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Transcriptomic responses of sponge holobionts to in situ, seasonal anoxia and hypoxia

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- 17 inverse was used on a previous paper. Both authors have the right to list their name first on their
- 18 CVs.

19 Abstract

20 Deoxygenation can be fatal for many marine animals; however, some sponge species are 21 tolerant of hypoxia and anoxia. Indeed, two sponge species, Eurypon sp. 2 and Hymeraphia 22 stellifera, survive seasonal anoxia for months at a time. To understand their tolerance 23 mechanisms, we performed differential gene expression analyses on the sponges, their mitochondria and their microbial symbionts under in situ conditions of normoxia, hypoxia and 24 25 anoxia. Each species possessed a unique microbiome, but the microbiomes of each species 26 were dominated by a species-specific Thaumarchaeon and a Gammaproteobacterium. 27 Holobiont gene expression was species- and oxygen-level dependent, though there were some 28 shared interspecific responses to deoxygenation. In general, few changes occurred in the 29 expression of sponge metabolic genes as a function of oxygenation level, indicating that they 30 may remain metabolically active under anoxia. However, ATP synthesis genes were 31 significantly upregulated under hypoxia when compared to normoxia, and genes for DNA 32 replication were downregulated. Mitochondrial gene expression was effectively unchanged 33 under both hypoxia and anoxia. Nevertheless, both anoxia and hypoxia caused upregulation of heat shock proteins (HSPs), indicating cellular level adaptations to deoxygenation stress. A 34 meta-analysis demonstrated that sponge transcriptional responses to anoxia were distinct from 35 those displayed by other invertebrates while dormant, and the hypothesis of sponge dormancy 36 37 under anoxia was not supported. Thaumarchaeota symbionts also upregulated stress response genes in hypoxia, while maintaining expression of oxygen-dependent metabolic pathways under 38 39 hypoxia and anoxia. Gammaproteobacteria symbionts showed relatively few noteworthy 40 changes in gene expression in response to anoxia but decreased metabolic gene expression in hypoxia. There was no clear evidence of upregulated anaerobic respiration in the 41 42 transcriptomes of the sponge holobionts under anoxia or hypoxia. The tolerance of some 43 sponges to prolonged anoxia warrants further investigation and could give them an advantage 44 in future oceans following climate change as well as in ancient oceans when oxygen

45 concentrations were lower than at present.

46 Introduction

47

48 Ocean oxygen levels are predicted to decline by 1–7% over the next century (Keeling,

- 49 Körtzinger, and Gruber 2010). Climate change is expanding marine oxygen-minimum zones,
- 50 while enhanced nutrient runoff is causing coastal eutrophication and subsequent deoxygenation
- 51 (Kirchman 2021). Deoxygenation is expected to negatively affect the physiology, metabolism
- 52 and survival of many marine organisms (Diaz and Rosenberg 2008). As a result of
- 53 deoxygenation and other anthropogenic stressors, we are facing large scale habitat and
- 54 biodiversity losses, but the full effects of these stressors are still unknown.
- 55
- 56 Sessile organisms that cannot move or migrate in response to deoxygenation are particularly
- vulnerable. Sponges (Phylum: Porifera Grant, 1836), sessile, ecologically important filter-
- 58 feeding animals, are hypothesized to suffer from increasing hypoxic/anoxic areas worldwide
- 59 (Breitburg et al. 2018; Levin 2018; Stramma et al. 2008), and some *in situ* mortality of sponges
- 60 (along with other taxa) following deoxygenation has been reported (Altieri et al. 2017;
- 61 Stachowitsch 1984). However, species-specific responses to deoxygenation in Porifera are
- 62 variable, and some species are tolerant of hypoxia. Lab experiments have shown that the
- 63 demosponge Halichondria (Halichondria) panicea Pallas, 1766 can survive low oxygen levels
- 64 down to 0.5–4% of air saturation (a.s.) (Mills et al. 2014), and *Tethya wilhelma* can survive down
- to 0.25–2% a.s. for 4 days and <0.05% a.s. for at least one hour (Mills et al. 2018). Additional
- 66 demosponge species that tolerate similar levels of hypoxia include *Geodia barretti* Bowerbank,
- 1858 with 7% a.s. (20 μ M O2, Leys and Kahn 2018) and Haliclona pigmentifera Dendy, 1905
- 68 (0.50-0.68% a.s., Gunda and Janapala 2009). In a recent experimental study, four
- 69 taxonomically distinct sponge species (Cliona celata Grant, 1826, Suberites carnosus Johnston,
- 70 1842, Polymastia crocea Kelly-Borges and Bergquist, 1997 and Suberites australiensis
- Bergquist, 1968) were exposed to various levels of hypoxia (1.5–40% a.s., 0.13–3.3 mg $O_2 L^{-1}$)
- in aquaria. These sponges exhibited species-specific physiological, morphological and
- behavioral responses to deoxygenation. Sub-lethal oxygen thresholds for these sponges were
- in the range of 6–20% a.s. $(0.48-1.56 \text{ mg O}_2 \text{ L}^{-1})$ while mortality was only observed in one of the
- two species (*P. crocea*) exposed to severe hypoxia lower than 5% a.s. (0.13 mg $O_2 L^{-1}$)
- 76 (Micaroni et al. 2021a). The sponge species most sensitive to deoxygenation in controlled
- experiments, *P. crocea*, had a median lethal time (time until death, LT50) of 286 h at ~1.5% a.s.
- $(0.13 \text{ mg O}_2 \text{ L}^{-1})$, (Micaroni et al. 2021a), which was still longer than most marine organisms
- ⁷⁹ under comparable conditions (reviewed in Micaroni et al. 2021a and Hughes et al. 2020).
- 80
- 81 Few studies have investigated the deoxygenation tolerance of sponges *in situ*. Bayer et al.
- 82 (2020) found that the glass sponge Vazella pourtalesii Schmidt, 1870 lives in reduced oxygen
- conditions (< 4 mL/L) on the Scotian Shelf, Canada. Schuster et al. (2021) reported at least nine
- 84 encrusting demosponge species of the orders Axinellida Lévi, 1953 and Poecilosclerida
- Topsent, 1928 that tolerate seasonal anoxia in a Marine Lake in Lough Hyne, Ireland for ~three
- 86 months at a time in the summer, and there are more sponge species at this site (Bell and
- 87 Barnes 2000). The anoxia-tolerant sponges of Lough Hyne therefore have LT50s that exceed
- their ca. three month exposure to anoxia, and, compared to other animals, their tolerance is
- rivaled only by the anemone *Bunodosoma cavernatum* Bosc, 1802 (LT50 in anoxia >1.5
- 90 months, (Ellington 1982). Moreover, the abundance (percent cover) of encrusting sponges in

the seasonally anoxic site of Lough Hyne only slightly decreased over 30 years despite 91

