

1 **Functional genomic analysis of corals from natural CO₂-seeps reveals core molecular**
2 **responses involved in acclimatization to ocean acidification**

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4 Running head: Core coral molecular response to CO₂-seeps

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16 **KEYWORDS:** *Acropora millepora*, *Symbiodinium*, RNA-seq, gene expression, carbon

17 dioxide, lipid metabolism, symbiosis, adaptation

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31 **ABSTRACT**

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33 Little is known about the potential for acclimatization or adaptation of corals to ocean
34 acidification and even less about the molecular mechanisms underpinning these processes.
35 Here we examine global gene expression patterns in corals and their intracellular algal
36 symbionts from two replicate population pairs in Papua New Guinea that have undergone
37 long-term acclimatization to natural variation in pCO₂. In the coral host, only 61 genes were
38 differentially expressed in response to pCO₂ environment, but the pattern of change was
39 highly consistent between replicate populations, likely reflecting the core expression
40 homeostasis response to ocean acidification. Functional annotations highlight lipid
41 metabolism and a change in the stress response capacity of corals as a key part of this
42 process. Specifically, constitutive downregulation of molecular chaperones was observed,
43 which may impact response to combined climate-change related stressors. Elevated CO₂ has
44 been hypothesized to benefit photosynthetic organisms but expression changes of *in hospite*
45 *Symbiodinium* in response to acidification were greater and less consistent among reef
46 populations. This population-specific response suggests hosts may need to adapt not only to
47 an acidified environment, but also to changes in their *Symbiodinium* populations that may not
48 be consistent among environments. This process adds another challenging dimension to the
49 physiological process of coping with climate change.

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62 INTRODUCTION

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64 Increasing atmospheric carbon dioxide concentration contributes to global warming
65 and alters ocean carbonate chemistry in the process known as ocean acidification (Sabine *et*
66 *al.*, 2004). Elevated atmospheric CO₂ increases the hydrogen ion concentration [H⁺], thereby
67 reducing ocean pH. This excess H⁺ reacts with carbonate ions [CO₂³⁻] to form bicarbonate
68 [HCO₃⁻], lowering the saturation state of carbonate minerals, such as calcite and aragonite
69 (Feely *et al.*, 2009). Many marine taxa rely on carbonate minerals to build their calcium
70 carbonate [CaCO₃] skeletons. Increasing H⁺ and concomitant reductions in pH increase the
71 potential for dissolution of present skeletons (van Woesik *et al.*, 2013). Simultaneous
72 reductions in the bioavailability of carbonate ions also increase the difficulty of depositing
73 new skeleton (Kleypas *et al.*, 1999). Ocean acidification has been predicted to have major
74 consequences for marine calcifying organisms, such as reef-building corals through this
75 combination of effects (Hoegh-Guldberg *et al.*, 2007).

76 Scleractinian corals form the basis of the most biodiverse marine ecosystems on the
77 planet: tropical coral reefs (Caley & St John, 1996, Idjada & Edmunds, 2006). They also
78 provide important ecosystem services, such as habitat for fisheries species and shore
79 protection (Sheppard *et al.*, 2005). Consequently, investigation of coral responses to
80 acidification has received substantial attention in recent years. The majority of empirical
81 work has focused on relatively short-term (days to months) exposure of corals to simulated
82 acidification in aquaria and the reported fitness consequences have been mixed. A recent
83 meta-analysis found that for every unit decrease in the saturation state of aragonite, coral
84 calcification declines by 15% on average, though individual studies report more significant
85 declines or even increases (Chan & Connolly, 2013), which may be attributable to
86 differences in tolerance among species (Albright, 2011, Erez *et al.*, 2011, Jokiel, 2011).

87 Natural CO₂-seep environments provide an attractive alternative to aquarium-based
88 experiments aimed at understanding coral resilience potential: no experimental manipulations
89 are necessary and *in situ* populations have likely already undergone some level of
90 acclimatization or adaptation to be able to inhabit low-pH environments. Work by Fabricius
91 *et al.* (2011) on corals at volcanic CO₂-seeps in Papua New Guinea (PNG) has provided
92 support for the mixed effects observed in laboratory experiments. Naturally acidified
93 environments drastically alter the coral community, but some species, like massive *Porites*,
94 appear unaffected, while others, such as Acroporids, are significantly less common or even
95 absent (Fabricius *et al.*, 2014). Population reductions *in situ*, combined with observations of

96 negative physiological impacts, including declines in calcification under elevated pCO₂
97 (Strahl *et al.*, 2015) strongly suggests that acidification imposes selection pressure on less
98 resilient taxa, such as Acroporids. Consequently, *Acropora* spp. are predicted to be ecological
99 ‘losers’ under future acidification scenarios (Schoepf *et al.*, 2013). However, the fact that
100 some *Acropora* spp. can still be found in seep environments indicates that standing genetic
101 variation for acidification tolerance may already exist within these less resilient species,
102 similar to recent work in analogous natural systems investigating variation in coral thermal
103 tolerance (D’Croz & Maté 2004, Kenkel *et al.*, 2013a, Oliver & Palumbi, 2011) and its
104 mechanistic basis (Barshis *et al.*, 2013, Dixon *et al.*, 2015, Kenkel & Matz, 2016).

105 Transcriptome sequencing has become a powerful tool for investigating physiological
106 plasticity and adaptive evolution in a changing environment and can provide insight into the
107 mechanistic basis of population-level variation (DeBiasse & Kelly, 2016). We used RNA-
108 seq to investigate the core genomic response underpinning long-term acclimatization to
109 acidification in *Acropora millepora* populations in the PNG seep system. In addition to
110 significant population declines and reduced rates of net calcification at CO₂-seep sites
111 compared to paired non-impacted reefs (Fabricius *et al.*, 2014, Strahl *et al.*, 2015), coral-
112 associated microbial communities also differ significantly in this species. In particular, *A.*
113 *millepora* at seep sites exhibit a 50% reduction in symbiotic *Endozoicomonas*, a putative
114 mutualist and generally dominant component of the coral microbiome (Morrow *et al.*, 2015,
115 Neave *et al.*, 2017). We evaluated global gene expression profiles in adult corals and their
116 algal endosymbionts, *Symbiodinium* spp., from replicate pairs of control and seep
117 environments at two different reefs in the PNG system: Dobu (control pH = 8.01, 368 µatm
118 pCO₂; seep pH = 7.72, 998 µatm pCO₂) and Upa-Upasina (control pH = 7.98, 346 µatm
119 pCO₂; seep pH = 7.81, 624 µatm pCO₂) (Fabricius *et al.*, 2014). We interpret consistent shifts
120 in expression among seep-site populations in the two replicate reef systems to reflect the core
121 molecular response involved in long-term acclimatization and/or adaptation to ocean
122 acidification.

123

124 **METHODS**

125

126 *Sampling Collection and Processing*

127 Small tips of coral branches were collected individually from 15 *A. millepora* colonies
128 each at the CO₂ seep and control sites of both Dobu and Upa-Upasina Reefs, Milne Bay
129 Province, Papua New Guinea, at 3 m depth, under a research permit by the Department of

130 Environment and Conservation of Papua New Guinea as described previously (Fabricius *et*
131 *al.*, 2014, Fabricius *et al.*, 2011). Samples were snap-frozen in liquid nitrogen within minutes
132 of collection and maintained at temperatures <-50°C until further processing.

