PLOS CLIMATE

RESEARCH ARTICLE

# Understanding differential heat tolerance of the threatened mountainous star coral *Orbicella faveolata* from inshore and offshore reef sites in the Florida Keys using gene network analysis

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**Citation:** Aguilar C, Enochs IC, Cohen K, Chomiak L, Kolodziej G, Baker AC, et al. (2024) Understanding differential heat tolerance of the threatened mountainous star coral *Orbicella faveolata* from inshore and offshore reef sites in the Florida Keys using gene network analysis. PLOS Clim 3(11): e0000403. https://doi.org/10.1371/journal.pclm.0000403

**Editor:** Johanna E. Johnson, James Cook University, AUSTRALIA

**Received:** March 11, 2024

**Accepted:** October 9, 2024

**Published:** November 27, 2024



**Data Availability Statement:** Data associated with this project are available at coris.noaa.gov, project ID 31197.

**Funding:** This work was supported by NOAA's Coral Reef Conservation Program (grant #31197 to DPM) and a NOAA Omics Program grant to DPM, ICE. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Abstract

Climate change driven ocean warming is causing widespread degradation of coral reefs. In the Florida Keys, many reefs have lost much of their coral cover, yet some inshore reefs have maintained higher coral cover and exhibited higher bleaching resistance and resilience than reefs offshore during marine heatwave events. To explore the molecular mechanisms underlying the higher heat tolerance observed on inshore reefs, we subjected three inshore and four offshore genotypes of the coral *Orbicella faveolata* to 30, 31, 32, or 33˚C for 31 days and measured photochemical efficiency (Fv/Fm), the species and relative abundance of dinoflagellate endosymbionts, and gene expression of the host and symbiont. All inshore coral genotypes, regardless of symbiont species, were significantly more thermotolerant than offshore genotypes based on comparatively smaller declines in photochemical efficiently. The most heat-tolerant inshore genotype (In1) was dominated by the symbiont *Durusdinium trenchii;* all other genotypes, both inshore and offshore, were *Breviolum minutum*-dominated, suggesting local adaptation or acclimatization contributes to the heat tolerance of inshore genotypes not dominated by *D. trenchii*. After 31 days of heat stress, all coral genotypes (except In2) had lost most of their *B. minutum* and became dominated by *D. trenchii*. Host genotype In1 presented unique expression patterns of genes involved in heat shock response, immunity, and protein degradation. There were fewer changes in the symbiont gene expression of inshore corals under heat stress when compared to the offshore colonies, which experienced significant changes, including increases in ribosomal and photosynthetic proteins. These data show that the differential thermotolerance between inshore

**Competing interests:** The authors have declared that no competing interests exist.

and offshore *O. faveolata* in the Florida Keys is associated with statistically significant differences in both host and symbiont gene expression that provide insights into the mechanisms underlying holobiont heat tolerance.

## Introduction

Coral bleaching events are increasing in severity, magnitude, and scale due to climate change driven ocean warming [1–3]. Bleaching is the process by which the endosymbiotic dinoflagellates within corals are expelled, leaving corals with a pale or white appearance [4]. This expulsion of symbionts (i.e., algal endosymbionts within the family Symbiodiniaceae) occurs when seawater temperatures remain elevated by $> 1^{\circ}$C above the climatological maximum monthly mean for a month or longer [5]. The coral response is a function of the magnitude and duration of the thermal anomaly, such that greater heat stress exacerbates the bleaching response and increases the likelihood of total or partial mortality of the affected coral [4]. Recovery can occur if the thermal anomaly is not too prolonged or severe, but corals that survive bleaching may be biologically compromised, with reduced growth and reproductive capacity, as well as increased susceptibility to disease for years after recovery [6–9].

Since the early 1980s, the coral reefs of the Florida Keys, like much of the wider Caribbean, have experienced dramatic losses in coral cover due to bleaching and disease [10–12]. Reefs in the region have experienced nine mass bleaching events from 1987–2023 [3,13,14]. Most Florida reefs have low coral cover ($< 5\%$), although some inshore patch reefs have maintained much higher coral cover (15–35%) [15–17]. Coral calcification rates at these inshore sites are higher than at offshore sites and recover more rapidly following both warm and cold stress [15,18–20]. The incidence and severity of coral disease is also lower inshore [21,22], which is unexpected because the inshore sites experience marginal conditions for reef development, including increased thermal variability, turbidity, sedimentation, and nutrients [15], all off which have been implicated in higher disease prevalence [23].

Corals in inshore reef habitats may be locally adapted and/or acclimatized to recurrent heat stress because of chronically higher and more variable temperature regimes [16,24,25]. This hypothesis is supported by the greater bleaching resistance and more rapid recovery of inshore corals vs. offshore during the back-to-back bleaching events of 2014 and 2015 [16]. For example, at one inshore patch reef, survivorship across both events was high (94.7% of $>$ 4,000 colonies), and there was lower bleaching severity and mortality during the second, stronger thermal anomaly in 2015, suggesting that compensatory mechanisms may be possible [13].

Coral bleaching resistance is closely linked to the species and abundance of symbionts, which potentially modulate the heat stress response of the host [26]. Corals that associate with symbionts in the genus *Durusdinium* are generally more thermally tolerant [27,28], and this was the case for the threatened coral *Orbicella faveolata* during the Florida Keys-wide 2015 bleaching event, when $>$ 90% of the unbleached corals were dominated by *Durusdinium trenchii* [16]. However, not all genotypes that were dominated by *D. trenchii* were equally resistant to bleaching, which suggests a role of the coral host in determining how much heat tolerance is conferred by the association with *D. trenchii* [29]. Indeed, recent work has highlighted the role of the coral host (in addition to symbiont species) in bleaching resistance [30–33]. In the Florida Keys, for example, inshore colonies of *Porites astreoides* were more heat tolerant than those offshore even though they hosted the same symbiont species [24].

Variation in heat tolerance among different coral genotypes has driven research into the molecular mechanisms of resilience. Kenkel and Matz [25] used gene network analysis to

investigate differences in *P. astreoides* populations across the Florida Keys and found that offshore corals had less gene expression plasticity than inshore corals. Moreover, work on *Acropora hyacinthus* in American Samoa found that corals under extreme conditions are more resistant than corals under moderate conditions when exposed to heat stress [34]. The more thermally tolerant corals were hypothesized to "frontload" specific genes because they expressed higher levels under control conditions and these genes were ultimately less up regulated during stress. However, for *O. faveolata* in the Florida Keys, although there is a clear linkage between bleaching resistance, symbiont community, and location, the molecular mechanisms behind the variability among host genotypes are still unknown [16]. In this study, we investigated the molecular underpinnings of bleaching resistance by exposing inshore and offshore *O. faveolata* to heat stress and relating differences in physiological performance (photochemical efficiency and calcification) to patterns of host and symbiont gene expression.

## Materials and methods

Six to eight cores (5 cm diameter) were removed from 18 colonies of *O. faveolata* (130 coral fragments) spanning three sites (2 inshore, 1 offshore) in the Florida Keys in July 2017 (Fig 1). Depths of the inshore sites ranged from 3.0–3.4 m, whereas the offshore site was 5.5 m. Some colonies were used as test samples for physiological measurements throughout the experiment but not included in the gene expression analysis. A total of 15 colonies from seven genotypes (three inshore and four offshore), previously identified in [16], were assigned letter/number labels (S1 Table). Four genotypes (O1, O2, O4, O5) were collected from the same offshore site (UKO2), and several clones of three genotypes (In1, In2, In3) were collected from two inshore reefs (UKI1 and UKI2) (Fig 1, see Table 1 for RNAseq sample distribution). Samples were transported back to the University of Miami CIMAS and NOAA AOML's Experimental Reef

