1 *Symbiodinium* functional diversity and clade specificity under global change stressors
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3 Running Title (50 characters max): *In-hospite Symbiodinium* transcriptomes under stress

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5 Davies S.W.^{1,2,3+}, Ries J.B.^{2*}, Marchetti A^{1*}, Rafaela Granzotti¹, and K.D. Castillo¹

6

7 ¹University of North Carolina at Chapel Hill, Department of Marine Sciences; Chapel
8 Hill, NC

9 ²Northeastern University, Department of Marine and Environmental Sciences; Nahant,
10 MA

11 ³Boston University, Department of Biology, Boston, MA

12

13 ⁺Corresponding author:

14 Sarah W. Davies, 617-353-8980, daviessw@bu.edu

15 Department of Biology, 5 Cummington Mall, Boston University, Boston, MA 02215,
16 tel-512-609-9134, fax-617-353-6340

17 *these authors contributed equally to this work

18

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20 stress, transcriptomics, functional variation, photosynthesis

21

22 ABSTRACT

23 Coral bleaching episodes are increasing in frequency, demanding examination of the
 24 physiological and molecular responses of corals and their *Symbiodinium* to climate
 25 change. Here we quantify bleaching and *Symbiodinium* photosynthetic performance of
 26 *Siderastrea siderea* from two reef zones after long-term exposure to thermal and CO₂-
 27 acidification stress. Molecular response of *in hospite* *Symbiodinium* to these stressors was
 28 interrogated with RNAseq. Elevated temperatures reduced photosynthetic efficiency,
 29 which was highly correlated with bleaching status. However, photosynthetic efficiencies
 30 of forereef symbionts were more negatively affected by thermal stress than nearshore
 31 symbionts, indicating greater thermal tolerance in nearshore corals. At control
 32 temperatures, CO₂-acidification had little effect on symbiont physiology, although
 33 forereef symbionts exhibited greater photosynthetic efficiencies than nearshore
 34 symbionts. Transcriptome profiling revealed that *S. siderea* were dominated by clade C
 35 *Symbiodinium*, except under thermal stress, which caused shifts to thermotolerant clade
 36 D. Comparative transcriptomics of conserved genes across symbiotic partners revealed
 37 few differentially expressed *Symbiodinium* genes when compared to corals. Instead of
 38 responding to stress, clade C transcriptomes varied by reef zone, with forereef
 39 *Symbiodinium* exhibiting enrichment of genes associated with photosynthesis. Our
 40 findings suggest that functional variation in photosynthetic architecture exists between
 41 forereef and nearshore *Symbiodinium* populations.

INTRODUCTION

Dinoflagellates are ubiquitous unicellular algae that can occur as free-living individuals, endosymbionts, or parasites across a wide variety of marine organisms. The most recognized among endosymbionts is the genus *Symbiodinium*, which establish obligate relationships with many reef-building corals (Trench and Blank 1987). Photosynthetically-derived nutrients translocated from *Symbiodinium* to the host can comprise up to 100% of the coral energy budget (Muscatine and Cernichiaro 1969, Muscatine 1990), allowing for prolific coral growth in shallow oligotrophic tropical waters. In turn, *Symbiodinium* gain a protected microenvironment enriched in light, inorganic nutrients, and dissolved inorganic carbon as provided by the host (Barott et al 2015, Muscatine and Cernichiaro 1969, Yellowlees et al 2008). Although initially considered a single species, advancements in genetics have uncovered nine divergent clades within *Symbiodinium*, designated A-I (Coffroth and Santos 2005, Pochon and Gates 2010, Thornhill et al 2014), with growing evidence for additional within-clade genetic diversity (Davies et al 2016b, Howells et al 2012, Pettay and Lajeunesse 2013, Thornhill et al 2017).

Increasing atmospheric $p\text{CO}_2$ has reduced seawater pH, which impairs reef accretion (Hoegh-Guldberg et al 2007), and has increased sea surface temperatures, which can cause a breakdown of the coral-algal symbiosis—termed coral bleaching (Baker 2003, Hoegh-Guldberg 1999, Hoegh-Guldberg and Bruno 2010, Weis 2008). During bleaching, the coral is deprived of symbiont-derived organic carbon, which may lead to reduced growth or mortality. Bleaching events have been recognized as the primary driver of recent global reef declines and have been increasing in frequency and

severity (Hughes et al 2017, Pandolfi et al 2011). However, the rich genetic diversity of *Symbiodinium* often reflects their functional diversity, which could confer resilience to changing environments. For example, within-host *Symbiodinium* communities can shift after bleaching disturbances (Baker 2001, Jones et al 2008, Kemp et al 2014, Silverstein et al 2015, Thornhill et al 2006), which can strongly influence coral gene expression and future bleaching susceptibility (DeSalvo et al 2010a, Howells et al 2012, Jones and Berkelmans 2010). In order to predict coral reef responses to global change, there is an urgent need for a more comprehensive understanding of how coral-*Symbiodinium* associations respond to ocean warming and acidification.

Studies of *Symbiodinium* physiology document a wide array of stress responses to environmental change, including impairment/inactivation of photosynthesis at high temperatures (Iglesias-Prieto et al 1992, Iglesias-Prieto and Trench 1994), increased production of reactive oxygen species (ROS) and antioxidant activity in response to thermal and UV exposure (Gardner et al 2017, Lesser 1996, Suggett et al 2008), and reduced symbiont pigment concentrations at low pH (Tremblay et al 2013). In contrast to these strong physiological responses, there is mounting evidence that *Symbiodinium* lack strong transcriptional responses to global change stressors, which starkly contrasts their host's transcriptional response (Barshis et al 2014, Leggat et al 2011). Leggat et al. (2011) found that *Symbiodinium* exhibit few changes in expression of stress response genes under thermal stress when compared to these same genes in their coral host. A lack of transcriptomic responses has also been observed in both *Symbiodinium* D2 and C3K following heat exposure (Barshis et al 2014), which contrasts wide-spread transcriptomic shifts observed in the coral host in response to heat stress (Barshis et al 2013). Despite

the unresponsiveness of *Symbiodinium* gene expression, transcriptome profiles were clade-specific, regardless of experimental treatment, supporting prior evidence of functional differences amongst clades (Barshis et al 2014). Within clade functional variation has also been documented in clade B strains: when transcriptomes were compared, lineage-specific differences in expression were observed (Parkinson et al., 2016), providing additional evidence that substantial functional variation exists amongst *Symbiodinium* clades.

