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Functional genomic analysis of corals from natural CO_2-seeps reveals core molecular responses involved in acclimatization to ocean acidification

Carly D. Kenkel[1,2] | Aurelie Moya[3] | Julia Strahl[1,4] | Craig Humphrey[1] | Line K. Bay[1]

[1]Australian Institute of Marine Science, Townsville, Qld, Australia

[2]Department of Biological Sciences, University of Southern California, Los Angeles, CA, USA

[3]ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Qld, Australia

[4]Carl von Ossietzky University of Oldenburg, Oldenburg, Germany

Correspondence
Carly D. Kenkel, University of Southern California, Los Angeles, CA, USA.
Email: ckenkel@usc.edu

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Abstract

Little is known about the potential for acclimatization or adaptation of corals to ocean acidification and even less about the molecular mechanisms underpinning these processes. Here, we examine global gene expression patterns in corals and their intracellular algal symbionts from two replicate population pairs in Papua New Guinea that have undergone long-term acclimatization to natural variation in pCO_2. In the coral host, only 61 genes were differentially expressed in response to pCO_2 environment, but the pattern of change was highly consistent between replicate populations, likely reflecting the core expression homeostasis response to ocean acidification. Functional annotations highlight lipid metabolism and a change in the stress response capacity of corals as key parts of this process. Specifically, constitutive downregulation of molecular chaperones was observed, which may impact response to combined climate change-related stressors. Elevated CO_2 has been hypothesized to benefit photosynthetic organisms but expression changes of *in hospite Symbiodinium* in response to acidification were greater and less consistent among reef populations. This population-specific response suggests hosts may need to adapt not only to an acidified environment, but also to changes in their *Symbiodinium* populations that may not be consistent among environments, adding another challenging dimension to the physiological process of coping with climate change.

KEYWORDS

Acropora millepora, adaptation, carbon dioxide, gene expression, lipid metabolism, RNA-seq, *Symbiodinium*, symbiosis

1 | INTRODUCTION

Increasing atmospheric carbon dioxide concentration contributes to global warming and alters ocean carbonate chemistry in the process known as ocean acidification (Sabine et al., 2004). Elevated atmospheric CO_2 increases the hydrogen ion concentration $[H^+]$, thereby reducing ocean pH. This excess H^+ reacts with carbonate ions $[CO_2^{3-}]$ to form bicarbonate $[HCO_3^-]$, lowering the saturation state of carbonate minerals, such as calcite and aragonite (Feely, Doney, & Cooley, 2009). Many marine taxa rely on carbonate minerals to build their calcium carbonate $[CaCO_3]$ skeletons. Increasing H^+ and concomitant reductions in pH increase the potential for dissolution of present skeletons (Van Woesik, Van Woesik, Van Woesik, & Van Woesik, 2013). Simultaneous reductions in the bioavailability of carbonate ions also increase the difficulty of depositing new skeleton (Kleypas et al., 1999). Ocean acidification has been predicted to have major consequences for marine calcifying organisms, such as reef-building corals, through this combination of effects (Hoegh-Guldberg et al., 2007).

Scleractinian corals form the basis of the most biodiverse marine ecosystems on the planet: tropical coral reefs (Caley & St John, 1996; Idjada & Edmunds, 2006). They also provide important ecosystem services, such as habitat for fisheries species and shore

protection (Sheppard, Dixon, Gourlay, Sheppard, & Payet, 2005). Consequently, investigation of coral responses to acidification has received substantial attention in recent years. The majority of empirical work has focused on relatively short-term (days to months) exposure of corals to simulated acidification in aquaria and the reported fitness consequences have been mixed. A recent meta-analysis found that for every unit decrease in the saturation state of aragonite, coral calcification declines by 15% on average, though individual studies report more significant declines or even increases (Chan & Connolly, 2013), which may be attributable to differences in tolerance among species (Albright, 2011; Erez, Reynaud, Silverman, Schneider, & Allemand, 2011; Jokiel, 2011).

Natural CO_2-seep environments provide an attractive alternative to aquarium-based experiments aimed at understanding coral resilience potential: no experimental manipulations are necessary and in situ populations have likely already undergone some level of acclimatization or adaptation to be able to inhabit low-pH environments. Work by Fabricius et al. (2011) on corals at volcanic CO_2-seeps in Papua New Guinea (PNG) has provided support for the mixed effects observed in laboratory experiments. Naturally high pCO_2/low-pH environments drastically alter the coral community, but some species, like massive *Porites*, appear unaffected, while others, such as Acroporids, are significantly less common or even absent (Fabricius, De'ath, Noonan, & Uthicke, 2014). Population reductions in situ, combined with observations of negative physiological impacts, including declines in calcification under elevated pCO_2 (Strahl et al., 2015) strongly suggest that low pH imposes selection pressure on less resilient taxa within the PNG system, such as Acroporids. Consequently, *Acropora* spp. are predicted to be ecological "losers" under future acidification scenarios (Schoepf et al., 2013). However, the fact that some *Acropora* spp. can still be found in seep environments indicates that genetic variation for low-pH tolerance may already exist within these less resilient species, in line with the findings of recent studies in analogous natural systems investigating variation in coral thermal tolerance (D'croz & Maté, 2004; Kenkel et al., 2013; Oliver & Palumbi, 2011) and its mechanistic basis (Barshis et al., 2013; Dixon et al., 2015; Kenkel & Matz, 2016).

Transcriptome sequencing has become a powerful tool for investigating physiological plasticity and adaptive evolution in a changing environment and can provide insight into the mechanistic basis of population-level variation (Debiasse & Kelly, 2016). We used RNA-seq to investigate the core genomic response underpinning long-term acclimatization to high pCO_2/low pH in *Acropora millepora* populations in the PNG seep system. In addition to significant population declines and reduced rates of net calcification at CO_2-seep sites compared to paired nonimpacted reefs (Fabricius et al., 2014; Strahl et al., 2015), coral-associated microbial communities also differ significantly in this species. In particular, *A. millepora* at seep sites exhibit a 50% reduction in symbiotic *Endozoicomonas*, a putative mutualist and generally dominant component of the coral microbiome (Morrow et al., 2015; Neave et al., 2017). We evaluated global gene expression profiles in adult corals and their algal endosymbionts, *Symbiodinium* spp., from replicate pairs of control and seep environments at two different reefs in the PNG system: Dobu (median control pH = 8.01, 368 μatm pCO_2; seep pH = 7.72, 998 μatm pCO_2) and Upa-Upasina (median control pH = 7.98, 346 μatm pCO_2; seep pH = 7.81, 624 μatm pCO_2; see Fabricius et al., 2014 for additional carbonate chemistry parameters). We interpret consistent shifts in expression among seep-site populations in the two replicate reef systems to reflect the core molecular response involved in long-term acclimatization and/or adaptation to ocean acidification.

2 | MATERIALS AND METHODS

2.1 | Sampling collection and processing

Small tips of coral branches were collected by SCUBA divers from the middle of 15 *A. millepora* colonies each at the CO_2 seep and control sites of both Dobu and Upa-Upasina Reefs, Milne Bay Province, Papua New Guinea, at 2–6 m depth (Table S1), under a research permit by the Department of Environment and Conservation of Papua New Guinea as described previously (Fabricius et al., 2011, 2014). Though seeps are volcanic in origin, water temperatures do not differ among seep and control sites (Uthicke & Fabricius, 2012) and were identical at the time of sample collection (Table S1). Photographs of sampled colonies are available for download from the Dryad Digital Repository (https://doi.org/10.5061/dryad.k57p6). Samples were snap-frozen in liquid nitrogen within 15 min of collection and maintained at temperatures <−50°C until further processing.

