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Shuffling between *Cladocopium* and *Durusdinium* extensively modifies the physiology of each symbiont without stressing the coral host

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Abstract

As sea surface temperatures increase, many coral species that used to harbour symbionts of the genus *Cladocopium* have become colonized with the thermally tolerant genus, *Durusdinium*. Here, we asked how gene expression in the symbionts of one genus changes depending on the abundance of another symbiont genus within the same coral host, and what effect this interaction has on the host. Symbiont gene expression was overwhelmingly driven by whether the genus was the minority or the majority within the host, which affected 79% (*Durusdinium*) and 96% (*Cladocopium*) of all genes. Particularly strong effects in both genera were observed for photosynthesis components (upregulated in the minority state) and proteins putatively associated with cell motility (upregulated in the majority state). Importantly, there was no distinct gene expression signature associated with the mixed symbiosis state when both genera were represented in comparable proportions within the host, which could lead to more intense competition. The mixed symbiosis was also not associated with elevated host stress: in fact, after heat treatment, stress signatures were the lowest in mixed-symbiosis corals compared to both *Cladocopium*- and *Durusdinium*-dominated corals. In conclusion, during shuffling between *Cladocopium* and *Durusdinium* both symbiont genera go through extensive and largely reciprocal physiological transitions, but there is no evidence of intensifying antagonistic interactions that are detrimental to the host. Unless the mixed-symbiosis corals in this study are not representative of the typical transition between *Cladocopium* and *Durusdinium*, the process of shuffling from one symbiont genus to another appears to be cost-free for the coral host, and even appears to be associated with lower stress susceptibility. This raises optimism for the future corals, which will probably have to rely on symbiont shuffling more and more to withstand environmental challenges.

1 | INTRODUCTION

Reef building corals get up to 90% of their energetic requirements for growth and calcification through symbiosis with dinoflagellate algae of the family Symbiodiniaceae (Falkowski et al., 1984). Coral bleaching is the breakdown of this symbiosis, and often occurs when water temperatures exceed a certain threshold. The symbionts, formerly delineated as clades A–I, have now been divided into many genera (Lajeunesse et al., 2018; Nitschke, et al., 2020). In the Great Barrier Reef, the majority of scleractinian corals of the genus *Acropora* have historically engaged in symbiosis with symbionts of the genus *Cladocopium*. However, as ocean temperatures continue to rise and bleaching events become more frequent, these corals are becoming colonized by relatively opportunistic, thermotolerant symbionts of the genus *Durusdinium*. This can have a profound effect on the bleaching tolerance of the coral: in the some species, the bleaching severity during a natural bleaching event can vary more depending on the genus of the symbiont than on the host genetics (Fuller, 2020; Glynn et al., 2001; Rowan et al., 1997). While each coral colony is typically dominated by a single Symbiodiniaceae genus (e.g., Baker, 2003), background levels of other genera are common (Silverstein, et al., 2012), and after bleaching events, their prevalence in coral colonies may increase (e.g., Cunning, Gillette, et al., 2015; Cunning, Silverstein, et al., 2015; Jones, et al., 2008; Thornhill et al., 2006), up to the point of nearly complete replacement of the typical symbionts (e.g., Manzello et al., 2019). Still, after the stress abates, corals often return to the originally-hosted symbiont genus (Thornhill et al., 2006). The influential "adaptive bleaching" hypothesis" postulated that corals could survive heat waves by replacing their symbionts with a newly acquired, putatively better adapted strain (Buddemeier & Fautin, 1993), however, this exact mechanism could not be experimentally validated. Instead, it turned out that corals undergo "symbiont shuffling," temporarily replacing the heat-sensitive symbionts with heat-tolerant ones that were already present within the host. This happens during and shortly after heat stress (e.g., Berkelmans and Oppen, 2006; Cunning, Gillette, et al., 2015; Cunning, Silverstein, et al., 2015; Jones, 2008; Ladner et al., 2012; Mieog, et al., 2007). Once the stress abates, the symbiont composition slowly reverts back to the original state (e.g., Thornhill et al., 2006). This acclimatization mechanism was called "the nugget of hope" for corals during climate change (Berkelmans and Oppen, 2006). The goal of this study was to investigate whether the process of transition from one symbiont genus to another might be costly to the coral host, presumably because of intensifying antagonistic interactions between the two symbiont genera when one of them replaces another.

Comparison of orthologous genes has demonstrated that the two symbiont genera have different expression patterns in genes associated with thermotolerance (Barshis et al., 2014), though symbionts show limited or no transcriptomic response to heat stress (Barshis et al., 2014; Leggat, et al., 2011; Putnam, et al., 2012). Despite this, it has been shown that following periods of thermal stress, *Durusdinium*-dominated colonies do not bleach as frequently as other colonies (e.g., Berkelmans and Oppen, 2006; Cunning, Gillette, et al., 2015; Cunning, Silverstein, et al., 2015; Glynn et al., 2001; Manzello et al., 2019). Although *Durusdinium* symbionts may confer bleaching resistance, it comes at the cost of reduced growth (e.g., Cunning, Gillette, et al., 2015; Cunning, Silverstein, et al., 2015; Jones and Berkelmans, 2010; Little, et al., 2004; Pettay et al., 2015). Other physiological trade-offs have been observed, including reduced fecundity, reduced carbon acquisition (Cantin et al., 2009; Matthews et al., 2018), reduced calcification (Pettay et al., 2015), and higher disease susceptibility (Shore-Maggio et al., 2018). Furthermore, it was found that in the Caribbean coral *Montastraea cavernosa*, *Durusdinium* dominance is associated with differential expression of stress-related genes in the host: having *Durusdinium* appears to stress the host (Cunning and Baker, 2020).