- 92 widespread sponge losses in the well oxygenated parts of the lough (Micaroni et al. 2021b),
- 93 indicating that these sponges are uniquely suited to their seasonally anoxic niche.
- 94

95 Many invertebrates exposed to harsh conditions like anoxia or hypoxia have evolved molecular 96 response pathways to survive in these environments. For instance, under anoxia, marine 97 mollusks shift from lipid, carbohydrate and amino acid metabolism to carbohydrate catabolism 98 as the primary fuel for ATP generation (Larade and Storey 2009). Some mollusks also maximize 99 anoxic survival time (mollusc LT50s at 0 mg L⁻¹: 360–672 h, see Micaroni, Strano, et al. 2021b) 100 by building up a fixed reserve of internal fuels (primarily glycogen) to increase ATP output and 101 to reduce acidity. A small suite of upregulated genes also plays a critical role in cell 102 preservation, facilitating long-term viability under hypoxia (Larade and Storey 2009). Some anemones may even have elements of a facultative anaerobic metabolism (Sassaman and 103 104 Mangum 1972). 105

106 Only one study has investigated the transcriptomic response of a sponge to hypoxia. After four days of exposure to hypoxia (0.25-2% a.s.), 128 genes within the sponge T. wilhelma were 107 108 differentially expressed compared to controls, but none of these genes were clearly related to 109 metabolic or stress responses (Mills et al. 2018). However, one hour exposure to near anoxia (<0.05% a.s., 105 nM O₂) elicited stress responses in *T. wilhelma*'s transcriptome (Mills et al. 110

111 2018), demonstrating that transcriptomic responses were mostly observed under very low oxygen levels.

112

113

114 In most metazoans, the hypoxia-inducible factor (HIF) pathway is essential for maintaining oxygen homeostasis in response to hypoxia (Samenza 2007). However, in sponges and 115 116 ctenophores, the likely sister-lineages to other metazoans (Feuda et al. 2017), key components 117 of the HIF pathway, including orthologs for HIFa and HIFb, are absent (Mills et al. 2018). HIFa is 118 a transcription factor responsible for triggering metabolic adaptation to deoxygenation in most 119 metazoans (Samenza 2007). Due to the absence of HIFa in sponges, the mechanisms of 120 tolerance to hypoxia and anoxia are largely unknown (Mills et al. 2018).

121

The sponge microbiome may play a role in sponge deoxygenation tolerance. Sponges can host 122 diverse microbiomes that perform vital functions and can constitute large portions of their 123 124 biomass (Wilkinson 1978). The host and microbiome together are referred to as a 'holobiont' 125 (Webster and Taylor 2012). Some sponge symbionts have anaerobic capabilities, including sulfate reduction (Hoffmann et al. 2005), denitrification, and anaerobic ammonium oxidation 126 (anammox) (Hoffmann et al. 2009). In V. pourtalesii, the microbial community is thought to be 127 128 specialized for the low-oxygen conditions in which it lives (Bayer et al. 2020). 129

130 A previous study has shown that the microbiomes of anoxia-tolerant sponges from Lough Hyne

131 were unchanged under anoxia, hypoxia and normoxia despite drastic changes in the microbial

communities of the water and sediment, indicating that the holobiont as a whole is tolerant to 132

133 deoxygenation (Schuster et al., 2021). Specifically, the microbiomes of the anoxia-tolerant

134 sponge species Eurypon sp. 2 and Hymeraphia stellifera Bowerbank, 1864 were both

135 dominated by two microbes - one *Thaumarchaeon* and one *Gammaproteobacterium*, though

each sponge hosted a different species from each clade. Eurypon sp. 2 harbors the 136 137 Thaumarchean OTU1 (closest related isolate: Nitrosopumilus adriaticus strain NF5), and H. stellifera harbors OTU3 (closest related isolate: Nitrosopumilus oxyclinae strain HCE1). For the 138 139 Gammaproteobacteria, Eurypon sp. 2 contains OTU2 (closest related isolate: Arenicella chitinivorans strain ALEN 1); whereas H. stellifera contains OTU7 (closest related isolate: A. 140 141 chitinivorans strain ALEN 1, (Schuster et al. 2021). Although their location within Lough Hyne sponges has not yet been determined, Gammaproteobacteria and Thaumarchaeota symbionts 142 143 are abundant and widely distributed throughout the sponge tissue in other species (Moeller et al. 2019). These symbionts also participate in integrated metabolic processes with their host 144 145 species (Moitinho-Silva et al. 2017). 146 147 Schuster et al. (2021) hypothesized potential metabolic pathways within these two symbiont taxa that might function under deoxygenation. Based on the genomes of their close relatives, 148 the *Thaumarchaeota* in these sponges were hypothesized to have components of a facultatively 149 anaerobic metabolism, including fumarate, nitrite, and sulfite respiration and the ability to 150 151 ferment (Bayer et al. 2020), which could aid in deoxygenation tolerance. A recent study demonstrated that another close Thaumarchaeota relative. Nitrosopumilus maritimus, can 152 153 produce trace levels of oxygen (100-200 nM) under environmental anoxia (Kraft et al. 2022). 154 The pathway of oxygen production and the genes involved are not currently known. However, comparative proteomics suggested that proteins upregulated during oxygen production included 155 156 some AsnC family proteins and nitroreductases (Kraft et al. 2021). We therefore hypothesized that nitroreductases and AsnC family proteins would be upregulated under hypoxia and anoxia 157 158 in sponge Thaumarchaeota. Gammaproteobacteria symbionts were hypothesized to use sulfide 159 or thiosulfate as an electron donor anaerobically coupled to nitrate reduction, removing 160 exogenous, toxic sulfide from the holobiont under anoxia (Schuster et al. 2021). 161 162 To understand the mechanisms of sponge holobiont tolerance and survival under extended 163 anoxia, we performed differential gene expression analysis of the sponges Eurypon sp. 2 and H. stellifera as well as their respective mitochondria and microbial symbionts including 164 Thaumarchaeota and Gammaproteobacteria. Sponges were collected from Lough Hyne under 165 in situ normoxia, hypoxia and anoxia. Since neither of these holobionts are established model 166 167 species, we generated reference transcriptomes for the sponges and assembled the genomes of their symbionts and mitochondria from metagenomic sequences. Additionally, we performed 168 a meta-analysis of the sponge differential expression data to determine if the responses of 169 170 Lough Hyne sponges to deoxygenation are similar to those of *T. wilhelma* under deoxygenation, 171 other invertebrates under heat stress (Dixon et al. 2015), in a dauer state (Sinha et al. 2012), and during diapause (Gong et al. 2013). Finally, we considered the implications of sponge 172 173 deoxygenation tolerance for future oceans as well as early animal evolution under low 174 atmospheric oxygen concentrations.