Samples were crushed in liquid nitrogen and total RNA was extracted individually from 59 samples using a slightly modified RNAqueous kit protocol (Ambion, Life Technologies), and DNAse treated as in Kenkel et al. (2011). Briefly, samples homogenized in lysis buffer were centrifuged for 2 min at 16,100 rcf to pellet skeleton fragments and other insoluble debris and 700 μl of supernatant was used for extraction following the manufacturers' instructions, with one additional modification: in the final elution step, the same 25 μl of elution buffer was passed twice through the spin column to maximize the concentration of eluted RNA. RNA quality was assessed through gel electrophoresis and evaluated based on the presence of the ribosomal RNA bands. One μg of RNA per sample was prepared for tag-based RNA-seq as in (Lohman, Weber, & Bolnick, 2016; Meyer, Aglyamova, & Matz, 2011), with modifications for sequencing on the Illumina HiSeq platform (e.g., different adapter sequences to be compatible with the different sequencing chemistry; full protocols available at: https://github.com/z0on/tag-based_RNAseq).

2.2 | Symbiont typing

Additional samples from the same coral colonies were analyzed by Noonan, Fabricius, and Humphrey (2013) using gel-based DGGE and direct Sanger sequencing to determine *Symbiodinium* types. Three dominant banding patterns were recovered for *A. millepora* in the PNG seeps system, termed Am1, Am2, and Am3 (Noonan et al., 2013). The majority of corals from both reefs (15/15 Upa-Upasina

control and 15/15 Upa-Upasina seep corals; 13/15 Dobu-control and 10/14 Dobu-seep corals) hosted the Am1 variant, which differed by 2-bp from both a Genbank Clade C1 (JQ180021) and Clade C3 (FJ224005) reference (Noonan et al., 2013; Table S2). Two of fifteen Dobu-seep corals hosted the Am3 variant, which is 7-bp different from both the C1 and C3 references (Noonan et al., 2013; Table S2). The remaining 2/15 seep and 2/13 control corals from Dobu hosted the Am2 variant (Noonan et al., 2013; Table S2). To verify continuity of symbiont clade types within coral colonies, we mapped reads for each sample against a reference that included *A. millepora* concatenated to *Symbiodinium* clades A, B, C and D. More than 90% of *Symbiodinium* reads were assigned to clade C across all samples (Table S3). A parallel RFLP digest (Palstra, 2000; Van Oppen, Palstra, Piquet, & Miller, 2001) of LSU types confirmed that all coral fragments used hosted C1 (Fig. S1), however one sample from the Dobu CO_2-seep also appeared to have some amplifiable level of D-type symbionts, therefore to be conservative, this sample was discarded from the *Symbiodinium* expression analysis dataset.

2.3 | Bioinformatic processing

Fifty-nine libraries were sequenced on two lanes of the Illumina HiSeq2500 at the University of Texas at Austin Genome Sequencing and Analysis Facility. On average, 5.4 million sequences were generated per library (range: 2.5–16.3 million), for a total of 316.8 million raw reads. A custom perl script was used to discard duplicate reads sharing the same degenerate primer (i.e., PCR duplicates) and trim the 5′-Illumina leader sequence from remaining reads. The *fastx_toolkit* (http://hannonlab.cshl.edu/fastx_toolkit) was used to remove additional reads with a homo-polymer run of "A" $\geq$ 8 bases, retain reads with minimum sequence length of 20 bases, and quality filter, requiring PHRED quality of at least 20 over 90% of the sequence. *Bowtie 2* (Langmead & Salzbert, 2012) was used to map filtered reads to a combined transcriptome reference: a concatenated *Acropora millepora* reference transcriptome (Moya et al., 2012) and a *Symbiodinium* Clade C reference transcriptome (Ladner, Barshis, & Palumbi, 2012). Read counts were assembled by isogroup (i.e., groups of sequences putatively originating from the same gene, or with sufficiently high sequence similarity to justify the assumption that they serve the same function) for both the host and symbiont transcriptomes using a custom perl script, discarding reads mapping equally well to multiple isogroups (Dixon et al., 2015). For the host transcriptome, on average, 811,704 reads per library (range: 414,605–2,102,534) were mapped to 45,442 unique isogroups. For the symbiont transcriptome, 277,517 reads per library (range: 96,025–571,019) were mapped to 24,076 unique isogroups.

2.4 | Statistical analyses

Analyses were carried out in the R statistical environment (R Development Core Team, 2013). Outlier analyses were conducted using the package ARRAYQUALITYMETRICS (Kauffmann, Gentleman, & Huber, 2009). Four outliers were identified in the coral host dataset, while only one was detected in the symbiont dataset. All outlier samples were discarded. Count data for the remaining host samples (Dobu-Seep = 14, Dobu-Control = 14, Upa-Upasina-Seep = 14, Upa-Upasina-Control = 13) and symbiont samples (Dobu-Seep = 13, Dobu-Control = 15, Upa-Upasina-Seep = 14, Upa-Upasina-Control = 15) were analyzed using the package DESEQ (Anders & Huber, 2010). Dispersion estimates of raw counts were obtained by maximizing a Cox-Reid adjusted profile likelihood of a model specifying population origin and seep environment for each sample and the empirical dispersion value was retained for each gene. Low-expression genes were excluded from subsequent analyses by removing isogroups with read count standard deviations in the bottom 60% quantile of both datasets, which were identified as the filter statistics best satisfying the assumptions of independent filtering as implemented in the package GENEFILTER (Gentleman, Carey, Huber, & Hahne, 2016). This left 18,177 highly expressed isogroups in the coral host dataset and 9,629 isogroups in the symbiont dataset. In each dataset, expression differences were evaluated with respect to reef site (Upa-Upasina/Dobu), and pCO_2 environment (Seep/Control) and the interaction using a series of generalized linear models implemented in the function fitNbinomGLMs. Multiple test correction was applied using the method of Benjamini & Hochberg (1995). Analyses were also repeated independently for each reef site population (Dobu-seep/control only and Upa-Upasina seep/control only) to verify candidate gene significance with respect to seep environment.

Functional enrichment analyses were conducted using the package GO-MWU (Voolstra et al., 2011) to identify over-represented gene ontology (GO) terms with respect to origin and seep environment using both the classical categorical test and a rank-based methodology (Dixon et al., 2015). The package MADE4 (Culhane, Thioulouse, Perriere, & Higgins, 2005) was used to conduct a between-groups analysis of seep and control samples within each dataset to identify the most discriminatory genes in terms of differential expression between reef environments. A permutation test was used to evaluate whether there were significantly more differentially expressed genes in the symbiont dataset relative to the coral host dataset. Since FDR-correction is partially based on the number of tests conducted, we created 1,000 random 9,629 gene subsets of the host 18,177 gene dataset and repeated FDR-correction on this reduced sample. We then compared the distribution of significant tests obtained in the subsample to the observed symbiont gene set to obtain an estimate of significance.