133 Samples were crushed in liquid nitrogen and total RNA was extracted individually
134 from 59 samples using a slightly modified RNAqueous kit protocol (Ambion, Life
135 Technologies), and DNase treated as in Kenkel *et al.* (2011). Briefly, samples homogenized
136 in lysis buffer were centrifuged for 2 minutes at 16100 rcf to precipitate skeleton fragments
137 and other insoluble debris and 700 µl of supernatant was used for extraction following the
138 manufacturers' instructions, with one additional modification: in the final elution step, the
139 same 25 µl of elution buffer was passed twice through the spin column to maximize the
140 concentration of eluted RNA. RNA quality was assessed through gel electrophoresis and
141 evaluated based on the presence of the ribosomal RNA bands. One µg of RNA per sample
142 was prepared for tag-based RNA-seq as in (Lohman *et al.*, 2016, Meyer *et al.*, 2011), with
143 modifications for sequencing on the Illumina HiSeq platform (e.g. different adapter
144 sequences to be compatible with the different sequencing chemistry; full protocols available
145 at: https://github.com/z0on/tag-based_RNAseq).

146 Noonan *et al.* (2013) demonstrated with gel-based DGGE and direct Sanger
147 sequencing that *Symbiodinium* types do not differ between corals found in CO₂ seep and
148 control environments and that *Acropora millepora* host variants of clade C, closely related to
149 C1 and C3, in the PNG seep system. To confirm this result, we mapped reads for each sample
150 against a reference that included *A. millepora* concatenated to *Symbiodinium* clades A, B, C
151 and D. More than 90% of *Symbiodinium* reads were assigned to clade C across all samples
152 (Table S1). A parallel RFLP digest (Palstra, 2000, van Oppen *et al.*, 2001) of LSU types
153 confirmed that all corals used hosted C1 (Fig. S1), however one sample from the Dobu CO₂-
154 seep also appeared to have some amplifiable level of D-type symbionts, therefore to be
155 conservative, this sample was discarded from the *Symbiodinium* expression analysis dataset.

156

157 *Bioinformatic Processing*

158 A total of 59 libraries were sequenced on two lanes of the Illumina HiSeq2500 at the
159 University of Texas at Austin Genome Sequencing and Analysis Facility. On average, 5.4
160 million sequences were generated per library (range: 2.5-16.3 million), for a total of 316.8
161 million raw reads. A custom perl script was used to discard duplicate reads sharing the same
162 degenerate primer (i.e. PCR duplicates) and trim the 5'-Illumina leader sequence from
163 remaining reads. The *fastx_toolkit* (http://hannonlab.cshl.edu/fastx_toolkit) was used to

164 remove additional reads with a homo-polymer run of ‘A’ ≥ 8 bases, retain reads with
165 minimum sequence length of 20 bases, and quality filter, requiring PHRED quality of at least
166 20 over 90% of the sequence. *Bowtie 2* (Langmead & Salzberg, 2012) was used to map
167 filtered reads to a combined transcriptome reference: a concatenated *Acropora millepora*
168 reference transcriptome (Moya *et al.*, 2012b) and a *Symbiodinium* Clade C reference
169 transcriptome (Ladner *et al.*, 2012). Read counts were assembled by isogroup (i.e. groups of
170 sequences putatively originating from the same gene, or with sufficiently high sequence
171 similarity to justify the assumption that they serve the same function) for both the host and
172 symbiont transcriptomes using a custom perl script, discarding reads mapping equally well to
173 multiple isogroups (Dixon *et al.*, 2015). For the host transcriptome, on average, 811,704
174 reads per library (range: 414,605 – 2,102,534) were mapped to 45,442 unique isogroups. For
175 the symbiont transcriptome, 277,517 reads per library (range: 96,025 – 571,019) were
176 mapped to 24,076 unique isogroups.

177

178 *Statistical Analyses*

179 Analyses were carried out in the R statistical environment (R Development Core
180 Team 2013). Outlier analyses were conducted using the package *arrayQualityMetrics*
181 (Kauffmann *et al.*, 2009). Four outliers were identified in the coral host dataset, while only
182 one was detected in the symbiont dataset. All outlier samples were discarded. Count data for
183 the remaining host samples (Dobu-Seep = 14, Dobu-Control = 14, Upa-Upasina-Seep = 14,
184 Upa-Upasina-Control = 13) and symbiont samples (Dobu-Seep = 14, Dobu-Control = 15,
185 Upa-Upasina-Seep = 14, Upa-Upasina-Control = 15) were analyzed using the package
186 *DESeq* (Anders & Huber, 2010). Dispersion estimates of raw counts were obtained by
187 maximizing a Cox-Reid adjusted profile likelihood of a model specifying population origin
188 and seep environment for each sample and the empirical dispersion value was retained for
189 each gene. Low-expression genes were excluded from subsequent analyses by removing
190 isogroups with read count standard deviations in the bottom 60% quantile of both datasets,
191 which were identified as the filter statistics best satisfying the assumptions of independent
192 filtering as implemented in the package *genefilter* (Gentleman *et al.*). This left 18,177 highly
193 expressed isogroups in the coral host dataset and 9,629 isogroups in the symbiont dataset. In
194 each dataset, expression differences were evaluated with respect to reef site (Upa-
195 Upasina/Dobu), and pCO₂ environment (Seep/Control) and the interaction using a series of
196 generalized linear models implemented in the function *fitNbinomGLMs*. Multiple test
197 correction was applied using the method of Benjamini and Hochberg (1995). Analyses were

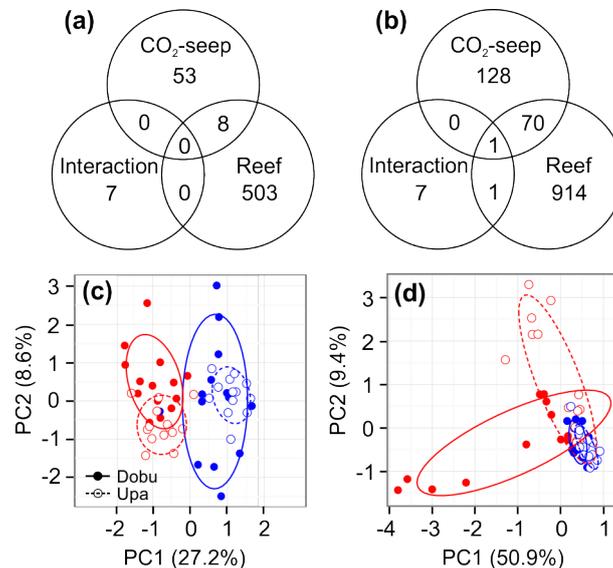
198 also repeated independently for each population to verify candidate gene significance with
199 respect to seep environment.

200 Functional enrichment analyses were conducted using the package GO-MWU
201 (Voolstra *et al.*, 2011) to identify over-represented gene ontology (GO) terms with respect to
202 origin and seep environment using both the classical categorical test and a rank-based
203 methodology (Dixon 2015). The package *made4* (Culhane *et al.*, 2005) was used to conduct a
204 between-groups analysis of seep and control samples within each dataset to identify the most
205 discriminatory genes in terms of differential expression between reef environments. A
206 permutation test was used to evaluate whether there were significantly more differentially
207 expressed genes in the symbiont dataset relative to the coral host dataset. Since FDR-
208 correction is partially based on the number of tests conducted, we created 1,000 random
209 9,629 gene subsets of the host 18,177 gene dataset and repeated FDR-correction on this
210 reduced sample. We then compared the distribution of significant tests obtained in the
211 subsample to the observed symbiont gene set to obtain an estimate of significance.

212

213

214 RESULTS



215

216 Figure 1. Venn diagrams of differentially expressed genes by factor
217 (FDR-adjusted $P < 0.1$) for host (a) and symbiont (b). Principal
218 components analysis of top 50 most significantly differentially
219 expressed genes by CO₂-seep (red=seep, blue=control) and reef origin
220 for host (c) and symbiont (d).