Most corals associate with a single symbiont type at a time, with only background levels of other symbionts present in host tissues (e.g., Baker, 2003), except at the young recruit stage (Little, 2004), which suggests that either mixed symbiosis is somehow disadvantageous or that one symbiont rapidly outcompetes the other. Still, mixed symbioses are well documented in a variety of organisms. In legume root nodules, for example, evenly mixed symbiont communities may include mutualistic partners as well as parasitic ones (e.g., Denison, 2000; Friesen and Mathias, 2010; Thrall et al., 2000). In some cases, such as yuccas and yucca moths, mutualists and cheaters are able to coexist (Pellmyr and Huth, 1994). In other cases, such as legumes and rhizobia, host-sanctioning mechanisms prevent symbiosis with parasitic partners (e.g., Lerouge et al., 1990; Mutch and Young, 2004; Oldroyd et al., 2011). Acroporid corals from the Great Barrier Reef typically host *Cladocopium* symbionts with background levels of *Durusdiunium*, or the reverse, with more even mixtures being relatively rare (e.g., Innis et al., 2018; Ulstrup and Oppen, 2003). Although it has been shown that the dominant symbiont type has an effect on host gene expression (e.g., Barfield et al., 2018; Cunning and Baker, 2020), whether these two symbiont genera interact in the host tissues is presently unknown.

Here, we analysed existing gene expression data from two studies with a combined total of 181 *Acropora hyacinthus* samples (Barshis et al., 2013; Seneca & Palumbi, 2015). Both of these studies investigated the association between host gene expression and bleaching susceptibility. Barshis et al. (2013) identified genes that were upregulated in resilient corals but not in susceptible corals in the absence of stress. During heat treatment, these same "front-loaded" genes were less upregulated in resilient corals than susceptible corals, suggesting that the host may "tune" the physiology of corals to tolerate stress. Similarly, Seneca and Palumbi (2015) exposed susceptible and resilient corals to heat stress over a 20 h period to capture the transcriptomic and bleaching responses. Like Barshis et al. (2013), they found a difference in the gene expression of susceptible and resilient corals: the return of gene expression to normal levels was associated with less severe bleaching. In addition, Rose et al. (2016) applied gene network analysis to the same data and identified two gene network modules, "module 10" and "module 12," expression of which 5 h after heat stress was associated with bleaching severity 15 h later: more severe bleaching was about to happen when module 10 was low and module 12 was high. Finally, in a single study of symbiont gene expression based on the same data, Barshis et al., 2014 characterized genus-specific gene expression patterns and found the surprising lack of response to heat stress in either symbiont genus.

We capitalized on the fact that corals from these studies had greater than 90% *Cladocopium*, greater than 90% *Durusdinium*, or a mixture of both symbionts. First, we predicted that symbionts change their gene expression depending on whether they are a minority or a majority within the host. Second, we expected that there would be evidence of intensifying competition when symbionts of different genera are at comparable proportions within the same host, with detrimental effects on the host. Competition between symbionts might cause them to become more virulent towards the host, prioritizing their own proliferation by sequestering more nutrients and translocating fewer photosynthates to the host (e.g., Baker et al., 2018; Lesser et al., 2013; Morris et al., 2019). Specifically, we expected that corals hosting mixed symbiont communities would be more susceptible to heat stress and would show altered expression of generalized stress response genes (Dixon et al., 2020) and gene network modules associated with more or less severe bleaching outcomes (Rose et al., 2016). If the mixed-symbiosis state was indeed associated with intense competition between symbionts and virulence towards the host, this would imply that the process of symbiont shuffling would incur fitness cost to the coral host and therefore may not be the a particularly easily available acclimatization solution.

2 | MATERIALS AND METHODS

2.1 | Data sources

We chose two studies (Barshis et al., 2013; Seneca & Palumbi, 2015) for this analysis by searching for the genus, *Acropora*, in the NCBI SRA database. The BioProjects are PRJNA274410 and PRJNA177515, respectively. Combined, these studies consist of 181 coral samples, of which 172 were chosen for the analysis (after removal of nine samples showing strong sequencing batch effect, D. Barshis. personal communication). These studies were selected because they involved similar heat stress experiments on *Acropora hyacinthus* corals. In both studies, *Acropora hyacinthus* colonies were collected from the tide pools on the south side of Ofu island, American Samoa. Seneca and Palumbi, (2015) maintained the temperature of the control tanks at 29°C and raised the experimental treatment temperature from 29°C to 35°C over 3 h, held the peak temperature for 1 h, and then allowed the temperature to return to 29°C. In this experiment, samples were collected twice, after 5 and 20 h. The 5 h time point was taken 1 h after peak temperature to capture the heat stress response, while the 20 h time point was taken to capture the onset of the bleaching response. Barshis et al. (2013) had temperatures ranging from 24.8–34.5°C, with a mean temperature of 29.2°C ($n = 2$). Barshis et al. (2013) raised the experimental treatment temperature by about 2.7°C over the ambient temperature and took samples after 72 h. This time was chosen because it induced mortality in corals from one pool while corals from the other pool remained resilient.