3 | RESULTS

In total, 571 isogroups (genes) were differentially expressed at the FDR cut-off level $P_{adj} < 0.1$ in the coral host (3% of total, Figure 1a). The majority of these differences were due to reef origin (Dobu vs. Upa-Upasina, 503 genes, Table S4). Only 61 genes were differentially regulated between corals originating from control and seep environments, 53 of which exhibited consistent differences irrespective of reef origin (Figure 1a,c, Table S5). Significantly more

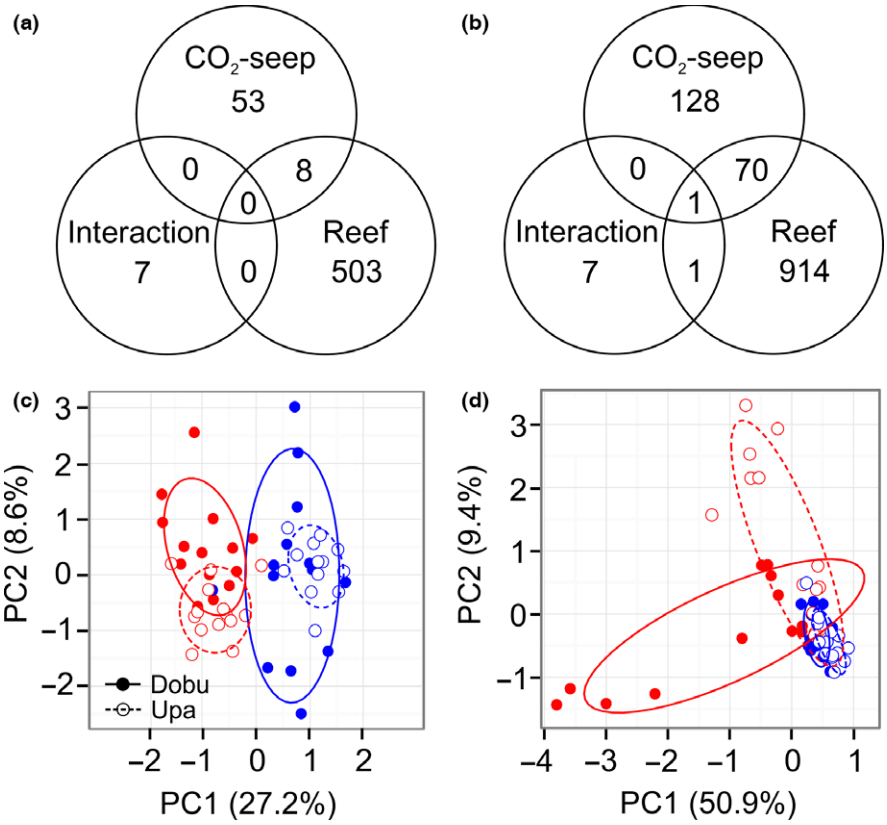

FIGURE 1 Venn diagrams of differentially expressed genes by factor (FDR-adjusted $p < .1$) for host (a) and symbiont (b). Principal components analysis of top 50 most significantly differentially expressed genes by CO_2-seep (red = seep, blue = control) and reef origin for host (c) and symbiont (d)

expression changes were detected in *Symbiodinium* populations ($P_{per\text{-}mutation} < 0.0001$) where a total of 1,123 genes were differentially expressed ($P_{adj} < 0.1$, 12% of total, Figure 1b). Again, the majority of these changes were attributable to differences in reef origin (Table S6), but 201 genes exhibited altered expression in seep environments relative to controls (Figure 1b, Table S7). Expression changes in symbionts were also less consistent between populations (Figure 1d) and this pattern was not attributable to differences in symbiont type (Fig. S2). The purpose of this study was to evaluate expression differences following lifelong acclimatization to elevated pCO_2 in corals; therefore, we focus on genes regulated with respect to seep environment. Differential expression patterns for genes responding to reef origin and associated functional enrichments can be found in the supplementary material (Tables S2and S4, Fig. S3) and discussion of potential environmental drivers of reef site-specific differences in coral physiology can be found in Strahl et al. (2015).

3.1 | Differential expression of coral host genes by CO_2 seep environment

Of the 61 genes showing common population-level responses to the CO_2-seep environment, 53 exhibited consistent baseline expression levels between corals from the different reef locations (Figure 1a, "CO_2 seep"). Of these, 26 were upregulated and 27 were downregulated in CO_2-seep environments. Roughly half (51%) of these genes have no annotation, and thus their functions cannot be determined. We report expression patterns among annotated candidates only but the data for all differentially expressed genes can be found in Table S5. We first consider individual candidate genes and then describe altered functional processes identified through enrichment analyses.

3.1.1 | De novo candidate genes

Among annotated genes significantly upregulated in seep-site corals, three associated with transcriptional regulation were also identified in a between-groups analysis as the most discriminatory genes between seep and control samples (Figure 2a). Two code for transcriptional regulators (ig19425, ig12770, 1.08-fold and 1.09-fold, respectively) and the third for a transcription factor in the basic leucine-zipper superfamily (ig10473, 1.2-fold). In the entire *A. millepora* transcriptome, 26 genes are annotated as "transcriptional regulators" and another eight are bZIP transcription factors. A methyl-CpG binding transcriptional regulator (ig9532) was also upregulated by 1.05-fold in corals from seep sites, but showed an additional effect of host origin, with corals from Dobu having higher baseline expression than corals from Upa-Upasina (Figure 2c). This methyl-CpG binding regulator was one of only three genes with this annotation in the entire *A. millepora* transcriptome; the other two (ig16785 and ig21898) were not found in the final expression set. A transcriptional repressor in the hairy/E (spl) family (ig7904) was among the most discriminatory genes and downregulated in response to seep