Clade C strains are the most derived *Symbiodinium* lineage and exhibit higher within-clade diversity when compared to other, more basal clades (Lesser et al 2013, Pochon et al 2006, Pochon and Gates 2010). Understanding how this diversity affects a coral's response to global change stressors is of great interest because clade-specific responses to stress are well documented. Transcriptomes of two divergent clade C1 lineages previously shown to exhibit distinct thermal tolerances (Howells et al 2012) revealed divergent expression patterns in response to heat stress, providing insights into how functional variation could lead to variation in holobiont (coral + *Symbiodinium*) thermal tolerance (Levin et al 2016). Given that coral host-*Symbiodinium* interactions ultimately determine how the holobiont will respond to global change, it is critical to determine how each symbiotic partner responds to multiple stressors. However, few studies have explored whole transcriptome responses of different *Symbiodinium* populations to ocean warming and acidification *in hospite* and even fewer have compared the responses of both symbiotic partners in parallel.

Here, we exposed the resilient and ubiquitous Caribbean reef-building coral *Siderastrea siderea* and its clade C *Symbiodinium* from two different reef zones

(nearshore and forereef) to thermal ($T = 25, 28, 32^{\circ}\text{C}$) and CO_2 -induced acidification ($p\text{CO}_2 = 324, 477, 604, 2553 \mu\text{atm}$) stress for 95 days. Our previous work has shown that both elevated temperature and $p\text{CO}_2$ elicited strong but divergent responses of the host's transcriptome (Davies et al 2016a) with increased temperatures substantially reducing calcification rates, with smaller declines observed in response to elevated $p\text{CO}_2$ (Castillo et al 2014). Here, we build on these studies by quantifying holobiont bleaching response, *Symbiodinium* photosynthetic efficiencies (F_v/F_m), and transcriptomic responses of *Symbiodinium in hospite*. We disentangle the response of the holobiont by isolating the responses of the host coral and *Symbiodinium* to thermal and acidification stress, which advances our understanding of their potential ecological trajectories in response to these global change stressors.

MATERIALS AND METHODS

Experimental design

Physiological measurements, transcriptomes and gene expression analyses presented here build upon experiments previously published in Castillo et al. (2014) and Davies et al. (2016). Briefly, whole *S. siderea* colonies from nearshore ($N=6$) and forereef ($N=6$) reef zones were collected from the Belize Mesoamerican Barrier Reef (total colonies = 12) in July 2011 and transported to the University of North Carolina at Chapel Hill (UNC) Aquarium Research Center. At UNC, whole coral colonies were sectioned into 18 fragments, which were maintained across six experimental treatments ($N=3$ tanks per treatment) that were illuminated with $250 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ on a 12-hour light-dark cycle for 95 days. Partial pressures of CO_2 corresponded to near-pre-industrial (324 ± 89

μatm , 28.14 ± 0.27 °C), near-present-day ($p\text{CO}_2$ 477 ± 83 μatm , 28.16 ± 0.24 °C),
 predicted end-of-century (604 ± 107 μatm , 28.04 ± 0.28 °C), and an extreme mid-
 millennium scenario (2553 ± 506 μatm , 27.93 ± 0.19 °C). Seawater temperatures
 approximated monthly minimum (25.01 ± 0.14 °C, $p\text{CO}_2$ 515 ± 92 μatm), mean ($28.16 \pm$
 0.24 °C, $p\text{CO}_2$ 477 ± 83 μatm), and maximum seawater temperatures (32.01 ± 0.17 °C,
 $p\text{CO}_2$ 472 ± 86 μatm) over the interval from 2002 to 2014 (Castillo and Helmuth 2005,
 Castillo and Lima 2010, Castillo et al 2012). Upon completion of the experiment,
 holobiont tissue (coral + symbiont) was extracted by water pick, immediately preserved
 in RNAlater, and stored at -80°C until RNA was extracted.

Symbiodinium physiology: tissue bleaching and photosynthetic efficiency

Coral fragments were photographed at the end of the 95-day experiment prior to tissue
 extraction. To quantify coral color differences associated with reduced chlorophyll and
 symbiont densities, Adobe Photoshop balance exposures were standardized across
 photographs using a white standard. Total red, green, blue and sums of all color channel
 intensities (red, green, blue) were calculated for 10 quadrats of 25×25 pixels within each
 coral fragment as a measure of brightness, with higher brightness indicating a reduction
 in algal pigment (i.e., reduced symbiont density or bleaching). This analysis was
 performed following Winters et al (2009) using the MATLAB macro “AnalyzeIntensity”
 and resulting data were inverted so that higher values represented increased coral
 pigmentation.

Photosynthetic efficiencies of the symbionts’ photosystem II (F_v/F_m) were
 quantified for each experimental fragment using pulse amplitude modulated fluorometry

(PAM; Diving-PAM, Waltz). Reductions in F_v/F_m values are indicative of sustained damage to the photosystem. F_v/F_m measurements were collected just prior to completion of the experiment and were made following dark adaptation one hour after sunset, which was when the lights turned off (n=3 measurements per coral fragment).