Both studies extracted RNA using modified TRIzol (GibcoBRL/Invitrogen) protocol (Barshis et al., 2013). Seneca and Palumbi (2015) constructed 152 libraries (76 heated and 76 control samples) using TruSeq RNASample Prep v2 (LS) protocol (Illumina). Barshis et al. (2013) constructed 31 libraries (14 heated and 14 control samples) and sequenced using Illumina Genome Analyser II (Illumina) at three different sequencing facilities. The original authors of Barshis et al.(2013) found that 11 samples, which were all sequenced at one facility, had a strong batch effect and removed them from the study (leaving 20 samples: 11 control and nine heated). We also removed these samples. The SRA metadata tables for each study are shown in Table S1.

2.2 | Sequence data processing and symbiont genus determination

Detailed descriptions of the data processing pipeline are on Github (https://github.com/evelynabbott/mixed_symbiosis.git). The fastq files from both studies were downloaded using the SRA toolkit. Adapter trimming was done in paired-end mode using cutadapt (Martin, 2011), with a minimum length of 20 bp and a PHRED quality cutoff set to 20. FASTQC (Andrews, 2010) was used to assess the quality of a subset of 10,000 reads before and after trimming. Reads were then mapped to a combined reference comprising of a *Cladocopium* transcriptome, a *Durusdinium* transcriptome (Ladner et al., 2012), and an *Acropora millepora* genome (Fuller, 2020) using bowtie2 with the --local option. The resulting alignment files were then split into three files, one for each organism. PCR duplicates were identified after alignment using MarkDuplicates from the Picard Toolkit (Broad Institute, 2019). Samtools (Li et al., 2009) was used to sort and convert from sam files to bam files. FeatureCounts (Liao et al., 2014) was used to count reads mapping to annotated gene boundaries. The number of host reads per sample ranged from 79,551 to 3,663,620. The number of *Cladocopium* reads per sample ranged from 2181 to 1,295,046. The number of *Durusdinium* reads per sample ranged from 2142 to 1,649,264. The relative proportions of reads mapping to the two symbiont genera differed among samples most mostly with respect to the source colony and did not align with the study, outplanting location, or experimental treatment (Figure S1).

2.3 | Symbiont gene expression

For symbionts the challenge was to avoid artefacts due to variation in total counts between minority and majority-states. To do this, we resampled the gene counts to 25,000 total counts per sample, by drawing gene counts without replacement with the probability proportional to the initial representation of a gene in the sample. The samples that originally had less than 25,000 counts (60 samples for *Cladocopium* and 27 samples for *Durusdinium*) were excluded from the differential gene expression analysis. We retained genes that had mean count of 0.5 in the samples where the focal symbiont genus comprised >90% of all symbiont reads (97 samples for *Durusdinium* and 42 samples for *Cladocopium*), or mean count of 0.25 among samples where the symbiont genus comprised less than 10% of total symbiont reads (15 samples for *Durusdinium* and 37 samples for *Cladocopium*, after excluding the samples with less that 25,000 original counts). These criteria retained 11,365 genes for *Durusdinium* and 10,888 genes for *Cladocopium*. The counts were imported into DESeq2 (Love et al., 2014) and per-gene log-fold changes with respect to the genus' proportion within the host were computed. Ten resampled data sets were produced for each genus, and per-gene log-fold changes were averaged among replicates.

To account for the effect of sampling time point and simultaneously the effect of study, a covariate "time" was included with the value of "0 h" for Barshis et al. (2013) data and "5 h" and "20 h" for Seneca and Palumbi (2015) data. Another covariate, "pool," was included to account for the effect of outplanting location. We did not include the effect of heat treatment into the model because it has been reported not to affect symbiont gene expression in these samples (Barshis et al., 2014), which we have confirmed in our preliminary analysis.

For heatmap plotting (Figure 1a,b), we have once again resampled the counts data to 25,000 but this time we retained samples with less-than 25,000 total counts, with their original counts. The resampled data were read into DESeq2 and then the function DESeq2::vst was used to produce normalized variance-stabilized data, which were averaged across 10 count-resampling replicates. Then, the logarithm of the total resampled counts per sample was removed as a covariate using function limma::removeBatchEffect, to account for the residual effect of samples with lower than 25,000 original counts. The data set columns were sorted in the order of increasing *Durusdinium* proportion, and then the heatmaps of the resulting data tables were plotted using the function pheatmap::pheatmap, clustering all genes (rows) into 250 groups of similar expression using k-means algorithm (pheatmap option kmeans_k = 250). Such k-means-clustered heatmaps are visually indistinguishable from full heatmaps with a row for every gene (where rows are clustered according to euclidean distance and the whole heatmap is scaled to the same size) but they make much smaller image files and are easier for plotting software to handle.

