

1 Microbiome diversity and host immune functions may define the fate of sponge holobionts
2 under future ocean conditions

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4 Running title: Sponge holobionts under future ocean conditions

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24 **Abstract**

25

26 The sponge-associated microbial community contributes to the overall health and
27 adaptive capacity of the sponge holobiont. This community is regulated by the
28 environment, as well as the immune system of the host. However, little is known about
29 the effect of environmental stress on the regulation of host immune functions and how
30 this may, in turn, affect sponge-microbe interactions. In this study, we compared the
31 microbiomes and immune repertoire of two sponge species, the demosponge,
32 *Neopetrosia compacta* and the calcareous sponge, *Leucetta chagosensis*, under varying
33 levels of acidification and warming stress. *Neopetrosia compacta* harbors a diverse
34 bacterial assemblage and possesses a rich repertoire of scavenger receptors while *L.*
35 *chagosensis* has a less diverse microbiome and an expanded range of pattern recognition
36 receptors and proteins with immunological domains. Upon exposure to warming and
37 acidification, the microbiome and host transcriptome of *N. compacta* remained stable,
38 which correlated with high survival. In contrast, the bacterial community of *L. chagosensis*
39 exhibited drastic restructuring and widespread downregulation of host immune-related
40 pathways, which accompanied tissue necrosis and mortality. Differences in microbiome
41 diversity and immunological repertoire of diverse sponge groups highlight the central role
42 of host-microbe interactions in predicting the fate of sponges under future ocean
43 conditions.

44

45 **Introduction**

46

47 Since the industrial revolution, the ocean has taken up a substantial amount of CO₂
48 that has led to an increased marine inorganic carbon concentration, reduced pH, and
49 decreased calcium carbonate saturation state (1). This global change in ocean chemistry,
50 exacerbated by sea surface warming, can affect many organismal processes,
51 consequently disrupting the reef population dynamics and ecosystem functioning (2). A
52 meta-analysis of climate change-associated studies of abundant benthic groups revealed
53 that sponges are likely winners under future climate scenarios (3).

54 Sponges (Porifera) are a major component of the benthic ecosystem and are
55 responsible for many ecological processes, such as nutrient cycling, ecosystem
56 structuring, reef consolidation, and bio-erosion (4). Sponges are generally thought to
57 possess exceptional ecological adaptability, stemming from a complex physiology and
58 diverse associated microbiome, that allows them to thrive even in extreme and perturbed
59 environments (5). However, while most siliceous demosponges exhibit resistance and
60 may even benefit from acidified ocean conditions, calcareous sponges are vulnerable
61 when exposed to lower pH levels (6).

62 Poriferans forge a close relationship with diverse groups of microorganisms to form
63 a complex structured ecosystem referred to as the holobiont (7). The microbial
64 complement, which can constitute up to 35% of sponge biomass (8), has key roles in
65 nutrient assimilation and metabolism, vitamin synthesis, and defense (9). However, the
66 species-specific bacterial assemblage in sponges can undergo restructuring under
67 drastic environmental perturbations (5). For example, elevated temperatures disrupt
68 symbiotic functions in the demosponge, *Rhopaloeides odorabile* Thompson, Murphy,
69 Bergquist & Evans, 1987, leading to holobiont destabilization and dysbiosis (10). While

70 this pattern of bacterial community dynamics usually precedes mass mortalities and is
71 widely observed in holobiont systems under stress (11), alternative trajectories of
72 microbiome plasticity promote rapid organismal adaptation (12, 13). For example, the
73 metagenomic profile of the microbiome of *Coelocarteria singaporensis* (Carter, 1883) at
74 CO₂ seeps have enhanced capacity to utilize the abundant inorganic carbon and exhibit
75 metabolic features that are necessary for efficient carbon fixation and nitrogen
76 metabolism in acidified conditions compared to individuals at control sites (14).
77 Differences in the response of the bacterial community in the context of the organismal
78 stress response determine the impact of environmental perturbations on different marine
79 organisms (5).

80 The bacterial complement is shaped by various ecological selective forces acting
81 within a holobiont. Resource limitation coupled with antagonistic interactions, such as
82 interspecific competition and immune functions, fine-tune microbial populations that
83 maintain holobiont homeostasis (15). However, the balance of these control mechanisms
84 may be disrupted in disturbed conditions allowing the proliferation of opportunistic
85 microbial taxa. The host's innate immune system, which is involved in sensing microbial
86 cells and activating phagocytosis, cell death, or production of antimicrobial molecules,
87 has been shown to respond to different stress signals in marine invertebrates (16-18).
88 For example, elevated temperature induced the coordinated expression of pattern
89 recognition receptors (PRRs), immune-related signaling cascades, and apoptosis
90 regulators in the demosponge, *Haliclona (Reniera) tubifera* (George & Wilson, 1919) (19).
91 Interestingly, the immune gene repertoire varies among sponges from different taxa (20,
92 21) or in species with different microbiome composition (18, 22). For example, *Stylissa*

93 *carteri* (Dendy, 1889), a low microbial abundance (LMA) sponge, possesses an expanded
94 family of proteins with scavenger receptor cysteine rich (SRCR)-like domains relative to
95 the high microbial abundance (HMA) sponge, *Xestospongia testudinaria* (Lamarck, 1815)
96 (22). Moreover, a survey of sponge transcriptomes revealed the absence of certain
97 immune pathway components, such as myeloid differentiation primary response 8
98 (*MyD88*), in the calcarean, *Sycon ciliatum* (Fabricus, 1780) (20). Distinct combinations of
99 immune molecules may influence microbiome control in the sponge holobiont. Thus,
100 elucidating the links between immune system functions and bacterial community
101 structuring may provide a better understanding of inter-species differences in the
102 tolerance of sponges to environmental stressors (23).

