Putative Functions of Immune & Stress Response Genes

Figure Legends

Figure 1 – Impact of elevated seawater temperatures and bacterial challenges on *Symbiodinium*. **(A)** Principal Coordinate Analysis plot representing the differences in the overall gene expression patterns in *Symbiodinium* endosymbionts following exposure to elevated seawater temperatures and bacterial challenges. **(B)** Venn diagram depicting the number of differentially expressed genes between the three temperature treatment comparisons. **(C)** Global profile of the differentially expressed genes in *Symbiodinium* at 32°C. **(D-E)** Gene ontology biological processes categories significantly enriched with genes either positively (red) or negatively (blue) responding to elevated temperatures at **(D)** 32°C and **(E)** 29°C. The dendrogram depicts the sharing of genes between GO categories, and font type indicates the multiplicity-corrected p-value.

Figure 2 – Impact of elevated seawater temperatures and bacterial challenges on the coral host *Montipora aequituberculata*. **(A)** Principal Coordinate Analysis plot representing the differences in the overall gene expression patterns in *M. aequituberculata* following exposure to elevated seawater temperatures and bacterial

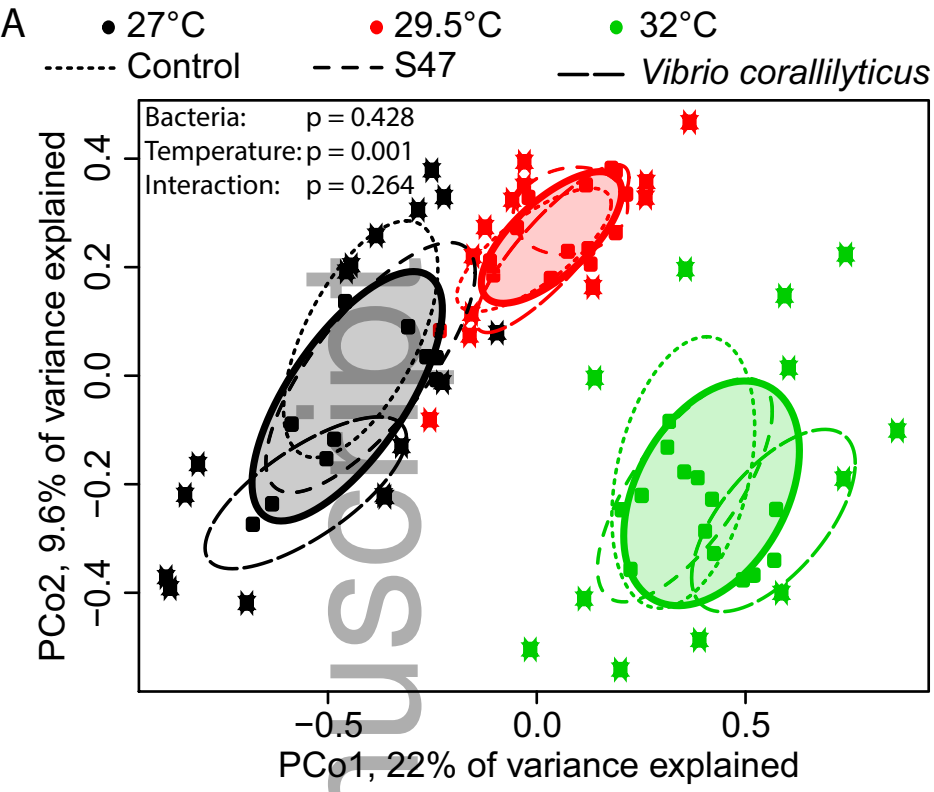
challenges. **(B)** Venn diagram depicting the number of differentially expressed genes between the three temperature treatment comparisons. **(C)** Global profile of the differentially expressed genes in *M. aequituberculata* at 32°C. **(D)** Gene ontology biological processes categories significantly enriched with genes either positively (red) or negatively (blue) responding to elevated temperatures at 32°C. Font type indicates the multiplicity-corrected p-value. The dendrogram depicts the sharing of genes between GO categories, and font type indicates the multiplicity-corrected p-value.