2.4 | Weighted gene coexpression network analysis

This analysis (WGCNA) (Langfelder and Horvath, 2017) was used to explore major patterns of gene expression variation in the symbionts in an unsupervised way. The input for this analysis were the matrices of normalized variance stabilized counts obtained with R function DESeq2::vst(), averaged across 10 counts-resampling replicates, excluding the samples that had less than 25,000 total counts, and from which the same covariates described in the previous section were removed using the R function

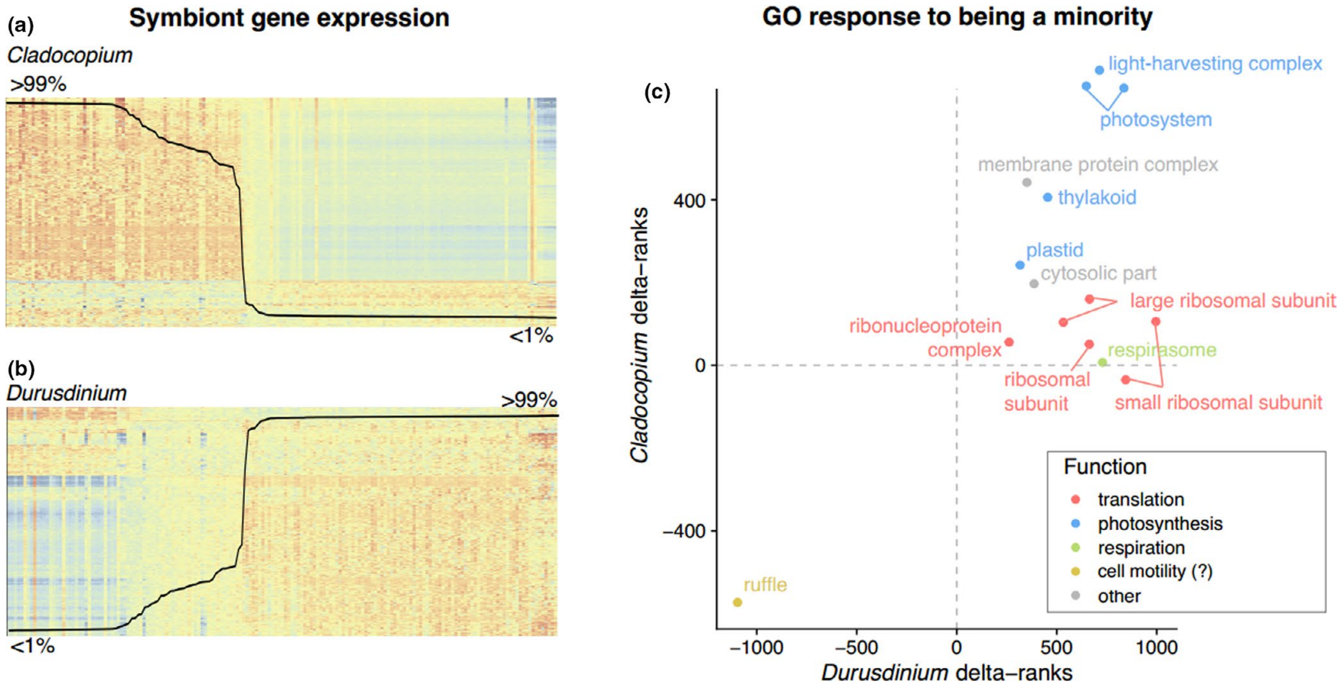

FIGURE 1 Minority-majority difference is the predominant driver of gene expression in the symbionts. (a and b) Show clustered gene expression heatmaps for *Cladocopium* (a) and *Durusdinium* (b): rows are genes clustered according to the euclidean distance of their expression across samples, and columns are samples arranged in the order of increasing *Durusdinium* proportion. The overlaid line graph on each heatmap shows the proportion of the focal symbiont genus. Red colour indicates upregulation, blue colour indicates downregulation, yellow colour indicates intermediate expression level (the scale is arbitrary). (c) Delta rank comparison of GO categories of the "Cellular component" division that were highly significant ($p < .01$) in either *Cladocopium* or *Durusdinium*. Higher delta rank indicates upregulation in the minority state, and lower delta rank indicates downregulation in the minority state [Colour figure can be viewed at wileyonlinelibrary.com]

limma::removeBatchEffect. We ran WGCNA with a soft threshold power of 10, minimal module size of 30, and no module merging for both symbiont genera.

2.5 | Host data set

For the host gene expression, we were interested in comparing three symbiotic states: *Cladocopium*-dominated (more than 90% of reads mapping to *Cladocopium*), *Durusdinium*-dominated (more than 90% of reads mapping to *Durusdinium*), and mixed (the remainder). The original data set was unbalanced with respect to these groups, containing 98 *Durusdinium*-dominated samples, 42 *Cladocopium*-dominated samples, and 32 mixed samples. Each of the symbiosis states were also split nearly equally in half by the control versus heat treatment, so the state:treatment group size ranged from 15 (mixed:control) to 50 (*Durusdinium*:control). To avoid artefacts due to unbalanced design and at the same time obtain resampling-based confidence estimates, we randomly drew 10 samples from each of the state:treatment groups to create 100 data sets containing 60 samples each. The resampled data sets were imported into DESeq2 to compute variance-stabilized normalized data (function DESeq2::vst), which were then used to examine expression of genes associated with the generalized stress response (GSR, Dixon et al., 2020) with respect to symbiont state and heat treatment. The same "pool" and "time" covariates as for the symbiont data (described above) were removed from the variance-stabilized data. Similar resampled data sets were generated only for samples from Seneca and Palumbi (2015) study, to examine the behaviour of bleaching-associated gene network modules identified by Rose et al. (2016) based on counts from Seneca and Palumbi (2015).