175

176 Materials and Methods

177

178 Sampling

- 179 Sponges were sampled between July 2018 and August 2019 at Labhra Cliff (Lough Hyne
- 180 Nature Reserve, Ireland; N51°30.05309 W9°18.17679) as described in Schuster et al. (2021)

181 under permit no. R23-27/2018 issued by the Irish Department of Environment, Heritage, and

- 182 Local Governments. Samples were taken under various in situ oxygen conditions that were
- categorized as follows: normoxic (5.3–12 mg L⁻¹, 49.3–111% a.s.), hypoxic (1.30–3.56 mg L⁻¹, 183
- 184 12.1–33.1% a.s.), and anoxic (0.00–0.01 mg L⁻¹, 0.00–0.93% a.s., i.e. instrument detection
- limit). All samples were frozen in liquid nitrogen within ~15 minutes of collection and kept at 185
- liquid nitrogen temperatures in a dry shipper until returned to the laboratory where samples 186
- 187 were stored in a -80°C freezer until extraction. The sponges were identified using molecular
- 188 barcodes (see Schuster et al. 2021) as Eurypon sp. 2 and H. stellifera.
- 189

190 **DNA/RNA** extraction

- Total RNA was isolated using TRIzol[™] Reagent from Invitrogen (ThermoFisher Scientific, 191
- Denmark). For RNA sequencing (RNAseq), eight samples of H. stellifera (anoxic = 2, hypoxic = 192
- 5, normoxic = 1) were taken, and sixteen samples of Eurypon sp. 2 (anoxic = 2, hypoxic = 7, $\frac{1}{2}$) 193
- 194 normoxic = 7) were collected (n = 24 in total). Metadata for individual samples, including oxygen
- concentration, season, depth and collection date are included in Supplemental Table 1. Since 195
- 196 these two species are indistinguishable in situ, the samples taken for each species were
- unequal in number. Remaining DNA in the RNA extracts were removed using the DNase MAX 197 198 kit (Qiagen).
- 199

Additionally, high molecular weight (HMW) DNA was extracted from one individual of each 200

- 201 species using the protocol from Vargas et al. (2021) for metagenomic sequencing. The Eurypon
- sp. 2 sample used for metagenomic sequencing was taken under anoxia (DC24), and RNA was 202
- also sequenced from this sample. Due to limited availability of samples and DNA, a sample of 203
- 204 H. stellifera that was not included in the RNAseq (DC297, sampled under normoxia) was used
- for metagenome sequencing. DNA and RNA yields were determined using a Qubit[™] (Thermo 205
- 206 Fisher Scientific) and a NanoDrop[™] spectrophotometer (Thermo Fisher Scientific). RNA quality
- 207 was evaluated on an Agilent TapeStation using an RNA ScreenTape (Agilent Technologies).
- 208

Reference transcriptomes and metatranscriptomes 209

All RNA samples were sequenced twice with two different library preps - one for polyadenylated 210 (poly A) messenger RNA (mRNA, from eukaryotes) and one for total RNA (to also capture 211 212 prokaryotic RNA). Library preparation for the eukaryotic (sponge) RNA was done using

- NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England Biolabs, USA).
- 213
- For the prokaryotic RNAsed, the supernatant from the poly A enrichment was treated with 214
- 215 NEBNext® rRNA Depletion Kit (Bacteria) and then NEBNext® rRNA Depletion Kit
- (Human/Mouse/Rat, New England Biolabs, USA) to eliminate ribosomal RNA (rRNA) that 216
- causes sequence biases. This combination of poly A exclusion and rRNA depletion eliminated 217
- 218 the most rRNA compared to other combinations of methods (or single methods in isolation) in preliminary tests, yielding sequences with only $\sim 25\%$ rRNA. For the eukaryotic (n = 24) and 219
- prokaryotic (n = 24) RNA samples, library preparation was completed using the NEBNext Ultra II 220
- 221 Directional RNA library preparation kit (New England Biolabs, Denmark). Libraries pooled in
- equimolar concentrations were sequenced (5M reads per sample, 2 x 50 bp) at the Center for 222
- 223 Functional Genomics and Tissue Plasticity (ATLAS) at SDU, using a NovaSeq 6000 (Illumina,
- 224 USA).
- 225

226 To ensure good coverage for the final reference transcriptomes, two samples from each species 227 were sequenced deeper in additional runs. For H. stellifera, the sample for deeper sequencing 228 (DC129, sampled under normoxia) was also present in the sample set above. In contrast, for 229 Eurypon sp. 2 another sample (DC128, sampled under normoxia) was used due to the limited availability of sample tissue. As in the sequencing above, two libraries were created and 230 sequenced for each sample - one with eukaryotic RNA and one with prokaryotic RNA. For this 231 232 deeper sequencing, mRNA in the sponge transcriptomes was purified using the NucleoTrap mRNA kit (Macherey-Nagel, Germany). Prokaryotic RNA was rRNA depleted using the Ribo-233 234 zero Magnetic kit (Illumina, USA). An additional purification step was performed for all RNA 235 samples using the standard protocol for the CleanNGS kit (CleanNA, Netherlands) before 236 sequencing preparation using the NEBNext Ultra II Directional RNA library preparation kit (New 237 England Biolabs, Denmark). Samples were diluted in equimolar concentrations, pooled and sequenced (2 x 150 bp) on a HiSeq X platform (Illumina, USA) by DNASense ApS (Aalborg, 238 239 Denmark).

240

For the eukaryotic RNA, shallow (2 x 50 bp) sequencing generated 9.20 x 10⁸ and 4.38 x 10⁸

reads for *Eurypon* sp. 2 and *H. stellifera*, respectively, across all sampled sponges, and deeper

sequences (2 x 150 bp) generated 2.05 x 10^8 and reads 3.45 x 10^8 for each sponge of the two

species, respectively. For the prokaryotic RNA, shallow sequencing yielded 1.27×10^9 and 6.68×10^8 reads for *Eurypon* sp. 2 and *H. stellifera*, respectively. The deeper prokaryotic sequencing generated 1.52×10^8 and 1.20×10^8 for *Eurypon* sp. 2 and *H. stellifera*, respectively (totals are

- 247 shown in Supplemental Table 2).
- 248

249 To obtain reference transcriptomes for each sponge species without prokaryotic symbionts, all eukaryotic RNA sequences were combined into a single file. These sequences were trimmed for 250 251 guality control using public scripts (Matz 2015; Meyer 2016) and assembled with Trinity v 2.8.5 252 (Grabherr et al. 2011). This assembly was filtered for contaminants and annotated using egg-NOG mapper (Huerta-Cepas et al. 2019). The final sponge transcriptomes included only genes 253 with metazoan annotations or no annotations. For a detailed description of all filtering and 254 255 assembly steps, see Strehlow et al. (2020) and (https://zenodo.org/badge/latestdoi/573040118) 256 for all scripts used in the current paper. Sponge reference transcriptomes fit within the expected range of size, gene number, and N50 (Supplemental Table 2) of published sponge 257 transcriptomes (Strehlow et al. 2020; Riesgo et al. 2014). Additionally, sponge reference 258 259 transcriptomes were effectively complete based on Benchmarking Universal Single-Copy 260 Orthologs (BUSCO) analysis (Supplemental Table 2, Simão et al. 2015) and were therefore 261 suitable for use for subsequent differential expression analysis (see below). Sponge reference 262 transcriptome assemblies are available through NCBI under BioProject number PRJNA89319. 263 **Reference Metagenomes** 264