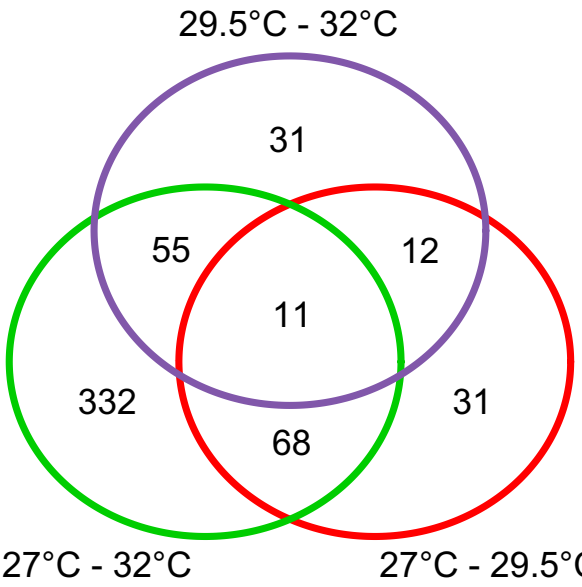
Figure 3 – Transcriptomic response of the coral host *Montipora aequituberculata* to bacterial challenges. Heatmap of the expression profile of the 10 annotated differentially expressed genes following bacterial challenges with Oceanospirillales S47 or *Vibrio coralliilyticus*.

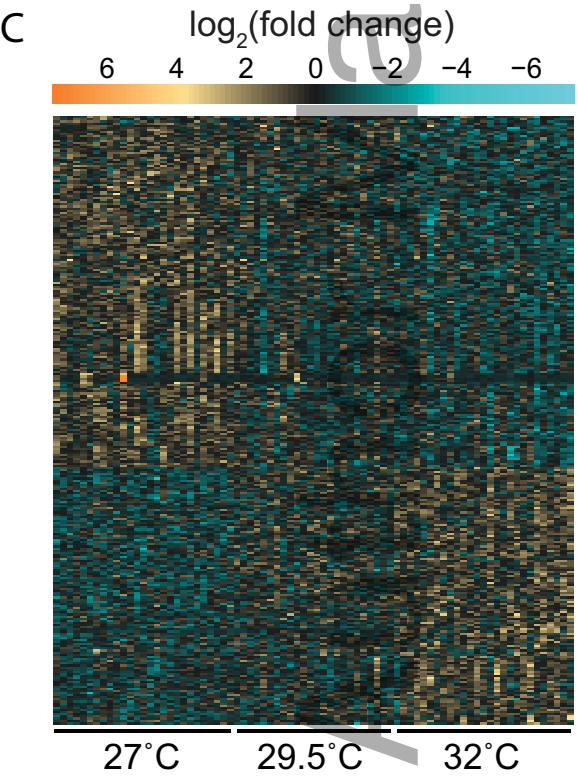
Figure 4 – Composition of coral-associated bacterial communities and the impacts of heat stress and bacterial challenges on the microbiome. **(A)** Relative contributions of the most abundant bacterial taxonomic classes (>0.1%) to the bacterial communities of *M. aequituberculata* under experimental treatments over time. **(B)** Principal coordinate analysis of beta diversity based on Bray-Curtis dissimilarity matrices, showing changes in the coral microbiome over time.