All statistical analyses were implemented in R (R Development Core Team 2015) using the ANOVA function based on log-transformed sum of all color channels and F_v/F_m data. The sum of all channels was chosen as the best color metric proxy since these data correlated best with F_v/F_m (Adjusted r^2 : 0.7366, p -value<0.001). Experimental treatment and reef zone were modeled as fixed effects and significant differences across levels within factors were evaluated using post-hoc Tukey's HSD tests. All assumptions of parametric testing were explored using diagnostic plots in R.

Transcriptome assembly, annotation and Symbiodinium identification

Detailed descriptions of sequence library preparations, holobiont transcriptome assembly and separation of host and symbiont contigs can be found in Davies *et al.* (2016a). Briefly, RNA was isolated from 94 holobiont fragments and pooled by reef zone within each experimental treatment, yielding a total of twelve sequencing libraries (Table 1), each of which contained RNA from at least six holobionts. Since each coral holobiont can host a community of *Symbiodinium*, RNA pooling is not expected to yield any additional complications compared to preparing each holobiont independently. Pooled libraries were prepared and sequencing was performed using four lanes of *Illumina HiSeq* 2000 at the University of North Carolina High Throughput Sequencing Facility, which yielded paired-end (PE) 100bp reads (Table 1).

Table 1: Summary of RNA libraries, including the number of holobionts from which RNA was pooled ('Pool'), reef zone where holobionts were collected (forereef = 'FR' and nearshore = 'NS'), temperature (°C) $\pm$ SD, $p\text{CO}_2$ (μatm) $\pm$ SD, raw 100bp paired-end reads ('PE Reads'), mapped holobiont reads ('Mapped') and total number of *Symbiodinium*-specific counts ('Symbiont'). Shaded samples were excluded from analyses due to low counts as a result of bleaching for a total of 235.1 million reads used in downstream analyses.

Sample	Pool	Reef zone	Temp (°C)	$p\text{CO}_2$ (μatm)	PE Reads (10^6)	Mapped (10^6)	Symbiont (10^6)
FR_P324	6	FR	28.14 $\pm$ 0.27	324 $\pm$ 89	63.6	59.2	24.1
FR_P604	6	FR	28.04 $\pm$ 0.28	604 $\pm$ 107	77.1	72.3	16.8
FR_P2553	7	FR	27.93 $\pm$ 0.19	2553 $\pm$ 506	48.1	44.7	17.1
NS_P324	6	NS	28.14 $\pm$ 0.27	324 $\pm$ 89	88.3	82.4	35.0
NS_P604	8	NS	28.04 $\pm$ 0.28	604 $\pm$ 107	48.5	45.3	10.5
NS_P2553	6	NS	27.93 $\pm$ 0.19	2553 $\pm$ 506	69.2	64.6	23.3
FR_T25	11	FR	25.01 $\pm$ 0.17	515 $\pm$ 92	50.9	47.4	20.5
FR_Control	14	FR	28.16 $\pm$ 0.24	477 $\pm$ 83	63.2	58.8	24.3
FR_T32	9	FR	32.01 $\pm$ 0.17	472 $\pm$ 86	50.0	47.2	1.2
NS_T25	7	NS	25.01 $\pm$ 0.17	515 $\pm$ 92	100.0	93.1	39.9
NS_Control	7	NS	28.16 $\pm$ 0.24	477 $\pm$ 83	56.7	52.7	23.6
NS_T32	7	NS	32.01 $\pm$ 0.17	472 $\pm$ 86	54.7	51.5	3.9
TOTAL					770.3	719.2	235.1

Transcriptome *de novo* assembly of over 770 million PE reads was completed using Trinity (Grabherr et al 2011) and resulting contigs were assigned as either coral- or *Symbiodinium*-derived contigs using a translated blast search (tblastx) against host and symbiont specific databases (Davies et al 2016a). *Symbiodinium*-specific contigs were annotated by BLAST sequence homology searches against UniProt and Swiss-Prot NCBI NR protein databases with an e-value cutoff of e^{-5} (Consortium 2015). Annotated sequences were then assigned to Gene Ontology (GO) categories. Transcriptome contiguity analysis (Martin and Wang 2011) and Benchmarking Universal Single-Copy Orthologs v2 (BUSCO; Simao et al 2015) were used to assess *Symbiodinium* transcriptome quality and completeness.

Symbiodinium community composition

To compare *Symbiodinium* expression across reef zones and experimental treatments, it was first necessary to determine which *Symbiodinium* clades were present in each library. Reads were trimmed with Fastx_toolkit (<20 bp length cutoff and bp quality score >20) and resulting quality filtered reads were then mapped to an ITS2-specific database (Franklin et al 2012) using Bowtie2.2.0 with the $-a$ flag to search all possible alignments (Langmead and Salzberg 2012). All possible alignments were identified and then the read was quantified in the analysis if all possible alignments for that read mapped to a single clade. This analysis determined that 96.7 to 100% of mapped reads from 10 of the 12 libraries exclusively aligned to a single *Symbiodinium* clade, with remaining libraries (NS_T32 and FR_T32) hosting divergent communities. However, these two libraries hosting divergent communities were excluded from downstream expression analysis due to the temperature-induced bleaching in these treatments yielding low concentrations of algal cells, which ultimately led to insufficient quantities of mapped *Symbiodinium* reads (1.2 and 3.9 million).