221 In total, 571 isogroups (genes) were differentially expressed at the FDR cut-off level
222 $P_{\text{adj}} < 0.1$ in the coral host (3% of total, Fig. 1a). The grand majority of these differences were
223 due to reef origin (Dobu vs. Upa-Upasina, 503 genes, Table S2). Only 61 genes were
224 differentially regulated between corals originating from control and seep environments, 53 of
225 which exhibited consistent differences irrespective of reef origin (Fig. 1a,c, Table S3).
226 Significantly more expression changes were detected in *Symbiodinium* populations
227 ($P_{\text{permutation}} < 0.0001$) where a total of 1123 genes were differentially expressed ($P_{\text{adj}} < 0.1$, 12%
228 of total, Fig 1b). Again, the majority of these changes were attributable to differences in reef
229 origin (Table S4), but 201 genes exhibited altered expression in seep environments relative to
230 controls (Fig. 1b, Table S5). Expression changes in symbionts were also less consistent
231 between populations (Fig. 1d). The purpose of this study was to evaluate expression
232 differences following lifelong acclimatization to elevated pCO₂ in corals. Therefore we focus
233 on genes regulated with respect to seep environment, although differential expression
234 patterns for genes responding to reef origin and associated functional enrichments can be
235 found in the supplementary material (Tables S2, S4, Fig S2).

236

237 ***Differential expression of coral host genes by CO₂ seep environment***

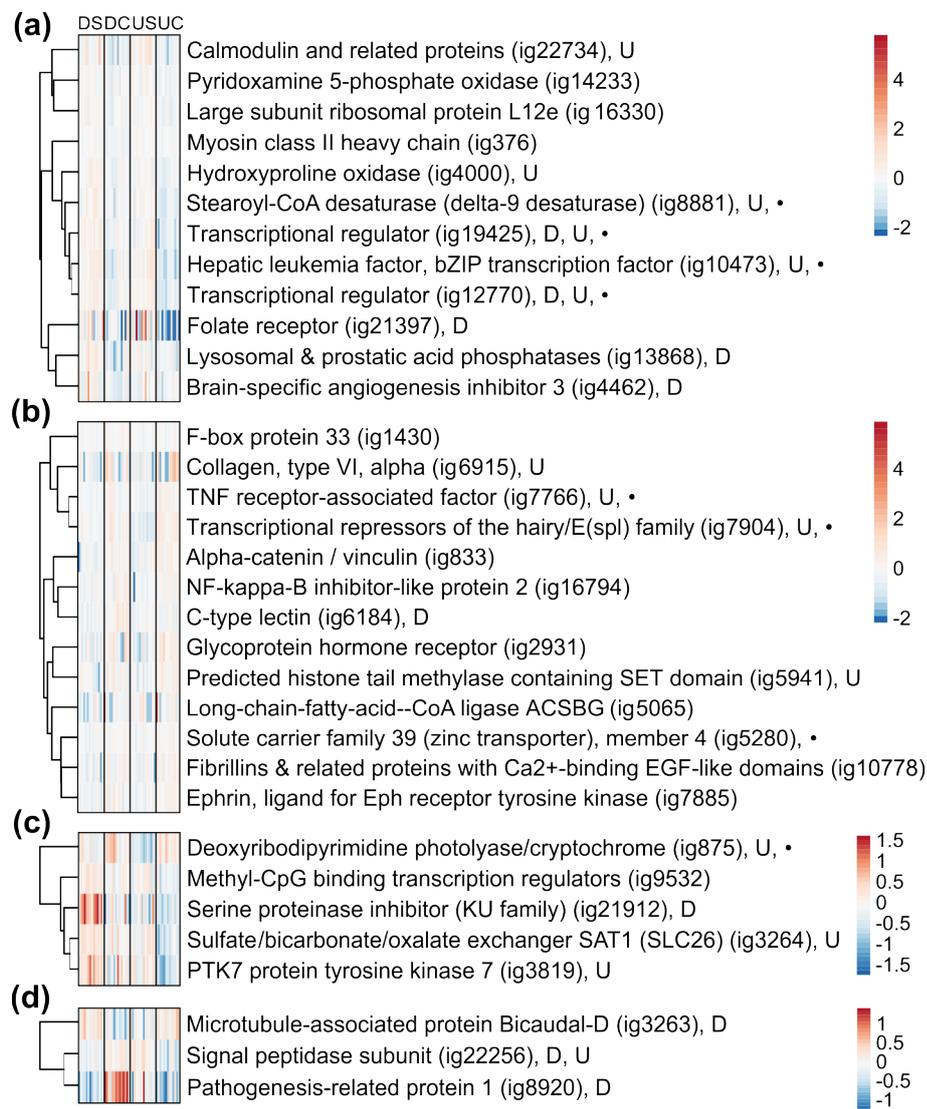
238 Of the 61 genes showing common population-level responses to the CO₂-seep
239 environment, 53 exhibited consistent baseline expression levels between corals from the
240 different reef locations (Fig. 1a, 'CO₂ seep'). Of these, 26 were upregulated and 27 were
241 downregulated in CO₂-seep environments. Roughly half (51%) of these genes have no
242 annotation, and thus their functions cannot be determined. We report expression patterns
243 among annotated candidates only but the data for all differentially expressed genes can be
244 found in Table S3. We first consider individual candidate genes and then describe altered
245 functional processes identified through enrichment analyses.

246

247 *De novo candidate genes*

248 Among annotated genes significantly upregulated in seep-site corals, three associated
249 with transcriptional regulation were also identified in a between-groups analysis as the most
250 discriminatory genes between seep and control samples (Fig. 2a). Two are transcriptional
251 regulators (ig19425, ig12770, 1.08-fold and 1.09-fold, respectively) and the third is a
252 transcription factor in the basic leucine-zipper superfamily (ig10473, 1.2-fold). In the entire
253 *A. millepora* transcriptome, 26 genes are annotated as 'transcriptional regulators' and another
254 8 are bZIP transcription factors. A methyl-CpG binding transcriptional regulator (ig9532)

255 was also upregulated by 1.05-fold in corals from seep sites, but showed an additional effect
 256 of host origin, with corals from Dobu having higher baseline expression than corals from
 257 Upa-Upasina (Fig. 2c). This methyl-CpG binding regulator was one of only three genes with
 258 this annotation in the entire *A. millepora* transcriptome, the other two of which (ig16785 and
 259 ig21898) were not found in the final expression set. A transcriptional repressor in the hairy/E
 260 (spl) family (ig7904) was among the most discriminatory genes and down-regulated in
 261 response to seep environments by 1.16-fold (Fig. 2b), again suggesting some role for
 262 transcriptional regulation, though 13 isogroups in the transcriptome also have this same
 263 annotation.



264
 265 Figure 2. Heatmaps of annotated genes (FDR-adjusted P<0.1) in the coral
 266 host that showed upregulation in response to seep environment (a),
 267 downregulation in response to seep environment (b), an effect of reef
 268 origin in addition to an effect of seep environment (c) or a reef origin x

269 seep environment interaction (d). D=FDR-adjusted $P < 0.1$ in Dobu-only
270 dataset; U=FDR-adjusted $P < 0.1$ in Upa-Upasina-only dataset; •=Top
271 discriminatory gene as identified via between-groups analysis for seep
272 environment. DS=Dobu-seep, DC=Dobu-control, US=Upa-Upasina-seep,
273 UC=Upa-Upasina-control.

274

275 A TNF receptor-associated factor (ig7766) is also a top discriminatory gene. This
276 family, involved in the innate immune response, recently came to prominence given its
277 putative role in the coral stress response (Barshis *et al.*, 2013). Its downregulation, together
278 with an NF-kappa-B inhibitor (ig16794) and a c-type lectin (ig6184, Fig. 2b), highlight a
279 potential impact of elevated pCO₂ on the innate immune response. However, 104, 49 and 143
280 isogroups respectively have identical annotations in the *A. millepora* transcriptome.

281 An alpha-catenin/vinculin isoform (ig833), one of three genes with this annotation, is
282 downregulated in seep site corals by 1.15-fold (Fig. 2b). The other two isoforms (ig1210 and
283 ig21857) are not differentially expressed and not included in this expression dataset.