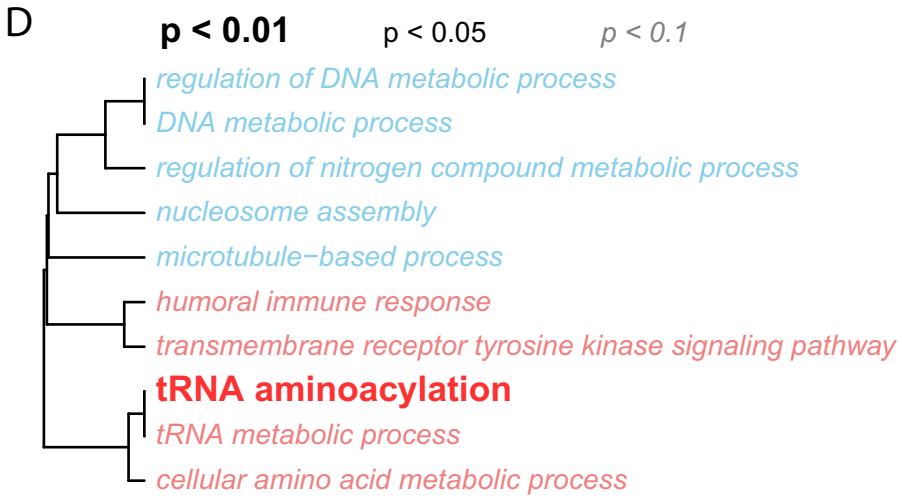
Figure 5 – Overview of the putative innate immune responses by *Montipora aequituberculata* under heat stress**.** (I) MAMP-activated Toll-like and NOD-like receptors as well as TNFα-activated tumor necrosis factor (TNF) receptors induce various signal transduction pathways via TRAFs, including NF-κB and MAPK (e.g. JNK and MAPK p38) pathways, resulting in transcription of immune genes (II). In addition, apoptosis may be induced via a caspase-mediated pathway. Products of transcribed immune genes may have intracellular functions or are exocytosed (III). Exocytosed cytokines have immunomodulatory functions, regulating immune gene expression and providing a chemotactic gradient for immune cell recruitment via cytokine receptor signalling (IV). In addition, activated phenoloxidase (PO) forms a microbe-immobilising barrier of melanin and produces cytotoxic compounds (V). The lectin-complement system (VI) is initiated by binding of a lectin to MAMPs and results in the proteolytic cleavage of C3 into C3b, which is deposited onto the

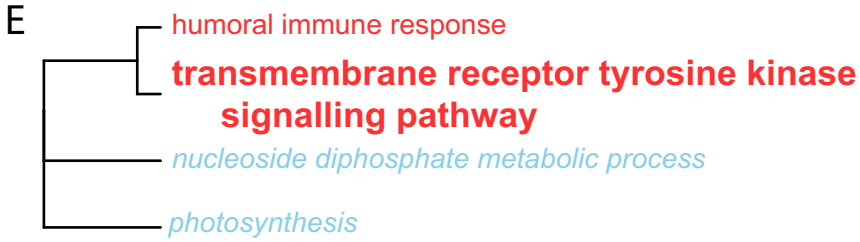
microbe. Via C3-specific receptors, C3b may induce phagocytosis of the microbe (VIII). Similarly, scavenger receptors may bind to microbes and induce phagocytosis (VII). Maturation of the phagosome leads to the formation of a microbicidal phagolysosome (IX). Destructive reactive radicals are neutralised by antioxidants to prevent host damage (X). Potential viral infections may be repressed by RNA interference against viral mRNA transcripts and direct inhibition of the virus productive cycle through DNA modification and inhibition of viral proteins (XI). Abbreviations: MAMP, microbe-associated molecular pattern; TLR, Toll-like receptor; NLR, nucleotide-binding oligomerisation domain (NOD-like) receptor; TNFR, tumor necrosis factor receptor; TRAF, TNF receptor-associated factor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa B; proPO, pro-phenoloxidase; C3, complement C3; NO, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidase; AMP, anti-microbial peptide.