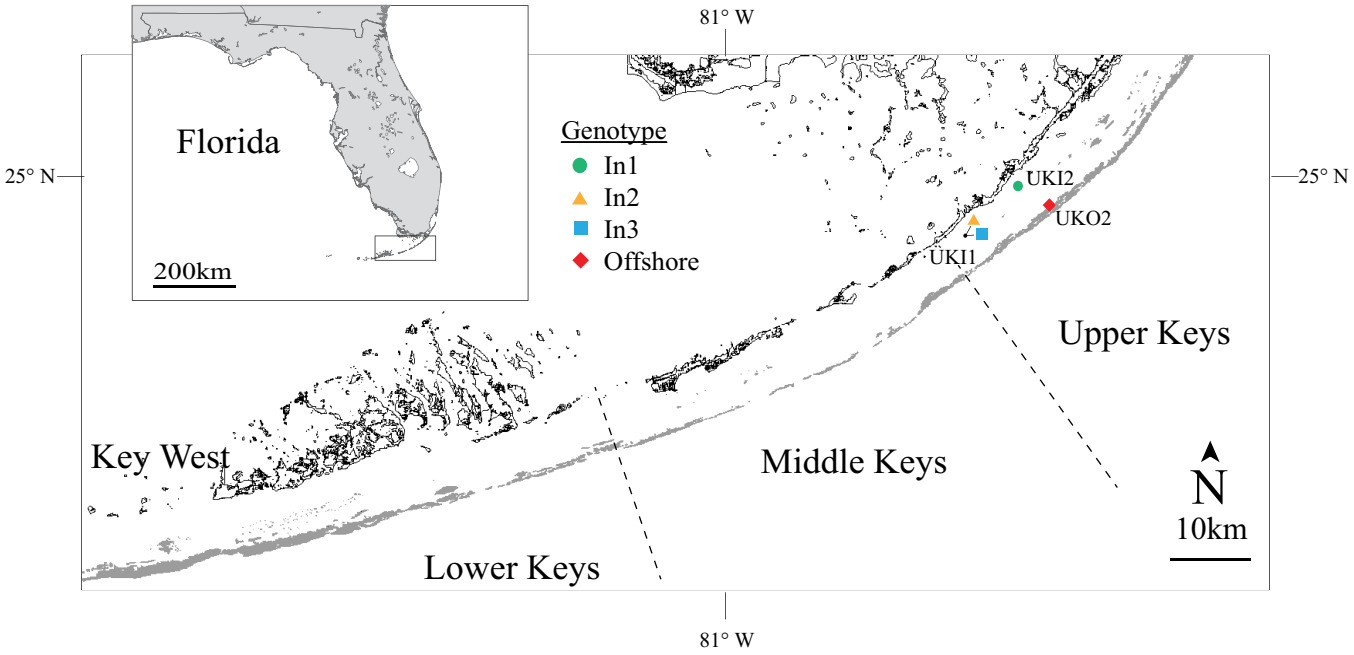


**Fig 1. Map of the three sites in the upper Florida Keys in Florida.** Indicating the location of the three inshore genotypes (UKI1 = Upper Keys Inshore 1 and UKI2 = Upper Keys Inshore 2) and offshore site were all four offshore genotypes that were sampled (UKO2 = Upper Keys Offshore 2). This map was constructed in ArcGIS using Florida's Unified Reef Map from the Florida Fish and Wildlife Conservation Commission. The base layer and terms of use can be found here: https://myfwc.com/research/gis/fisheries/unified-reef-map/.

https://doi.org/10.1371/journal.pclm.0000403.g001

**Table 1. Coral samples collected for the RNAseq.**

| Time | Treatment | Genotype | Location | Collection Site | Colony ID |
|---|---|---|---|---|---|
| 5 days | 30˚C | In1 | Inshore | UKI2 | A3, A4, A6 |
| | | In2 | Inshore | UKI1 | B5, B6 |
| | | In3 | Inshore | UKI1 | D3, D5, D6 |
| | | O1 | Offshore | UKO2 | C1 |
| | | O2 | Offshore | UKO2 | C2 |
| | | O5 | Offshore | UKO2 | C5 |
| | 33˚C | In1 | Inshore | UKI2 | A3, A4, A6 |
| | | In2 | Inshore | UKI1 | B5, B6 |
| | | In3 | Inshore | UKI1 | D3, D5, D6 |
| | | O1 | Offshore | UKO2 | C1 |
| | | O2 | Offshore | UKO2 | C2 |
| | | O5 | Offshore | UKO2 | C5 |
| 31 days | 30˚C | In1 | Inshore | UKI2 | A2, A4, A5 |
| | | In2 | Inshore | UKI1 | B5 |
| | | In3 | Inshore | UKI1 | D4, D5, D6 |
| | | O2 | Offshore | UKO2 | C2 |
| | | O4 | Offshore | UKO2 | C4 |
| | | O5 | Offshore | UKO2 | C5 |
| | 32˚C | In1 | Inshore | UKI2 | A2, A4, A5 |
| | | In2 | Inshore | UKI1 | B5 |
| | | In3 | Inshore | UKI1 | D3, D4, D5 |
| | | O2 | Offshore | UKO2 | C2 |
| | | O4 | Offshore | UKO2 | C4 |
| | | O5 | Offshore | UKO2 | C5 |

https://doi.org/10.1371/journal.pclm.0000403.t001

Laboratory, where one or two fragments per colony were randomly assigned to one of 12 independent aquarium systems (150 L, sump plus tank) [35] and acclimated for seven days at control conditions (30˚C) (See S2 Table for experimental design and sample distribution by tank and treatment). Following acclimation, temperatures in the experimental tanks were increased by 0.14˚C, 0.29˚C, 0.43˚C per day to reach 31˚C, 32˚C and 33˚C, respectively, over an additional 7-day period, while the control (30˚C) remained constant. Coral replicates were maintained at these temperatures for four weeks. Corals were fed twice a week at dusk with Reef-Roids (~1.2 g in 20 mL per tank, Polyp Lab).

A diel irradiance regime was programmed using 135W LED arrays (Hydra 52 HD, Aqua Illumination) to simulate natural conditions. Photosynthetically active radiation (PAR, 400–700 nm) increased in intensity starting at 06:00 h local time, reached a peak of 516 μmol $m^{-2}$ $s^{-1}$ at 12:00 h, and then declined until simulated dusk at 19:00 h. These values were based on the average daily dose of PAR (11.9 mol $m^{-2}$ $d^{-1}$) measured at the sampled offshore site during August 2016 using a submersible PAR logger (EcoPAR, Wet Laboratories) as described in [16]. We matched the average daily PAR dosages from the offshore site (UKO2) because paired light sensor deployment at inshore and offshore sites revealed higher PAR inshore. Leading up to our collection in Spring 2016, the mean ± SE daily dose of PAR was 16.2 ± 0.68 mol $m^{-2}$ $d^{-1}$ at one of our inshore sites (UKI2) and 13.8 ± 0.48 mol $m^{-2}$ $d^{-1}$ at UKO2 [16]. Maximum daily dose PAR values during this time at UKI2 and UKO2 were 24.2 and 19.7 mol $m^{-2}$, respectively. Given that the severity of bleaching depends on the interaction between high temperature and high light, we reasoned that, if offshore colonies were exposed to more light than what they

normally experience, this may have unnaturally exacerbated their apparent heat sensitivity. Therefore, using the offshore values provided the most conservative approach. The seawater pH in all aquaria included natural diurnal variability as described in [35]. Daily mean values were $pH_T = 8.05$ with a diel range of 0.05 pH units.

Calcification rates (mg $CaCO_3$ $cm^{-2}$ $d^{-1}$) were measured 22 and 36 days after the start of the experiment using the buoyant weight methodology [36]. Surface area was calculated geometrically from the average height and radius of the coral samples. Chlorophyll fluorometry was used to measure the maximum photochemical efficiency of PSII of symbionts using an imaging-pulse-amplitude-modulated (IPAM) fluorometer (MAXI M-series, WALZ). These measurements were taken at the end of the acclimation period and then weekly until the end of the experiment, and were recorded at 19:20 h, following 20 min of dark adaptation.

Calcification and photochemical efficiency of PSII data were analyzed using R version 3.4.3 car 3.0–3 package [37,38]. A repeated measures ANOVA was used to test for differences among genotypes per treatment for each time point. A two-way ANOVA was used to test for the interaction between genotype and treatment per time point for both datasets. In our analysis we treated each individual coral colony as the sampling unit (N) and each fragment from a colony as a replicate. For instance, colonies A1, A2, A3 etc. were treated as separate coral colonies even though post-hoc genetic analyses revealed they were the same genotype [16]. Although this violates the assumption of independence, we nevertheless proceeded with parametric ANOVAs because *O. faveolata* exhibits significant phenotypic variability within colonies due to variation in both symbiont community structure [39,40] and function [41]. Consequently, although some individual colonies were the same genotype, we opted to treat them as different sampling units due to the considerable biological variability among colonies.