2.6 | Expression of stress- and bleaching-associated gene networks in the coral host

Lists of genes comprising the generalized stress response and bleaching-associated modules (modules 10 and 12 from Rose et al., 2016) were downloaded from the Supporting Information data of the corresponding papers. To summarize the behaviour of these gene groups across resampled data sets, we used the eigengene approach (Langfelder and Horvath, 2017): we have computed the first principal component of these genes' expression across samples by applying the function vegan::rda to the variance-stabilized dataset containing only the genes belonging to the network module of interest, and adjusted the sign such that positive scores corresponded to elevated expression of module genes. The variation in these eigengene expression scores across symbiont states was then analysed using a linear model with symbiont state as the predictor. We have computed model-predicted values of the eigengene expression across symbiont states for both treatments for all 100 resampled replicates. The resampling support was calculated as the fraction of replicates supporting the particular direction of difference between model-predicted values for experimental groups.

2.7 | Comparing the effect of mixed symbiosis with effect of *Durusdinium* dominance

For this analysis, we chose the *Cladocopium*-dominated state as the baseline and compared per-gene log-fold changes due to mixed symbiosis (C + D) to log-fold changes due to *Durusdinium* dominance. This analysis was performed on resampled data containing either only heated samples or only control samples. The per-gene log-fold changes were computed in DESeq2 with the symbiont state as the predictor, and two covariates, "pool" and "time", described above for the symbiont gene expression analysis. The log-fold changes were averaged across 30 replicates (we have confirmed that the results hold for the number of replicates as low as 10).

2.8 | Functional enrichment tests

We used a gene ontology (GO) enrichment analysis that utilizes the Mann-Whitney U (MWU) test (Wright et al., 2017) to identify significant functional differences among up- and downregulated genes associated with symbiosis state. This test compares log-fold change ranks among genes to determine whether the ranks of genes in a GO category diverge significantly from ranks of other genes. The key test statistic for GO analyses is the delta-rank, which is the difference between the median rank of all genes annotated with a specific GO term and all other genes. A positive delta-rank indicates upregulation of genes annotated with the term, and conversely, a negative delta-rank indicates downregulation of these genes. The statistical test to determine the significance of the delta-ranks is the Mann-Whitney U test. Importantly for the current study, delta-ranks can be used to compare functional results across studies and even across highly divergent organisms (Strader et al., 2016), because delta-ranks are properties of universal annotation terms (GO categories) rather than organism-specific genes. This approach is particularly suitable for comparing responses in highly divergent lineages such as *Cladocopium* and *Durusdinium*, because it does not restrict the analysis to the small subset of genes for which orthologous relationship can be inferred (Ladner et al., 2012) and instead utilizes all annotated genes in each organism.

In this study, for all gene ontology analyses we have used DESeq2-derived log-fold changes (averaged across resampling replicates) instead of *p*-value-based measures (as in, for example, Dixon et al., 2015) to avoid bias towards abundantly expressed genes. Such genes are measured with better precision and thus get stronger *p*-values compared to other genes. This bias can become particularly strong for datasets with low counts, such as the resampled symbiont data sets used here, where all genes except the most highly expressed ones are measured with a great deal of noise. We have used resampling and averaging to obtain more robust per-gene log-fold change estimates despite the sampling noise.

3 | RESULTS

3.1 | Symbiont gene expression

From the clustered heatmaps of resampled counts, variance-stabilized gene expression data (Figure 1a,b) it is visually clear that the minority-majority gradient is the overwhelming driver of gene expression in both symbiont genera. This result was also more formally supported by WGCNA: it identified only two gene network modules in *Cladocopium*, one of which was upregulated and another downregulated when the genus was the minority (Figure S2A). In *Durusdinium*, five gene network modules were identified, one of which was associated with the minority state and the remaining four, with the majority state (Figure S2B). Importantly, neither clustered heatmaps nor WGCNA analysis identified gene groups associated with the transitional, mixed-symbiosis state.

DESeq2 analysis confirmed the trends that were visually apparent in clustered heatmaps (Figure 1a,b): 91% of all genes in *Cladocopium* and 73% in *Durusdinium* were significantly upregulated in the majority state, but there were also genes significantly upregulated in the minority state (5% in *Cladocopium* and 6% in *Durusdinium*).

For functional analysis of these differences we used per-gene log-fold-changes calculated by the DESeq2 model, averaged across 10 counts-resampling replicates. We used the rank-based gene ontology (GO) analysis implemented in the GO_MWU package (Wright et al., 2017), which identifies GO categories significantly enriched with up- or downregulated genes. We focus on results for the GO division "cellular component" since they were the most easily interpretable. There were nine GO terms that were significantly ($p < .05$) enriched with differentially expressed genes in *Cladocopium* and 15 for *Durusdinium*. To compare these functional signals we compared the delta-ranks returned by the GO_MWU analysis for all terms that were highly significant ($p < .01$) in at least one of the genera (Figure 1c). In both *Cladocopium* and *Durusdinium*, being a minority was associated with elevated expression of genes belonging to four partially overlapping (i.e., gene-sharing) GO terms related to photosynthesis: light-harvesting complex, photosystem, thylakoid, and plastid. Additionally, there were four terms related to translation and one term related to respiration that were enriched with upregulated genes only in *Durusdinium* (Figure 1c). Lastly, there was one term, "ruffle" (the leading edge of a crawling cell), containing five genes for *Cladocopium* and 10 genes for *Durusdinium*, which were downregulated in the minority state in both genera (Figure 1c and Figure S3).