Mapping and differential expression analysis for clade C Symbiodinium

Raw reads across samples ranged from 48.1 to 100.0 million PE 100bp sequences. Quality filtered reads were mapped to the holobiont transcriptome (*Siderastrea siderea* + clade C *Symbiodinium*) and *Symbiodinium* mapped reads ranged from 10.5 (NS_P604) to 39.9 (NS_T25) million (Table 1). Differential gene expression analyses were performed on raw counts with *DESeq2* (v. 1.6.3; Love et al 2014) in R (v. 3.1.1; R Development Core Team 2015) using the model: design ~ reef zone + treatment. Counts were

normalized for size factor differences and pairwise contrasts were computed for each treatment relative to the control and between the two reef zones. A contig was considered significantly differentially expressed if it had an average basemean expression >5 and an FDR adjusted p -value < 0.05 (Benjamini and Hochberg 1995). Raw counts were then *rlog* normalized, a principle coordinate analysis was performed and the *adonis* function tested for overall expression differences across treatments and reef zones (Oksanen et al 2013). Gene expression heatmaps with hierarchical clustering of expression profiles were created with the *pheatmap* package in R (Kolde 2012). Gene ontology (GO) enrichment analysis was performed using the GO_MWU method, which uses adaptive clustering of GO categories and Mann-Whitney U tests (Voolstra et al 2011) based on a ranking of signed log p -values (Dixon et al 2015). Results were plotted as a dendrogram, which traces the level of gene sharing between significant categories and lists the proportion of genes in the dataset with raw p -values < 0.05 relative to the total number of genes within the entire expression dataset.

Expression comparison across holobiont partners

In order to compare expression across symbiotic partners, a subset of highly conserved genes (HCG) from the coral host (*Siderastrea siderea*) and clade C *Symbiodinium* were mined based on conserved gene annotation (N=2,862 genes). Differential gene expression analyses were performed with *DESeq2* using only data from the HCG set (design ~ reef zone + partner.treatment). Counts were normalized for size factor differences and pairwise contrasts were computed for each treatment relative to the control for each symbiotic partner. A contig was considered significantly differentially expressed if it had

an average basemean expression > 5 and an FDR adjusted p -value < 0.05 (Benjamini and Hochberg 1995). Significant DEGs were then compared across symbiotic partners. In order to confirm that *Symbiodinium* gene expression results were not confounded by strong reef zone differences in expression, heatmaps were generated on the HCG panel that was significantly differentially expressed in the host but not the symbiont.

RESULTS

Reef zone variation in Symbiodinium physiology

Long-term thermal stress treatments resulted in significantly reduced F_v/F_m of *Symbiodinium* ($p < 0.001$; Fig. 1A), which was highly correlated with bleaching status ($p < 0.001$, $r^2 = 0.74$; Fig. 1B). Interestingly, *Symbiodinium* originating from nearshore habitats had significantly higher F_v/F_m under thermal stress when compared to forereef *Symbiodinium* (Tukey's HSD $p = 0.008$), suggesting increased resilience to warmer temperatures. However, nearshore *Symbiodinium* only exhibited significantly higher F_v/F_m compared to forereef symbionts in 32°C treatments; in all other experimental treatments, forereef symbionts had constitutively higher photosynthetic efficiencies (Fig. 1A; $p < 0.001$).

Figure 1: Physiological measurements of *in hospite Symbiodinium*. (A) Photosynthetic efficiency of *Symbiodinium* photosystem II (PSII) (F_v/F_m) after 95 days in experimental treatments. Blue circles: forereef population average ($\pm$ SE); red triangles: nearshore population average ($\pm$ SE). Asterisk (*) indicates a significant interaction between treatment and reef zone. Treatment pairs lacking a letter in common indicate a statistically significant difference between those treatments pursuant to Tukey's Honest Significant Difference tests. High temperatures reduce F_v/F_m , however, symbionts from nearshore environments are less affected than those from the forereef, while forereef symbionts exhibit higher F_v/F_m in all other treatments. (B) Maximum F_v/F_m of *Symbiodinium* vs. mean coral bleaching status after 95 days in experimental treatments (T25= 25.01 $\pm$ 0.14 $^{\circ}$ C, 515 $\pm$ 92 μ atm; Control= 28.16 $\pm$ 0.24 $^{\circ}$ C, 477 $\pm$ 83 μ atm; P324= 28.14 $\pm$ 0.27 $^{\circ}$ C, 324 $\pm$ 89 μ atm; P604= 28.04 $\pm$ 0.28 $^{\circ}$ C, 604 $\pm$ 107 μ atm; P2553= 27.93 $\pm$ 0.19 $^{\circ}$ C, 2553 $\pm$ 506 μ atm; T32= 32.01 $\pm$ 0.17 $^{\circ}$ C, 472 $\pm$ 86 μ atm), as measured by the sum of all color intensities in the red, green and blue channels in standardized coral photographs across all treatments. This relationship demonstrates that F_v/F_m values and bleaching status are highly correlated.

Clade C *Symbiodinium* transcriptome

Although several clade C *Symbiodinium* transcriptomes are publicly available, we assembled a novel transcriptome because our samples were derived from *Siderastrea siderea* and therefore likely divergent from the previously assembled transcriptomes of clade C *Symbiodinium* hosted by the Pacific coral *Acropora hyacinthus* (Barshis et al 2014b) and the Caribbean anemone *Discosoma sanctithomae* (Rosic et al 2015). After adapter trimming and quality filtering, a total of 1,255,626,250 reads were retained

(81.5%; 69.6% paired, 11.9% unpaired). The resulting holobiont metatranscriptome contained 333,835 contigs (N50 = 1673), of which 65,838 were unambiguously assigned as *Symbiodinium* specific contigs, with an average length of 1,482 bp and an N50 of 1746. Among symbiont contigs, 45,947 unique isogroups were obtained, of which 22,239 (48.4%) had gene annotations based on sequence homology. Thirty-nine percent of *Symbiodinium* contigs had protein coverage exceeding 0.75 (Supplementary Figure 1) and results from BUSCO suggest that 81.9% of complete and fragmented BUSCOs were present (77.6% complete, 4.3% fragmented), indicating that the transcriptome was fairly comprehensive. All raw reads are archived in the National Center for Biotechnology Information Short Read Archive (SRA) under accession number PRJNA307543, with transcriptome assembly and annotation files available at <https://davieslab.wordpress.com/data/> and www.bco-dmo.org/project/635863.