265 Two reference metagenomes were sequenced for *Eurypon* sp. 2 and *H. stellifera* at DNASense

ApS (Aalborg, Denmark). DNA was fragmented to ~550 bp (M220 Focused-ultrasonicator,

267 Covaris, USA) and used for library preparation following the NEBNext Ultra II DNA library

preparation kit. The DNA library was paired-end sequenced (2 x 150 bp) on a NovaSeq platform

- 269 (Ilumina, USA).
- 270

- 271 Sequences were assembled using Megahit v.1.2.9 (Li et al. 2016). The final contig fasta files
- were used to plot GC (%) vs. mean coverage of mapped reads to identify individual bins. For
- this, we used the custom python scripts spadescontigstocovgc.py (Francis 2020a) as well as the
- 274 R script contig_gc_coverage.R (Francis 2020b) to extract bins for the *Thaumarchaeota* and
- 275 *Gammaproteobacteria* symbiont of each sponge species. Final statistics for each genome are
- shown in Supplemental Table 2. Raw sequences are available from NCBI under BioProject
- 277 number PRJNA893197. Metagenome assemblies are available on
- 278 <u>https://github.com/bstrehlow/Sponge-transcriptomic-responses-to-seasonal-anoxia</u>.
- 279

280 Mitogenomes

- Using only the deep sequences of total ('prokaryotic') RNA, the mitochondrial genomes of single individuals of *Eurypon* sp. 2 (DC128) and *H. stellifera* (DC129) were assembled following the
- 283 pipeline in Plese et al. (2019).
- 284
- For future utilization, single contigs that likely represent the mitochondrial genomes of *Eurypon*
- sp. 2 and *Hymeraphia stellifera* were retrieved from the reference metagenome in addition to
- the coding regions used for differential expression below. The assembled contigs for each
- metagenome were blasted against the mitochondrial genome of DC128 (*Eurypon* sp. 2) using
- blastn, with an e-value cutoff of 1×10^{-50} for *Eurypon* sp. 2 (DC97) and 1×10^{-30} for *Hymeraphia*
- stellifera (DC297). In general, there was only a single hit for each protein coding gene,
- 291 confirming a single contig as the mitochondrial genome for each sample. Based on the blast,
- these two contigs contained all protein coding genes retrieved from the transcriptome that were
- the focus of this analysis. These 'mitogenomes' can be accessed here:
- 294 https://zenodo.org/badge/latestdoi/573040118.
- 295

296 Differential Expression Analysis

- 297 Trimmed reads from the shallow sequencing for each individual were mapped back to their 298 respective reference transcriptome or genome using bowtie2 (version 2.3.5.1, Langmead and 299 Salzberg 2012) and mapped reads were counted for each contig using samcount.pl (see Matz 300 2015). Eukaryotic reads were mapped to the sponge reference transcriptome for each species. Prokaryotic reads were mapped to their respective metagenomic bins - metagenome assembled 301 302 genomes (MAGs), i.e. Thaumarchaeota or Gammaproteobacteria, within each sponge species. 303 The mitochondrial reference was broken into its 14 genes and prokaryotic (total RNA) reads 304 from both species were mapped to their matching mitochondrial reference and counted as 305 above.
- 306
- 307 Analyses were performed to compare the gene expression of each target (sponge species, 308 symbiont species, and mitochondria; n=8) under the three different oxygen conditions (anoxia, hypoxia and normoxia). Differential gene expression analysis was performed with the package 309 DESeg2 v. 1.22.2 (Love et al. 2016) in R (R Core Team 2016) with (RStudio Team 2020). Three 310 311 pairwise comparisons of oxygen condition (normoxia vs. hypoxia, normoxia vs. anoxia, and anoxia vs. hypoxia) were computed from DESeq2 results. Significantly differentially expressed 312 313 genes between each of these pairwise comparisons were identified using a false discovery rate 314 (FDR) adjusted p-value threshold of 0.1. The ReportingTools software package (Huntley et al. 2013) was used to create PDF pages displaying 4 plots of significantly differentially expressed 315

316 genes and their annotations in various oxygen conditions, as well as box plots, p-values, and

LFCs (https://zenodo.org/badge/latestdoi/573040118). These files are searchable in case

particular genes are of interest to future studies. We note that comparisons to *H. stellifera* under

normoxia were made with only one normoxic sample due to sampling restrictions. Despite this

sampling limitation, the normoxic data for *H. stellifera* are still shown as a baseline and

reference. For each sponge species, log fold changes (LFC, from DESeq2) in all genes were

322 calculated so that positive LFCs were upregulated and negative values were downregulated in

hypoxia or anoxia relative to normoxia. For the comparisons between hypoxia and anoxia,

- 324 positive LFCs were upregulated in anoxia and vice versa.
- 325

326

327 KOG analysis

328 EuKaryotic Orthologous Groups (KOG class) assignments were used to compare large scale

329 trends and potential correlations in differential gene expression between Lough Hyne sponges

330 under varied oxygen conditions and external gene expression data from other species and

331 conditions. Delta-ranks, a measure of each KOG class enrichment with up or downregulated

332 genes, were calculated from LFC data using the KOGMWU package in R (Dixon et al. 2015),

and delta-ranks were plotted using the package pheatmap (Kolde 2013). While it is also

334 possible to calculate delta-ranks with signed p-values (see KOGMWU supporting

documentation), LFC was used here to include changes between normoxia and other conditions

- for *H. stellifera*. Since there was only one normoxic replicant for this species, LFC was a more
- appropriate metric than signed p values. Within each pairwise comparison (i.e. hypoxic, anoxic,
- and anoxic vs hypoxic), significantly enriched KOGs were also calculated using KOGMWU (FDR-adjusted p < 0.05).
- 340

341 These KOG enrichments were also compared to those calculated from the sponge *Tethya*

342 *wilhelma* after four days of exposure to hypoxia and one hour of exposure to anoxia (Mills et al.

2018). To test whether KOG enrichment in sponges exposed to variable oxygen conditions was

344 correlated with gene expression in other invertebrate stress responses, delta rank data was
 345 included from adult *Acropora millepora* coral exposed to heat stress (31.5 °C for three days,

346 (Dixon et al. 2015). Similarly, to test the similarity of the sponge data to noted decreases in

347 metabolic activity in invertebrates, KOGs enriched in the dauer (dormant) state in

348 *Caenorhabditis elegans* (Sinha et al. 2012) and the diapause state of the midge Sitodiplosis

349 mosellana (Gong et al. 2013) were added using delta rank tables calculated by Strader et al.