284 Additional cytoskeletal components including a collagen (ig6915) and fibrillin (ig10778) are
285 also downregulated by 1.23 and 1.17-fold respectively, although these annotations are fairly
286 common (86 and 372 isogroups in the transcriptome, respectively).

287

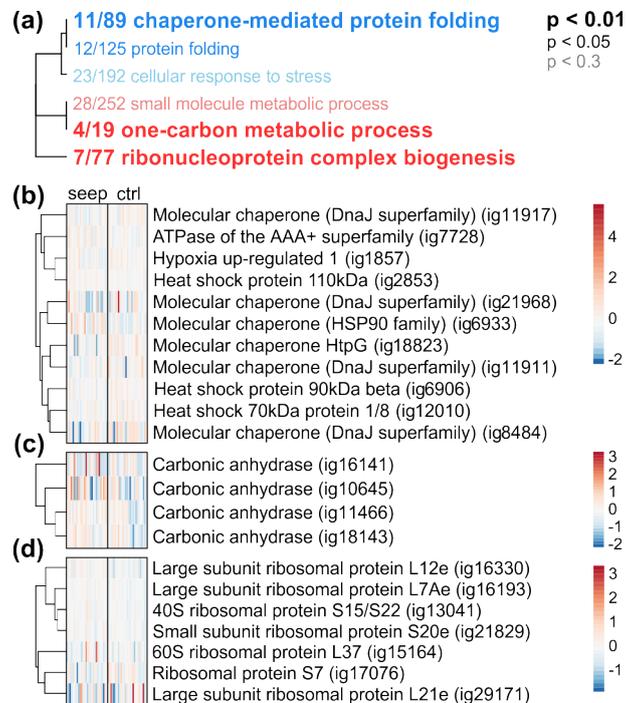
288 *Categorical Functional Enrichments*

289 A categorical functional enrichment analysis did not reveal any statistically
290 significant candidates following FDR-correction. The top three ‘biological process’
291 enrichments were ‘small molecule biosynthetic process’ (GO:0044283, $P_{\text{Raw}} = 0.1$), ‘fatty
292 acid metabolic process’ (GO:0006631, $P_{\text{Raw}} = 0.3$) and ‘small molecule catabolic process’
293 (GO:0044282, $P_{\text{Raw}} = 0.3$), which resulted from a set of four candidate genes. Pyridoxamine
294 5-phosphate oxidase (ig14233, upregulated by 1.06-fold in seep-site corals, GO:0044283,
295 Fig. 2a), an enzyme catalyzing the rate-limiting step in vitamin B₆ metabolism is an
296 annotation only assigned to one other gene in the host transcriptome (ig27779) that was not
297 differentially expressed with respect to either seep environment or reef origin.

298 Hydroxyproline oxidase (ig4000, GO:0044283, GO:0044282, Fig 2a), hypothesized to play a
299 role in activation of the apoptotic cascade (Cooper *et al.*, 2008), is also upregulated by 1.07-
300 fold in seep site corals. The only other gene of the transcriptome with this annotation
301 (ig1278) is differentially regulated with respect to reef origin, showing 1-fold upregulation in
302 corals from Dobu ($P_{\text{Reef}} < 0.1$, Table S2).

303 The remaining two genes are primarily involved in fatty-acid metabolism. Stearoyl-
 304 CoA desaturase (ig8881, GO:0044283, GO:0006631) is upregulated in seep sites by 1.15-
 305 fold. There are only 5 isogroups in the transcriptome with this annotation, 3 occur in the final
 306 expression list, but this isoform is the only one differentially expressed. The other candidate,
 307 long-chain-fatty-acid—CoA ligase, or long-chain acyl-CoA synthetase (ig5065,
 308 GO:0006631, GO:0044282), is downregulated by 1.19-fold and is one of only seven isoforms
 309 with this annotation. One other isoform is differentially expressed with respect to reef origin,
 310 with greater expression in Dobu-origin corals (ig3997, $P_{\text{Reef}} < 0.1$, Table S2), but remaining
 311 isoforms (ig2622, ig2781, ig5009, ig5135, ig12633) were not differentially expressed.
 312

313 Rank-based Functional Enrichments



314
 315 Figure 3. Hierarchical clustering of enriched gene ontology terms
 316 (‘biological process’) among upregulated (red) and downregulated
 317 (blue) genes in the coral host with respect to CO₂-seep (a). Font
 318 indicates level of statistical significance (FDR-corrected). Term names
 319 are preceded by fraction indicating number of individual genes within
 320 each term differentially regulated with respect to seep site (unadjusted
 321 $P < 0.05$). Heatmaps of these ‘good gene’ fractions are shown for
 322 ‘chaperone-mediated protein folding’ (b), ‘one-carbon metabolic
 323 process’ (c) and ‘ribonucleoprotein complex biogenesis’ (d).

324 Given the low number of candidate genes that passed the FDR threshold, a rank-based
325 methodology was also used to determine functional enrichments among generally
326 upregulated (red) and downregulated (blue) ontologies in corals from CO₂-seep environments
327 (Fig. 3a). ‘Chaperone-mediated protein folding’ (GO:0061077) was the top enrichment
328 among genes downregulated in CO₂-seep sites (Fig 3a, b). ‘Ribonucleoprotein complex
329 biogenesis’ and ‘one-carbon metabolic process’ were the top two most enriched functional
330 ontologies among genes upregulated in seep sites (GO:0022613 and GO:0006730,
331 respectively, Fig. 3a, c, d). Interestingly, the most significantly differentially regulated genes
332 within ‘one-carbon metabolic process’ are all individually annotated as carbonic anhydrases
333 (Fig 3c).