***Symbiodinium* community shifts from clade C to D in response to thermal stress**

Clade-level analysis of sequencing libraries for *Symbiodinium* maintained at the low (25°C) and control (28°C) temperature treatments (for all $p\text{CO}_2$ treatments) revealed that 96.7-100% of reads mapped exclusively to one clade (N=4-436) aligned to clade C *Symbiodinium* (Fig. 2). Analysis of sequencing libraries for *Symbiodinium* maintained at the high temperature treatment (32°C; NS_T32 and FR_T32) consisted of divergent *Symbiodinium* communities hosting 50-75% clade D *Symbiodinium* (Fig. 2). These two libraries were removed from all gene expression analyses due to their divergent clade-level communities, which have been shown to exhibit strong transcriptomic differences (Barshis et al 2014). In addition, these libraries also had the lowest mapped

308 *Symbiodinium* reads (Table 1) due to low sample yield resulting from bleaching under
309 thermal stress.

310 Figure 2: Proportion of RNAseq reads mapping exclusively to one *Symbiodinium* clade
311 lineage (A, B, C, D) for each transcriptome library, with independent columns
312 representing a unique transcriptome library. Number at top of bar indicates total number
313 of reads mapping exclusively to one clade within that library. Red and blue blocks
314 indicate that the library originated from nearshore and forereef corals, respectively.
315 Treatment conditions are noted below the bar (T25= 25.01 ± 0.14 °C, 515 ± 92 µatm;
316 Control= 28.16 ± 0.24 °C, 477 ± 83 µatm; P324= 28.14 ± 0.27 °C, 324 ± 89 µatm; P604=
317 28.04 ± 0.28 °C, 604 ± 107 µatm; P2553= 27.93 ± 0.19°C, 2553 ± 506 µatm; T32= 32.01
318 ± 0.17 °C, 472 ± 86 µatm). All libraries were dominated by clade C with the exception of
319 T32 treatments where dominated by clade D.

320
321 *Minimal response to acidification stress, with population-specific differences in*
322 *expression of genes associated with photosynthesis*

323 Without consideration of the high temperature treatment libraries that were excluded due
324 to low mapped reads, transcriptomic analysis of *Symbiodinium* gene expression across the
325 remaining treatments exhibited few differentially expressed genes (DEGs) relative to the
326 control treatment, with 92 (25°C), 40 (324 µatm), 576 (604 µatm) and 23 (2553 µatm)

DEGs observed for the respective treatments, representing a total of 1.69% of the entire transcriptome responding to any treatment (Fig. 3A). In contrast, 24.45% of genes were differentially expressed with respect to reef zone, with 3,792 upregulated in nearshore specimens and 6,441 upregulated in forereef specimens, regardless of treatment condition—demonstrating a strong whole-transcriptomic response to reef zone (Fig. 3B; Fig. 4A, $p=0.016$). No differences in whole-transcriptome response were detected for treatment ($p=0.33$; Fig. 3B). Gene ontology enrichment analysis across reef zones revealed many significantly enriched GO terms within cellular components, which were dominated by GO terms associated with photosynthesis in forereef *Symbiodinium* [e.g., *thylakoid part* (GO:0044436), *photosystem* (GO:0009521), *light-harvesting complex* (GO:0030076), *plastid part* (GO:0044435)], suggesting fundamentally different photosynthetic architectures across reef zones (Fig. 4B).

Figure 3: Global RNA-Seq patterns for *in hospite* clade C *Symbiodinium* under different $p\text{CO}_2$ (28 °C) and low temperature (25 °C) treatments. (A) Venn diagram of the number of differentially expressed *Symbiodinium* genes (out of 45,947, FDR=0.05) in experimental conditions relative to control conditions. Orange numbers indicate overrepresented genes and turquoise numbers indicate underrepresented genes, but overall very little transcriptomic response is observed. B: Principal coordinate analysis of all *r-log* transformed isogroups clustered by experimental treatment and reef zone, demonstrating significantly different transcriptome profiles across *Symbiodinium* from different locations, regardless of experimental treatment (NS= nearshore and FR= forereef; T25= 25.01 ± 0.14 °C, 515 ± 92 µatm; Control= 28.16 ± 0.24 °C, 477 ± 83 µatm; P324= 28.14 ± 0.27 °C, 324 ± 89 µatm; P604= 28.04 ± 0.28 °C, 604 ± 107 µatm; P2553= 27.93 ± 0.19°C, 2553 ± 506 µatm).

Figure 4: Differentially expressed genes (DEGs) of *Symbiodinium* across reef zones and treatments. (A) Heatmap for the top 300 DEGs for reef zone where each row is a gene and each column is a unique transcriptome library. The color scale is in log₂ (fold change relative to the gene's mean) and genes and samples are clustered hierarchically based on Pearson's correlation of their expression across samples. Red and blue blocks indicate that libraries originated from nearshore and forereef corals, respectively. Treatment conditions are noted below the bar (T25= 25.01 ± 0.14 °C, 515 ± 92 μatm; Control= 28.16 ± 0.24 °C, 477 ± 83 μatm; P324= 28.14 ± 0.27 °C, 324 ± 89 μatm; P604= 28.04 ± 0.28 °C, 604 ± 107 μatm; P2553= 27.93 ± 0.19°C, 2553 ± 506 μatm). Hierarchical clustering of libraries (columns) demonstrates strong reef zone differences in expression. (B) Gene ontology (GO) enrichment of the 'Cellular component (CC)' category derived from the transcriptomic differences across reef zones. Dendrograms depict sharing of genes between categories (categories with no branch length between them are subsets of each other), with the fractions corresponding to proportion of genes with an unadjusted *p* < 0.05 relative to the total number of genes within the category. Text size and boldness indicate the significance (Mann-Whitney U tests) of the term. Blue categories are enriched in forereef *Symbiodinium* while red categories are enriched in the nearshore. Forereef *Symbiodinium* exhibit enrichment of GO categories associated with photosynthesis.