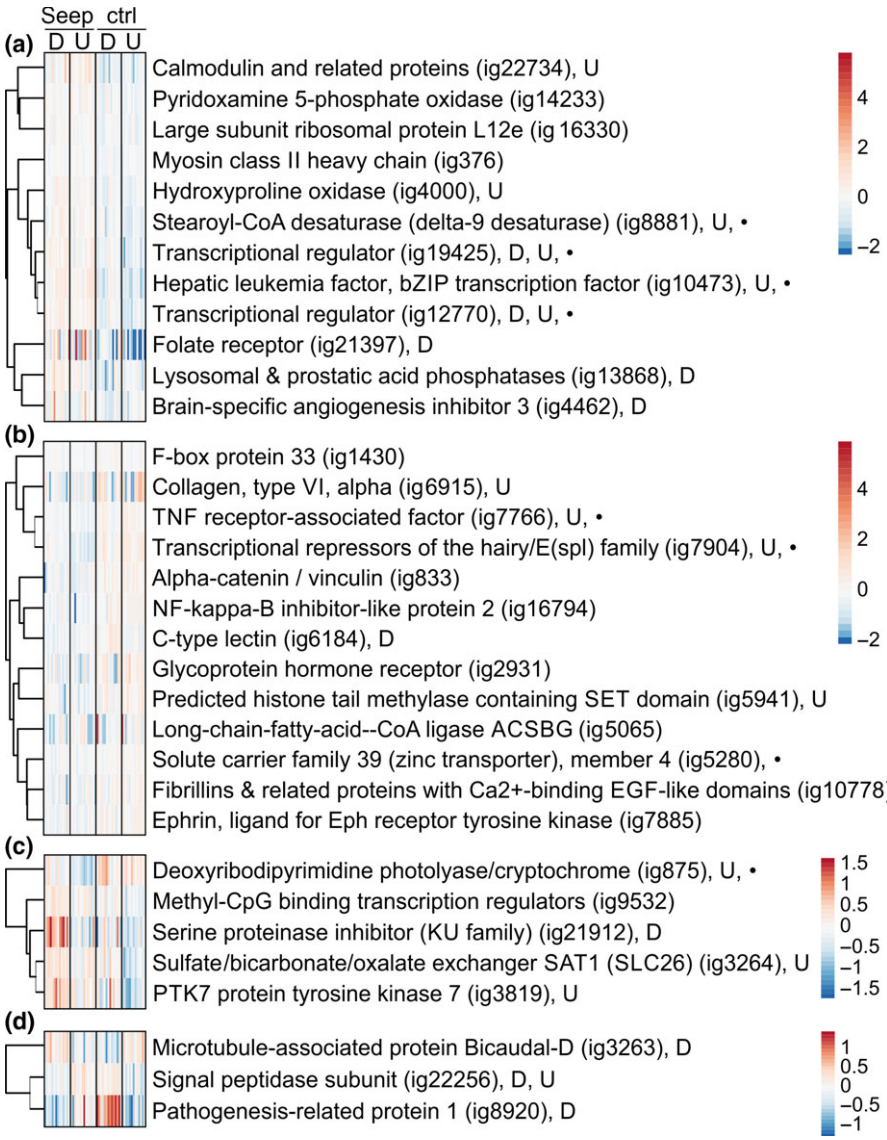

FIGURE 2 Heatmaps of annotated genes (FDR-adjusted $p < .1$) in the coral host that showed upregulation in response to seep environment (a), downregulation in response to seep environment (b), an effect of reef origin in addition to an effect of seep environment (c) or a reef origin x seep environment interaction (d). D = FDR-adjusted $p < .1$ in Dobu-only dataset; U = FDR-adjusted $p < .1$ in Upa-Upasina-only dataset; ● = Top discriminatory gene as identified via between-groups analysis for seep environment. Column labels: D = Dobu, U = Upa-Upasina

environments by 1.16-fold (Figure 2b), again suggesting some role for transcriptional regulation, though 13 isogroups in the transcriptome also have this same annotation.

A TNF receptor-associated factor (ig7766) is also a top discriminatory gene. This family, involved in the innate immune response, recently came to prominence given its putative role in the coral stress response (Barshis et al., 2013). Its downregulation, together with an NF-kappa-B inhibitor (ig16794) and a c-type lectin (ig6184, Figure 2b), highlight a potential impact of elevated pCO_2 on the innate immune response. However, 104, 49 and 143 isogroups respectively have identical annotations in the *A. millepora* transcriptome.

An alpha-catenin/vinculin isoform (ig833), one of three genes with this annotation, is downregulated in seep-site corals by 1.15-fold (Figure 2b). The other two isoforms (ig1210 and ig21857) are not differentially expressed and not included in this expression dataset. Additional cytoskeletal components including a collagen (ig6915) and fibrillin (ig10778) are also downregulated by 1.23 and 1.17-fold respectively, although these annotations are fairly common (86 and 372 isogroups in the transcriptome, respectively).

3.1.2 | Categorical functional enrichments

A categorical functional enrichment analysis did not reveal any statistically significant candidates following FDR-correction. The top three "biological process" enrichments were "small molecule biosynthetic process" (GO:0044283, $P_{Raw} = 0.1$), "fatty-acid metabolic process" (GO:0006631, $P_{Raw} = 0.3$) and "small molecule catabolic process" (GO:0044282, $P_{Raw} = 0.3$), which resulted from a set of four candidate genes. Pyridoxamine 5-phosphate oxidase (ig14233, upregulated by 1.06-fold in seep-site corals, GO:0044283, Figure 2a), an enzyme catalyzing the rate-limiting step in vitamin B_6 metabolism is an annotation only assigned to one other gene in the host transcriptome (ig27779) that was not differentially expressed with respect to either seep environment or reef origin. Hydroxyproline oxidase (ig4000, GO:0044283, GO:0044282, Figure 2a),

hypothesized to play a role in activation of the apoptotic cascade (Cooper, Pandhare, Donald, & Phang, 2008), is also upregulated by 1.07-fold in seep-site corals. The only other gene in the transcriptome with this annotation (ig1278) is differentially regulated with respect to reef origin, showing 1.07-fold upregulation in corals from Dobu ($P_{Reef} < 0.1$, Table S4).

The remaining two genes are primarily involved in fatty-acid metabolism. Stearoyl-CoA desaturase (ig8881, GO:0044283, GO:0006631) is upregulated in seep sites by 1.15-fold. There are only five isogroups in the transcriptome with this annotation, three occur in the final expression list, but this isoform is the only one differentially expressed. The other candidate, long-chain-fatty-acid—CoA ligase, or long-chain acyl-CoA synthetase (ig5065, GO:0006631, GO:0044282), is downregulated by 1.19-fold and is one of only seven isoforms with this annotation. One other isoform is differentially expressed with respect to reef origin, with greater expression in Dobu-origin corals (ig3997, $P_{Reef} < 0.1$, Table S4), but remaining isoforms (ig2622, ig2781, ig5009, ig5135, ig12633) were not differentially expressed.

3.1.3 | Rank-based functional enrichments

Given the low number of candidate genes that passed the FDR threshold, a rank-based methodology was also used to determine functional enrichments among generally upregulated (red) and downregulated (blue) ontologies in corals from CO_2-seep environments (Figure 3a). "Chaperone-mediated protein folding" (GO:0061077) was the top enrichment among genes downregulated in CO_2-seep sites (Figure 3a, b). "Ribonucleoprotein complex biogenesis" and "one-carbon metabolic process" were the top two most enriched functional ontologies among genes upregulated in seep sites (GO:0022613 and GO:0006730, respectively, Figure 3a,c,d). Interestingly, the most significantly differentially regulated genes within "one-carbon metabolic process" are all individually annotated as carbonic anhydrases (Figure 3c).

3.2 | Differential expression of *Symbiodinium* genes by CO_2 seep environment

Of the 201 genes differentially expressed in response to CO_2-seep environment, 128 exhibited similar baseline expression levels between symbionts in corals from the different reef locations (Figure 1b, "CO_2 seep", Table S7). Of these, 96 were upregulated and 32 were downregulated in CO_2-seep environments. Only 40% of these genes were annotated, and we again report expression patterns among these candidates only, but include the data for all differentially expressed genes in Table S7. To enhance the sparse knowledge on *Symbiodinium* responses to high pCO_2/low pH *in hospite*, we report altered functional processes identified through categorical and rank-based enrichment analyses.

3.2.1 | Categorical functional enrichments

The relatively small number of genes responding to seep site and a lack of annotations resulted in no statistically significant ontology terms following FDR-correction of a categorical enrichment analysis. The top three "biological process" enrichments were "regulation of chromosome organization" (GO:0033044, $P_{Raw} = 0.005$), "response to bacterium" (GO:0009617, $P_{Raw} = 0.03$) and "regulation of organelle organization" (GO:0033043, $P_{Raw} = 0.05$), which resulted from a set of six candidate genes. A peptidyl-prolyl cis-trans isomerase in the Ess family, matching Ess1 (c78122, GO:0033044, GO:0033043 Figure 4a) was upregulated by 1.05-fold at seep sites. In the *Symbiodinium* Clade C transcriptome 62 clusters are annotated as PPIs, which catalyze the *cis–trans* isomerization of peptide bonds N-terminal to proline residues in polypeptide chains, but this is the only cluster to have homology with Ess1. An E3 SUMO-protein ligase pli1 (c28523, GO:0033044, GO:0033043, Figure 4c) was also upregulated in seep-site corals by 1.1-fold, but shows an additional effect of reef origin, with *Symbiodinium* in Dobu corals having higher baseline expression than *Symbiodinium* in Upa-Upasina corals. This annotation occurred twice in the transcriptome, but the other gene (c71663) was not included in the final expression set. The third gene in this regulatory group, the meiosis protein mei2 (c26263, GO:0033043, Figure 4a) was also upregulated by 1.1-fold at seep sites. Three other clusters in the transcriptome were assigned this annotation (c_sym_78605, c49233_81271, c94595), two of which were in the final expression set analyzed here and one was significantly differentially expressed with respect to reef origin (c94595, 1.02-fold, Table S5).