A total of 42 samples from 15 colonies were collected for gene expression analyses after five days (T1, 33˚C) and 31 days (T2, 32˚C) of heat stress, as well as from control conditions (T1 and T2, 30˚C) (Table 1). The initial plan was to sample the corals exposed to 33˚C after one week and one month. However, the offshore corals bleached so rapidly that these corals were sacrificed after just five days because they were not anticipated to survive a full week at 33˚C. Consequently, after one month, the warmest treatment was 32˚C. Three inshore (In1, In2, and In3) and four offshore (O1, O2, O4, O5) genotypes were collected for each treatment (heated vs. control) per time point. See S1 Table for the sample distribution.

Whole coral fragments were snap-frozen in liquid nitrogen and stored in a -80˚C freezer prior to analysis. Total RNA was extracted using the total RNAqueous kit (AM1912, Life Technologies), according to the manufacturer's instructions. RNA was treated with Turbo DNA-free (AM1907, Life Technologies) and quantified using the Qubit RNA BR assay kit (Q10210, Life Technologies) in a Qubit 3.0 fluorometer (ThermoFisher Scientific). Total RNA was sent on dry ice to the Duke Center for Genomic and Computational Biology (GCB) for library preparation and sequencing. Sample quality was assessed on a 2100 Bioanalyzer (Agilent Technologies) and Qubit 2.0 (ThermoFisher Scientific). RNA-seq libraries were prepared using the commercially available KAPA Stranded mRNA-Seq Kit following the manufacturer's protocol. Libraries were sequenced in a NextSeq500 High Output flow cell at 75bp paired end. Samples were preprocessed for library prep using polyA-tail capture.

Adapters and low-quality reads were removed in Trimmomatic v0.36 (phred33, quality score $> 20$, 4 bp sliding window; [42]. These reads were used for *de novo* assembly in Trinity v2.5.1 (https://github.com/trinityrnaseq/trinityrnaseq/wiki) [43]. After the assembly, a Trinity script (get_longest_isoform_seq_per_trinity_gene.pl) was used to select the longest isoform per gene. The metatranscriptome was filtered by performing BLASTx (e-value $< 1e^{-5}$) searches against two coral proteomes, and BLASTn (e-value $< 1e^{-5}$) searches against four Symbiodiniaceae genomes and transcriptomes (as of June, 2018) (corals: *Acropora digitifera*

genome and transcriptome, http://marinegenomics.oist.jp/coral/viewer/download?project_id=3, [44]; *Orbicella faveolata* genome, https://www.ncbi.nlm.nih.gov/genome/?term=txid48498[orgn], [45]. Symbiodiniaceae: *Symbiodinium microadriaticum* genome, http://smic.reefgenomics.org/download/, [46]; *Breviolum minutum*, http://marinegenomics.oist.jp/symb/viewer/download?project_id=21 genome and transcriptome, [47,48]; *Cladocopium goreaui* genome, http://symbs.reefgenomics.org/download/, [49]; *Durusdinium trenchii* genome, https://doi.org/10.48610/27da3e7 [50]. Transcripts that had a hit to both coral and symbiont proteomes were sorted based on their lowest e-value and highest bit score. The symbiont transcripts were further divided into the two transcriptomes based on their best hit to either *B. minutum* or *D. trenchii* as the proportion of reads that matched to the *S. microadriaticum* and *C. goreaui* genomes were less than 1% (see results below). In addition, the results from a nr (non-redundant) database search (e-value $< 1e^{-4}$) were used to identify contigs with matches to metazoan proteomes as candidate coral transcripts, and identify matches with the eukaryote proteomes as candidate symbiont transcripts. Transcripts with no hits to the nr database were also kept if they matched the coral, *B. minutum* and *D. trenchii* databases in each transcriptome.

After separating the host, *B. minutum*, and *D. trenchii* transcripts, the completeness of each transcriptome was determined using BUSCO (Benchmarking Universal Single-Copy Orthologs) [51] in the gVolante server [52] selecting metazoan orthologs for the coral host, and eukaryotes for the symbionts. The transcriptome annotations were completed according to the Trinotate v3.1.1 pipeline (https://github.com/Trinotate/Trinotate/wiki) [53]. BLASTx and BLASTp searches to the uniprot database were done using an e-value cut-off = $1e^{-4}$. A script (extract_GO_for_BiNGO.pl) within Trinotate program was used to generate the gene annotation and ontology files for each transcriptome [53].

The proportion of each symbiont genus was calculated by quantifying the number of reads mapped to each sample following the methods described in [54]. These methods were used to detect the dominant Symbiodiniaceae genus in each sample. In brief, sample reads were mapped using Bowtie2 v.2.3.4 [55] to a combination of the coral reference genomes, and symbiont genomes and transcriptomes (*S. microadriaticum*, *B. minutum*, *C. goreaui*, and *D. trenchii*). A custom perl script zooxType.pl [55] was then used to count the relative proportions of reads producing highly unique matches (mapping quality 40 or higher) to each symbiont genus.

For gene expression analysis, Trinity scripts [43] were used to map the reads back to each of the three transcriptomes using the alignment-free abundance estimation method kallisto and to build an expression matrix from the transcript abundance files. Expression matrices were used for downstream analyses in R [37]. First, the package arrayQualityMetrics [56] was used to check for outliers and counts (variance-stabilizing transformation, VST) were visualized using principal component analysis (PCA). Two samples from the same colony were detected as outliers from each of the host dataset (genotype O4), these samples were excluded from the analyses, leaving a total of 40 samples (S1 Fig). Differential gene expression analysis was performed in the DESeq2 v.1.18.1 package [57] to test the effects of high temperature treatment in each time point and sub-setting by four groups (In1, In2, In3 and offshore), using a design: ~ genotype + genotype:treatment.

The default functions performed by DESeq2 were used to estimate size factors, dispersion, calculate negative binomial GLM fit, and Wald statistics to obtain a list of log2 fold changes (log2FC) when comparing control vs. temperature treatment for each of the four groups described above. False discovery rate (FDR) adjusted p-values for each gene were controlled at 5% for the host and 1% for the symbionts [58].

To examine the correlation between phenotypic traits and gene expression, data were analyzed using the weighted gene co-expression network analysis (WGCNA v1.66) package in R

was done for the host [59]. A matrix of sample traits was made using genotype, time, treatment, *D. trenchii* proportion, and photochemical efficiency as factors. Photochemical efficiency was determined by binning the Fv/Fm values into six quantiles. Transcript count data were input after using variance-stabilized transformation and following WGCNA tutorials. A soft thresholding power of 13 was used for the host to run a signed automatic network construction (module size $\geq$ 40). The output of the host modules was used to: (1) calculate the "eigengene" expression per module (moduleEigengenes from WGCNA); (2) calculate the correlations between module eigengenes and sample traits; and (3) examine the response of specific groups of genes within each module by calculating the eigengene expression of selected genes of known function as described in [25]. Analysis of the correlation of each of these groups of genes to the genotype was done using the cor.test in R [37].

Statistically over-represented gene ontology (GO) categories (hypergeometric test and FDR $<$ 0.01) were used to identify classes of genes over-represented in a large set of genes using BiNGO [60] in Cytoscape 3.1.1 [61]. To run this analysis, we used the gene annotation file created for each transcriptome (created in Trinotate as indicated above) and a gene ontogology file was downloaded from https://geneontology.org/docs/download-ontology/ (2018-12-04).

The subset of genes from each species that were used as input data came from two different analysis: (1) the set of genes that were differentially expressed (FDR $<$ 0.05) to the treatment per genotype from DESeq2; and (2) the set of genes within each module from the WGCNA analysis for the host.

## Results

After five days at 33˚C, severe bleaching was observed in the offshore colonies (O1, O2, O4, O5), such that they appeared completely white with no pigment. Most of the inshore genotypes (In2, In3) were visibly pale, but still pigmented. In contrast, genotype In1 did not show any visible signs of bleaching and all sampled colonies except one did not start to bleach until they experienced 15 days of sustained exposure to 33˚C. One In1 colony (A5) began bleaching after one week at 33˚C. After 26 days at the 32˚C treatment, all replicates of the offshore corals were completely white with no pigment.