Coral host elicits stronger transcriptomic response than *Symbiodinium*

When the highly conserved gene (HCG) set from the coral host (*Siderastrea siderea*) and clade C *Symbiodinium* were compared, coral hosts were far more transcriptionally responsive when compared to their *Symbiodinium* partners (Fig. 5). Among these HCGs

(N=2,862), corals modified expression by 3.7-15.7% across experimental treatments, while *Symbiodinium* only modified expression by 0.05-2.8%, demonstrating that coral hosts were far more responsive to low temperature and variable acidification stressors when compared to their algal symbionts (Fig. 5). Heatmaps also confirmed that the lack of *Symbiodinium* HCG expression was not driven by strong differences in *Symbiodinium* gene expression across reef zones. Instead genes that were highly differentially expressed in the host showed no differences in expression across reef zones for *Symbiodinium* (Supplementary Fig. S1A-D).

Figure 5: Barplot showing numbers of significantly ($p < 0.05$) differentially expressed genes (DEGs) for the host (left) and its algal symbiont (right) across the four experimental treatments relative to the control for the 2862 highly conserved genes (HCG) across host and symbiont. Orange bars represent enriched genes and turquoise bars represent depleted genes in the treatment (T25= 25.01 ± 0.14 °C, 515 ± 92 μ atm; P324= 28.14 ± 0.27 °C, 324 ± 89 μ atm; P604= 28.04 ± 0.28 °C, 604 ± 107 μ atm; P2553= 27.93 ± 0.19 °C, 2553 ± 506 μ atm) relative to the control (28.16 ± 0.24 °C, 477 ± 83 μ atm). Overall, hosts exhibit a much stronger response across HCGs when compared to *in hospite* *Symbiodinium*, regardless of experimental treatment.

DISCUSSION

Increased thermotolerance of Siderastrea siderea from more thermally variable environments

Reductions in *Symbiodinium* photosynthetic ability have been strongly correlated with a lack of thermal tolerance and increased susceptibility to bleaching (Howells et al 2012, Takahashi et al 2009, Warner et al 1999). Here we observed strong reductions in photosynthetic efficiency in response to the high temperature treatment (Fig. 1A), which has been consistently observed for *Symbiodinium* exposed to thermal stress (Roth 2014, Warner et al 1996). Strong reductions in calcification rate (Castillo et al 2014) and overall homeostatic disturbance of host gene expression (Davies et al 2016a) was previously observed for these same coral specimens in response to the prescribed thermal stress. These severe reductions in holobiont fitness in response to chronic high temperatures might therefore be driven by the decreases in symbiont photosynthetic function observed here (Fig. 1A).

Notably, we observed that reductions in *S. siderea* symbiont photophysiology at 32°C were dependent on natal reef location, where nearshore *Symbiodinium* were less negatively affected by thermal stress than forereef *Symbiodinium* (Fig. 1A), with photosynthetic efficiency being highly correlated with the coral's bleaching status (Fig. 1B). Since *Symbiodinium* photophysiology is expected to be a reliable proxy for thermal tolerance (Ragni et al 2010, Wang et al 2012, Warner et al 1996, Warner et al 1999), these fluctuating responses may be due to local adaptation of *Symbiodinium* to more thermally variable nearshore environments (Baumann et al 2016, Castillo and Helmuth 2005). Indeed, Castillo et al. (2012) observed reductions in linear extension

rates of forereef *S. siderea* colonies over the past three decades of anthropogenic warming, while nearshore and backreef colonies exhibited constant (nearshore) or increasing (backreef) extension rates over the same interval. Castillo et al (2012) attributed these differential trends in linear extension to thermal preconditioning of backreef and nearshore corals, to the extent that nearshore and backreef environments experience more extreme baseline diurnal and seasonal fluctuations in seawater temperature compared to more thermally stable forereef environments.

The potential for local adaption of *Symbiodinium* is thought to be high given their haploid genomes and short generation times (Correa and Baker 2011, Santos and Coffroth 2003). Howells et al. (2012) observed increased thermotolerance of clade C1 *Symbiodinium* originating from warmer reef locations and Hume et al. (2016) detected strong selection in *S. thermophilum*, a thermotolerant lineage found in extreme temperatures of the Persian Gulf. In addition, recent assisted evolution efforts have shown that *Symbiodinium* can rapidly evolve heat tolerance in culture (Chakravarti et al 2017), providing further evidence of local adaptation in *Symbiodinium*.

Chronic high temperatures cause shifts to more thermotolerant Symbiodinium

Symbiodinium genetic diversity has been reported to vary not only among different coral host species and environments but also within a single coral species (Baker 2003, Coffroth and Santos 2005, Thornhill et al 2014, Thornhill et al 2017). This genetic diversity has been shown to drive physiological variation within *Symbiodinium* (Iglesias-Prieto and Trench 1994, Iglesias-Prieto et al 2004, Warner et al 1999) and numerous studies have demonstrated that genetically distinct

Symbiodinium lineages can exhibit a range of thermotolerances (Robison and Warner 2006, Suggett et al 2008). Consequently, some *Symbiodinium* appear less affected by thermal stress, while others are impacted by even small changes in temperature (Berkelmans and van Oppen 2006, Hume et al 2016, Jones and Berkelmans 2010, Rowan 2004). Here, we observed that long-term thermal stress induced shifts in *Symbiodinium* communities from clade C to D in *S. siderea*, regardless of reef zone origin (Fig. 2). However, this result is at least partially due to a loss of symbiosis with clade C, rather than clade D becoming more successful *in hospite* under increased temperatures, since the corals were still severely bleached (Fig. 1A).