103 Here, we characterized the microbiomes and repertoire of immune-related genes in
104 *Neopetrosia compacta* (Ridley & Dendy, 1886) (class Demospongiae, order
105 Haplolsclerida, family Petrosiidae) and *Leucetta chagosensis* Dendy, 1913 (class
106 Calcarea, order Clathrinida, family Leucettidae). We examined the response of these
107 genes and of the sponge-associated microbial communities to varying stress conditions.
108 In addition, we compared the immune gene repertoire and microbial diversity of other
109 sponge species to elucidate common trends. We hypothesized that changes in sponge
110 microbiome structure will correlate with changes in the expression of certain immune
111 response genes. Our findings highlight the importance of host-microbe interactions in
112 predicting the fate of marine sponges in the face of a rapidly changing ocean.

113

114 **Material and Methods**

115

116 Sponge sampling and culture

117

118 Six specimens each of *N. compacta* and *L. chagosensis* were collected from the
119 Bolinao-Anda Reef Complex in Pangasinan, northwestern Philippines (16.296° N,
120 120.014° E), in September 2018 with permission from the Philippines Department of
121 Agriculture (Gratuitous Permit No. 0169-19). Sponge identities were confirmed by their
122 morphology (24) and 28S rRNA gene analyses (Fig. S1). Donor sponges were cut into
123 twelve ($\approx 1 \text{ cm}^3$) fragments using a sterile razor and allowed to heal *in situ* for 30 days.
124 Healed fragments were brought to the Bolinao Marine Laboratory and allowed to
125 acclimatize for seven days in aquaria receiving flow-through seawater under ambient
126 conditions of pH 8.0 and 28°C.

127

128 Stress response experiments

129

130 Stress response experiments were conducted in independently aerated 10L aquaria
131 with flow-through seawater. Temperatures were regulated using 300W submersible
132 heaters, levels of injected CO₂ manipulated with mass flow controller, and the illumination
133 followed a 12:12 light: dark photoperiod using daylight LED lamps. Conditions were
134 designed to simulate the present day and predicted 2100 Representative Concentration
135 Pathway (RCP) 6.0 and 8.5 scenarios (25). Treatment conditions included (i) pH 8.0, 28°C
136 (Present Day), (ii) pH 7.6, 28°C (Acidification), (iii) pH 8.0, 32°C (Warming), (iv) pH 7.8,
137 30°C (RCP 6.0), and (v) pH 7.6, 32°C (RCP 8.5). Each treatment was represented by four
138 independent replicate aquaria containing three fragments of each sponge species.

139 Temperature and pH levels were changed gradually (temperature: +1°C/day, pH: -
140 0.5/day) until the desired conditions were reached (Fig. S2). Treatment conditions were
141 maintained for up to three days when the experiment was terminated because tissue
142 necrosis had begun to manifest in some fragments. Tissues of surviving sponges were
143 washed with UV filtered seawater, flash-frozen in liquid nitrogen, and stored at -80°C.
144 Light and temperature in the tanks were monitored using submersible loggers (HOBO
145 pendant, Onset Computer Corp., Bourne, MA, USA), pH was measured using a SevenGo
146 Duo Pro pH meter (Mettler Toledo, Columbus, OH, USA), and DO and salinity were
147 measured using a multiparameter meter (Pro 2030, YSI Inc., Yellow Springs, OH, USA).
148 Dissolved inorganic carbon and total alkalinity (TA) were quantified using a TA Analyzer
149 (Kimoto Electric, ATT-05, Japan). Seawater carbonate chemistry parameters were
150 calculated from pH, TA, temperature, and salinity data using the CO2SYS package (Table
151 S1) (26).

152

153 16S rRNA gene sequencing and analysis

154

155 Total genomic DNA was extracted from sponge tissues (three biological replicates
156 per species for each treatment) using DNeasy PowerSoil Pro Kit (Mo Bio, Carlsbad, CA,
157 USA). DNA concentration was quantified using a NanoDrop spectrophotometer (Thermo
158 Fisher Scientific). DNA extracts were sent to Macrogen, South Korea, for sequencing.
159 Bacterial 16S rRNA V3-V4 hypervariable region was amplified from the extracted DNA
160 using barcoded primers Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') and Bakt_805R
161 (5'-GACTACHVGGGTATCTAATCC-3') (27). Paired-end sequencing (300 bp) was

162 performed on the Illumina MiSeq platform following the dual-index sequencing strategy.

163 Sequences were processed using QIIME2 version 2019.7(28). The DADA2 package
164 (29) was used to remove chimeric sequences and singletons, and to correct amplicon
165 errors. The denoised forward and reverse reads were assembled into single contigs. The
166 taxonomic assignment of processed sequences was carried out using a Naïve Bayes
167 classifier trained on SILVA version 132 (30). The classifier was set to include V3-V4
168 regions of 16S rRNA genes at 99% sequence similarity. Sequence reads from
169 chloroplasts and mitochondria were removed from the final set of Amplicon Sequence
170 Variants (ASVs). Raw sequence reads can be accessed in the NCBI Short Read Archive
171 database under the BioProject ID PRJNA689294.

172

173 Microbial community composition analysis

174

175 Rarefied ASV libraries were produced through random down-sampling to the
176 identified smallest library size. Alpha diversity indices were computed using Phyloseq
177 (31). Community distance matrices based on Bray-Curtis dissimilarity index were
178 estimated using vegan (32) and visualized by non-metric multidimensional scaling.
179 ADONIS and ANOSIM tests were performed to evaluate changes in the structure and
180 composition of bacterial communities across treatments. Responsive ASVs were
181 described through pairwise comparisons between Present Day samples versus samples
182 subjected to (i) Acidification, (ii) Warming, (iii) RCP 6.0, and (iv) RCP 8.5. Differentially
183 abundant ASVs (log fold change $\geq |2|$, Benjamini-Hochberg-adjusted p-value ≤ 0.1) were
184 identified across treatments using Phyloseq-DESeq (33) implemented in R.