3.2 | Mixed symbiosis and association with host stress

To examine the potential effects of hosting two symbiont genera at comparable proportions on the coral stress and bleaching responses, we analysed the behaviour of three previously identified gene network modules associated with these processes. The first one included 634 genes comprising the generalized stress response (GSR, Dixon et al., 2020), which are upregulated in *Acropora* sp. corals under any kind of high-intensity stress. The other two were the two modules from (Rose et al., 2016), module 10 (181 genes, negatively associated with bleaching severity) and module 12 (201 genes, positively associated with bleaching severity). We compared the eigengene expression of these modules in corals dominated by *Cladocopium*, dominated by *Durusdinium*, and in mixed-symbiosis corals under control conditions and after heat stress (Figure 2). As expected, heat stress resulted in strong upregulation of GSR and module 12 (Figure 2a,c) and strong downregulation of module 10 (Figure 2b). After heat stress, corals dominated by *Cladocopium* symbionts had the highest GSR signature, *Durusdinium*-dominated corals were the next-highest, and the mixed-symbiosis corals had the lowest GSR, although only the difference

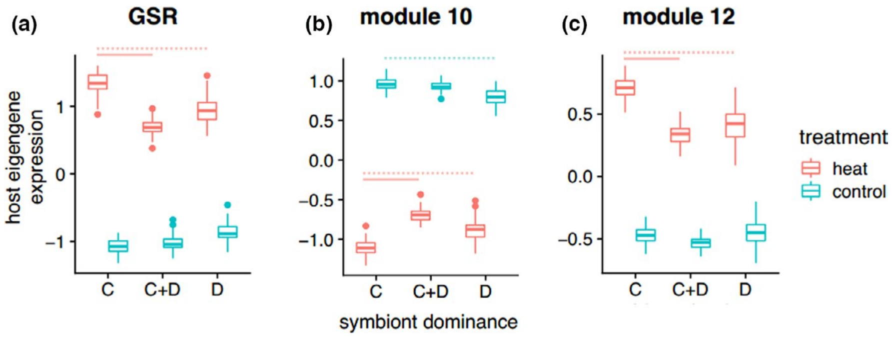

FIGURE 2 Expression of generalized stress response (GSR) gene network module (a) and bleaching- associated gene network modules (b, c) depending on the symbiosis state ("C", *Cladocopim*-dominated; "D", *Durusdinium*-dominated; "C + D", mixed) and heat stress treatment. The y-axis is the host eigengene expression (i.e., PC1 score) of the group of genes comprising the gene network module, in units reflecting the proportion of variance explained by the eigengene. Boxplots are based on mean eigengene expression for 100 resampling replicates. Differences across stress treatments have 100% resampling support in all cases. Within treatments, differences with >95% resampling support are indicated by solid horizontal bars above boxplots, differences with 90%–95% support are indicated by dotted bars [Colour figure can be viewed at wileyonlinelibrary.com]

between mixed symbiosis and *Cladocopium* dominance had >95% resampling support (Figure 2a). Under control conditions, GSR did not significantly differ between symbiosis states but there was a trend towards higher GSR expression in *Durisdinium*-dominated corals compared to mixed-symbiosis and *Cladocopium*-dominated corals (Figure 2a). Exactly the same pattern, but in reverse, was observed for module 10 (Figure 2b). Module 12 eigengene expression matched GSR, although without any notable trends in control (Figure 2c).

3.3 | Mixed-symbiosis corals compared to *Durusdinium*-dominated corals

To see if the host response to mixed symbiosis was nothing more than a less pronounced response to *Durusdinium* dominance, we compared the gene expression differences observed when either mixed symbiosis or *Durusdinium* dominance are contrasted against *Cladocopium* dominance. Plotting the log-fold-changes in response to mixed symbiosis against the changes in response to *Durusdinium* dominance reveals clear positive correlation both under control and heated condition (Figure 3a,c, $p < 2e\text{-}16$ in both cases); however, this correlation was far from perfect ($R^2 = 0.18$ under control and $R^2 = 0.31$ under heated conditions). Functional analysis of the difference between these two responses revealed many cellular component GO terms that were highly significantly ($p < .01$) enriched with genes that were upregulated more strongly or weakly during transition to mixed symbiosis (Figure 3b, 16 terms under control and 13 terms under heated conditions). These GO terms included some common and condition-specific ones (Figure 3b,d, Figure S4). The common ones were the two groups of overlapping (i.e., gene-sharing) terms related to chromosome structure (upregulated in mixed symbiosis) and plasma membrane components (downregulated in mixed symbiosis). Condition-specific responses to mixed symbiosis included upregulation of ribosomal genes under control conditions and downregulation of Golgi components under heated conditions.