The last three genes were all involved in bacterial response (GO:0009617) and all upregulated in seep sites. One of the genes codes for a 14-3-3-like protein (c50996, 1.06-fold, nine genes with this annotation in transcriptome, Figure 4a). The second codes for an ankyrin repeat domain-containing protein 2 (c26861, 1.02-fold, nine genes with this annotation, Figure 4a). The last codes for a tartrate resistant acid phosphatase type 5 (c26554, 1.1-fold, Figure 4c, 11 genes with this annotation) which also showed an effect of origin, with *Symbiodinium* in Dobu corals having higher baseline expression than *Symbiodinium* in Upa-Upasina corals.

3.2.2 | Rank-based functional enrichments

The only significant functional enrichment identified with rank-based analysis was "translation" (GO:0006412, Figure 5a) which was enriched among genes upregulated in seep sites. Individual genes within this term were primarily annotated as ribosomal proteins (Figure. 5b).

4 | DISCUSSION

The aim of this study was to investigate the genomic basis of acclimatization to chronic exposure to high pCO_2/low pH in a reef-building coral through a comparison of closely situated control and CO_2 impacted sites, which are separated by 500 m and 2,500 m at Upa-Upasina and Dobu, respectively, with 30 km between the two

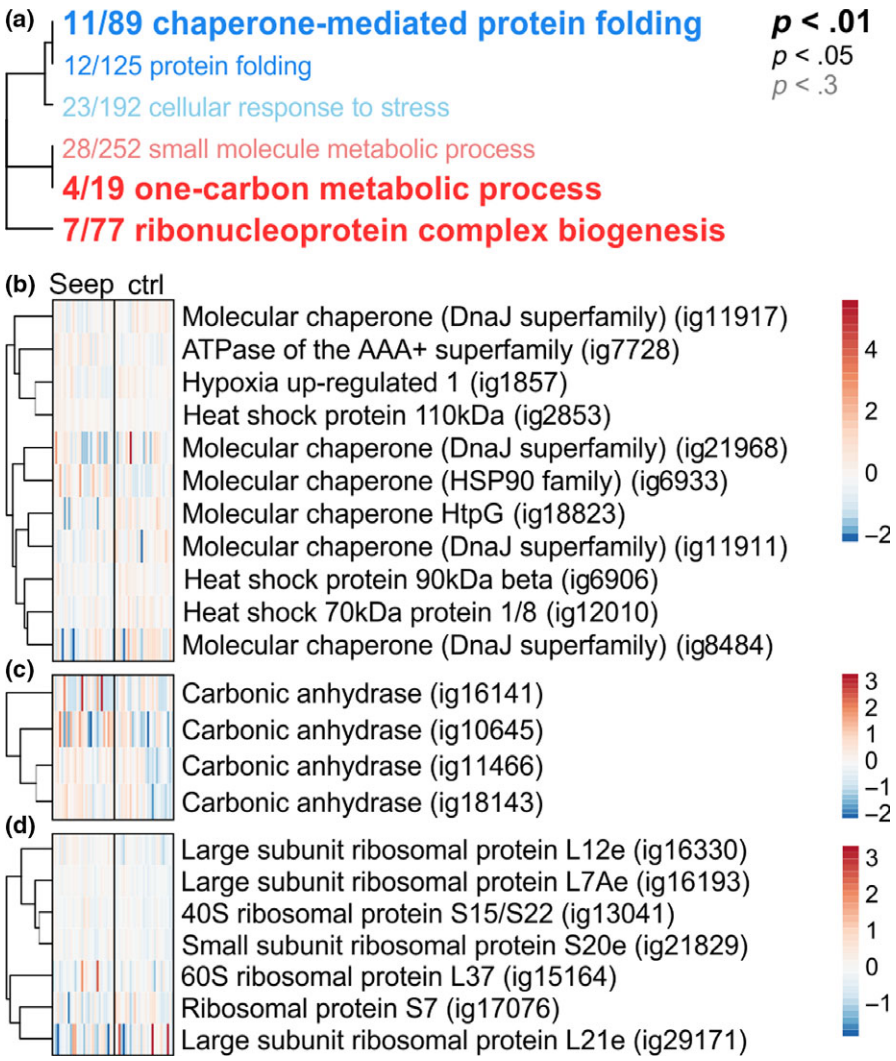

FIGURE 3 Hierarchical clustering of enriched gene ontology terms ("biological process") among upregulated (red) and downregulated (blue) genes in the coral host with respect to CO_2-seep (a). Font indicates level of statistical significance (FDR-corrected). Term names are preceded by fraction indicating number of individual genes within each term differentially regulated with respect to seep site (unadjusted $p < .05$). Heatmaps of these "good gene" fractions are shown for "chaperone-mediated protein folding" (b), "one-carbon metabolic process" (c) and "ribonucleoprotein complex biogenesis" (d)

populations. Uniquely, we report global gene expression profiles in both the coral host and their *in hospite Symbiodinium* that have undergone life-long acclimatization to naturally low-pH environments. Previous population-level studies (Fabricius et al., 2014; Morrow et al., 2015; Strahl et al., 2015) strongly suggest that low-pH environments impact the fitness of *Acropora millepora*. Despite this, we found very few consistent changes in global gene expression patterns between control and seep sites (Figure 1). This may be because gene expression changes did not reflect actual protein content or because of post-translational regulation (Greenbaum, Colangelo, Williams, & Gerstein, 2003). It is also possible that substantial interindividual variation in expression (e.g., Bay, Nielsen, Jarmer, Seneca, & Van Oppen, 2009; Csaszar, Seneca, & Van Oppen, 2009) masked the detectability of expression differences in response to environmental pCO_2. On the other hand, important biochemical health measures related to cell protection and cell damage were unaffected in *A. millepora* in response to elevated pCO_2 up to 800 μatm at the same sites studied here (Strahl et al., 2015), consistent with our findings of a minimal expression response.

The absence of significant gene expression changes may not necessarily be surprising if low pH is a chronic stressor for the corals. Cellular stress gene expression responses are transient and nonspecific (Kültz, 2005). Once immediate damage is repaired, a secondary, permanent cellular homeostasis response occurs, which is specific to the triggering stressor and which facilitates the maintenance of homeostasis under the new environmental regime (Kültz, 2003). It is likely that *A. millepora* exhibits open populations in this system given the broadcast spawning behavior of this species and the close proximity of study sites. Newly recruited juvenile corals may have exhibited an initial stress response, but their gene expression baselines could have shifted with age in order to acclimate to their local environment. Moya et al. (2015) previously reported dampened expression responses in a time-series exposure of juvenile *A. millepora* to elevated pCO_2, consistent with this hypothesis. Therefore, the small but constitutive differences in expression detected here, in two replicate populations (n = 13–15 colonies per site) acclimatized to CO_2-seep environments, likely reflects the core expression homeostasis response to ocean acidification.