350 (2016). Correlations between the KOG delta ranks in different datasets were performed using

351 Pearson's Correlation in KOGMWU. Within the significantly enriched KOGs 'replication,

recombination and repair' and 'energy production and conversion,' genes that were significantly

353 differentially expressed were plotted in heatmaps to further investigate sponge transcriptomic

responses to deoxygenation at the gene level in Lough Hyne sponges.

355

KOG enrichment analysis was performed as above for each of the two major symbionts within the two sponge species; however, Clusters of Orthologous groups (COGs) were used since

the two sponge species, nowever, clusters of Onnologous groups (COGs) were used sinc they are designed for prokaryotes rather than eukaryotic KOGs and are fewer in number.

360 Gene ontology (GO) term enrichment analysis

361 Log fold changes calculated above were used for rank-based gene ontology (GO) analysis with

adaptive clustering (GO_MWU, Wright et al. 2015) to determine what GO terms were

363 significantly enriched under various oxygen conditions. These analyses were run individually for

- each pairwise comparison of oxygen condition in both sponge species. These analyses were
- not completed for the two symbiont types because GO terms were not available for thesemicrobes.
- 366 367

368 **Phylogenetic positions of nitroreductase proteins**

369 To determine if nitroreductases in sponge symbionts are related to genes that may be involved

- in oxygen production in *N. maritimus,* nitroreductase proteins from the *Thaumarchaeota*
- 371 symbionts of *H. stellifera* and *E.* sp. 2 were extracted using annotations assigned by egg-NOG.
- Phylogenetic position of nitroreductase proteins from *H. stellifera* and *E.* sp. 2 were investigated
- by aligning them to other known nitroreductase proteins from Genbank using Mafft v7 (Katoh
- and Standley 2013). The ModelTest-NG v.0.1.7 function (Edler et al. 2020) implemented within
- RaxmIGUI 2.0 was used to calculate the evolutionary model. According to AIC, LG+I+G was
- 376 chosen as the best-fitting model. Maximum Likelihood (ML) phylogeny was then reconstructed
- using raxmIGUI v.2.0.6 (Edler et al. 2020) with 100 (rapid) bootstraps. The resulting protein tree
- 378 was rendered by FigTree v.1.4.4 (<u>http://tree.bio.ed.ac.uk/software/figtree/</u>).
- 379

380 **Results**

381

382 Sponge differential expression

There were three pairwise comparisons for significantly differentially expressed genes - hypoxia 383 384 vs. normoxia, anoxia vs. normoxia, and anoxia vs. hypoxia. The number of significantly 385 differentially expressed genes in these comparisons for sponges, their mitochondria and their 386 symbionts are summarized in Table 1. In the sponge Eurypon sp. 2, 737, 1,376 and 1,090 genes were significantly differentially expressed under hypoxia (vs. normoxia), anoxia (vs. 387 normoxia), and anoxia vs. hypoxia, respectively (p < 0.1, Table 1). The differentially expressed 388 genes under each oxygen condition ranged between 1.5–2.8% of the genes of the Eurypon sp. 389 390 2 reference transcriptome, and 41-48% of these genes are annotated, which reflects the overall annotation percentage of 39.7% for the whole transcriptome (Supplemental Table 2). In 391 hypoxia, the majority of these differentially expressed genes were upregulated (68%). Under 392 393 anoxia, on the other hand, the majority of genes were downregulated (60%). In anoxia vs. 394 hypoxia, 57% of genes were upregulated in anoxia compared to hypoxia in Eurypon sp. 2 395 (Table 1).

397

- 398 **Table 1.** Number of significantly differentially expressed genes (DEGs) under deoxygenation in the
- 399 sponge holobionts summarized per reference genome or transcriptome. The direction (up or down)
- 400 within each paired oxygen condition comparison is relative to normoxia or in the case of anoxia vs.
- 401 hypoxia, hypoxia. The number of annotated genes is shown in parentheses.
- 402
- 403

			Hypoxia		Anoxia		Anoxia vs hypoxia	
Sponge	Reference	Total genes in Reference	Up	Down	Up	Down	Up	Down
Eurypon sp. 2	sponge	49,911	499 (239)	238 (112)	551 (228)	825 (340)	622 (273)	468 (205)
H. stellifera	sponge	62,494	465 (125)	58 (9)	40 (11)	244 (87)	1531 (438)	623 (286)
<i>Eurypon</i> sp. 2	mitochondria	14	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)
H. stellifera	mitochondria	14	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>Eurypon</i> sp. 2	Thaumarchaeota	1,556	19 (14)	1 (1)	3 (3)	0 (0)	0 (0)	0 (0)
H. stellifera	Thaumarchaeota	1,551	17 (12)	0 (0)	0 (0)	0 (0)	1 (0)	27 (20)
Eurypon sp. 2	Gammaproteobacteria	2,751	3 (3)	29 (29)	0 (0)	0 (0)	0 (0)	0 (0)
H. stellifera	Gammaproteobacteria	3,057	0 (0)	0 (0)	0 (0)	0 (0)	20 (20)	416 (401)

404

In the sponge H. stellifera, 523, 284, and 2,154 genes were significantly differentially expressed 405 in hypoxia, anoxia, and in anoxia vs. hypoxia, respectively (p < 0.1, Table 1). Differentially 406 407 expressed genes in the different oxygen conditions corresponded to 0.42-3.4% of the total number of genes in the H. stellifera transcriptome. Approximately 25-37% of the differentially 408 409 expressed genes were annotated, which, as in the other sponge species, roughly corresponded 410 to the global percentage (37.0%) of genes annotated within the H. stellifera reference 411 transcriptome. Under hypoxia in this species, the vast majority of differentially expressed genes (89%) were upregulated. As in Eurypon sp. 2, the majority of differentially expressed genes 412 413 (84%) were downregulated in anoxia in *H. stellifera*. The majority of differentially expressed genes between anoxia and hypoxia were upregulated in anoxia (71%, Table 1). 414 415 416 KOG enrichments in sponges

The global gene expression patterns of Lough Hyne sponges in response to deoxygenation

- 419 were compared across the two species by assessing up and down regulation of KOG classes
- 420 (broad functional annotations assigned to genes). Within these sponges, KOG expression in
- 421 response to hypoxia and anoxia (versus normoxia) were largely distinct in both species (Figure
- 1A). However, KOG expression in *H. stellifera* in hypoxia vs. anoxia was significantly positively
- 423 correlated, i.e. similar, to that of *Eurypon* sp. 2 under hypoxia (r = 0.43, p < 0.05, Figure 1E), but
- 424 global expression patterns were only similar between the two species in this single case.
- 425
- 426
- 427
- 428