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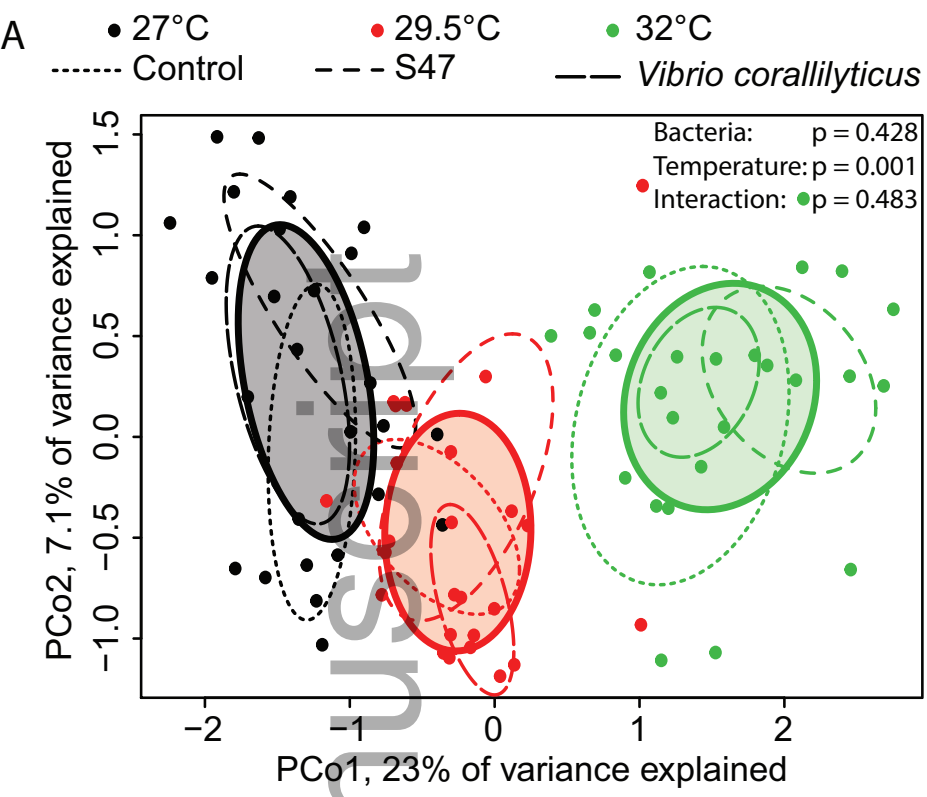