In the coral host, this core response involves changes in gene regulation involved in fatty-acid (FA) metabolism (Figure 2). Differential regulation of stress response genes also occurred and specifically, corals from seep environments constitutively downregulated

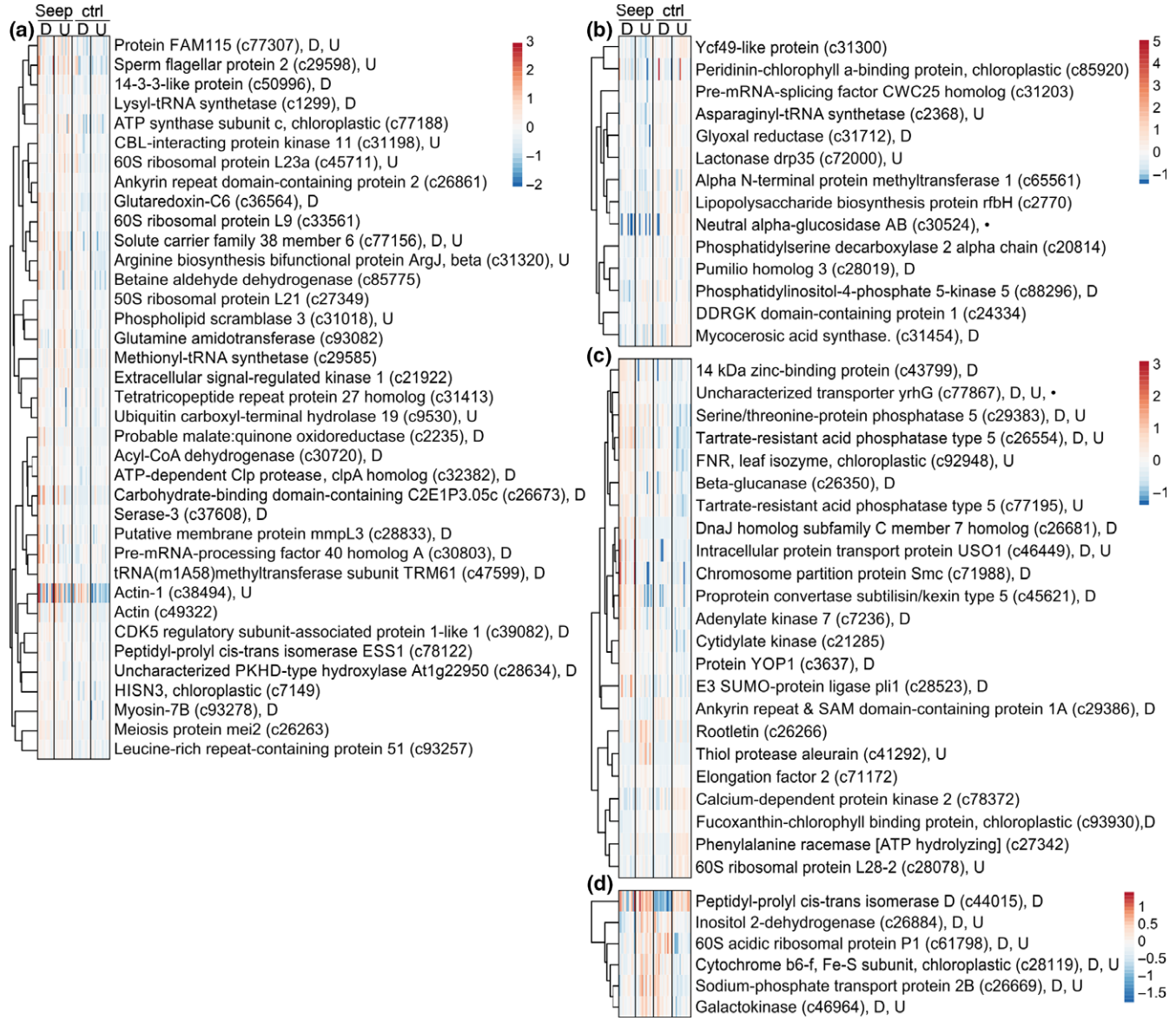

FIGURE 4 Heatmap of annotated genes (FDR-adjusted $p < .1$) in *Symbiodinium* that showed upregulation in response to seep environment (a), downregulation in response to seep environment (b), an effect of reef origin in addition to an effect of seep environment (c) or a reef origin x seep environment interaction (d). D = adjusted $p < .1$ in Dobu-only dataset; U = adjusted $p < .1$ in Upa-Upasina-only dataset; ● = Top discriminatory gene as identified via between-groups analysis for seep environment. Column labels: D = Dobu, U = Upa-Upasina

expression of molecular chaperones (Figure 3a,b). Interestingly, we did not find explicit signatures indicating altered expression in calcification-related genes, though some carbonic anhydrase isoforms did appear to be constitutively upregulated in seep environments (Figure 3c), but those could also be involved in cellular pH homeostasis. Finally, expression changes *in hospite Symbiodinium* were greater, and unlike patterns in their coral hosts, were not consistent in seep habitats at the different reef sites (Figure 1c,d), which may have implications for the symbiosis.

4.1 | Differential regulation of fatty-acid metabolism

The combined upregulation of a FA synthesis gene (Stearoyl-CoA desaturase) and downregulation of a FA catabolism gene (Long-chain-fatty-acid-CoA ligase), both key enzymes in their respective functional pathways (Dobrzyn et al., 2004; Watkins, 1997) and fairly rare annotations within the *A. millepora* transcriptome, suggest that coral lipid metabolism is modified in the process of acclimatization to low pH. Recent work on the transcriptomic response of urchins (*Strongylocentrotus purpuratus*) to experimental ocean acidification found that populations which naturally experience more frequent low-pH conditions also differentially regulated fatty-acid metabolic pathways (Evans, Pespeni, Hofmann, Palumbi, & Sanford, 2017). Interestingly, differential regulation of lipid metabolism genes was also observed in prior laboratory experiments on corals exposed to acute low-pH stress (Moya et al., 2012), but this particular functional process was not specifically discussed. Eleven clusters encoding fatty-acid synthases were found in the *A. millepora* transcriptome,