334

335 ***Differential expression of Symbiodinium genes by CO₂ seep environment***

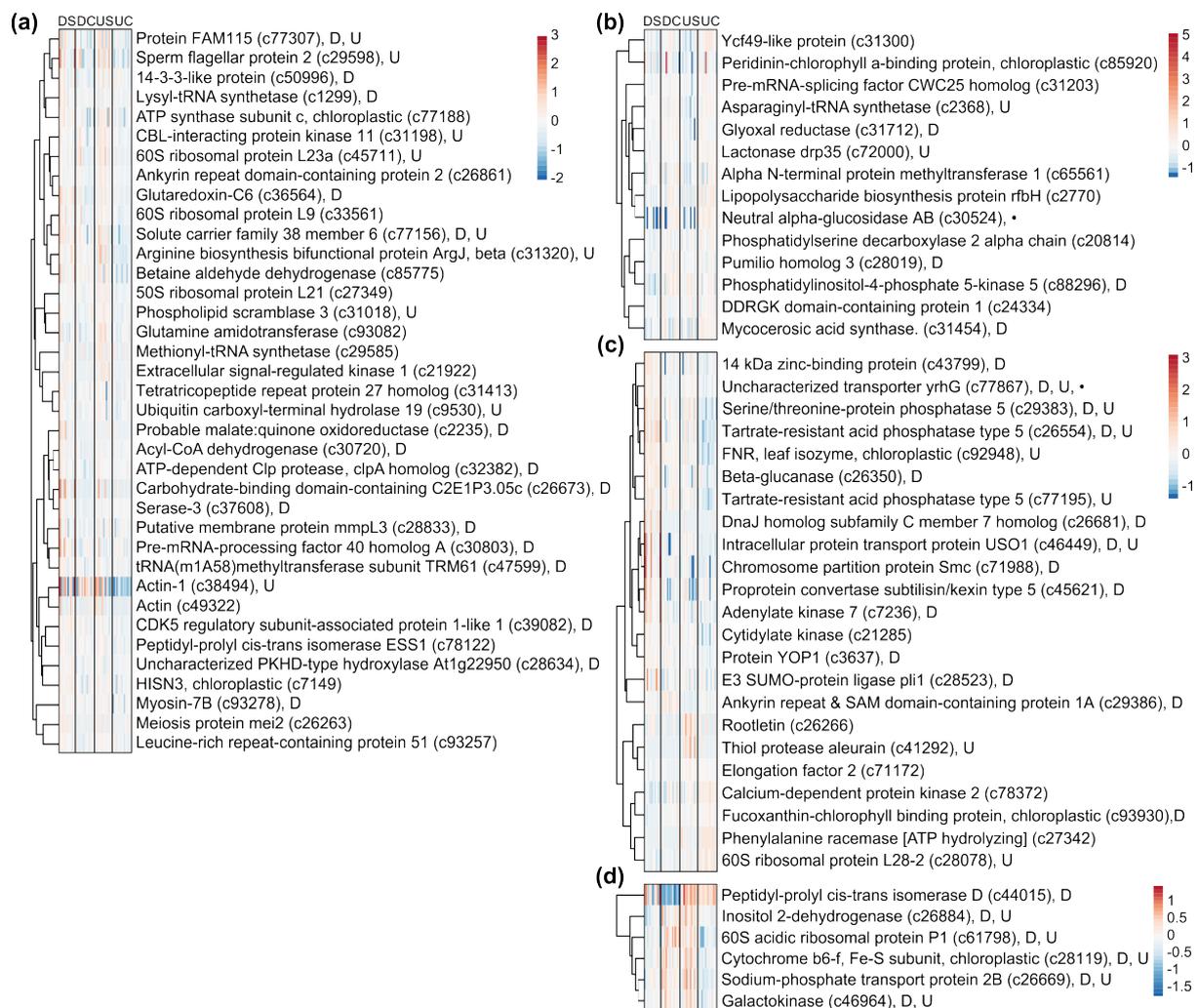
336 Of the 201 genes differentially expressed in response to CO₂-seep environment, 128
337 exhibited similar baseline expression levels between symbionts in corals from the different
338 reef locations (Fig. 1b, ‘CO₂ seep’, Table S5). Of these, 96 were upregulated and 32 were
339 downregulated in CO₂-seep environments. Only 40% of these genes were annotated, and we
340 again report expression patterns among these candidates only, although the data for all
341 differentially expressed genes can be found in Table S5. To enhance the sparse knowledge on
342 *Symbiodinium* responses to acidification, we report altered functional processes identified
343 through categorical and rank-based enrichment analyses.

344

345 ***Categorical Functional Enrichments***

346 The relatively small number of genes responding to seep site and a lack of annotations
347 resulted in no statistically significant ontology terms following FDR-correction of a
348 categorical enrichment analysis. The top three ‘biological process’ enrichments were
349 ‘regulation of chromosome organization’ (GO:0033044, P_{Raw} = 0.005), ‘response to
350 bacterium’ (GO:0009617, P_{Raw} = 0.03) and ‘regulation of organelle organization’
351 (GO:0033043, P_{Raw}=0.05), which resulted from a set of six candidate genes. A peptidyl-
352 prolyl cis-trans isomerase in the Ess family, matching Ess1 (c78122, GO:0033044,
353 GO:0033043 Fig. 4a) is upregulated by 1.05-fold at seep sites. In the *Symbiodinium* Clade C
354 transcriptome 62 clusters are annotated as PPIs, which catalyze the *cis–trans* isomerisation of
355 peptide bonds N-terminal to proline residues in polypeptide chains, but this is the only cluster
356 to have homology with Ess1. An E3 SUMO-protein ligase pli1 (c28523, GO:0033044,
357 GO:0033043, Fig 4c) was also upregulated in seep site corals by 1.1-fold, but shows an

358 additional effect of reef origin, with *Symbiodinium* in Dobu corals having higher baseline
 359 expression than *Symbiodinium* in Upa-Upasina corals. This annotation occurred twice in the
 360 transcriptome, but the other gene (c71663) was not included in the final expression set. The
 361 third gene in this regulatory group, the meiosis protein mei2 (c26263, GO:0033043, Fig 4a)
 362 was also upregulated by 1.1-fold at seep sites. Three other clusters in the transcriptome were
 363 assigned this annotation (c_sym_78605, c49233_81271, c94595), two of which were in the
 364 final expression set analyzed here and one was significantly differentially expressed with
 365 respect to reef origin (c94595, 1.02-fold, Table S3).

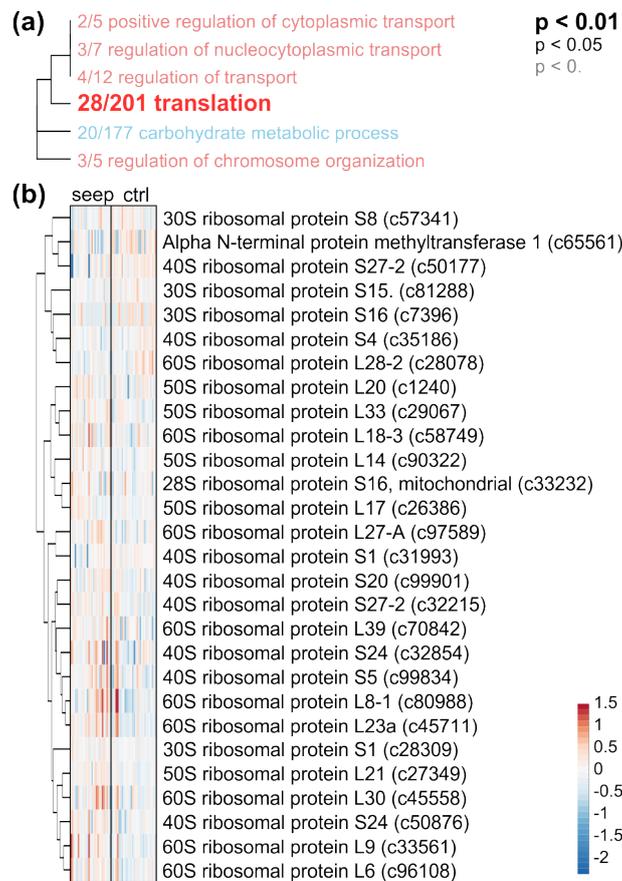


366
 367 Figure 4. Heatmap of annotated genes (FDR-adjusted $P < 0.1$) in *Symbiodinium* that showed
 368 upregulation in response to seep environment (a), downregulation in response to seep
 369 environment (b), an effect of reef origin in addition to an effect of seep environment (c) or a
 370 reef origin x seep environment interaction (d). D=adjusted $P < 0.1$ in Dobu-only dataset;
 371 U=adjusted $P < 0.1$ in Upa-Upasina-only dataset; •=Top discriminatory gene as identified via
 372 between-groups analysis for seep environment. DS=Dobu-seep, DC=Dobu-control, US=Upa-
 373 Upasina-seep, UC=Upa-Upasina-control.

374 The last three genes were all involved in bacterial response (GO:0009617) and all
 375 upregulated in seep sites. One of the genes was a 14-3-3-like protein (c50996, 1.06-fold, 9
 376 genes with this annotation in transcriptome, Fig. 4a). The second was an ankyrin repeat
 377 domain-containing protein 2 (c26861, 1.02-fold, 9 genes with this annotation, Fig. 4a). The
 378 last was tartrate resistant acid phosphatase type 5 (c26554, 1.1-fold, Fig 4c, 11 genes with
 379 this annotation) and also showed an effect of origin, with *Symbiodinium* in Dobu corals
 380 having higher baseline expression than *Symbiodinium* in Upa-Upasina corals.

381

382 Rank-based Functional Enrichments



383

384 Figure 5. Hierarchical clustering of enriched gene ontology terms
 385 ('biological process') among upregulated (red) and downregulated
 386 (blue) symbiont genes with respect to CO₂-seep (a). Font indicates
 387 level of statistical significance (FDR-corrected). Term names are
 388 preceded by fraction indicating number of individual genes within
 389 each term differentially regulated with respect to seep site (unadjusted
 390 P<0.05). A heatmap of this 'good gene' fraction is shown for
 391 'translation' (b).