185

186 Functional prediction and analyses

187

188 Phylogenetic Investigation of Communities by Reconstruction of Unobserved
189 States (PICRUSt2) (34), installed as QIIME2 plugin, and Tax4Fun2 (35) were used to
190 predict the functional profiles of the bacterial communities. These tools use marker genes,
191 such as 16S rRNA, to predict community gene counts based on ASV taxon affiliations.
192 The weighted Nearest Sequenced Taxon Index (NSTI) cut-off score was set to 2.0 to
193 increase accuracy of PICRUSt2 predictions. The NSTI score summarizes the relatedness
194 of the ASVs in the sample to the closest available reference genome and serves as a
195 basis for assessing the quality of prediction. Low NSTI values indicate higher similarity to
196 the reference genomes and, thus, more accurate functional gene prediction (34). KEGG
197 ortholog (KO) prediction and abundance estimation was performed and associated high-
198 level functions were determined using the `pathway_pipeline.py` script with a KEGG
199 pathways mapping file. In Tax4Fun, representative sequences were searched against the
200 Ref100NR database to find the closest reference genome using NCBI blast+. Thereafter,
201 KO counts and KEGG pathway profiles were predicted using the
202 `makeFunctionalPrediction` command.

203 Changes in the predicted functional potential of the bacterial communities were
204 described through pairwise comparisons between Present Day samples versus samples
205 subjected to the other treatments. Differentially abundant KOs (Benjamini-Hochberg-
206 adjusted p -value ≤ 0.05) were identified across treatments using Phyloseq-DeSeq (33)
207 implemented in R. The combined set of differentially abundant KOs across treatments

208 was searched against the KEGG database using the KEGG mapper-search pathway
209 mapping tool. The relative abundance of the retrieved KEGG pathways was then
210 calculated from the sum of the relative abundance of the associated KOs.

211

212 Transcriptome sequencing, assembly, and annotation

213

214 Total RNA was extracted from *N. compacta* and *L. chagosensis* samples using
215 TRIzol (Invitrogen, Waltham, MA, USA). Contaminating DNA was removed using TURBO
216 DNA-free kit (Invitrogen). RNA concentration was determined using a NanoDrop
217 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of RNA
218 extracts was evaluated using gel electrophoresis on 1% agarose in 1x TBE and the
219 Agilent 2200 TapeStation System (Agilent Technologies, Santa Clara, CA, USA).
220 Libraries were prepared from three samples per treatment, except for the *L. chagosensis*
221 Warming and RCP 6.0 treatments, for which we were only able to obtain high quality RNA
222 for two samples each. Barcoded libraries were prepared at Macrogen, South Korea, using
223 the Truseq RNA library preparation kit (Illumina, San Diego, CA, USA). mRNA-enriched
224 libraries were sequenced on the Illumina Novaseq 6000 platform to generate 100 bp
225 paired-end reads.

226 Raw sequence reads were visualized with FastQC v0.11.8 (Babraham
227 Bioinformatics) and trimmed using Trimmomatic v0.32 (36). Filtering included the removal
228 of poor quality bases (quality score < 3) at the start and end of the reads, scanning the
229 read with a 4-base sliding window, trimming if the average per-base quality is below 20,
230 excluding reads below 36 bases long, and cutting 15 bases from the start of the reads.

231 *De novo* transcriptome assembly was carried out on Trinity (37). Transcripts with
232 90% sequence similarity were clustered and the longest representative contigs (>300bp)
233 were retained. Reads were mapped back to the assembled transcriptomes and isoforms
234 with zero isoform percentage (IsoPct) were removed to filter out putative misassembled
235 transcripts. Isoforms with the highest combined IsoPct or longest length were retained for
236 each transcript to generate a reference transcriptome for each species. The non-
237 redundant transcriptomes of *N. compacta* and *L. chagosensis* are composed of 69 202
238 (N50= 1 150) and 92 629 (N50=1 475) transcripts, respectively. The quality of the
239 assembled transcriptome is comparable to other Poriferan transcriptomes (20, 38) as
240 assessed through Bowtie (39), Detonate (40), and Transrate (41) (Table S2). Highly
241 expressed transcripts were also determined to have high contig length (Fig. S3). The
242 assembly contains more than 90% of the metazoan core genes measured through
243 BUSCO (42) with Metazoa *odb9* dataset (Table S2). Raw sequence reads were deposited
244 in the NCBI Short Read Archive database under BioProject ID PRJNA689294. The
245 reference transcriptomes used in this study has been deposited at DDBJ/EMBL/GenBank
246 under the accession GIYW000000000 (*N. compacta*) and GIYV000000000 (*L.*
247 *chagosensis*). The version described in this paper
248 are the first versions, GIYW01000000 and GIYV01000000, respectively.

249 *N. compacta* and *L. chagosensis* peptides were predicted using the Transdecoder
250 package in Trinity. Peptides were mapped against the UniProtKB/Swiss-Prot database
251 (April 2020). To predict gene ontology (GO) annotations, the top Blastp hit for each
252 sequence was used as input into Blast2GO (43), while protein domains were annotated

253 by mapping the peptide sequences against Pfam 32.0 database (44) using HMMER v3.3
254 (45).

255

256 Expression analysis

257

258 Reads were mapped to the assembled reference transcriptomes to estimate
259 transcript abundance using RNA-Seq by Expectation Maximization (RSEM) (46) with
260 bowtie alignment method (39). Differentially expressed transcripts were identified using
261 the edgeR (47) package in R. Expected counts were converted to counts per million
262 (CPM) and only genes with >10 CPM in at least two libraries were included in the analysis.