430 Figure 1. KOG expression profiles of sponges under varied oxygen concentrations compared to each 431 other and external datasets. A. Clustered heatmap (based on correlation) of KOG class (rows) 432 enrichments for upregulated or downregulated genes. Highlighted columns correspond to data 433 generated in this study (Purple: Hymeraphia stellifera [Hs], Cyan: Eurypon sp. 2 [Es]). Unhighlighted 434 columns represent external datasets. Up or down regulation with respect to normoxia is indicated in 435 'Hypox' (hypoxia) and 'Anox' (anoxia) columns, and differential regulation with respect to hypoxia is 436 shown in 'Hypox vs. Anox' columns, i.e. upregulated KOGs (red delta ranks) are upregulated in anoxia 437 with respect to hypoxia. Within each column on the heatmap, KOGs that are significantly enriched (FDR-438 adjusted p < .05) are marked with asterisks. KOG enrichments from the sponge Tethya wilhelma after 439 four days exposure to hypoxia (Tw Hypox Long) and one hour exposure to anoxia (Tw Anox Short), the 440 coral Acropora millepora after three days of exposure to heat stress (Am Coral Heat), Caenorhabditis 441 elegans in a dauer state (Ce Dauer), and the midge *Sitodiplosis mosellana* in diapause (Sm Diapause) 442 from external studies were included for comparative purposes. B-E. Significant (p < 0.05) correlations 443 between the KOG enrichments for different species and oxygen levels from this study. F-J. Significant 444 correlations between KOG enrichments in this study and those of external datasets. All other possible

- 445 comparisons between the various datasets were not significant.
- 446

Within *Eurypon* sp. 2, KOG class expression patterns in hypoxia vs. anoxia were significantly positively correlated to those of hypoxia (r = 0.71, p < 0.01, Figure 1B). Similarly, in *H. stellifera*, KOG enrichment in hypoxia vs. anoxia was positively correlated to that of hypoxia (r = 0.72, p < 0.001, Figure 1C) and anoxia (r = 0.46, p < 0.05, Figure 1D). All significant correlations between datasets are shown in (Figure 1B-J), and all relationships that were not significantly correlated are not shown.

453

454 At the level of individual KOGs, the KOG class 'replication recombination and repair' (RRR) was 455 significantly enriched in all pairwise comparisons of oxygen conditions for both sponge species 456 ($P_{adj} < 0.05$), though in different directions depending on oxygen. It was significantly upregulated 457 in anoxia and anoxia vs. hypoxia but downregulated in hypoxia in both species (Figure 1A).

458

In Eurypon sp. 2, five genes with RRR KOG annotations were significantly upregulated in 459 hypoxia, e.g. HELZ and HELZ2 transcription associated helicases, and 11 genes were 460 significantly downregulated (p < 0.1, Supplemental Figure 4A), including five genes from the 461 462 minichromosome maintenance (MCM) protein complex. In anoxia, two genes were significantly 463 upregulated within RRR in this species - 'siz regulation of ARF protein signal transduction' and DNA polymerase epsilon catalytic subunit (p < 0.1, Supplemental Figure 4B). The majority of 464 significantly differentially expressed RRR genes (five) were downregulated in anoxia (p < 0.1. 465 466 Supplemental Figure 4B). The majority of RRR genes (nine) were also downregulated in anoxia vs. hypoxia, with only two significantly upregulated in anoxia (Supplemental Figure 4B). The 467 genes NBN and RFC1 were downregulated in anoxia compared to both normoxia and hypoxia 468 469 (Supplemental Figure 4B,C). Similarly, the gene 'siz regulation of ARF protein signal transduction' was upregulated in anoxia compared to both normoxia and hypoxia (Supplemental 470 471 Figure 4B,C).

In *H. stellifera*, three genes were significantly differentially expressed in the RRR KOG class in

- 474 hypoxia, and all were upregulated (p < 0.1, Supplemental Figure 4D). No genes within this class
- 475 were significantly differentially expressed in anoxia (vs. normoxia). Eleven genes were
- 476 upregulated and eight genes were downregulated in anoxia vs. hypoxia (Supplemental Figure
- 477 4E). Similar to *Eurypon* sp. 2, HELZ2 was downregulated in anoxia vs. hypoxia in *H. stellifera*.
- In contrast to *Eurypon* sp. 2 a distinct MCM gene (MCM6) was downregulated in anoxia vs.
- 479 hypoxia. Additionally, four genes from the structural maintenance of chromosomes protein 6
- 480 (SMC6) complex were downregulated in anoxia vs. hypoxia.
- 481

Like RRR, the KOG class 'energy production and conversion' (EPC) was significantly enriched ($P_{adj} < 0.05$) between all conditions except for anoxia vs. hypoxia in *H. stellifera* ($P_{adj} = 0.07$). EPC was significantly upregulated in hypoxia in *H. stellifera* but downregulated in all remaining

- 485 conditions in both sponge species (Figure 1A).
- 486

Within *Eurypon* sp. 2 specifically, four genes were significantly downregulated and twelve genes
were upregulated within the EPC class in hypoxia (p < 0.1, Supplemental Figure 5A). Various
nuclear genes for processes in the mitochondria were differentially expressed in hypoxia.
SLC25A27 (a mitochondrial membrane uncoupling protein), ndufs8 (a NADH dehydrogenase

491 core subunit in the mitochondrial membrane - complex I), and Surf1 (part of the complex that

492 regulates cytochrome c oxidase assembly) were downregulated in hypoxia (Supplemental

- Figure 5A). However, other nuclear mitochondrial genes were upregulated, including four
- 494 subunits of ATP synthase and two additional genes with annotations corresponding to ATP
 495 synthesis (Supplemental Figure 5A). In anoxia, fourteen genes were upregulated and five genes
 496 were downregulated in the EPC class (Supplemental Figure 5B).
- 497

498 As in hypoxia, ndufs8 was downregulated, but no other significant DEGs were shared across 499 the two oxygen levels. The following genes were also downregulated in anoxia: mitochondrial sulfite oxidase (SUOX), malate dehydrogenase (MDH1), and two genes belonging to the ATP 500 gaunido phosphatase family (Supplemental Figure 5B). Conversely, four genes involved in 501 phosphoenolpyruvate carboxykinase activity were significantly upregulated in anoxia (p < 0.1, 502 503 Supplemental Figure 5B). Additionally, three mitochondrial membrane transporter proteins from 504 the SLC family, two CYB5R4 cytochrome reductase genes, a mitochondrial carrier protein, and 505 fdx1 cytochrome reductase were upregulated in anoxia. In anoxia vs. hypoxia in Eurypon sp. 2, 506 ten genes were upregulated in anoxia (Supplemental Figure 5C). Upregulated genes included 507 all of the same genes that were significantly upregulated in anoxia, except for the 508 phosphoenolpyruvate carboxykinase associated genes. Six genes were significantly 509 downregulated in anoxia vs. hypoxia. Of these, three were also downregulated in anoxia (vs. 510 normoxia) and two genes were significantly upregulated in anoxia (Supplemental Figure 5A-C). It is noteworthy, however, that all of the upregulated genes in anoxia vs. normoxia and hypoxia 511 512 were only strongly upregulated in one of the two anoxia replicates (DC56, Supplemental Figure 513 5 B,C).