392 The only significant functional enrichment identified with rank-based analysis was
393 ‘translation’ (GO:0006412, Fig. 5a) which was enriched among genes upregulated in seep
394 sites. Individual genes within this term were primarily annotated as ribosomal proteins (Fig.
395 5b).

396

397

398 **DISCUSSION**

399 The aim of this study was to investigate the genomic basis of acclimatization to
400 chronic exposure to ocean acidification in a reef-building coral through a comparison of
401 closely situated control and CO₂ impacted sites (500 m and 2500 m at Upa-Upasina and
402 Dobu, respectively, with 30 km between the two populations). Uniquely, we report global
403 gene expression profiles in both the coral host and their *in hospite Symbiodinium* that have
404 undergone life-long acclimatization to naturally acidified environments. Previous population
405 level studies (Fabricius *et al.*, 2014, Morrow *et al.*, 2015, Strahl *et al.*, 2015) strongly suggest
406 that acidified environments impact the fitness of *Acropora millepora*. Despite this, we found
407 very few consistent changes in global gene expression patterns between control and seep sites
408 (Fig. 1). This may be because gene expression changes did not reflect actual protein content
409 or because of post-translational regulation (Greenbaum *et al.*, 2003). It is also possible that
410 substantial inter-individual variation in expression (e.g. Bay *et al.*, 2009, Csaszar *et al.*, 2009)
411 masked the detectability of expression differences in response to environmental pCO₂. On the
412 other hand, important biochemical health measures related to cell protection and cell damage
413 were unaffected in *A. millepora* in response to elevated pCO₂ up to 800 µatm at the same
414 sites studied here (Strahl *et al.* 2015), consistent with our findings of a minimal expression
415 response.

416 The absence of significant gene expression changes may not necessarily be surprising
417 if acidification is a chronic stressor for the corals. Cellular stress gene expression responses
418 are transient and non-specific (Kültz, 2005). Once immediate damage is repaired, a
419 secondary, permanent cellular homeostasis response occurs, which is specific to the
420 triggering stressor and which facilitates the maintenance of homeostasis under the new
421 environmental regime (Kültz, 2003). It is likely that *A. millepora* exhibits open populations in
422 this system given the broadcast spawning behavior of this species and the close proximity of
423 study sites. Newly recruited juvenile corals may have exhibited an initial stress response, but
424 their gene expression baselines shifted with age in order to acclimate to their local
425 environment. Moya *et al.* (2015) previously reported dampened expression responses in a

426 time-series exposure of juvenile *A. millepora* to elevated pCO₂, consistent with this
427 hypothesis. Therefore, the small but constitutive differences in expression detected here, in
428 two replicate populations (n = 13 - 15 colonies per site) acclimatized to CO₂-seep
429 environments, likely reflects the core expression homeostasis response to ocean acidification.

430 In the coral host, this core response involves changes in gene regulation involved in
431 fatty acid (FA) metabolism (Fig. 2). Differential regulation of stress response genes also
432 occurred: specifically, corals from seep environments constitutively down-regulate
433 expression of molecular chaperones (Fig 3a, b). Interestingly, we did not find explicit
434 signatures indicating altered expression in calcification related genes, though some carbonic
435 anhydrase isoforms did appear to be constitutively upregulated in seep environments (Fig 3c),
436 but those could also be involved in cellular pH homeostasis. Finally, expression changes in *in*
437 *hospite Symbiodinium* were greater, and unlike patterns in their coral hosts, were not
438 consistent in seep habitats among reefs (Fig. 1c, d), which may have implications for the
439 symbiosis.

440

441 *Differential regulation of fatty acid metabolism*

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

455 Our results indicate a metabolic shift in CO₂-seep site corals in favor of increasing
456 lipid storage. This is supported by findings of Strahl *et al.* (2015), who detected slightly
457 higher ratios of storage to structural lipids in *A. millepora* at seep vs. control sites at Dobu
458 and Upa-Upasina. Stearoyl-CoA desaturase catalyzes the rate-limiting step in the synthesis of
459 unsaturated fatty acids, which are components of both structural (e.g. membrane

460 phospholipids) and storage lipids (e.g. triacylglycerol, wax esters, sterol ester), and the
461 disruption of genes encoding this enzyme in mice leads to reduced body adiposity (Ntambi *et*
462 *al.*, 2002). Long-chain-fatty-acid-CoA ligase, on the other hand, activates the first step of
463 fatty acid metabolism or β -oxidation (Watkins, 1997), when lipids are being broken down.
464 Storage lipids such as wax esters, triacylglycerol and free fatty acids are critical components
465 of corals' energetic status (Edmunds & Davies, 1986, Harland *et al.*, 1993) and depletions in
466 lipid stores can impact long-term survival and reproduction (Anthony *et al.*, 2009).
467 Furthermore, genes involved in lipid metabolism were found to exhibit significantly elevated
468 rates of protein evolution in Acroporids, but the authors were unable to speculate about
469 putative adaptive roles for lipid metabolism (Voolstra *et al.*, 2011).

470 Recently, Strahl *et al.* (2016) found that *A. millepora* from the Dobu seep site tend to
471 have elevated levels of total lipid and protein, as well as elevated levels of FAs (including
472 polyunsaturated FA) relative to control site corals, in support of observed expression
473 differences. Other studies have also found significant changes in lipid content in response to
474 acidification. In two separate aquarium-based acidification experiments, lipid content in *A.*
475 *millepora* was found to increase following exposure to elevated pCO₂ (Kaniewska *et al.*,
476 2015, Schoepf *et al.*, 2013). Behavioral changes may also be involved in this pattern as both
477 feeding rate and lipid storage increased in *Acropora cervicornis* under simulated acidification
478 (Towle *et al.*, 2015). Whether the mechanism is behavioral plasticity or adaptive genetic
479 change in lipid metabolic capacity, the combined evidence suggests that lipid metabolism
480 likely plays a role in a coral's capacity to withstand ocean acidification and future work
481 should aim to investigate the mechanistic basis of this process.

482

483 *Downregulation of chaperones*

484 Upregulation of chaperones is a hallmark of the acute cellular stress response (Gasch
485 *et al.*, 2000), but is usually transient as constitutive upregulation of heat shock proteins is
486 costly and can result in decreased growth and fecundity (Sørensen *et al.*, 2003). In
487 *Drosophila* and soil isopods exposed to chronic stress, Hsp70 expression is reduced rather
488 than elevated (Köhler & Eckwert, 1997, Sørensen *et al.*, 1999). HSPs are also known to be
489 constitutively downregulated following long-term thermal stress in corals (Kenkel *et al.*,
490 2013b, Meyer *et al.*, 2011, Sharp *et al.*, 1997). Short-term laboratory manipulations suggest
491 that exposure to acidification prompts expression of immediate stress response genes, like
492 HSPs (Moya *et al.*, 2012b, Moya *et al.*, 2015); and Kaniewska *et al* (2012) observed
493 downregulation of chaperones following one month of elevated pCO₂ exposure. Therefore,

494 acute exposure to acidification conditions is stressful for *A. millepora* and the constitutive
495 downregulation of HSPs observed here is likely a consequence of chronic exposure to
496 elevated pCO₂ at the seep sites.