263 Genes were considered differentially expressed if up or downregulation was greater than
264 4-fold relative to the controls with a Benjamini-Hochberg-adjusted p-value $<1 \times 10^{-5}$.

265 Pairwise comparisons were conducted between control samples (Present Day) and
266 samples subjected to the other treatments. Functional enrichment analysis for
267 differentially expressed transcripts was done using the topGO package (48) in R. Only
268 GO terms with a p-value <0.05 were considered significantly enriched. Protein-protein
269 interactions for sponge homologs of genes involved in the human innate immune
270 response were retrieved from the STRING v.11 database (49). Interaction networks were
271 visualized using Cytoscape v.3.7.2 (50). Relative expression of sponge gene homologs
272 in each treatment relative to the Present Day control was computed as the average sum
273 of \log_2 transformed transcripts per million (TPM).

274

275 Comparative analysis of bacterial communities and predicted metagenomes

276

277 Selected datasets from the Sponge Microbiome Project (51) were retrieved from the
278 Qiita database under Study ID: 10 793 (March 2020). Sequences from healthy adult
279 individuals viz. Demospongiae (n = 1441), Calcarea (n = 20), Homoscleromorpha (n =
280 41), and Hexactinellida (n = 2) were included in the analysis. Alpha diversity indices were
281 computed with Phyloseq (31). The predicted functions of 128 sponge microbiomes with
282 known LMA-HMA status viz. Demospongiae-LMA (n = 69), Demospongiae-HMA (n = 48),
283 Calcarea-LMA (n = 4), Homoscleromorpha-LMA (n = 1), Homoscleromorpha-HMA (n =
284 5), Hexactinellida-LMA (n = 1), as described in a previous study (52), were shared by
285 Miguel Lurgi (CNRS-Paul Sabatier University, France).

286

287 Comparative analysis of sponge immunological repertoire

288

289 Predicted peptide sequences of representative demosponges (*Amphimedon*
290 *queenslandica* Hooper & van Soest, 2006 (53), *H. tubifera* (38), *Petrosia* (*Petrosia*)
291 *ficiformis* (Poiret, 1789) (20)), calcareans (*S. ciliatum*, *Leucosolenia complicata* (Montagu,
292 1814) (54)), and homoscleromorph (*Oscarella carmela* Muricy & Pearse, 2004) were
293 annotated against the UniProtKB/Swiss-Prot (April 2020) and Pfam 32.0 (44) database.
294 Sponge peptide sequences were downloaded from Compagen (55), except for *A.*
295 *queenslandica*, which was retrieved from Ensembl Metazoa, and *P. ficiformis*, which was
296 shared by Ana Riesgo (Natural History Museum, London).

297 NACHT domain-containing genes, with bona fide or tripartite NLR gene architecture
298 (56), were identified from the sponge predicted peptides. Amino acid sequences

299 corresponding to the NACHT domain (PF05729) were used for phylogenetic
300 comparisons. Multiple sequence alignment was performed using Clustal Omega (57) and
301 the aligned sequences were manually trimmed. The best-fit substitution model (LG+G+F)
302 was identified based on Bayesian Information Criterion using protest v3.4.2 (58).
303 Bayesian inference analysis was performed in MrBayes v.3.2 (59) with two-independent
304 MCMC runs and four chains per run. The analysis was sampled every 100 trees until the
305 average standard deviation of split frequencies was <0.01. The first 25% of trees were
306 discarded as burn-in.

307

308 **Statistical analyses and visualization**

309

310 Community alpha diversity values and expression levels of PRRs were tested for
311 normality using Shapiro-Wilk test and homogeneity of variance through Levene's test.
312 Statistical differences were calculated using Welch's t-test or Wilcoxon test with p-values
313 <0.05 considered statistically significant. KEGG functions and immunological domains
314 that distinguished among sponge groups were identified using Linear Discriminant
315 Analysis effect size (LDA-LEfSe) (60) based on relative abundance values. All
316 visualizations were done using the ggplot2 package (61) in R.

317

318 **Results and Discussion**

319

320 Sponges exhibit differential survival under ocean warming and acidification

321

322 Fragments of the siliceous sponge, *N. compacta* (Fig. 1A), remained healthy under
323 variable levels of pH and temperature stress, as well as to combinations of these two
324 stressors (Fig. S4). In contrast, the calcareous sponge, *L. chagosensis* (Fig. 1B), showed
325 visible tissue necrosis under the warming, RCP 6.0, and RCP 8.5 conditions, but not in
326 the acidification only treatment (Fig. S4). While up to 97% of *N. compacta* fragments
327 survived the most extreme condition at RCP 8.5, only 25% of the *L. chagosensis*
328 fragments survived in the RCP 8.5 treatment after just two days of sustained exposure
329 (Fig. 1C). These observations are comparable to the findings of other studies that
330 reported the high survivorship of demosponges and the susceptibility of calcareous
331 sponges subjected to these climate change-associated stressors (19).

332

333 Bacterial community shifts in the stress response of sponges

334

335 Rarefaction curves indicate the completeness of detected ASVs present in *N.*
336 *compacta* (Fig. S5A) and *L. chagosensis* (Fig. S5B) across the different treatments.
337 *Neopetrosia compacta* is associated with a fairly diverse and heterogenous bacterial
338 assemblage (species richness = 188.00 ± 27.78 ; Shannon diversity index = 3.68 ± 0.02)
339 with enrichment of the Chloroflexi-related SAR202 clade (22.23%) and phototrophic
340 groups, including Nostocales (8.19%) and Synechococcales (0.12%). On the other hand,
341 *L. chagosensis* harbored a less diverse microbiome (species richness = 150.33 ± 53.58 ;
342 Shannon diversity index = 2.04 ± 0.66) composed primarily of Oceanospirillales (56.17%)
343 and Deltaproteobacteria SAR324 clade (27.26%) (Fig. 2A-B; Fig. S6, S7A).