4 | DISCUSSION

4.1 | Symbionts

As expected, the difference between majority and minority states was the primary driver of gene expression in both symbiont genera. Importantly, there was no evidence of a separate "transition state" in between that could be associated with elevated antagonistic

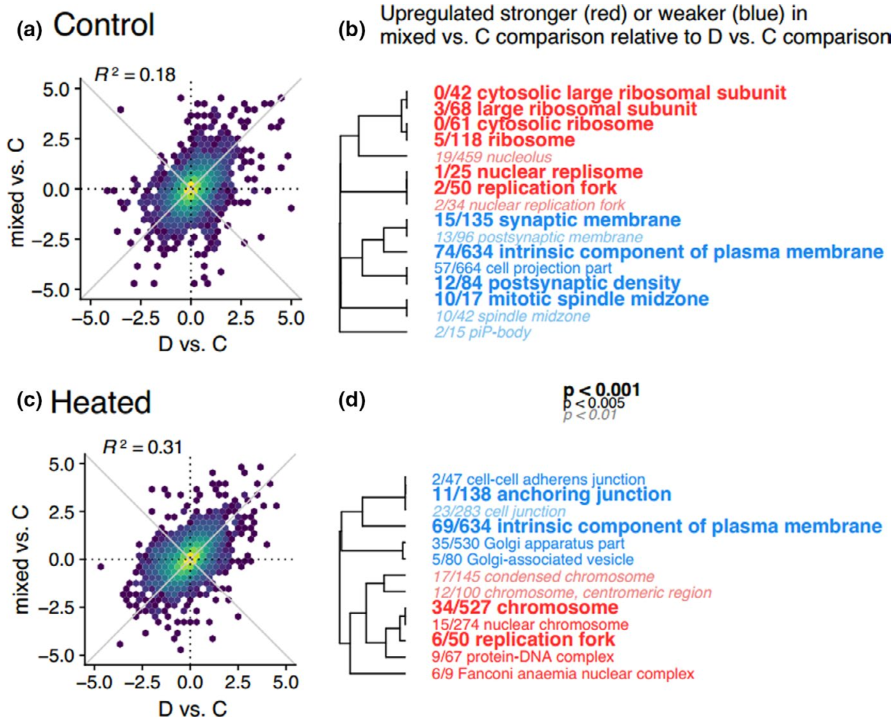

FIGURE 3 Comparison of gene expression differences under mixed symbiosis and differences under *Durusdinium* dominance relative to *Cladocopium*-dominated state, under control (a, b) and heated (c, d) conditions. (a, c) Per-gene log2-fold changes for mixed symbiosis versus *Cladocopium* dominance contrast (y-axis) are plotted against log2-fold changes for *Durusdinium* dominance versus *Cladocopium* dominance contrast (x-axis), averaged across 30 resampled replicates. The colour of the plot represents the density of the points, with the highest density in yellow and the lowest density in dark purple. (b, d) Gene ontology terms that become upregulated more strongly (red) or weakly (blue) when contrasting mixed symbiosis with *Cladocopium* dominance relative to the contrast between *Durusdinium* and *Cladocoipum* dominance. Font indicates the *p*-value adjusted for multiple testing over GO categories (see legend). Dendrograms represent hierarchical clustering of GO categories based on sharing of the genes among them. The fraction shows the number of genes with log2-fold difference exceeding 0.5 relative to the total number of genes annotated with a given term [Colour figure can be viewed at wileyonlinelibrary.com]

interactions (Figure 1a,b and Figure S2). Functional analysis revealed upregulation of photosynthesis machinery in both genera when they are the minority within the host (Figure 1c). This might indicate the emphasis on higher productivity to boost the minority-symbiont's competitiveness, and/or be a response to shading by the majority-symbionts, since upregulation of chloroplasts, photosynthetic pigments, and light harvesting complex is a common response to shading in plants (e.g., Fan et al., 2019; Gong et al., 2015; Lv, et al., 2020). Perhaps minority-symbionts constantly try to outgrow their competitors to escape shading in a manner similar to forest plants trying to outpace their neighbours for access to sunlight.

Importantly, a minority symbiont could not be shaded by a majority symbiont unless its physical location in the coral is different. This is because if symbionts are homogeneously mixed, the average amount of shading per cell – and indeed the complete average microenvironment - would be equal for all symbionts. Perhaps when a symbiont is a minority it could be restricted to a particular microhabitat within the coral tissue. For example, the poorer competitor may be more easily outcompeted in the upper, brighter tissue areas, but perhaps deeper, darker tissues are a specific microhabitat where they are able to remain competitive enough to avoid being completely displaced. In such a scenario, the functional differences associated with being a minority could actually be due to occupying a particular spatial microhabitat location/niche.

Interestingly, *Durusdinium* (but not *Cladocopium*) additionally upregulates respiratory components and ribosomes when it is the minority. High ribosome production is associated with elevated growth rate in a variety of organisms, including multicellular plants, green algae (e.g., Giordano et al., 2015), insects, crustaceans (e.g., Elser et al., 2003), bacteria (e.g., Bosdriesz et al., 2015), and yeast (e.g., López-Maury et al., 2008). Additional upregulation of ribosomes and oxidative metabolism therefore supports the hypothesis of the highly competitive minority state. The fact that only *Durusdinium* shows these additional upregulations aligns well with the notion that *Durusdinium* is a particularly competitive "weedy" symbiont, able to rapidly colonize recently bleached colonies and marginal habitats (Baker, 2003).