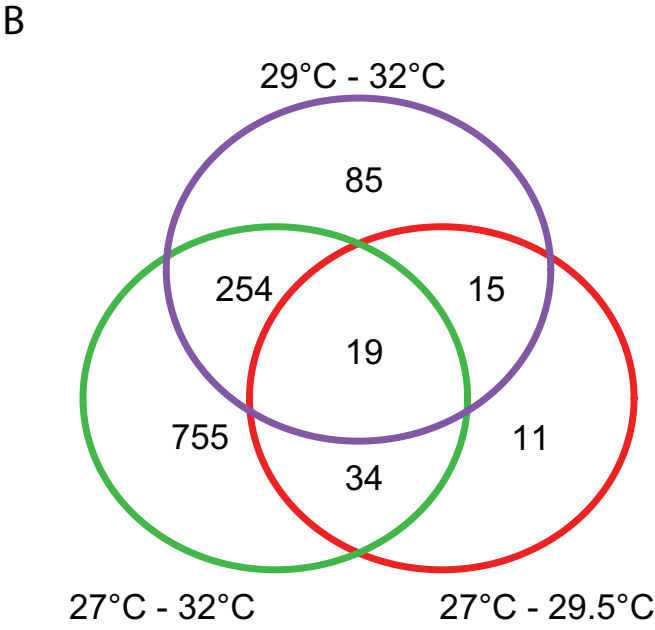

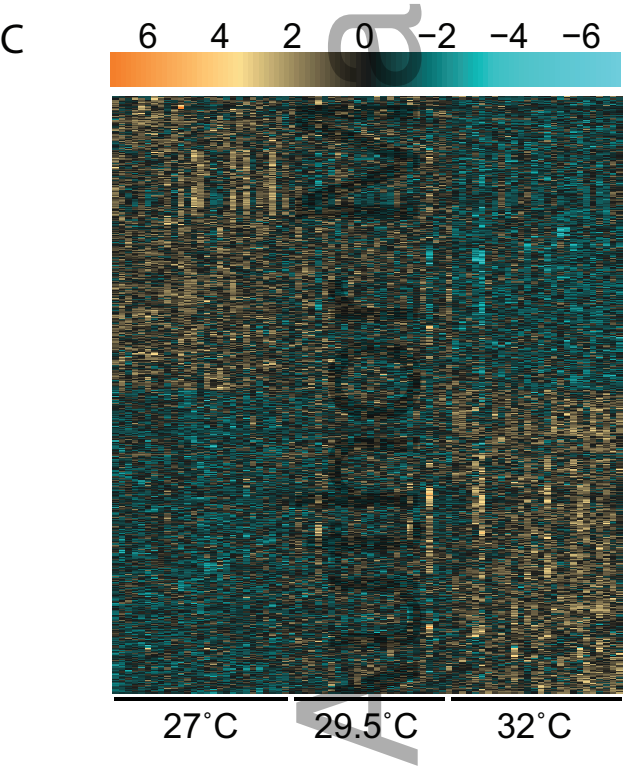

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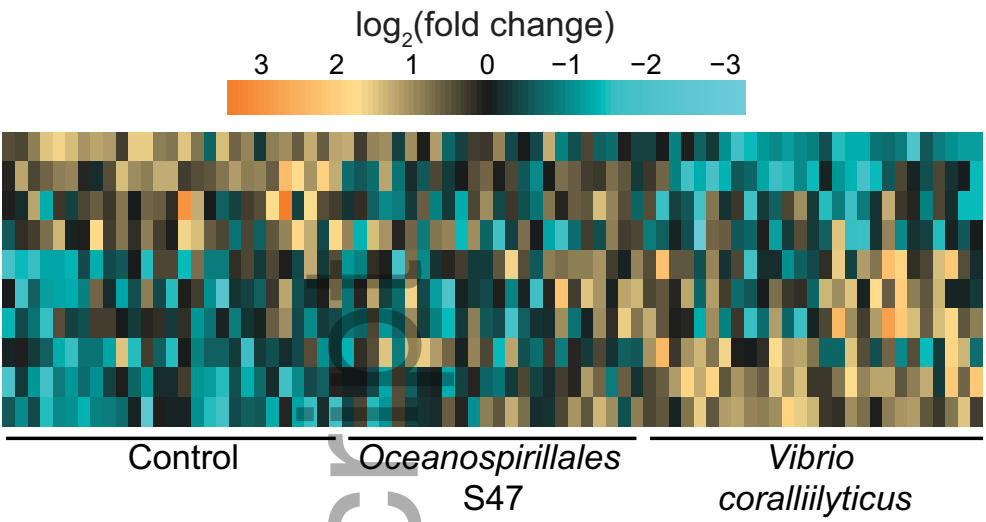

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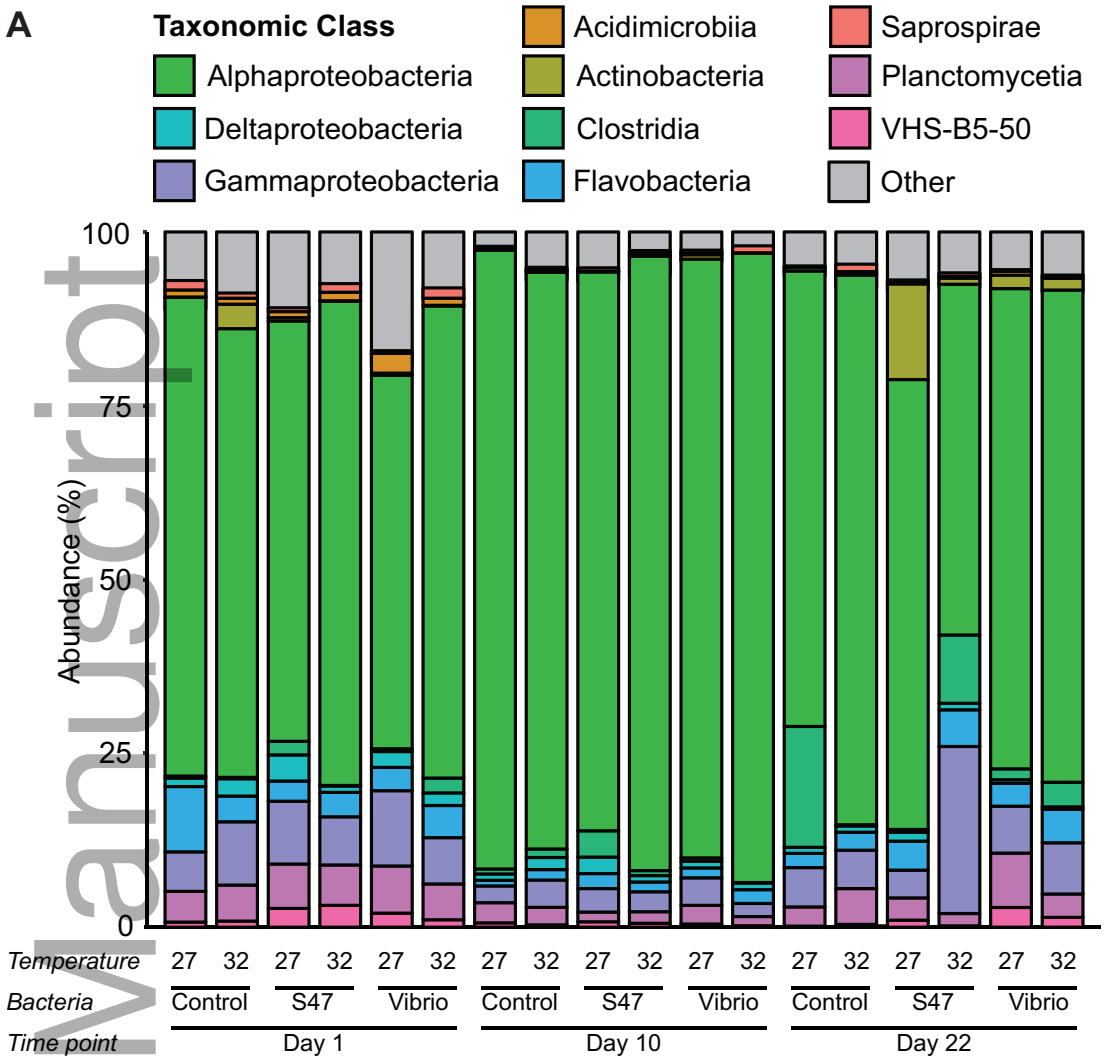