514

515 In *H. stellifera*, eight genes were upregulated and two genes were downregulated in hypoxia. As 516 in *Eurypon* sp. 2, the ATP5A1 ATP synthase subunit was upregulated in hypoxia. An additional

517 ATP synthase subunit (ATP6V0C) that was not significantly upregulated in *Eurypon* sp. 2 was

upregulated in *H. stellifera* in hypoxia (p < 0.1, Supplemental Figure 5D). Mitochondrial
sulfide:quinone oxidoreductase (SQRDL), three genes associated with phosphoenolpyruvate
carboxykinase, and a histone demethylase (KDM6A) were also significantly upregulated in
hypoxia in *H. stellifera*. Conversely, PCK1 (also associated with phosphoenolpyruvate
carboxykinase) and MDH1 were significantly downregulated in hypoxia (p < 0.1, Supplemental
Figure 5D). In anoxia in *H. stellifera*, ATP5A1 was significantly upregulated, and ATP6V0C,

- 524 PCK1, and MDH1 were significantly downregulated (Supplemental Figure 5E). Comparing
- 525 anoxia vs. hypoxia, eight genes were upregulated and five genes were downregulated
- 525 anoxia vs. hypoxia, eight genes were upregulated and live genes were downregulated 526 (Supplemental Figure 5F).
- 527
- 528 As in hypoxia, SQRDL, four genes associated with phosphoenolpyruvate carboxykinase activity,
- and KDM6A were upregulated in anoxia vs. hypoxia. Additionally, two unshared genes,
- 530 including the mitochondrial Succinyl-CoA:3-ketoacid coenzyme A transferase 1 (OXCT1) and a
- 531 mitochondrial carrier protein, were upregulated in anoxia vs. hypoxia (Supplemental Figure 5F).
- 532 Five genes were downregulated in anoxia vs. hypoxia in *H. stellifera*. Two of these four only
- 533 exhibited high expression levels in one hypoxic sample (DC105) that drove the observed
- 534 difference (Supplemental Figure 5F). The other three downregulated genes in anoxia vs.
- 535 hypoxia were mitochondrial creatine kinase (CKMT2), NAD P transhydrogenase (NNT), and
- 536 proto-oncogene tyrosine-protein kinase (Src).
- 537

538 The 'cytoskeleton' KOG class was also significantly enriched ($P_{adj} < 0.01$) in all but one oxygen 539 condition – anoxia in *Eurypon* sp. 2 ($P_{adj} = 0.1$). Interestingly, enrichment patterns in this KOG 540 were species specific, being significantly upregulated in hypoxia and anoxia vs. hypoxia in 541 *Eurypon* sp. 2 and downregulated in all conditions for *H. stellifera*.

542

543 The other twenty KOG classes were only significantly enriched in Lough Hyne sponges under a 544 few (or no) different oxygen conditions and patterns of significant enrichment were generally condition and species-specific (Figure 1A). With the exception of EPC, KOGs pertaining to 545 546 metabolic processes were rarely significantly enriched. Expression of the KOG classes 547 'carbohydrate metabolism' and 'amino acid transport and metabolism' were not significantly changed under any oxygen condition ($P_{adi} > 0.05$). There was some significant enrichment of 548 metabolic KOGs, but only in Eurypon sp. 2. 'Coenzyme transport and metabolism' and 549 550 'inorganic ion transport and metabolism' were both downregulated in anoxia, and 'nucleic acid 551 transport metabolism' was downregulated in hypoxia vs. anoxia. For both these conditions 552 (anoxia and hypoxia vs. anoxia), 'Lipid transport and metabolism' and 'Secondary metabolites 553 biosynthesis, transport and catabolism' were significantly downregulated. However, the transcription of the majority of genes labeled with metabolic KOGs remained unchanged under 554 555 anoxia and hypoxia.

- 556
- 557 Comparing the Lough Hyne dataset with external datasets of invertebrate KOG expression in
- response to stress, only two positive correlations were found. In *Eurypon* sp. 2, expression
- 559 patterns under anoxia vs. hypoxia were comparable to those of *T. wilhelma* under short-term
- anoxia (r = 0.44, p < 0.05, Figure 1H). The hypoxic response of *Eurypon* sp. 2 resembled that of
- diapause in S. mosellana (r = 0.68, p < 0.001, Figure 1F), but this was the only correlation found
- 562 between Lough Hyne sponge data and diapause or dauer states. Importantly, KOG enrichments

under anoxia (versus normoxia) were not positively correlated to any other external datasets foreither sponge species.

565

Three significant negative correlations, which corresponded to opposing KOG expression patterns, were observed between Lough Hyne sponges and external datasets. Hypoxia responses in *H. stellifera* were negatively correlated with those of *A. millepora* under heat stress (r = -0.56, p < 0.01, Figure 1G). The long exposure of *T. wilhelma* to hypoxia was negatively correlated to both anoxia (r = -0.64, p < 0.01, Figure 1J) and hypoxia vs. anoxia (r = -0.61, p <

- 571 0.01, Figure 1I) in *Eurypon* sp. 2.
- 572

573 GO term enrichments in sponges

574

575 Generally, significantly enriched GO terms between the different treatments were not shared between the two sponge species (Supplemental Figures 1-3). The strongest upregulated GO 576 enrichments (p < 0.01) in hypoxia in *Eurypon* sp. 2 were cellular signaling and 'lipoprotein 577 578 particle receptor binding' (Supplemental Figure 1A). For H. stellifera, on the other hand, the strongest upregulated enrichments (p < 0.01) in hypoxia included the terms 'protein tyrosine 579 580 kinase binding,' 'molecular adaptor,' 'structural molecule,' and 'structural constituent of 581 ribosome'; whereas the downregulation of the term 'motor' was strong (p < 0.01, Supplemental 582 Figure 1B).

583

584 Under anoxia, strong upregulation of the 'proteasome binding' term was observed in *Eurypon* 585 sp. 2 (p < 0.01), and the terms 'phosphoenolpyruvate carboxykinase', 'carbohydrate kinase,' 586 monovalent inorganic cation transmembrane transporter,' and 'sodium ion transmembrane 587 transporter' were highly downregulated (p < 0.01, Supplemental Figure 2A). In *H. stellifera* 588 under anoxia, the terms 'damaged DNA binding' and 'calcium-dependent phospholipid binding' 589 were highly upregulated, and four terms associated with various aspects of the cytoskeleton 590 were downregulated (p < 0.01, Supplemental Figure 2B).