Clade D *Symbiodinium* are generally associated with hosts from marginal reef environments exposed to increased thermal stress (Pettay et al 2011, Pettay and Lajeunesse 2013, Pettay et al 2015) and corals hosting predominantly clade D have been shown to exhibit greater resistance to bleaching (Berkelmans and van Oppen 2006, Jones et al 2008), while those hosting predominantly clade C benefit from higher rates of carbon fixation by the symbionts (Jones and Berkelmans 2010, Stat et al 2008). Although it is unclear from the data at hand if these communities would shift back to clade C dominant communities after alleviation of thermal stress (Sampayo et al 2016), these shifts could represent adaptive potential for corals exposed to future ocean warming (Cunning et al 2015, Silverstein et al 2015).

Symbiodinium gene expression and photosynthesis unresponsive to ocean acidification

Increases in atmospheric $p\text{CO}_2$ have been shown to reduce calcification rates of reef-building corals (e.g., Castillo et al 2014, Chan and Connolly 2013, Hoegh-Guldberg et al 2007, Kleypas et al 1999), but the effects of $p\text{CO}_2$ on the coral's symbiotic *Symbiodinium* are less clear. Some research suggests that elevated $p\text{CO}_2$ enhances *Symbiodinium* primary production and has proposed that dissolved inorganic carbon is the limiting substrate for photosynthesis, which is more available under high $p\text{CO}_2$ (Brading et al 2011, Crawley et al 2010). In contrast, others have observed negative effects of increased $p\text{CO}_2$ on *Symbiodinium* physiology, including reduced productivity, photosynthesis and calcification (Anthony et al 2008, Zhou et al 2016). Here, we observed minimal effects of long-term $p\text{CO}_2$ stress on *Symbiodinium* photosynthetic efficiencies across a wide range of $p\text{CO}_2$ conditions (Fig. 1A). This lack of response starkly contrasts the strong physiological response and community shift (clade C to D) of *Symbiodinium* under increased temperatures (Figs. 1 & 2). One explanation for this lack of physiological response to $p\text{CO}_2$ could be that the specific *Symbiodinium* lineage investigated here (clade C) simply does not respond to $p\text{CO}_2$ stress, which has been observed for other *Symbiodinium* strains (Brading et al 2011).

This lack of physiological response of *Symbiodinium* to acidification stress was also evident at the molecular level, where no significant responses were observed at the whole-transcriptome level (Fig. 3), even when reef zone-specific responses were considered (Fig. S1). This paucity of a transcriptional stress response in *Symbiodinium* is consistent with previous studies, which generally observe little to no transcriptional response to environmental stressors (Barshis et al 2014, Leggat et al 2011, Putnam et al 2013), with the exception of extreme heat stress (Baumgarten et al 2013, Gierz et al 2017,

Levin et al 2016)—which we were unable to investigate here due to a combination of severe bleaching (Fig. 1A) and shifts in *Symbiodinium* community (Fig. 2). Instead of responding transcriptionally, it has been proposed that *Symbiodinium* use post-transcriptional regulatory mechanisms, including translational regulation and post-translational modifications, to drive molecular responses. Evidence suggests that very few transcription factors are present in *Symbiodinium* transcriptomes and genomes (Bayer et al 2012, Shoguchi et al 2013). Instead, it has been proposed that these algae utilize small RNAs and microRNAs, (Baumgarten et al 2013, Lin et al 2015), RNA-editing (Liew et al 2017), and *trans*-splicing of spliced leader sequences (Lin et al 2010, Lin 2011, Zhang et al 2007) to regulate their environmental stress responses. Another possibility is that the timescale of this study (95 days) was not sufficient to trigger physiological and molecular responses in *Symbiodinium*. While the stability of dinoflagellate mRNA is known to be considerably longer than for other organisms (Morey and Van Dolah 2013), it is possible that physiological and transcriptomic responses were minimized by long-term acclimatization of *Symbiodinium* via phenotypic buffering (Reusch 2014). Lastly, and perhaps most likely, it could be that *Symbiodinium in hospite* simply do not respond to changes in $p\text{CO}_2$ because their position within host-derived tissue-bound space buffers the algae from external changes in pH (Barott et al 2015, Rands et al 1993, Venn et al 2009).

Functional differences in photosynthesis across divergent environments

Symbiodinium exists endosymbiotically across a variety of hosts and habitats, which presents these algae with diverse challenges with respect to photosynthesis and survival.

Photosynthetic efficiency of photosystem II (F_v/F_m) is widely used as an indicator of photosynthetic performance and stress (Murchie and Lawson 2013). Here, higher F_v/F_m values observed in forereef *Symbiodinium* relative to nearshore (Fig. 1A) might indicate variation in the rate of carbon translocation to hosts (Roberty et al 2014, Warner and Berry-Lowe 2006), since nearshore environments experience increased temperatures and nutrients along with reduced light levels relative to forereef habitats (Baumann et al 2016, Castillo and Lima 2010). These environmental differences may stimulate nearshore corals to rely more heavily on heterotrophy and dissolved/particulate organic matter relative to corals in light-rich forereef habitats (Grottoli et al 2006, Tremblay et al 2014). However, if forereef hosts were receiving increased quantities of photo-assimilates, increased calcification rates would also be expected in forereef corals—a trend that was not previously observed (Castillo et al 2014). Because the physiologies of symbionts from forereef and nearshore corals were divergent even after 95 days in common garden experiments, we propose that the environment has selected for distinct nearshore and forereef *Symbiodinium* ‘ecotypes’ (Chakravarti et al 2017, Howells et al 2012, Iglesias-Prieto et al 2004), although further work is required to test this hypothesis.