497 Given that HSP induction is critical for mounting a successful thermal stress response,
498 the suppression of baseline HSP expression levels induced by acidified environments may
499 impact the capacity of *A. millepora* to cope with the synergistic effects of global climate
500 change. Acidification is predicted to become a chronic stress on reefs worldwide if climates
501 continue to change (Hoegh-Guldberg *et al.*, 2007). While temperatures will simultaneously
502 increase, extreme thermal anomalies are also predicted to become more frequent and severe
503 (Frich *et al.*, 2002). Our results suggest that the combined effects of acidification and
504 temperature stress may be more detrimental than acidification alone because of the
505 dampening effects of chronic exposure on the cellular stress response. Some laboratory
506 manipulations have found synergistic negative impacts of combined acidification and
507 temperature; for example, calcification of *Stylophora pistillata* decreased by 50% under both
508 elevated temperature and pCO₂, but was unchanged under each stressor individually
509 (Reynaud *et al.*, 2003). However, bleaching surveys following a minor thermal stress event in
510 PNG did not indicate that acidified reefs suffered increased bleaching relative to control reefs
511 (Noonan & Fabricius, 2015). It will be critical to determine whether constitutive
512 downregulation of HSPs resulting from long-term pCO₂ exposure makes it more difficult for
513 a coral to subsequently upregulate HSPs to counter acute thermal stress, or if other
514 mechanisms or isoforms are employed to counter acute thermal stress in chronically acidified
515 environments.

516

517 *No significant differential regulation of calcification genes*

518 We did not observe functional enrichments indicating differential regulation of
519 calcification related genes overall in *A. millepora*, although some carbonic anhydrase
520 isoforms were constitutively upregulated in seep site corals (Fig. 3c). Experimentally, some
521 coral species have been shown to maintain (Reynaud *et al.*, 2003) and even increase (Castillo
522 *et al.*, 2014) calcification during laboratory acidification experiments and this effect has been
523 hypothesized to result from the ability of corals to alter carbonate chemistry at the site of
524 calcification (McCulloch *et al.*, 2012, Venn *et al.*, 2013). Our *a priori* expectation was that
525 expression patterns of calcification related genes should be altered to affect this physiological
526 rescue. *Pocillopora damicornis* were observed to upregulate HCO₃⁻ transporters at
527 moderately low pH (7.8 and 7.4; Vidal-Dupiol *et al.*, 2013), while *Siderastrea siderea*

528 upregulated expression of H⁺ ion transporters (Davies *et al.*, 2016) consistent with this
529 hypothesis.

530 However, *A. millepora* does not appear to conform to this expectation. Expression of
531 calcification related genes significantly changed in *A. millepora* following short-term 3-day
532 acidification stress exposure (Moya *et al.*, 2012b), but these effects dissipate when
533 experimental treatment periods are extended (Kaniewska *et al.*, 2012, Moya *et al.*, 2015,
534 Rocker *et al.*, 2015; 28, 9 and 14 days, respectively). Furthermore, *A. millepora* from PNG
535 seep sites had reduced levels of net calcification, resulting from decreases in dark
536 calcification, compared to neighboring control reef sites (Strahl *et al.*, 2015). This suggests
537 that *A. millepora* has a reduced capacity to actively alter pH at the site of calcification in the
538 absence of additional photosynthetic energy (i.e. in the dark, Strahl *et al.*, 2015). The
539 regulation of cellular pH at calcification sites is an energetically costly process (Al-Horani,
540 2005, Barnes & Chalker, 1988). Given that calcification related gene expression is plastic in
541 *A. millepora* on shorter time-scales (Moya *et al.*, 2012a), it is possible that the lack of
542 constitutive differential regulation under long-term acidification, and subsequent decrease in
543 net calcification, were not necessarily due to a lack of genetic variation in the ability to
544 actively regulate these genes, but a result of trade-offs in allocation of finite energetic
545 resources to other less costly processes that maximize net fitness under acidification stress.
546 Indeed, Strahl *et al.* (2016) hypothesized that *A. millepora* may invest in increased tissue
547 biomass rather than skeletal growth under acidified conditions based on prior experimental
548 observations of unchanged or increased biomass in combination with reduced calcification
549 (Krief *et al.*, 2010, Schoepf *et al.*, 2013, Strahl *et al.*, 2015).

550

551 *Inconsistent changes in Symbiodinium expression profiles*

552 More significant differences in gene expression were detected for *Symbiodinium* than
553 for host corals between control and elevated pCO₂ sites examined here. This corroborates
554 findings from *Pocillopora damicornis* where their *in hospite* clade C *Symbiodinium*,
555 symbionts demonstrated a more pronounced expression response following a 2-week
556 exposure to elevated temperature, although this difference was no longer evident after 36-
557 weeks (Mayfield *et al.*, 2014). Kaniewska *et al.* (2015) examined metatranscriptomic
558 expression responses of coral holobionts to future climate change scenarios, but their analysis
559 method did not explicitly compare host and symbiont. Kenkel and Matz (2016) reported
560 expression of both host and symbionts in *P. astreoides* corals reciprocally transplanted
561 between reef habitats, but again, their network-based analytical approach precludes a direct

562 comparison with results uncovered here. A reanalysis of their dataset with the method used
563 here found that 14.8% of the host transcriptome was significantly differentially expressed
564 with respect to transplant environment, while only 1.4% of the symbiont transcriptome was
565 altered (Kenkel, unpublished data). Given the paucity of studies examining global expression
566 of both partners in response to environmental stress (to our knowledge, the present study is
567 only to examine expression under elevated pCO₂), it is difficult to draw any conclusions
568 regarding the present patterns. More data are needed to determine whether there are any
569 consistent patterns in *Symbiodinium* gene expression responses relative to those of their host
570 corals.

571 Expression changes of *in hospite Symbiodinium* showed greater differences between
572 control and seep sites across reefs compared to the coral host (Fig. 1c vs. d). The dominant
573 *Symbiodinium* types did not differ among corals at control and seep sites (Table S1, Fig. S1,
574 Noonan *et al.*, 2013) but it is possible that undetected background *Symbiodinium* clades or
575 types impacted expression levels if reads from other types failed to map to the Clade C
576 reference transcriptome used here (Ladner *et al.*, 2012). However, if differences in rare
577 *Symbiodinium* types or their expression patterns were consistent among reefs, we would still
578 expect to observe consistent changes in expression with respect to seep environment.
579 Conversely, if there was an interaction between potential differences in background clades or
580 types and seep environment, this could explain the variation observed (Fig. 1d). Control site
581 expression profiles are remarkably similar between reef sites, and the major axis of variation
582 differentiated seep from control site populations. However, the second principal component
583 describes variation in expression that is largely the result of divergence between seep site
584 expression of Dobu and Upa-Upasina origin corals (Fig. 1d).

585 Consistent expression changes among control and seep site *Symbiodinium* implicated
586 an alteration of the biological process of translation: specifically, many ribosomal proteins
587 were constitutively upregulated at the seep sites (Fig. 5). Ribosome production is intimately
588 tied to cell growth, and known to regulate cell size and the cell cycle (Jorgensen & Tyers,
589 2004). Net photosynthesis was significantly elevated in *A. millepora* from CO₂-seep sites
590 (Strahl *et al.*, 2015), potentially as a result of enhanced *Symbiodinium* cell growth or division
591 (and hence elevated expression of ribosomal proteins) although these processes remain to be
592 quantified. Determining the mechanistic drivers of divergence between seep site populations
593 among Dobu and Upa-Upasina reefs is more difficult. Of the top 10 gene loadings for PC2
594 (Fig. 1d), 8 had no annotation, precluding speculation about function. Nevertheless, the
595 complexity of the response in *Symbiodinium* may have implications for the symbiotic

596 interaction, if the coral host has to respond to the dual impacts of changes in its external
597 environment, and its symbiont community. It is recognized that mutualisms are more
598 susceptible to climate change impacts because the inherent inter-dependency between species
599 means that even though stress only impacts one partner, both partners ultimately share the
600 cost (Kiers *et al.*, 2010). There are many knowledge gaps remaining for both major global
601 change stressors, however, our understanding of thermal stress impacts on the coral-algal
602 symbiosis far outstrips understanding of acidification impacts (Barshis, 2015). Filling this
603 gap will be critical for refining predictions of coral response to continued acidification and
604 the combined impacts of global climate change.