The GO term "ruffle" (the leading edge of a crawling cell), downregulated in both genera in the minority state, is somewhat of a puzzle because (as far as we know) Symbiodiniaceae cells do not crawl (Blank, 1987; Lajeunesse et al., 2018; Lee, et al., 2020; Trench et al., 1981). "Ruffle" genes are associated with a dynamically remodeled cell membrane, which in Symbiodiniaceae might be related to the flagellum or to the highly complex system of internal membranes surrounding and penetrating the nucleus (Blank, 1987; Taylor, 1969). It does seem plausible therefore that their regulation might be associated with some aspect of cell shape adjustment and motility, but confirming this would require a separate study.

4.2 | Coral host

Symbiodiniaceae are not always mutualistic: for example, under heat stress, they may start to parasitize the coral host by sequestering host resources and proliferating in host tissues without giving photosynthates to the host (e.g., Baker et al., 2018; Lesser et al., 2013; Morris et al., 2019). Generally, in symbiosis, higher competition between different genetically distinct symbionts typically results in higher virulence towards the host, incurring physiological costs (e.g., Bremermann and Pickering, ,1983; Chao, et al., 2000). We had initially hypothesized that the two symbiont genera would compete the most, potentially harming the host, when they are represented in comparable proportions inside the host, that is, in the "mixed-symbiosis" state. However, looking at the symbiont gene expression in this study, it seems more plausible that the most competitive state for a symbiont is the minority state. If so, the mixed-symbiosis might in fact be the most benign state for the host since neither symbiont is the minority. Indeed, the three signatures of heat stress - upregulations of the generalized stress response genes (GSR, Dixon et al., 2020) and modules 12 from (Rose et al., 2016) and downregulation of module 10 from (Rose et al., 2016) - were less pronounced in heat-stressed mixed-symbiosis corals compared to corals with any single symbiont type (Figure 2).

Another known effect of *Durusdinium* dominance is higher background expression of stress-related genes ("frontloading" sensu Barshis et al., 2013; Cunning and Baker, 2020) but lower magnitude of their response to actual stress. This effect is seen on Figure 2a,b, where expression of both GSR and module 10 in *Durusdinium*-dominated corals is shifted towards the stressed state under control and does not change as much after stress, compared to *Cladocopium*-dominated corals. In the mixed-symbiosis state under control, GSR and module 10 are not significantly different from in *Cladocopium*-dominated corals (resampling support 58% for GSR and 65% for module 10) but there is a trend toward stress-like expression, which can be the beginning of the frontloading effect of the increasing *Durusdinium* proportion. Still, this minor trend towards frontloading cannot account for the fact that the stress response in mixed-symbiosis corals tends to be even less pronounced than in *Durusdinium*-dominated corals (Figure 2a,b), which suggests that additional mechanisms are at play. Indeed, while the response to mixed symbiosis is positively correlated with the response to *Durusdinium* dominance (Figure 3a,c), there are many functional differences (Figure 3b,d). Perhaps the most notable of those is higher expression of replication fork genes under both control and stressed conditions and higher expression of ribosomal genes under control conditions in the mixed-symbiosis corals. Both of these signatures suggest elevated growth rate, which aligns well with the finding that mixed-symbiosis corals show the least signatures of stress compared to both single-symbiont states (Figure 2a,b).

4.3 | Caveats

This study was conceived and performed during the lockdown of 2020, when new data generation was impossible, and so several uncertainties remain that in another year could have been addressed with new field experiments. Some conjectures that are based solely on the gene expression patterns could have been verified, such as higher symbiont productivity in the minority state or higher growth rate of mixed-symbiosis corals. Perhaps the most important outstanding question is, does mixed symbiosis lead to better coral host health (higher stress resilience and potentially higher growth rate), or the other way around? Indeed, it is quite possible that mixed symbiosis is promoted by exceptionally good health of the host. This could explain why mixed symbiosis is relatively rare in nature (Lajeunesse et al., 2018; Lee, et al., 2016). For example, of the 172 corals in this study, only 20 had mixed symbiont communities in which the less common symbiont made up at least 20% of the symbiont reads. This brings us to the last important point that requires verification in the future studies: do the mixed-symbiosis corals from this study represent the typical transitional state of shuffling between *Cladocopium* and *Durusdinium*, or are they unusual in some way that results in stable mixed symbiosis? A study monitoring the actual symbiont shuffling process in time is needed to resolve this, which is quite a challenge to design (but see Cunning and Baker, 2020).

5 | CONCLUSIONS

Gene expression in the symbionts was overwhelmingly determined by whether the genus was the minority or the majority within the host, with signatures suggesting higher productivity in the minority state (especially in *Durusdinium*) which might indicate higher competitiveness. In the mixed-symbiosis state, there was no evidence of intensifying antagonistic interactions between symbionts, while the coral host showed diminished signatures of stress as well as gene expression signatures suggestive of faster growth. Overall, this suggests that symbiont shuffling from one genus to another is not costly to the coral host and might even be promoted via reduced stress susceptibility during the transition. Still, it is also possible that it is the faster host growth and reduced stress susceptibility that promotes mixed symbiosis. The direction of causation should be clarified in future studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Reads used in this study can be accessed in the SRA database accession PRJNA274410 and PRJNA177515. All scripts used in this analysis can be found on github at https://github.com/evelynabbott/mixed_symbiosis.git.

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SUPPORTING INFORMATION

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