344 To explore the possible roles of bacterial community dynamics in the stress
345 response of the two sponge holobionts, we described the shifts in the taxonomic profiles
346 of their bacterial associates following exposure to simulated conditions. The microbial
347 community of *N. compacta* showed little change in structure when subjected to the
348 various stressors (Fig. 2C). The few ASVs that showed a significant change in abundance
349 relative to the present day treatment include ASV749 (Microbacteriaceae), which
350 decreased in Acidification and RCP 6.0 conditions, and ASV842
351 (Endozoicomonadaceae), ASV2840 (Cellvibrionaceae), ASV73, ASV1088, ASV887,
352 ASV2836, ASV1181, and ASV2292 (Rhodobacteraceae), ASV2836
353 (Alteromonadaceae), ASV1181 (Nitrincolaceae), and ASV2292 (Colwelliaceae), which
354 increased in RCP 6.0 (Fig. S8).

355 In contrast, the bacterial assemblage of *L. chagosensis* exhibited apparent
356 restructuring with the treatments, although not statistically supported (Fig. 2D, Table S3).
357 A total of 37 ASVs exhibited changes in relative abundance, with 21 decreasing and 16
358 increasing (Fig. 2E). In the Acidification treatment, where 100% of the sponges survived,
359 a decrease in Bacteroidales and Clostridiales and an increase in the most dominant
360 symbiont, Oceanospirillales, was observed (Fig. S9B). On the other hand, in the
361 treatments with high sponge mortality (i.e. Warming, RCP 6.0, and RCP 8.5), there was
362 reduced abundance of ASV2477 (SAR324 clade) and ASV2219
363 (Endozoicomonadaceae). Presumptive opportunistic taxa, such as Vibrionales (ASV90,
364 ASV688), Rhodobacterales (ASV1216, ASV1190, ASV1738, ASV2661, ASV1725), and
365 Rhizobiales (ASV1658) (62), increased in relative abundance under these treatments
366 (Fig. 2E; Fig. S9B).

367 Taxa that proliferated in the microbial community of *L. chagosensis* under stress
368 conditions were predicted to invest more in the production of antimicrobial molecules (Fig.
369 2F), which may be advantageous in competitive colonization of the tissues of the sponge.
370 The increased abundance of opportunistic taxa may be due to their capacity to form
371 biofilms for efficient surface adhesion and active secretion of virulence factors to invade
372 the host cell (63). These traits, along with the ability to sense and respond to
373 environmental perturbations, as evidenced by enrichment of functions related to two-
374 component signaling and bacterial chemotaxis, may support successful proliferation of
375 certain taxa that will eventually outcompete other microbiome members (64). The
376 resulting large-scale changes in the bacterial community of *L. chagosensis* are predicted
377 to correlate to a shift in the functional and metabolic potential of the holobiont, which may
378 further contribute to the decline of the host.

379

380 Sponge immune response under ocean warming and acidification conditions

381

382 To determine how the host innate immune system is affected by acidification and
383 warming, we described the expression patterns of immune-related genes in *N. compacta*
384 and *L. chagosensis* using RNA-Seq. A total of 1 596 genes (Acidification = 74, Warming
385 = 308, RCP 6.0 = 501, RCP 8.5 = 713) were found to be differentially expressed in *L.*
386 *chagosensis*, whereas only 70 genes (Acidification = 3, Warming = 7, RCP 6.0 = 12, RCP
387 8.5 = 48) genes were differentially expressed in *N. compacta*.

388 Although reduced environmental pH levels have been reported to induce bacterial
389 virulence (65), gene ontology (GO) enrichment analysis of differentially expressed genes

390 in the calcareous sponge revealed that *L. chagosensis* is able to mount relevant
391 responses, including endosome organization and antibacterial humoral response to avoid
392 pathobiont invasion, under Acidification treatment (Fig. 3A). On the other hand, key
393 defense mechanisms against microbial perturbations were differentially regulated in *L.*
394 *chagosensis* under Warming, RCP 6.0, and RCP 8.5 conditions. Specifically, genes
395 implicated in recognition of microbe-associated molecular patterns (MAMPs) and
396 receptor-mediated endocytosis were repressed in *L. chagosensis*. In particular,
397 scavenger receptors (SRCRs), secretin G-protein coupled receptors (GPCRs), and
398 nucleotide-binding domain and leucine-rich repeat-containing genes (NLRs), exhibited
399 reduced expression under Warming, RCP 6.0, and RCP 8.5 conditions (Fig. 3B). The
400 reduction in the expression levels of these genes and the sensor proteins that they
401 encode, along with the repression of bactericidal permeability-increasing protein (*BPI*)
402 and lipopolysaccharide binding protein (*LBP*) (Fig. 3C), may result in impaired recognition
403 of microbial cells or molecules, which, in turn, influences the regulation of downstream
404 effectors of the immune response (66). Components of other principal machineries
405 involved in the response to pathogen infection, such as autophagy, inflammation, and
406 apoptosis, were similarly downregulated (Fig. 3A).