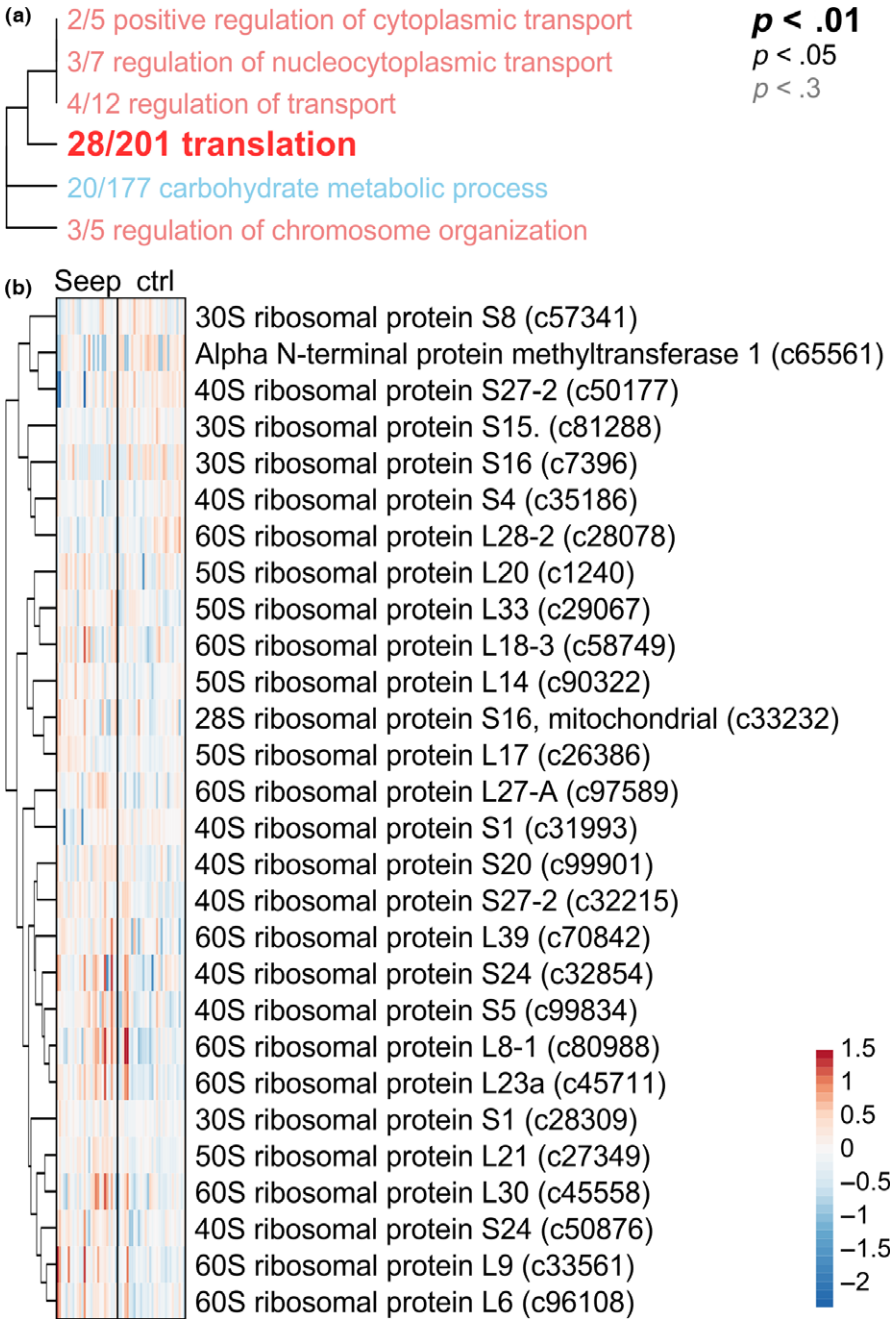

FIGURE 5 Hierarchical clustering of enriched gene ontology terms ("biological process") among upregulated (red) and downregulated (blue) symbiont genes with respect to CO_2-seep (a). Font indicates level of statistical significance (FDR-corrected). Term names are preceded by fraction indicating number of individual genes within each term differentially regulated with respect to seep site (unadjusted $p < .05$). A heatmap of this "good gene" fraction is shown for "translation" (b)

and most of them were upregulated in response to acute low-pH stress (A. Moya unpublished data).

Our results indicate a metabolic shift in CO_2-seep site corals in favor of increasing lipid storage. This is supported by the findings of Strahl et al. (2015), who detected slightly higher ratios of storage to structural lipids in *A. millepora* at seep vs. control sites at Dobu and Upa-Upasina. Stearoyl-CoA desaturase catalyzes the rate-limiting step in the synthesis of unsaturated fatty acids, which are components of both structural (e.g., membrane phospholipids) and storage lipids (e.g., triacylglycerol, wax esters, sterol ester), and the disruption of genes encoding this enzyme in mice leads to reduced body adiposity (Ntambi et al., 2002). Long-chain-fatty-acid-CoA ligase, on the other hand, activates the first step of fatty-acid metabolism or β-oxidation (Watkins, 1997), when lipids are being broken down. Storage lipids such as wax esters, triacylglycerol and free fatty acids are critical components of corals' energetic status (Edmunds & Davies, 1986; Harland, Navarro, Davies, & Fixter, 1993) and depletions in lipid stores can impact long-term survival and reproduction (Anthony, Hoogenboom, Maynard, Grottoli, & Middlebrook, 2009). Furthermore, genes involved in lipid metabolism were found to exhibit significantly elevated rates of protein evolution in Acroporids, but the authors were unable to speculate about putative adaptive roles for lipid metabolism (Voolstra et al., 2011).

Recently, Strahl, Francis, Doyle, Humphrey, and Fabricius (2016) found that *A. millepora* from the Dobu-seep site tend to have elevated levels of total lipid and protein, as well as elevated levels of

FAs (including polyunsaturated FA) relative to control site corals, in support of observed expression differences. Other studies have also found significant changes in lipid content in response to acidification. In two separate aquarium-based acidification experiments, lipid content in *A. millepora* was found to increase following exposure to elevated pCO_2 (Kaniewska et al., 2015; Schoepf et al., 2013). Behavioral changes may also be involved in this pattern as both feeding rate and lipid storage increased in *Acropora cervicornis* under simulated acidification (Towle, Enochs, & Langdon, 2015). Whether the mechanism is behavioral plasticity or adaptive genetic change in lipid metabolic capacity, the combined evidence suggests that lipid metabolism likely plays a role in a coral's capacity to withstand ocean acidification and future work should aim to investigate the mechanistic basis of this process.

4.2 | Downregulation of chaperones

Upregulation of chaperones is a hallmark of the acute cellular stress response (Gasch et al., 2000), but is usually transient as constitutive upregulation of heat shock proteins is costly and can result in decreased growth and fecundity (Sørensen, Kristensen, & Loeschcke, 2003). In *Drosophila* and soil isopods exposed to chronic stress, Hsp70 expression is reduced rather than elevated (Köhler & Eckwert, 1997; Sørensen, Michalak, Justesen, & Loeschcke, 1999). HSPs are also known to be constitutively downregulated following long-term thermal stress in corals (Kenkel, Meyer, & Matz, 2013; Meyer et al., 2011; Sharp, Brown, & Miller, 1997). Short-term laboratory manipulations suggest that exposure to high pCO_2/low pH prompts expression of immediate stress response genes, like HSPs (Moya et al., 2012, 2015); and Kaniewska et al. (2012) observed downregulation of chaperones following one month of elevated pCO_2 exposure. Therefore, acute exposure to low-pH conditions is stressful for *A. millepora* and the constitutive downregulation of HSPs observed here is likely a consequence of chronic exposure to elevated pCO_2 at the seep sites.

Given that HSP induction is critical for mounting a successful thermal stress response, the suppression of baseline HSP expression levels induced by low-pH environments may impact the capacity of *A. millepora* to cope with the synergistic effects of global climate change. Acidification is predicted to become a chronic stress on reefs worldwide if climates continue to change (Hoegh-Guldberg et al., 2007). While temperatures will simultaneously increase, extreme thermal anomalies are also predicted to become more frequent and severe (Frich et al., 2002). Our results suggest that the combined effects of acidification and elevated temperature stress may be more detrimental than acidification alone because of the dampening effects of chronic exposure on the cellular stress response. Some laboratory manipulations have found synergistic negative impacts of combined high pCO_2/low pH and temperature; for example, calcification of *Stylophora pistillata* decreased by 50% under both elevated temperature and pCO_2, but was unchanged under each stressor individually (Reynaud et al., 2003). However, bleaching surveys following a minor thermal stress event in PNG did not indicate that high pCO_2/low-pH reefs suffered increased bleaching relative to control reefs (Noonan & Fabricius, 2015). It will be critical to determine whether constitutive downregulation of HSPs resulting from long-term pCO_2 exposure makes it more difficult for a coral to subsequently upregulate HSPs to counter acute thermal stress, or if other mechanisms or isoforms are employed to counter acute thermal stress in chronically low-pH environments.