The photochemical efficiency (Fv/Fm) of the offshore corals declined in response to heat stress and was significantly lower than the inshore corals after just one day of exposure to 33˚C, as well as after seven, 14, and 21 days at 32˚C (TukeyHSD, p adj $<$0.05; S2 Fig and S3 Table). All offshore corals from the 33˚C treatment were sacrificed for RNA samples after five days, due to their extremely rapid bleaching response. Genotype In1 was the most heat tolerant and had significantly higher Fv/Fm than In3 after one and two weeks at 33˚C, and higher than In2 after one week at 33˚C (TukeyHSD, p adj $<$ 0.05; S2 Fig and S3 Table). After one month at 32˚C, genotype In1 had significantly higher Fv/Fm values than the offshore corals, but the other inshore genotypes were not significantly different than the offshore genotypes.

The calcification rates of inshore corals were significantly depressed at 33˚C when compared to 30˚C and 31˚C after two and four weeks (TukeyHSD, p adj$<$0.005; S3 Fig and S4 Table). Although genotype In1 maintained high Fv/Fm for longer at high temperatures (i.e., was more heat tolerant), it exhibited similar rates of calcification to the other genotypes that bleached more readily. After two weeks at 32˚C, genotype In3 had greater rates of calcification than the offshore corals and the inshore genotype In1 (TukeyHSD, p adj $<$ 0.05; S4 Table). Genotype In1 calcified at a similar rate over two and four weeks at 30, 31 and 32˚C and only declined significantly at 33˚C (TukeyHSD, p adj $<$ 0.05; S3 Fig, S4 Table).

All the corals in our study were dominated by either *B. minutum* or *D. trenchii*, although 30 samples (71% of 42 total samples) had both genera present (Fig 2). The highest proportion of

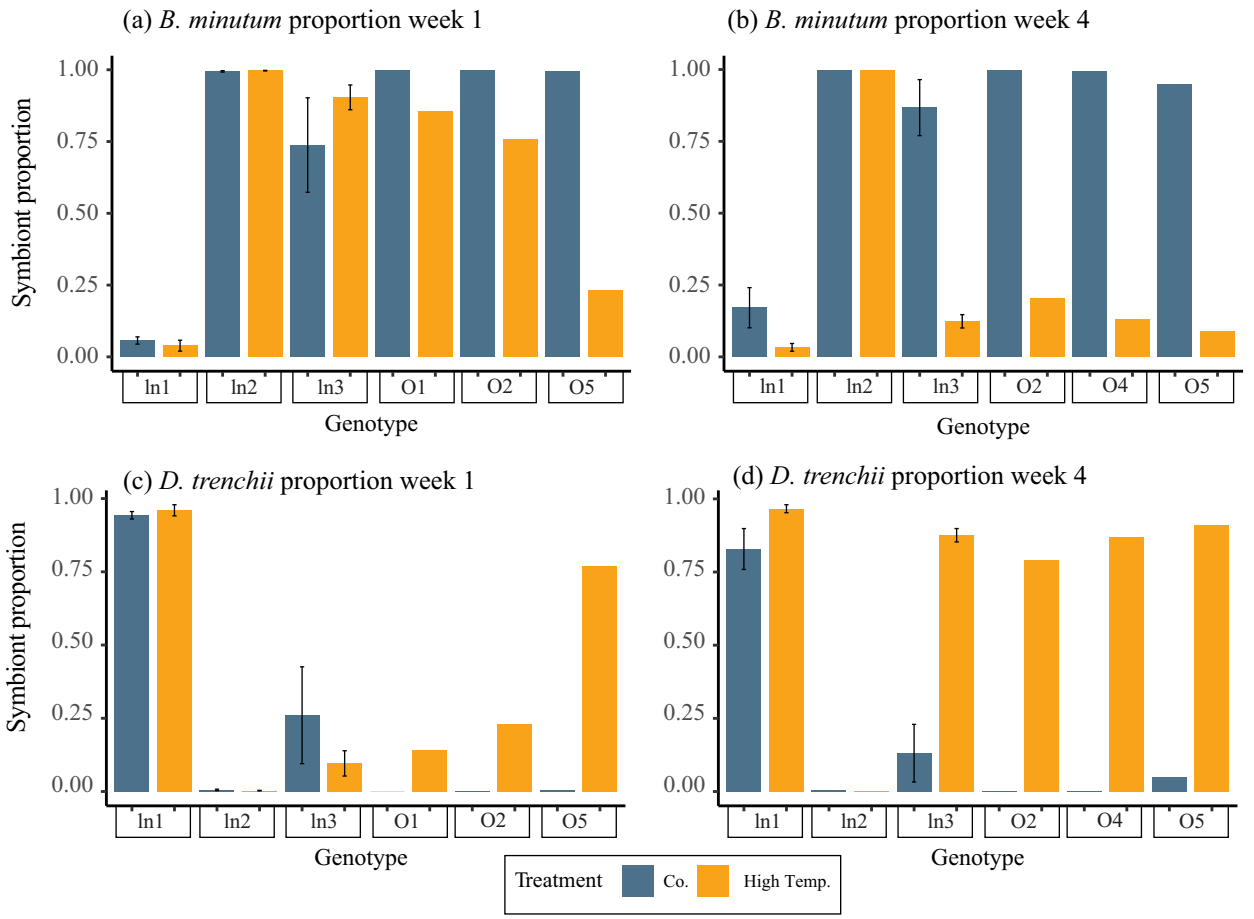


**Fig 2.** Proportion of *B. minutum* and *D. trenchii* in the *O. faveolata* inshore (In1, In2, In3) and offshore (O1, O2, O4, O5) genotypes per condition after (a and c) five days and (b and d) 31 days of treatment. Bars indicate the standard error, except for offshore samples since there is one sample per genotype.

https://doi.org/10.1371/journal.pclm.0000403.g002

reads that mapped to *S. microadriaticum* and *C. goreaui* in any samples was < 0.7% and <0.9%, respectively. *B. minutum* dominated the offshore corals and genotype In3 under control conditions, but *D. trenchii* increased its relative dominance in these corals after 31 days of heat stress. Genotype In1 was dominated by *D. trenchii* in both the controls and treatments throughout the experiment, whereas In2 was always dominated by *B. minutum*.

A total of 574 M reads were sequenced, of which 498 M were retained after trimming and used for the assembly. From these sequences, a total of 491,454 contigs (N50 = 1,192) were assembled in the metatranscriptome, of which 87,440 (N50 = 2,228) were assigned to *O. faveolata*, 42,761 to *B. minutum* (N50 = 1,780) and 52,359 to *D. trenchii* (N50 = 1,434) transcriptomes (Table 2). The coral transcriptome had 39% of contigs annotated to the uniprot database, while the symbionts had 31% for *B. minutum* and 22% for *D. trenchii*.

The PCA from the host reads showed that the three inshore genotypes (In1, In2, In3) grouped with their respective genotype and the offshore genotypes (O1, O2, O4, O5) grouped together (S4 Fig). After 31 days of stress, In2 and the offshore corals grouped close to one another, while In1 and In3 samples grouped by genotype (S4 Fig). A permutational multivariate analysis of VST data supported each of these four groups (In1, In2, In3, Offshore corals) as distinct ($p < 0.001$). Consequently, the gene expression experimental design (one sample per

**Table 2. Transcriptome assembly statistics.**

| | *O. faveolata* | *B. minutum* | *D. trenchii* |
|---|---|---|---|
| Mean GC content | 41.99 | 52.14 | 55.83 |
| N contigs | 87,440 | 42,761 | 52,359 |
| Average contig | 1,070 | 1,091 | 890 |
| N50 (bp) | 2,228 | 1,780 | 1,434 |
| % annotated_uniprot | 39 | 31 | 22 |
| % kegg | 29 | 26 | 20 |
| BUSCOs | | | |
| N core genes queried | 978 | 255 | 255 |
| N complete (%) | 811 (83%) | 114 (45%) | 112 (44%) |
| N single copy | 756 (77%) | 89 (35%) | 107 (42%) |
| N duplicate | 55 (6%) | 26 (10%) | 5 (2%) |
| N fragmented | 79 (8%) | 18 (7%) | 23 (9%) |
| N missing | 88 (9%) | 123 (48%) | 117 (46%) |

https://doi.org/10.1371/journal.pclm.0000403.t002

offshore genotype per condition; see Table 1) was done with three inshore genotypes while grouping the offshore ones. For the symbiont reads, the variation in the PCA was explained by symbiont identity within each sample (S4 Fig). For example, in the PCA of the *B. minutum* transcriptome after 1 week of stress, samples dominated by *B. minutum* grouped to the right of the plot and samples dominated by *D. trenchii* grouped to the left (S4 Fig). However, the data were analyzed based on host groupings to understand differences in the symbionts between these genotypes.