407 Decreased expression of genes involved in tumor necrosis factor (*TNF*) signaling
408 suggests that *L. chagosensis* may no longer be able to deploy synchronized expression
409 of effector molecules that mediate diverse aspects of innate immunity (67). While the *TNF*
410 receptor (*TNFR*) increased in expression, the *TNF* ligand, activator disintegrin and
411 metalloproteinase domain-containing protein 10 (*ADAM10*), and the adapter protein *TNF*
412 receptor-associated factor 5 (*TRAF5*) reduced in expression (Fig. 3C). The repression of

413 responses regulated through this pathway is further supported by the downregulation of
414 immune-related transcription factors, such as interferon regulatory factor 5 (*IRF5*) and
415 nuclear factor NF-kappa-B p105 subunit (*NFKB1*), coupled with the increased levels of
416 the *NF-kB* inhibitor (*IKB*) and inhibitor of *NF-kB* kinase (*IKK*) (68). Indeed, the
417 downregulation of macrophage-expressed gene protein 1 (*MPEG1*), allograft
418 inflammatory factor-1 (*AIF1*), initiator caspase *CASP2/9*, and executioner caspase
419 *CASP3/6/7*, suggest inhibited antimicrobial, inflammatory, and apoptotic mechanisms
420 (69-71). Genes with anti-apoptotic functions, including the apoptosis regulator (*BCL2*),
421 Bcl-2-like protein 1 (*BCL2L1*), and X-linked inhibitor of apoptosis (*XIAP*) were negatively
422 regulated, as well. These results generally suggest that *L. chagosensis* may not be able
423 to restore immune homeostasis under combined warming and acidification conditions.

424 In contrast to the calcareous sponge, the demosponge *N. compacta* exhibited
425 activation of the complement system and cytokine-induced processes under RCP 8.5
426 conditions (Fig. 3A). An increased level of *TNF*, along with the upregulation of interleukin-
427 1 receptor-associated kinase-4 (*IRAK4*), *TRAF5*, and *NFKB1*, indicates that the TNF-
428 NFkB and Myd88-dependent signaling pathways were activated (72, 73) (Fig. 3C). The
429 increased expression of *AIF1*, *CASP2/9*, and *CASP3/7*, along with the *BCL2* and *XIAP*,
430 suggest active inflammatory and apoptotic functions (69, 71). These indicate that *N.*
431 *compacta* may be able to sustain its ability for symbiont recognition and pathogen
432 clearance through the coordinated expression of signaling pathways and immune effector
433 mechanisms even under the most extreme conditions in this study.

434

435 Demosponges and calcareans are characterized by disparate bacterial communities and
436 immunological repertoires

437

438 Evaluation of the diversity patterns of bacterial communities in representatives
439 from different sponge classes showed that demosponges typically harbor bacterial
440 communities with a wide range of taxonomic diversity, whereas calcareans are generally
441 associated with less diverse bacterial communities (Fig. 4A). Low microbial abundance
442 has been consistently reported among calcareans through microscopy (74),
443 metagenomics (51), and culture-based techniques (74). Comparisons of predicted
444 microbiome functions indicate that there is functional differentiation among HMA and LMA
445 sponge microbiomes (Fig. 4B). Generally, the bacterial associates of calcareans, along
446 with LMA demosponges and homoscleromorphs, are enriched for functions related to
447 metabolism, whereas the bacterial assemblage of HMA demosponges and
448 homoscleromorphs are enriched for functions related to general cellular processes and
449 genetic information processing (Fig. 4B; Table S4). In particular, functions related to
450 cofactor and vitamin metabolism, transport, and catabolism are enriched in the
451 microbiomes of calcareans. LMA demosponge microbiomes are enriched for xenobiotic
452 biodegradation and metabolism, terpenoid and polyketides metabolism, and lipid
453 metabolism. In contrast, the bacterial associates of HMA demosponges and
454 homoscleromorphs are enriched with genes involved in transcription, translation, protein
455 processing, signal transduction, cellular community, cell motility, cell growth and death,
456 and energy metabolism. HMA species have been reported to host bacterial communities
457 with convergent functional potential, whereas LMA species exhibit greater microbiome

458 differentiation (52, 75). The functional variability of LMA sponge microbiomes is likely
459 shaped by the metabolic requirements of the holobiont under emerging environmental
460 conditions.

461 Host phylogeny influences the diversity of the sponge microbiome (76), which may,
462 in part, be due to species-specific immune receptor complements. Comparison of
463 immune-related protein domains in representative sponge species revealed lineage-
464 specific abundance patterns (Fig. 4C). SRCR domain-containing proteins were more
465 abundant in demosponges. These domains are part of PRRs that recognize a wide array
466 of bacterial ligands (77). SRCR-containing peptides in calcareans are associated with
467 diverse combinations of immune or cell-adhesion domains, whereas in demosponges,
468 the peptides consist mostly of multiple SRCR domains (Fig. 4D). Calcareans also
469 possess a higher number of genes with secretin GPCR domains (Fig. 4C), a membrane
470 receptor involved in sensing diverse physiological stimuli and in shaping immune
471 responses toward extracellular pathogens and danger molecules (78).

472 NLRs are a group of intracellular receptors that detect foreign microbes that are
473 able to evade extracellular defenses (79). These genes likely play a critical role in
474 mediating host-symbiont interactions and in differentiating pathogenic from symbiotic
475 microbes (80). Bona fide NLRs (NLRX) are characterized by both a central NACHT
476 domain and C-terminal leucine-rich repeats (LRRs) (81). Calcareans possess an
477 extensive family of NLRs, which group into a separate clade distinct from that of other
478 metazoan NLRs (Fig. 4E; Fig. S10) (56). Twenty NLRX genes were identified in *S.*
479 *ciliatum*, 29 in *L. complicata*, and 28 in *L. chagosensis*. In contrast, fewer NLRX genes
480 were detected in the demosponges, with six in *A. queenslandica*, one in *H. tubifera*, and

481 three in *N. compacta*. Among the 28 NLRX genes in *L. chagosensis*, 11 have a tripartite
482 architecture with either a CARD (NLRC, n = 7) or DEATH domain (NLRD, n = 4) at the N-
483 terminal. Other *L. chagosensis* NLRs that are phylogenetically related to NLRX genes
484 possess only the central NACHT domain alongside either a CARD (CARD-NACHT, n =
485 11) or DEATH domain (DEATH-NACHT, n = 3). The co-expansion of tripartite NLRC,
486 CARD-NACHT, and other CARD-containing genes in *L. chagosensis*, as well as in other
487 calcareans is indicative of enhanced signaling potential from homotypic interactions that
488 launch immune effector mechanisms (82). This lineage-specific expansion, coupled with
489 the rich complement of other surface receptors, may have evolved to facilitate the
490 maintenance of the low abundance and distinct microbiome in calcareans (52) through
491 efficient selection or phagocytic clearance of interacting microorganisms.