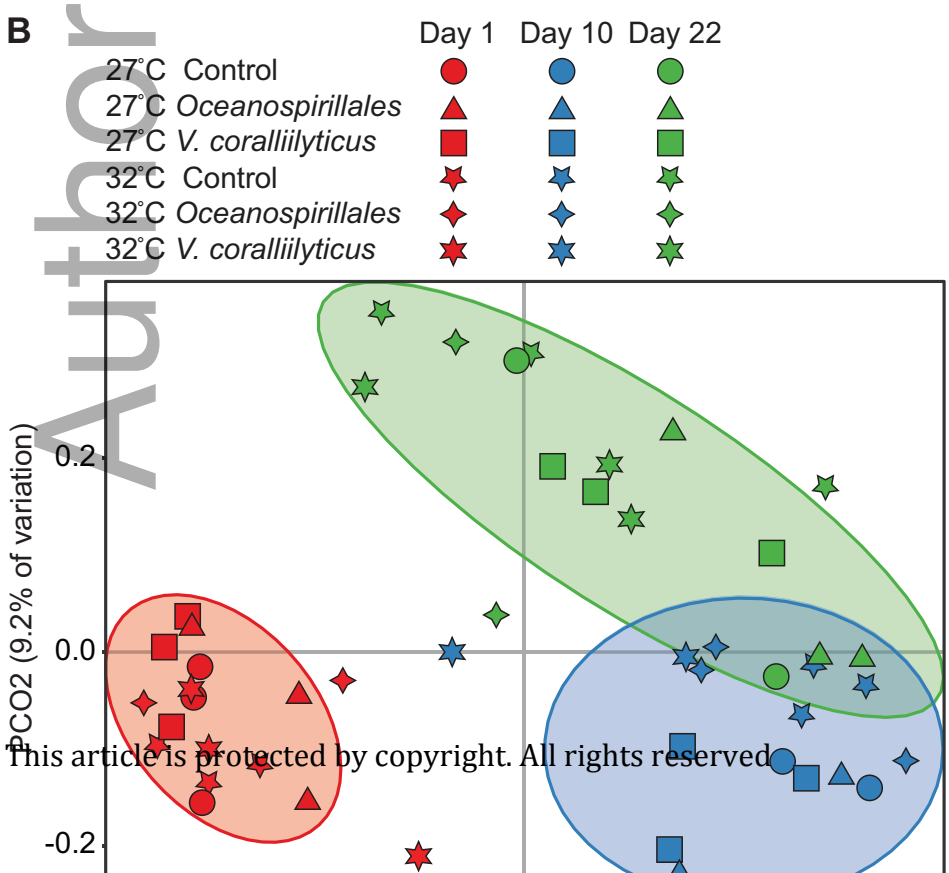

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