The number of differentially expressed coral genes (DEGs, FDR < 0.05) between the heat and control treatments decreased over time for all genotypes. Genotype In3 had the highest number of DEGs after five days at 33˚C (N = 1,295) while In2 had the lowest number (N = 281) (Fig 3E). There were 66 DEGs in common among all genotypes when concatenating the results for each genotype after 5 days of exposure to 33˚C (S5 Fig, S5 Table). Most of these genes were downregulated by heat stress, but had higher baseline levels in In1, the most heat tolerant genotype. One heat shock protein gene (*Hsp23*) was up regulated under heat stress relative to the control across all genotypes, but the increase was relatively less in genotype In1 (S5 Fig). The concatenation also revealed DEGs that were specific to each genotype (240 genes in genotype In1, 107 in In2, 568 in In3, and 570 in offshore colonies; S5 Fig), and included enriched terms for genotypes In1 and In3 (S5 Table). In contrast, offshore corals were not enriched for any GO terms, but the DEGs that responded to stress included a highly down-regulated ribosomal protein disulfide-isomerase (*PDI*; log2FC of -5.34) and three 60S ribosomal proteins (log2FC of -5.32, -4.87 and -4.47, FDR<0.05, S5 Table).

After 31 days at 32˚C, genotype In3 had the strongest response (359 DEGs) and genotype In1 had the weakest (30 DEGs) (Fig 3F). However, only two differentially expressed genes (a short-chain dehydrogenase/reductase family 16C [*SDR16C*] and a gene with no annotation) were shared across all genotypes (S5 Fig). Genotype In3 was the only genotype with enriched GO terms (N = 7), and these were mainly related to lipid homeostasis (S5 Table). Offshore colonies were characterized by 118 unique genes that included a highly up-regulated cytochrome c oxidase subunit 1(*COX-1*) (log2FC of 19.7, FDR<0.05) and a heat shock 70 kDa protein (*Hsp70*) (log2FC of 20.1, FDR<0.05, S5 Table).

Symbiont gene expression patterns revealed that the severely bleached offshore corals had the most significant response after five days of acute thermal stress, with 3,306 DEGs (FDR < 0.01, Fig 3A) in the *B. minutum*, and 260 DEGs (FDR < 0.01, Fig 3C) in the *D.*

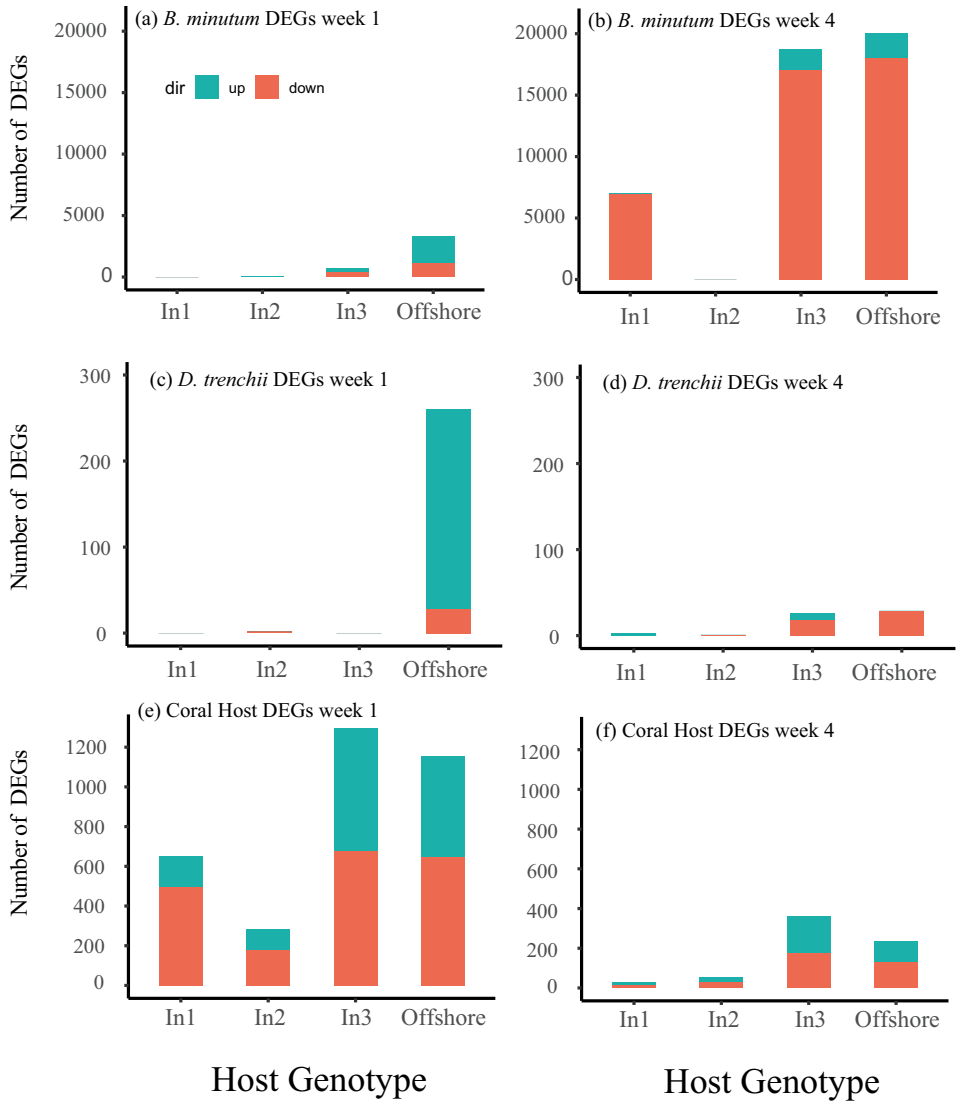


**Fig 3. Response of the symbiont and host to heat stress after five and 31 days of treatment (33˚C = five days, 32˚C = 31 days).** Differential gene expression analysis (DEGs) in heat stress treatment versus the control in: (a) *B. minutum* after five days, (b) *B. minutum* after 31 days, (c) *D. trenchii* after five days, (d) *D. trenchii* after 31 days, (e) coral host after five days, and (f) coral host after 31 days.

https://doi.org/10.1371/journal.pclm.0000403.g003

*trenchii*. Inshore genotypes In2 and In3, which were dominated by *B. minutum*, had the fewest DEGs in response to heat stress (759 DEGs in In3 and 59 DEGs in In2, Fig 3A). Gene ontology of the 2,129 genes that were up-regulated in response to heat stress in the *B. minutum* offshore corals had 14 over-expressed (FDR < 0.05) terms including: "RNA splicing", "translation", and "peptide biosynthetic process". There were four GO terms (FDR < 0.01) in the *D. trenchii* DEGs up-regulated in the offshore colonies that included: "protein chromophore linkage" and "photosynthesis".