Notably, reef-zone-specific photosynthetic efficiencies were also reflected at the transcriptomic level, where fixed differences in gene expression were observed (Fig. 3B, 4A). Divergent stable-state gene expression is perhaps not surprising given that transcriptomic differences across clades (Barshis et al 2014, Rosic et al 2015) and lineages within a clade (Parkinson et al 2016) have been previously observed. Gene ontology enrichment analysis detected strong upregulation of genes related to photosynthesis in forereef symbionts (Fig. 4B), corroborating our observation of higher

photosynthetic efficiencies for forereef *Symbiodinium*. These differences in regulation of photosynthesis-related genes could facilitate differences in thermal tolerance across ‘ecotypes’, as was observed here under heat stress (Fig. 1A). Thermally sensitive *Symbiodinium* have been shown to exhibit increased disruption of PSII photochemistry (Robison and Warner 2006, Warner et al 1999), which has been associated with variations in the regulation of genes involved in photosynthesis (McGinley et al 2012). Given that expression differences amongst *Symbiodinium* lineages are consistently enriched for photosynthesis-related genes (Barshis et al 2014, Baumgarten et al 2013, Parkinson et al 2016, Rosic et al 2015), we propose that *Symbiodinium* ‘ecotypes’ have evolved unique transcriptomic signatures for photosynthesis-related genes, which likely reflect functional differences in photosynthetic architectures across environments (Fig. 4B) and represent phenotypic variation upon which selection can act (Chakravarti et al 2017).

Coral hosts elicit stronger transcriptomic responses than Symbiodinium

Understanding how each partner of the coral–*Symbiodinium* symbiosis will respond to environmental stress is required to accurately predict future coral reef susceptibility to global oceanic change (Weis 2008). Although both partners exhibit a wide array of physiological stress responses, *Symbiodinium* are assumed to initiate symbiosis breakdown (Berkelmans and van Oppen 2006, Stat et al 2006, Stat and Gates 2011) due to their production of reactive oxygen species (ROS), which can damage the host (Lesser 1996, Weis 2008). In contrast, the present experiments show that *S. siderea* exhibit stronger transcriptomic responses across highly conserved genes (HCGs) than their

Symbiodinium symbionts to thermal and CO₂-acidification stress (Fig. 5), suggesting that symbiosis breakdown or ‘bleaching’ may be initiated by the host instead of the symbiont. Strong transcriptomic responses of coral hosts are well-documented (Davies et al 2016a, DeSalvo et al 2010b, Meyer et al 2011, Moya et al 2012, Seneca and Palumbi 2015), while *Symbiodinium* transcriptomic responses are generally more subtle (Barshis et al 2014, Baumgarten et al 2013, Leggat et al 2011). For example, Barshis et al. (2014) similarly observed few transcriptional changes across >50,000 genes in response to heat stress across two *Symbiodinium* lineages in symbiosis (D2, C3K), starkly contrasting the broad transcriptomic shifts observed in the symbiont’s host when exposed to identical conditions (Barshis et al 2013). As discussed above, *Symbiodinium* in our study may be unresponsive to the stressors investigated, or the stressors were not applied over a sufficient timescale to elicit a transcriptomic response. Alternatively, the transcriptomic stability of *Symbiodinium* could result from *in hospite* buffering of the symbiosome under pCO₂ stress, which has been observed in response to changes in seawater pH (Barott et al 2015, Rands et al 1993, Venn et al 2009). Although potentially costly to the host, manipulation of *Symbiodinium* responses to stress through active regulation of the symbiont’s environment might be favored over the potential chemical toxicity resulting from the release of reactive molecules by stressed *Symbiodinium* (e.g., Lesser 1996).

Conclusion

Acclimation and adaptation play critical roles in determining an organism’s ability to tolerate variable environments (Reusch 2014, Schlichting and Pigliucci 1996). Here, we demonstrate that coral-associated *Symbiodinium* exhibit immense capacity for adaptation

581 and acclimation to thermal and acidification stress. *Symbiodinium* from nearshore
 582 locations were more resilient to thermal stress than forereef symbionts, suggestive of
 583 local adaptation. We also observed differences in gene expression between *Symbiodinium*
 584 from nearshore and forereef environments that were coupled with differences in
 585 photosynthetic efficiencies, irrespective of treatment condition. These transcriptomic
 586 differences suggest that photosynthesis-related gene expression varies by habitat, which
 587 likely reflects adaptation to unique environments over potentially long timescales. Host
 588 retention of a more thermotolerant clade of *Symbiodinium* (clade D) under thermal stress
 589 was also documented, providing further evidence for a mechanism of acclimation to
 590 thermal stress. Acclimation to $p\text{CO}_2$ was also observed at both physiological and
 591 transcriptomic scales, the mechanisms of which have not yet been identified, although *in*
 592 *hospite* buffering remains a viable hypothesis. Overall, we find evidence for both
 593 adaptation and acclimation in the *S. siderea*-*Symbiodinium* holobiont that may explain the
 594 relative resilience of this coral species to global change stressors.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplemental Figure 1: Heatmaps for the expression of highly conserved genes (HCG) found to be differentially expressed in the coral host for each experimental treatment. The left four columns represent expression of these genes in the coral host (orange blocks) and the right four column represent expression of these same genes in *Symbiodinium* (green blocks) across the experimental treatments relative to the control (28.16 ± 0.24 °C, 477 ± 83 μ atm): (A) low temperature (T25 = 25.01 ± 0.14 °C, 515 ± 92 μ atm), (B) pre-industrial $p\text{CO}_2$ (P324 = 28.14 ± 0.27 °C, 324 ± 89 μ atm), (C) next-century $p\text{CO}_2$ (P604 = 28.04 ± 0.28 °C, 604 ± 107 μ atm), and (D) extreme-high $p\text{CO}_2$ (P2553 = 27.93 ± 0.19 °C, 2553 ± 506 μ atm). Red and blue blocks indicate that the library originated from nearshore and forereef reef zones, respectively. Each row is a gene and each column is a unique transcriptome library. The color scale is in $\log_2$ (fold change relative to the gene's mean) and genes are clustered hierarchically based on Pearson's correlation of their expression across samples. Results reveal lack of differential gene expression amongst experimental treatments relative to the control in *Symbiodinium*, compared to strong differential gene expression amongst treatments in the host, and that this result is not due to differential gene expression amongst reef zones in *Symbiodinium*.