605

606 DATA ARCHIVING

607

608 Raw RNA Tag-seq data have been uploaded to NCBI's SRA: PRJNA362652. R scripts and
609 input files for gene expression analyses will be archived on DRYAD upon manuscript
610 acceptance. R scripts for ontology enrichment analyses and directions for formatting input
611 files can be found at http://www.bio.utexas.edu/research/matz_lab/matzlab/Methods.html

612

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875 SUPPLEMENTARY FIGURES

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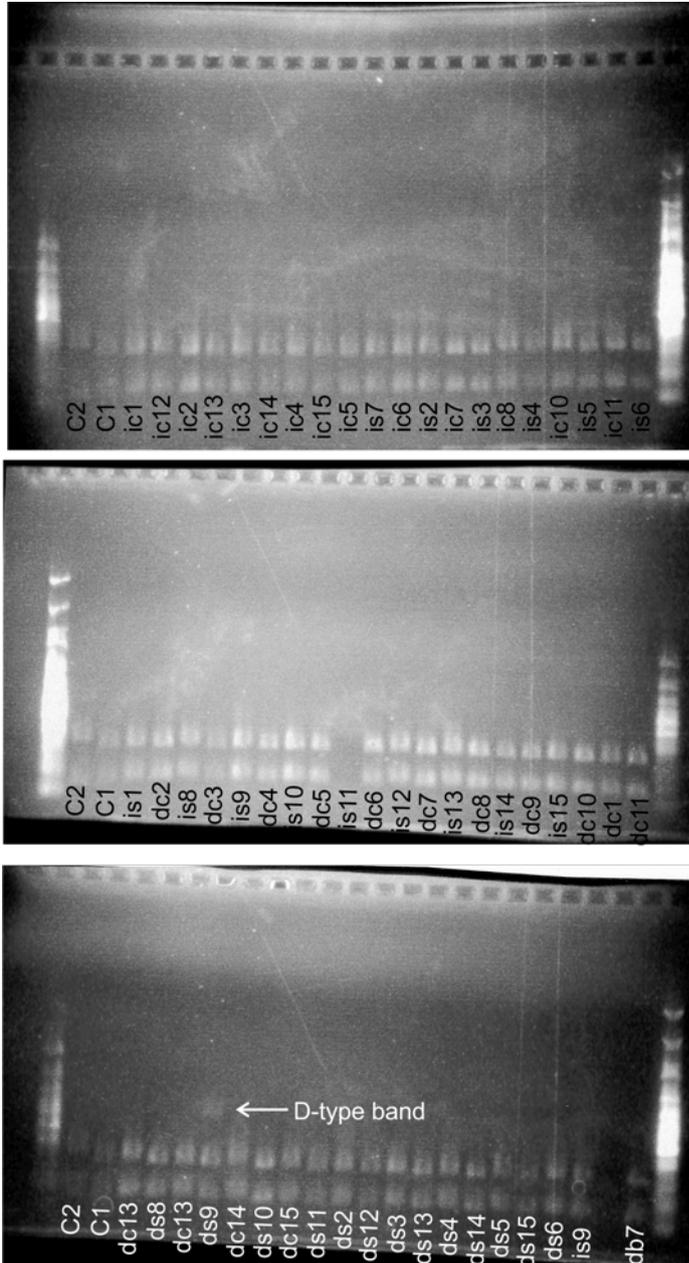
877 Figure S1. Electrophoresis gel showing digest of *Symbiodinium* Isu type for sampled corals.

878 All banding patterns match C1, save for sample ds9. C2 = *Symbiodinium* type C2 banding

879 pattern, C1= *Symbiodinium* type C1 banding pattern. ic=Upa-Upasina Control, is=Upa-

880 Upasina Seep, dc=Dobu Control, ds=Dobu Seep.

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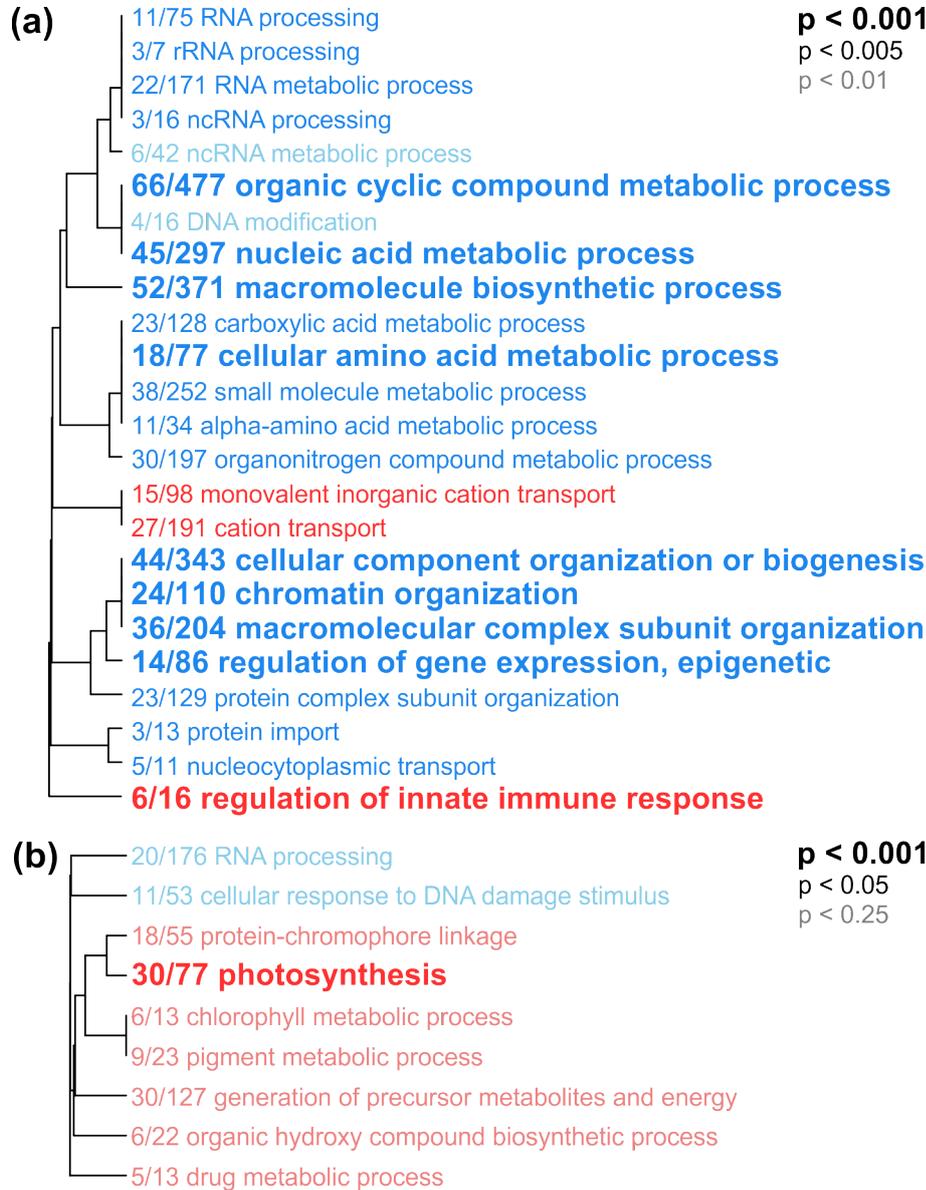
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890 Figure S2. Hierarchical clustering of enriched gene ontology terms ('biological process')
891 among differentially regulated genes by reef origin for the coral host (a) and symbiont (b).
892 Red indicates terms among genes upregulated in Upa-Upasina-origin corals relative to Dobu
893 corals and blue indicates terms among genes upregulated in Dobu corals relative to Upa-
894 Upasina corals.
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