After 31 days of stress, when all colonies showed signs of bleaching, the *B. minutum* gene expression profile was suppressed in genotypes In1 and In3, as well as the offshore colonies, while there were zero DEGs in In2 colonies, likely due to the low number of samples (N = 1 per condition; Fig 3B). There were 6,844 DEGs shared in the *B. minutum* after concatenating

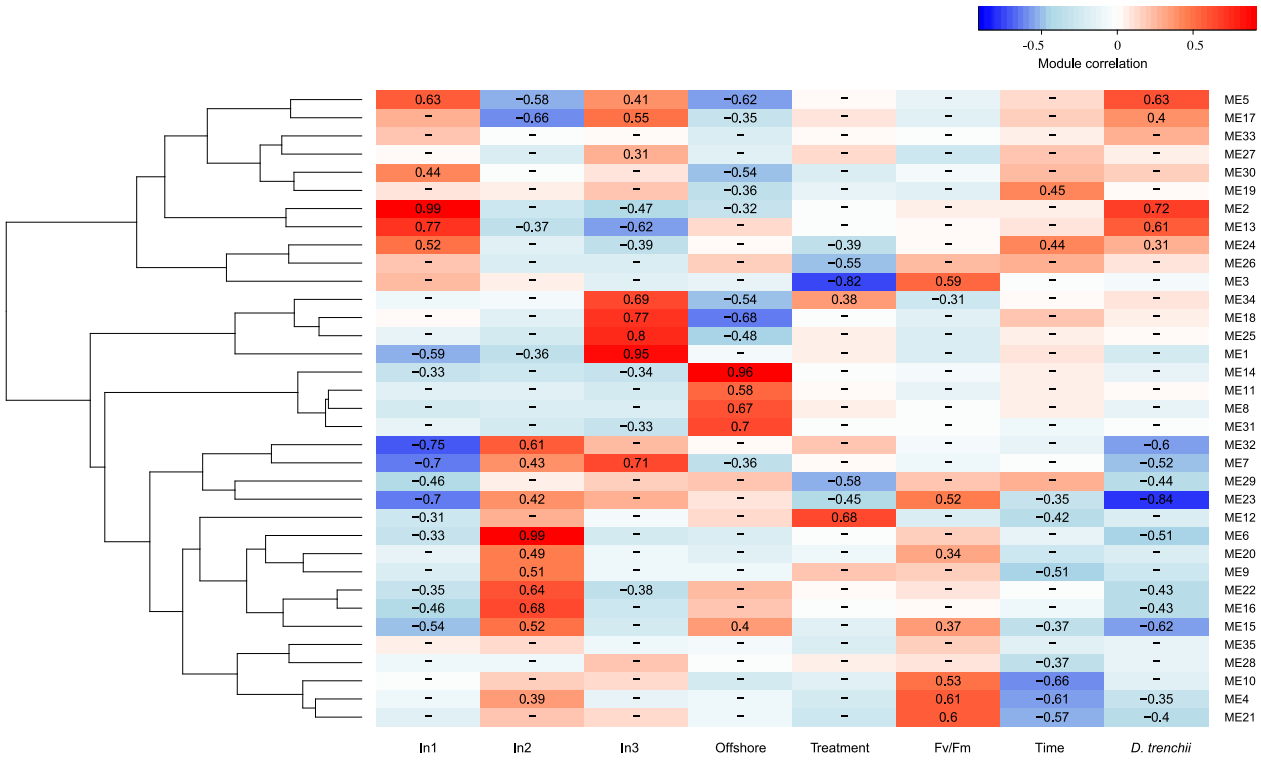


**Fig 4. Hierarchical clustering dendrogram of module-trait associations for the host transcriptome.** Each row corresponds to a module and the columns to a trait. Red are positive and blue are negative correlations with values corresponding to a significant Pearson's correlations ($P < 0.05$). Traits were input as follows: Fv/Fm score that was divided in quantiles groups (6 = higher yield to 1 = lower yield), time (1 = 5 days to 4 = 31 days), treatment (0 = control, 1 = treatment), inshore genotypes (In1, In2, In3; 1 = present, 0 = absent) and grouped offshore genotypes (1 = present, 0 = absent), and *D. trenchii* proportion values.

https://doi.org/10.1371/journal.pclm.0000403.g004

the DEGs to the heat treatment in In1, In3 and offshore genotypes at this time point (S6 Fig). The GO analysis of these (6,844 DEGs) shared genes included: "regulation of cell cycle", "photosynthesis", and "phosphorylation" (S5 Table). At this time point the *D. trenchii* transcriptome in In1 only had three genes differentially expressed (Fig 3D).

The host WGCNA analysis identified groups of co-regulated genes based on 40.4% of the transcriptome (35,310 out of 87,440 contigs) after filtering the low count reads. From this, 60.6% (21,404) were assigned to 35 modules (S6 Table). These modules ranged in size from 66 to 4,173 genes, and 21 of these modules were enriched with at least one biological process. Modules were associated with nine traits (Fv/Fm, treatment, time, *D. trenchii* proportion, three inshore genotypes, and offshore colonies), with 93% of the modules associated with at least one inshore or offshore genotype, while five modules were associated with treatment and a genotype (Fig 4).

Module 2 (M2) was the most positively correlated module to the proportion of *D. trenchii* and was also associated with the most resilient genotype In1 ($p = 0.72, 0.99$, Fig 4; TukeyHSD, p adj = 0, S7 Table). M2 was the second largest module with unique expression of 2,241 genes (S6 Table) and was enriched with 36 GO terms (FDR< 0.01), that included eight terms involved in immune and inflammation response. Overall, the eigengene expression of this module showed that genotype In1 had a higher expression of these genes compared to the other genotypes. This is evident in the expression of stress-related genes including six

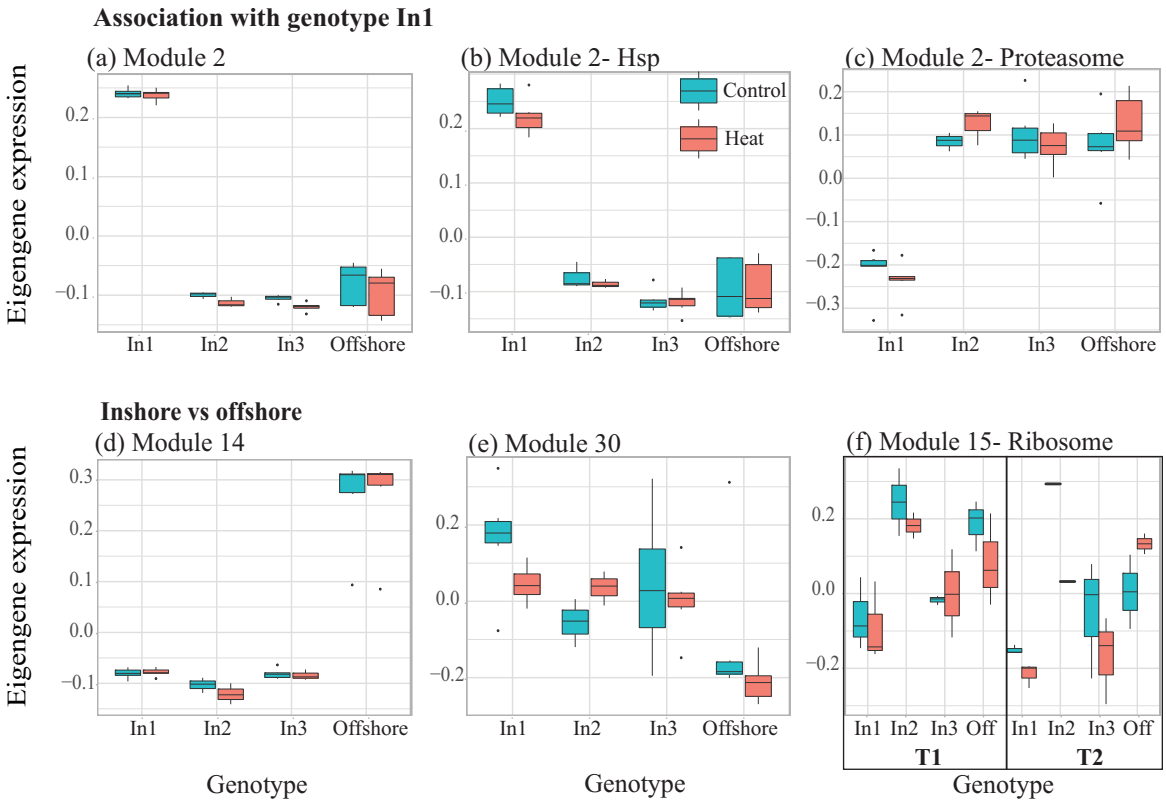


**Fig 5. Boxplots of the module eigengene expression (*y*-axis) with respect to the genotypes (*x*-axis) and condition for the host.** (a) module 2 (N = 2,241), (b) Hsp70 (N = 6, p-value = 6.90 e-11, cor = -0.82) in module 2, (c) proteasome activator complex (PSME, N = 11, p-value = 9.79 e-09, cor = 0.76) in module 2, (d) module 14 (N = 468), (f) module 30 (N = 123), and (e) 40S and 60S ribosomal proteins (N = 52; p-value = 2.09 e-2, cor = 0.36) in module 15.

https://doi.org/10.1371/journal.pclm.0000403.g005

belonging to the heat shock protein 70 (*Hsp70*) (Fig 5B) and one caspases-3 (*CASP3*) (S7 Fig). Conversely, genes associated with proteasome and immune response regulation that had a lower expression in genotype In1 compared to other genotypes included 11 proteasome activator complex (*PSME*) homologs (Fig 5C), and 11 NLR family CARD domain-containing protein 3 (*NLRC3*) homologs (S7 Fig, S6 Table).