492

493 Sponge holobionts in the future ocean

494

495 Understanding the persistence of sponge holobionts in perturbed and extreme
496 conditions requires the elucidation of the animal host, the bacterial partners, and their
497 interactions. Our study revealed that bacterial complement diversity may define the
498 adaptive capacities of sponge holobionts under future ocean conditions. The HMA
499 sponge *N. compacta* exhibited greater tolerance to stress compared with LMA sponge *L.*
500 *chagosensis*, which showed visible tissue necrosis and high mortality to the combined
501 effects of warming and acidification.

502 The stress tolerance of *N. compacta* was supported by a stable microbiome with
503 abundant phototrophic members (Fig. S7A, C). Other photosymbiotic sponges,

504 *Carteriospongia foliascens* (Pallas, 1766) and *Cymbastela coralliophila* Hooper &
505 Bergquist, 1992, have also been shown to have a higher resistance to future ocean
506 conditions due to enhanced productivity of their cyanobacteria symbionts under elevated
507 inorganic carbon concentration (83, 84). The notable increase in relative abundance of
508 photoheterotrophic Rhodobacteraceae (Fig. S8) and the stable population of other
509 photosymbionts in *N. compacta* (Fig. S9A) may have ameliorated the effects of stress
510 from acidification and warming.

511 On the other hand, we propose that the susceptibility of *L. chagosensis* to stressors
512 is linked to the instability of its microbiome, possibly stemming from low taxonomic
513 diversity (Fig. S6) and low functional redundancy (Fig. S6; Fig. S7B) (85). While
514 microbiome flexibility in low microbial abundant corals (86) and sponges (87) has been
515 proposed as a mechanism for rapid adaptation (13), unstable phases during community
516 restructuring may result in loss of essential functions and offer an opportunity for
517 pathogen invasion. Large-scale changes in the predicted metabolic capabilities and
518 pathogenic potential of the restructured *L. chagosensis* microbiome under stress is
519 consistent with observations on the dysbiotic metagenomes of *R. odorabile* (10) and the
520 coral, *Porites compressa* (88).

521 We speculate that the difference in bacterial community dynamics in the two
522 sponges may be underpinned by differences in the host's immune functions. Microbial
523 recognition in sponges, such as in *Dysidea avara* (Schmidt, 1862) and *Aplysina*
524 *aerophoba* (Nardo, 1833), involves the expression of genes encoding NLRs, SRCRs, and
525 GPCRs, along with the activation of apoptotic functions (18). Further, recent studies
526 provide evidence that symbiont recognition and maintenance among poriferans is

527 mediated by TNF-NF κ B dependent pathways (72, 89). Under the simulated stress
528 conditions, sustained levels of surface receptors and expression of immune effectors in
529 *N. compacta* may have allowed efficient symbiont recognition and pathogenic clearance,
530 whereas the broadscale suppression of immune pathways in *L. chagosensis* may have
531 disrupted the sponge-symbiont interactions and attenuation of the host's defense
532 mechanisms. Our results mirror reports on adaptive or dysbiotic events in other holobionts
533 challenged by various environmental perturbations. For instance, the coral,
534 *Montipora aequituberculata*, which had a stable bacterial community under elevated
535 temperatures, exhibited regulation of the complement system and phagocytosis (90),
536 while the dissociation of coral-algal symbiosis in *Orbicella faveolata* following a prolonged
537 thermal anomaly was accompanied by overall reduced expression of genes implicated in
538 the TNF pathway and apoptosis (17).

539 The HMA or LMA status of sponges correlates with differences in host physiology
540 (91) and holobiont strategies for nutrient assimilation and processing (92). Although
541 sponge pumping rates may vary across species and sponge body size (93), HMA species
542 generally have slower filtration rates and a denser mesohyl, while LMA demosponges
543 and calcareans can more rapidly take up large volumes of seawater through their tissues
544 (91, 94, 95). For example, *Leucetta* can filter about 4.56L hr⁻¹ (95) while *Neopetrosia*
545 *problematica* (de Laubenfels, 1930) can only take up 0.53L hr⁻¹ (94). Differences in
546 pumping rate and tissue density may influence the degree of exposure to stressors.
547 Species with higher pumping rates and lower tissue density may be more susceptible to
548 perturbations as they may have more frequent encounters with pathobionts and are less
549 protected against the external environment. Under elevated temperature and lowered pH

550 conditions, sponge pumping capacity and skeletal strength may also be adversely
551 affected, as observed in the glass sponge, *Aphrocallistes vastus* (Schulze, 1886) (96).
552 Given their LMA status and synapomorphic calcitic spicules, calcareans are likely to be
553 more negatively affected by future ocean conditions. It is worth noting, however, that *L.*
554 *chagosensis* survived under reduced pH, which corroborates with the reported survival
555 and proliferation of *L. complicata* at pH 7.7 (97). Indeed, elevated temperature seems to
556 be more detrimental to sponges compared to reduced pH (83). However, the predicted
557 co-occurrence of acidification and warming may cause the narrowing of organismal
558 thermal tolerance thresholds (98). Surprisingly, the calcaronean sponge, *Sycettusa*
559 *hastifera*, has been shown to tolerate thermo-acidic stress with little change to its
560 microbiome and spicules (99). The resistance of *S. hastifera* to perturbed environmental
561 conditions may be linked to its opportunistic and invasive traits (100).