4.3 | No significant differential regulation of calcification genes

We did not observe functional enrichments indicating differential regulation of calcification-related genes overall in *A. millepora*, although some carbonic anhydrase isoforms were constitutively upregulated in seep site corals (Figure 3c). Experimentally, some coral species have been shown to maintain (Reynaud et al., 2003) and even increase (Castillo, Ries, Bruno, & Westfield, 2014) calcification during laboratory acidification experiments and this effect has been hypothesized to result from the ability of corals to alter carbonate chemistry at the site of calcification (Mcculloch, Falter, Trotter, & Montagna, 2012; Venn et al., 2013). Our a priori expectation was that expression patterns of calcification-related genes should be altered to affect this physiological rescue. *Pocillopora damicornis* were observed to upregulate HCO_3^- transporters at moderately low pH (7.8 and 7.4; Vidal-Dupiol et al., 2013), while *Siderastrea siderea* upregulated expression of H^+ ion transporters (Davies, Marchetti, Ries, & Castillo, 2016) consistent with this hypothesis.

Acropora millepora does not appear to conform to the expectation of altered expression of calcification-related genes in response to long-term exposure to low pH. Expression of calcification-related genes significantly changed in *A. millepora* following short-term 3-day low-pH stress exposure (Moya et al., 2012), but these effects dissipate when experimental treatment periods are extended (Kaniewska et al., 2012; Moya et al., 2015; Rocker et al., 2015; 28, 9 and 14 days, respectively). Furthermore, *A. millepora* from PNG seep sites had reduced levels of net calcification, resulting from decreases in dark calcification, compared to neighboring control reef sites (Strahl et al., 2015). This suggests that *A. millepora* has a reduced capacity to actively alter pH at the site of calcification in the absence of additional photosynthetic energy (i.e., in the dark, Strahl et al., 2015). The regulation of cellular pH at calcification sites is an energetically costly process (Al-Horani, 2005; Barnes & Chalker, 1988). Given that calcification-related gene expression is plastic in *A. millepora* on shorter time-scales (Moya et al., 2012), it is possible that the lack of constitutive differential regulation under long-term high pCO_2/low-pH exposure, and subsequent decrease in net calcification, were not necessarily due to a lack of genetic variation in the ability to actively regulate these genes, but a result of trade-offs in allocation of finite energetic resources to other less costly processes that maximize net fitness under acidification stress. Indeed, Strahl et al. (2016) hypothesized that *A. millepora* may invest in increased tissue biomass rather than skeletal growth under acidified conditions based on prior experimental observations of unchanged or increased

biomass in combination with reduced calcification (Krief et al., 2010; Schoepf et al., 2013; Strahl et al., 2015).

4.4 | Inconsistent changes in *Symbiodinium* expression profiles

More significant differences in gene expression were detected for *Symbiodinium* than for host corals between control and elevated pCO_2 sites examined here. This corroborates findings from *Pocillopora damicornis* where their in hospite clade C *Symbiodinium*, demonstrated a more pronounced expression response following a 2-week exposure to elevated temperature, although this difference was no longer evident after 36-weeks (Mayfield, Wang, Chen, Lin, & Chen, 2014). Kaniewska et al. (2015) examined metatranscriptomic expression responses of coral holobionts to future climate change scenarios, but their analysis method did not explicitly compare host and symbiont. Kenkel & Matz (2016) reported expression of both host and symbionts in *P. astreoides* corals reciprocally transplanted between reef habitats, but again, their network-based analytical approach precludes a direct comparison with results uncovered here. A reanalysis of their dataset with the method used here found that 14.8% of the host transcriptome was significantly differentially expressed with respect to transplant environment, while only 1.4% of the symbiont transcriptome was altered (C. D. Kenkel, unpublished data). Given the paucity of studies examining global expression of both partners in response to environmental stress (to our knowledge, the present study is only to examine expression under elevated pCO_2), it is difficult to draw any conclusions regarding the present patterns. More data are needed to determine whether there are any consistent patterns in *Symbiodinium* gene expression responses relative to those of their host corals.

Expression changes of *in hospite Symbiodinium* showed greater differences between control and seep sites across reefs compared to the coral host (Figure 1c vs. d). Control site expression profiles are remarkably similar between reef sites. However, the second principal component describes variation in expression that is largely the result of divergence between seep site expression of Dobu and Upa-Upasina origin *Symbiodinium* (Figure 1d). *A. millepora* in the PNG seeps system were found to host clade C-type *Symbiodinium* (Noonan et al., 2013). While intraclade differences in dominant *Symbiodinium* types were detected, there were no systematic differences between control and seep sites (Noonan et al., 2013; Tables S2and S3), nor do subtypes cluster together in terms of their expression profiles (Fig. S2). Therefore, the divergence in seep site expression of Dobu and Upa-Upasina *Symbiodinium* is not attributable to major differences in symbiont type. DGGE detection limits are typically 5%–10% (Correa, McDonald, & Baker, 2009), therefore, it is possible that undetected background *Symbiodinium* clades or types impacted expression levels (Ladner et al., 2012). If there was an interaction between potential differences in background clades or types and seep environment, this could explain the variation observed (Figure 1d).

Consistent expression changes among control and seep site *Symbiodinium* implicated an alteration of the biological process of translation: specifically, many ribosomal proteins were constitutively upregulated at the seep sites (Figure 5). Ribosome production is intimately tied to cell growth, and known to regulate cell size and the cell cycle (Jorgensen & Tyers, 2004). Net photosynthesis was significantly elevated in *A. millepora* from CO_2-seep sites (Strahl et al., 2015), potentially as a result of enhanced *Symbiodinium* cell growth or division (and hence elevated expression of ribosomal proteins) although these processes remain to be quantified. Determining the mechanistic drivers of divergence between seep-site populations among Dobu and Upa-Upasina reefs is more difficult. Of the top 10 gene loadings for PC2 (Figure 1d), eight had no annotation, precluding speculation about function. Nevertheless, the complexity of the response in *Symbiodinium* may have implications for the symbiotic interaction, if the coral host has to respond to the dual impacts of changes in its external environment, and its symbiont community. It is recognized that mutualisms are more susceptible to climate change impacts because the inherent interdependency between species means that even though stress only impacts one partner, both partners ultimately share the cost (Kiers, Palmer, Ives, Bruno, & Bronstein, 2010). There are many knowledge gaps remaining for both major global change stressors, however, our understanding of thermal stress impacts on the coral-algal symbiosis far outstrips understanding of acidification impacts (Barshis, 2015). Filling this gap will be critical for refining predictions of coral response to continued acidification and the combined impacts of global climate change.

DATA ARCHIVING

Raw RNA Tag-seq data have been uploaded to NCBI's SRA: PRJNA362652. Photographs of individual coral colonies at the time of sample collection, R scripts and input files for DESeq-based gene expression analyses can be obtained from DRYAD https://doi.org/10.5061/dryad.k57p6. R scripts for ontology enrichment analyses and directions for formatting input files can be found at http://www.bio.utexas.edu/research/matz_lab/matzlab/Methods.html.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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