The module with the strongest negative correlation to *D. trenchii* was module 23 (N = 238, p = -0.84), which was also correlated with genotype In1, In2, time, treatment, and Fv/Fm. The expression of this module showed a negative eigengene expression in coral samples with high *D. trenchii* communities, while corals dominated by *B. minutum* had a high expression of this group of genes (S8 Fig). Although this module was 59% annotated, there were no enriched gene ontology terms.

There were several (N = 14) modules with significant differences between the inshore and offshore genotypes (TukeyHSD, p adj = <0.001, S7 Table). Module 14 (M14) had the strongest positive correlation to offshore genotypes (p = 0.96), but it was not enriched with GO terms and had 177 annotated genes (Fig 5D). The offshore colonies had a positive eigengene expression in this module, while all the inshore genotypes had a negative expression. Within M14, the expression of specific stress immune-related genes (seven sacsin genes and two TNF receptor-associated factor 4 (*TRAF4*)) genes was lower in the offshore corals (S8 Fig).

Another module with significant differences between the offshore genotypes and inshore genotype In1 was module 30, with 45 annotated genes that were highly enriched with 94 GO

terms (Fig 5E; FDR <0.01, S6 Table). Most of the GO terms were related to immune and virus response and included the expression of four glutathione hydrolase (*GGT*) and four DEAD Box helicase 60 (*DDX60*) genes that had a lower expression in offshore colonies (S8 Fig, S6 Table).

Offshore colonies were also differentiated by a module enriched with protein biosynthesis terms (module 15), which was correlated with the sample Fv/Fm value and time. It had a positive correlation with genotype In2 and the offshore colonies, and a negative correlation to genotype In1 and In3 (Fig 4). This module had 32 GO enriched terms, that included 52 homologs to 40S and 60S ribosomal proteins (S6 Table). These genes had a higher expression in genotype In2 and the offshore corals after five days of treatment, and increased their expression after the four weeks treatment in the offshore corals (Fig 5F).

## Discussion

Genotypes of *O. faveolata* from inshore sites were significantly more heat tolerant than those from the offshore sites. However, there was significant variability between the inshore genotypes, with genotype In1 being the most thermally tolerant (i.e., bleaching-resistant). This is the same pattern observed in the field, as this genotype did not bleach during the second year of back-to-back bleaching in 2015 [16]. This field study also found that genotype In1 was dominated by *D. trenchii* in September 2015, May 2016, and remained dominated by this symbiont in July 2017. Genotype In1 was the only genotype found at UKI2 (Upper Keys Inshore 2, Fig 1), even though 20 colonies, located up to 40 m apart, were haphazardly sampled at this site, encompassing a total area of nearly 1,000 $m^2$ [16]. It is not clear if genotype In1 is the only genet at this location that has persisted through recent disturbance events, or if it is the lone immigrant that has been able to colonize this site. The presence of large, dead colonies of *O. faveolata* at UKI2 suggest the former scenario is more likely.

### Response of the most thermotolerant genotype

The unique host expression of heat shock proteins within genotype In1, plus the high correlation of module 2 with *D. trenchii*, provides important insights into the molecular underpinnings of the high heat tolerance found in this genet of *O. faveolata*. Higher constitutive expression of heat shock protein 70 (*Hsp70*) genes has been observed in other thermally tolerant corals, as well as in corals adapted to warmer temperatures [34,54]. While this approach of focusing on specific genes with potential linkages to thermal tolerance can have limitations because gene expression is not always correlated with protein expression and functional response [62,63], we nevertheless included it here to allow comparison with previous studies [25,34,64].

Module 2 was also enriched with several gene ontology terms related to "immune and inflammation response" that were differentiated in the most resilient genotype In1. This included a lower expression of Nod-like receptors C3 (*NLRC3*) genes, that have been well characterized in the *O. faveolata* genome and found to be involved in the activation of both the immune and apoptosis response [65]. One explanation of their role in both pathways was that lower levels of activation of these genes is indicative of immune promotion, while higher levels could indicate inflammatory cell death [65]. This could suggest that genotype In1 had higher immune promotion and less cell death, however is it hard to reach any further conclusions as the expression of other immune and apoptotic related genes (six *TNF* associated factor (*TRAF*) and three caspases) had both higher and lower levels in genotype In1 relative to the other genotypes (S7 Fig).

## Inshore vs offshore samples

In a similar study on *P. astreoides*, Kenkel et al. [24] found that inshore corals were more resistant to heat stress than offshore colonies. The thermal tolerance of the inshore corals was attributed to local adaptation of the hosts as there were no differences in the symbiont species between the inshore and offshore corals. In a companion study, it was shown that inshore corals had higher expression of specific metabolic genes (pyruvate carboxylase, phosphoenolpyruvate carboxykinase and the lipid beta-oxidation enzyme acyl-CoA dehydrogenase) when compared to offshore corals during heat stress [66]. Gene network analysis revealed that inshore corals had a higher expression of stress-related genes such as the molecular chaperones *DnaJ* and *Hsp70*, while offshore corals had higher expression of 55 small ribosome subunit genes [25].

Our gene network analysis of *O. faveolata* identified a module that correlated to several traits (In1, In2, offshore genotypes, Fv/Fm, and time) that included 24 and 28 homologs to 40S and 60S ribosomal proteins, respectively (Module 15, Figs 4 and 5F). The eigengene expression showed that the expression of these genes under stress was lower than the control samples after 31 days for the inshore corals, but in the offshore corals heat stress resulted in a higher expression of these genes compared to the control (Fig 5F). Ribosomal genes are involved in translation or protein synthesis and are essential for expression responses to changes in cellular conditions. Translation can be reduced in response to cellular stress in order to save energy [67]. However, studies in human cells have shown this disruption can switch and increase the translation of selective proteins that are required for cell survival during stress [68]. This mechanism is a possible explanation for the increase in the expression of ribosomal genes in the offshore corals after four weeks when colonies were completely bleached.

We also identified other modules that had clear differences between offshore and inshore corals (Fig 5D and 5E). Module 14 had seven genes homologous to sacsin genes that had lower expression in the offshore corals (S8 Fig). These genes are co-chaperones that can regulate *Hsp70* expression, and while the specific expression of several sacsin transcripts were found to increase under acute $pCO_2$ stress in the coral *Acropora millepora*, other co-chaperones such as *DnaJ* are well known to be involved in heat stress response [64,69,70]. Moreover, there were two homologs to *TRAF4* genes that had a lower expression in offshore colonies (S8 Fig). As mentioned above, these genes are known to be part of the *NF-kB* immune response in *O. faveolata* and part of the stress response in other coral species [34,71,72]. There were also differences involved in the oxidative stress response that included the expression of four glutathione hydrolase/gamma-glutamyltranspeptidase (*GGT*; Module 30, Figs 5E and S8). Overall, this shows that several stress response genes have lower baseline expression levels in the offshore coral genotypes, supporting the idea that higher constitutive levels of stress response genes are present in heat tolerant corals [34].

## Symbiont proportion

Field surveys have previously found that the bleaching resistant genotype In1 was dominated by *D. trenchii* [16]. The offshore colonies had mixed communities of *B. minutum* and *D. trenchii* during peak bleaching, but these communities reverted to *B. minutum* dominance during recovery. The gene expression patterns of these two dominant symbiont types differed between the offshore and inshore corals. After five days of heat stress, gene expression of *D. trenchii* had changed in the offshore colonies, but not in inshore colonies (Fig 3C). This response had "photosynthesis" as an over-expressed GO term that included five caroteno-chlorophyll a-c-binding (*CCAC*) protein transcripts, with an increase between 4.8–6.5 $\log_2$FC in the offshore colonies. *CCAC* genes are photosynthetic genes that were present in the

transcriptomes of all four symbiont genera (*Symbiodinium*, *Breviolum*, *Cladocopium*, and *Durusdinium*). They have been found to be up regulated in thermally stressed *Symbiodinium* cultures and up regulated in *Acropora aspera* symbionts under elevated nutrient conditions. This suggests that our observed increase in *CCAC* expression within the offshore *D*. *trenchii* symbionts represents increases in their photosynthetic capacity during the heat stress [73–75].