562 Comparison of the lineage-specific patterns of microbiome diversity and
563 immunological repertoire among poriferans provides broader insights into the adaptive
564 capacity of different sponge groups in perturbed ocean conditions. Although sponges are
565 generally predicted to be winners under future ocean scenarios, species and lineage-
566 specific holobiont features may define their susceptibility or tolerance to various stress
567 events. It is thus warranted to further investigate the roles of microbiome flexibility and
568 immune functions in the stress response of other sponge species representing diverse
569 groups with different evolutionary histories, morphologies, and microbiome densities.
570 Given that sponges are critical members of the reef ecosystem, with key roles in nutrient
571 cycling and substrate consolidation, revealing the mechanisms to their adaptive success
572 or failure is pivotal in projecting the reef landscape in the future ocean.

573

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580

581 **Competing Interests**

582 The authors declare that they have no competing interests.

583

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863 **Figure legends**

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865 Fig.1. Sponge holobiont features and survivorship under future ocean conditions.
866 Representative images of (A) *Neopetrosia compacta* and (B) *Leucetta chagosensis* in
867 their natural habitats. (C) Survival probability of *N. compacta* (top) and *L. chagosensis*
868 (bottom) throughout the duration of the experiment visualized using Kaplan-Meier survival
869 analysis.

870

871 Fig. 2. Bacterial community restructuring across stress treatments. (A) Relative abundance
872 of major ($\geq 1\%$) bacterial Orders in the *Neopetrosia compacta* and *Leucetta chagosensis*
873 microbiomes. (B) NMDS clustering of sponge-associated microbial communities. Diversity
874 and structure of (C) *N. compacta* and (D) *L. chagosensis* microbiomes under variable
875 stress conditions. Graphs show NMDS clustering of samples. Box plots of Simpson
876 diversity index ($1/D$) are shown above each graph. Colors represent different treatments
877 (blue, Present Day; skyblue, Acidification; yellow, Warming; orange, RCP 6.0; red,
878 RCP8.5). (E) Plot of differentially abundant ASVs in *L. chagosensis* relative to the Present
879 Day samples. (F) Bubble plot of differentially enriched KEGG pathways in *L. chagosensis*
880 relative to the Present Day samples. Bubble size indicates relative abundance.

881

882 Fig. 3. Immune responses to different treatment conditions. (A) Gene Ontology (GO)
883 enrichment analysis for the up and downregulated transcripts in *Neopetrosia compacta*
884 and *Leucetta chagosensis* under the different treatments. Only immune-related GO terms
885 are presented. (B) Expression levels of major pattern recognition receptors are presented

886 as the log₂ transformed TPM values in *N. compacta* (left) compared with *L. chagosensis*
887 (right). NLRX refers to NACHT-containing genes with bona fide NLR architecture. Colors
888 represent different treatments. Asterisks indicate significant change ($p < 0.05$) in
889 expression relative to the Present Day samples, as determined through Welch's t-test or
890 Wilcoxon test. (C) Protein interaction network of immune-related genes in *L. chagosensis*
891 (right) and *N. compacta* (left). Relative expression of genes was computed as the sum of
892 TPM values relative to the Present Day samples (blue, low; red, high; gray, no match).
893 Genes with at least one differentially expressed transcript are marked by black borders.
894 The network is based on human protein-protein interactions.

895

896 Fig. 4. Microbiomes and immunological repertoire of sponges. (A) Taxonomic diversity
897 (Shannon index) of bacterial communities of healthy sponge adults retrieved from the
898 Sponge Microbiome Project. Yellow and blue circles represent diversity of the bacterial
899 communities of *N. compacta* and *L. chagosensis*, respectively. (B) Enrichment of
900 predicted functions (red, high; blue, low) in the microbiome associated with LMA or HMA
901 sponges from different classes (Demospongiae, Demo; Calcarea, Calc;
902 Homoscleromorpha, Homo; Hexactinellida, Hexa). Asterisks denote significant
903 enrichment of functions in a specific sponge group, as determined through LDA-LEfSe.
904 (C) Abundance of peptides containing selected Pfam domains across species. Bubble
905 size indicates the percent of peptides containing a specific domain relative to the total
906 number of predicted peptides in each species. Colors represent sponge class (yellow,
907 Demospongiae; red, Homoscleromorpha; blue, Calcarea). Asterisks denote significantly
908 higher counts of PFAM domains for a specific sponge class, as determined through LDA-

909 LEfSe. (D) Abundance and diversity of SRCR-containing peptides across species. The
910 percent of SRCR-containing peptides (left) and the count of SRCR peptides associated
911 with other PFAM domains (right) are presented for each species. (E) Diversification of
912 bona fide NLRs in Demosponges and Calcareans. The phylogenetic tree was derived
913 from Bayesian analysis. Numbers on selected branches represent Bayesian posterior
914 probabilities. Outer and inner color strips indicate species and peptide architectures,
915 respectively. Species abbreviations: *Amphimedon queenslandica* (Aque), *Haliclona*
916 *tubifera* (Htub), *Petrosia ficiformis* (Pfic), *Neopetrosia compacta* (Ncom), *Oscarella*
917 *carmela* (Ocar), *Leucetta chagosensis* (Lcha), *Sycon ciliatum* (Scil), *Leucosolenia*
918 *complicata* (Lcom).