The *B*. *minutum* transcriptome showed a strong response (3,306 DEGs) in the offshore corals after five days of stress (Fig 3A). The GO analysis of the differentiated genes revealed an over-expression of protein synthesis genes, including the 43 ribosomal proteins homologs that were up-regulated in response to heat stress. Several coral host ribosomal proteins transcripts were also found to increase their expression in response to heat stress after 31 days in the offshore colonies. This indicates that protein translation may be a general response to stress in both the host and its symbionts that occurs first in the symbionts.

The notable changes in gene expression for both symbiont species within the offshore corals, but not the inshore corals, aligns with the observation that the offshore corals quickly bleached and had significantly lower Fv/Fm values after only one day of exposure to 33˚C (S2 Fig). Although genotypes In2 and In3 were not dominated by the heat tolerant symbiont *D*. *trenchii*, they were also initially resistant to high temperatures. This suggests that the elevated heat tolerance of the inshore corals is not solely a result of associating with *D*. *trenchii*, but that there is also some degree of local adaptation or acclimatization on the part of the coral host. It is also possible that there are locally adapted genotypes of *B*. *minutum* at the inshore sites that are more heat tolerant than those at the offshore site, as has been shown for other symbionts [76]. However, the most heat-resistant genotype was dominated by *D*. *trenchii* and had a different baseline gene expression of several host genes.

## Conclusions

This study sheds light on the molecular mechanisms by which corals from inshore environments in the Florida Keys can withstand higher temperatures than corals offshore. As previously shown for *O*. *faveolata* at these sites [16], dominance of the symbiont community by *D*. *trenchii* is associated with the greatest levels of heat tolerance. However, as was the case of the previous study, there is compelling evidence for host acclimatization or adaptation, as inshore colonies dominated by *B*. *minutum* were also significantly more heat tolerant than offshore colonies similarly dominated by this symbiont species. Here we show evidence that the gene expression of the most heat-resistant genotype was characterized by having a baseline expression of several genes (2.6% of the transcriptome) that differ from the other colonies, including genes that are known to be involved in heat stress response such as *Hsp70*. Moreover, it is evident from our work that the heat-sensitive offshore colonies had differences in their response to stress in both the host and the symbiont transcriptome when compared to inshore genotypes. *B*. *minutum* from the offshore colonies differentially expressed several genes during the heat treatment while *B*. *minutum* from the inshore colonies did not experience these changes. Future work is necessary to further understand the different contributions of the coral host and its symbionts to overall heat tolerance and whether these are fixed traits that might indicate these corals are valuable candidates for restoration efforts designed to increase climate resilience.

## Supporting information

**S1 Fig. Principal Component Analysis utilized to identify outliers in host transcriptome data.** Data shown with (a) and without outliers (b).
(EPS)

**S2 Fig. Photosynthetic efficiency of symbiont cells within adults of the coral *O. faveolata* under control and three heat stress conditions throughout the experiment.** Each shape represents the mean (±SD) per genotype (see S3 Table for samples number). Note. ANOVA results from significance among the genotypes are denoted with asterisk, significance code '*' $p<0.05$, '***' $p<0.0001$ (S3 Table).
(EPS)

**S3 Fig.** Boxplots of calcification rates per day after (a) 15 and (b) 29 days of heat stress. Note: ANOVA results from significance within the genotypes are denoted with asterisk, significance code '**' $p<0.05$ (S4 Table).
(EPS)

**S4 Fig. Principal component analysis (PCA) of normalized gene expression values for each inshore (In1, In2, In3) and offshore (O1, O2, O4, O5) *O. faveolata* genotypes.** (a) Coral host counts after five days of treatment, (b) coral host counts after 31 days of treatment, (c) *B. minutum* counts after five days of treatment, (d) *B. minutum* counts after 31 days of treatment, (e) *D. trenchii* counts after five days of treatment, and (f) *D. trenchii* counts after 31 days of treatment.
(EPS)

**S5 Fig. Differentially expressed genes (DEGs, FDR <0.05) to heat stress in the coral host.** Venn diagram after (a) 5 days (treatment = 33˚C) and (b) 31 days (treatment = 32˚C) between each inshore (In1, In2, In3) and offshore (O1, O2, O5) genotypes. Shared genes after 5 days between all samples (c) eigengene expression (*y*-axis) of the 66 shared transcripts, and (d) gene expression (*y*-axis) of the Hsp23 (N = 1) that responded to stress.
(EPS)

**S6 Fig. Venn diagram of the differentially expressed genes (DEGs, FDR <0.01) to heat stress in the *B. minutum* transcriptome after 31 days of stress between two inshore (In1 and In3) and three offshore (O2, O4, O5) genotypes.**
(EPS)

**S7 Fig. Expression of genes within host module 2 including.** (a) boxplot of the module eigengene expression (*y*-axis) of *NLRC3* (N = 11, p-value = 6.79 e-08, cor = 0.73) by genotype (*x*-axis), and boxplots of the gene expression (*y*-axis) per gene (*x*-axis) for: (b) six *TRAF* (p-value = 2.82 e-09, cor = -0.78), (c) three *CASP3* (p-value = 2.45 e-4, cor = -0.55), and (d) four *CHRNA7* homologues (p-value = 7.84 e-08, cor = -0.73).
(EPS)

**S8 Fig. Gene expression of host modules 14, 23 and 30.** Boxplots of the module eigengene expression (*y*-axis) per genotype (*x*-axis) for: (a) seven *sacsin* genes (p-value = 3.93 e-08, cor = -0.74) within module 14 and (b) module 23 including the time factor. Boxplots of the gene expression (*y*-axis) per gene (*x*-axis) of: (c) two *TRAF* genes (p-value = 5.15 e-07, cor = -0.70 in module 14, (d) four *DDX60* genes (p-value = 2.43 e-4, cor = -0.55) in module 30, and (e) four *GGT1* genes (p-value = 4.05 e-08, cor = -0.74) in module 30.
(EPS)

**S1 Table. RNAseq samples distribution.**
(XLSX)

**S2 Table. Sample distribution for coral colony/genotype by tank and treatment.**
(XLSX)

**S3 Table. Statistical significance tests of photosynthetic yield (imaging pulse amplitude modulated, iPAM) during thermal stress: a) one-way ANOVA, b) Tukey post-hoc test, c) two-way ANOVA, and d) Tukey post-hoc test.**
(XLSX)

**S4 Table. Statistical significance tests of buoyant weights data for the corals taken 15 and 29 days after acclimation period.** a) one-way ANOVA per genotype, b) TukeyHSD test, c) one-way ANOVA per treatment, d) TukeyHSD test, e) two-way ANOVA for genotype and treatment, and f) TukeyHSD test.
(XLSX)

**S5 Table. Host and symbionts differential gene expression results.** (a-c) Host differential gene expression analysis: (a) gene enrichment results, (b) log2FC of the transcripts differentiated after 5 days of heat stress and their annotation, and (c) log2FC of the transcripts differentiated after 31 days of heat stress and their annotation. (d-e) Symbionts gene expression analysis: (d) gene enrichment results from the symbiont, (e) log2FC of the transcripts differentiated after five and 31 days of heat stress and their annotation after concatenation.
(XLSX)

**S6 Table. Host WGCNA analyses results.** (a) host modules size, their percentage annotated based on the protein blast (evalue cut-off = 1e−4), and the number of GO terms associated, (b) gene enrichment results for each host module (FDR<0.01), (c) annotation of the relevant host modules (2, 14, 15, 23, 30).
(XLSX)

**S7 Table. Results from the analysis of variance to test for the relationship between the host module eigengene expression and the samples genotypes.** A) one-way ANOVA, and b) TukeyHSD for the significant ANOVA results.
(XLSX)

## Acknowledgments

Samples were collected under permit #FKNMS-2015-112-A1. We thank Joanne Delaney for her timely assistance with permitting. P. Jones helped with field and lab work. We thank Carly D. Kenkel for her advice with the co-expression network analysis, Mikhail V. Matz for his assistance with the symbiont community analysis, as well as Katherine Dougan and Mauricio Rodriguez-Lanetty for sharing the *D. trenchii* genome. The scientific results and conclusions, as well as any views or opinions expressed herein, are those of the author(s) and do not necessarily reflect the views of NOAA or the Department of Commerce.

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