591

592 In Eurypon sp. 2 under anoxia vs. hypoxia, the terms 'lipoprotein receptor binding' and 593 'proteasome binding' were strongly upregulated in anoxia, but no terms were strongly downregulated in anoxia versus hypoxia (p < 0.01, Supplemental Figure 3A). The former term 594 was also upregulated under anoxia vs. normoxia. Conversely, the strongest enrichments in H. 595 stellifera in anoxia vs. hypoxia were downregulated in anoxia and were composed of three of 596 597 the four cytoskeletal binding GO terms that were also downregulated in anoxia vs. normoxia 598 (see above, p < 0.01, Supplemental Figure 3B), but no strong upregulation of GO terms was 599 observed.

600

601 Considering GO enrichments at lower significance levels (0.01), within*Eurypon*sp. 2,602 the GO terms 'proteasome binding', 'lipoprotein particle receptor binding,' and 'Hsp70 binding'603 (HSP: Heat shock protein) were significantly upregulated in all cases of decreased oxygen albeit604 at varied significance levels (<math>p < 0.05, Supplemental Figures 1–3A). Between only two levels, 605 the terms 'transmembrane signaling receptor,' 'secondary active transmembrane transporter,' 606 and 'molecular transducer' were upregulated in hypoxia but downregulated in anoxia. However, 607 the term lipoprotein particle receptor binding was downregulated in anoxia and upregulated in bioRxiv preprint doi: https://doi.org/10.1101/2023.02.27.530229; this version posted October 26, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

608 hypoxia (p < 0.1, Supplemental Figures 1,2A). Additionally, the term 'structural constituent of 609 ribosome' was upregulated in anoxia but downregulated in anoxia vs. hypoxia (p < 0.1,

610 Supplemental Figures 1,3A). No additional GO terms other than the three present in all oxygen

611 levels for *Eurypon* sp. 2 were enriched in both the pairwise comparison of anoxia and anoxia vs.

612 hypoxia.

613

No GO terms were significantly up or downregulated in all three levels of oxygen conditions for

- 615 *H. stellifera*. However, some GO terms were significantly enriched in two levels. The term
- 616 'ATPase, coupled to transmembrane movement of ions, rotational mechanism' was
- downregulated in hypoxia but upregulated in anoxia (p < 0.1, Supplemental Figures 1B, 2B
- respectively). The GO terms 'I-SMAD binding', thioesterase binding,' and 'ubiquitin-like protein
- 619 ligase binding' were all upregulated in hypoxia (vs. normoxia) and anoxia vs. hypoxia, while the 620 'motor' term was downregulated (p < 0.1, Supplemental Figures 1B, 3B).
- 621
- There were only two shared GO enrichments between both sponge species for any given
- 623 oxygen condition. The terms 'protein tyrosine kinase binding' and 'structural constituent of the
- ribosome' were upregulated in both species in hypoxia (p < 0.1, Supplemental Figure 1),
- although they were enriched to different degrees of significance. No significantly enriched GO
- terms were shared between species in normoxia vs. anoxia or hypoxia vs. anoxia.
- 627
- At the gene level, significantly differentially expressed heat shock proteins (Hsps) in both
- sponges are shown in Supplemental Figure 8. In *Eurypon* sp. 2, one Hsp gene
- 630 (DNAJC8/Hsp40) was downregulated in hypoxia (p < 0.01), but four Hsp genes (HSPA8, two
- genes for HSP90B1, and Hsp70) were significantly upregulated in hypoxia (p < 0.1,
- 632 Supplemental Figure 8A). Additionally, HSPA8 and C11orf73 (Hsp70 binding protein) were
- upregulated in anoxia vs normoxia (p < 0.05, Supplemental Figure 8A). Another Hsp70 gene
- 634 was also significantly downregulated in anoxia vs. hypoxia (p < 0.05, Supplemental Figure 8A).
- In *H. stellifera*, HSP90A1 was upregulated in hypoxia and anoxia (p < 0.1, Supplemental Figure
- 836 8B). Two additional Hsp genes (both for HSP90B1) were also upregulated in anoxia. Finally, the
- two HSP90B1 genes were upregulated in anoxia vs. hypoxia, but HSPA5 was downregulated in anoxia vs. hypoxia (p < 0.05, Supplemental Figure 8B).
- 639

640 Mitochondrial gene expression

All 14 coding genes from the sponge mitochondria were transcribed in both species and in all
oxygen conditions. There was only one mitochondrial gene (nad2) that was significantly
differentially expressed but only in *Eurypon* sp. 2. This gene was significantly upregulated (p <
0.1) in anoxia vs hypoxia (Supplemental Figure 6A, Table 1). Similar counts for each individual
mitochondrial gene across both sponge species and all oxygen conditions were found. For
instance, counts for atp6 were consistently twice as high as counts for atp8 (Supplemental
Figure 6).

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- 650
- 651

652 Prokaryotic differential expression

653

654 *Thaumarchaeota* gene expression

For the Thaumarchaeota associated with Eurypon sp. 2, 20, 3 and 0 genes were differentially 655 656 expressed in hypoxia, anoxia, and hypoxia vs. anoxia, respectively (p < 0.1, Table 1). These genes accounted for 0-1.3% of this Thaumarchaeota genome, and 75-100% of the differentially 657 expressed genes were annotated (Table 1), compared to 69.2% annotated in the entire 658 reference genome. All but one of the differentially expressed genes in hypoxia were upregulated 659 (Table 1, Figure 2A). The downregulated gene belonged to the sodium solute symporter family 660 (SSF, TC 2.A.21, Figure 2A). The upregulated genes in hypoxia included three stress response 661 genes (COG0589 Universal stress protein [UspA], small heat shock protein [HSP20], and DnaJ 662 molecular chaperone homology domain), two CBS domains, a member of the lactate/malate 663 664 dehydrogenase (LDH MDH) superfamily, a tetratricopeptide (TPR) repeat, two transcription regulators, and a PII signal transduction protein. 665 666



- **Figure 2.** A-D. All significantly differentially expressed genes for the *Thaumarchaeota* in both sponge
- species (left: *E.* sp 2, right: *H. stellifera*. For *Thaumarchaeota* associated with *E.* sp. 2, only two pairwise
- 670 comparisons of oxygen conditions had significant differences: A. hypoxia vs. normoxia and C. anoxia vs.
- 671 normoxia. In symbionts associated with H. stellifera, there were significantly differentially expressed
- 672 genes in hypoxia vs. normoxia (B) and anoxia vs. hypoxia (D). There were no significant differences in the
- three genes involved in ammonia oxidation (AmoA, B and C) in either symbiont, but their heatmaps are
- shown in E and F for *E*. sp 2 and *H*. stellifera, respectively.
- 675

676 Only three genes were significantly differentially expressed in anoxia in Eurypon sp. 2's

677 Thaumarchaeota, and all were upregulated (Table 1, Figure 2C). The upregulated genes in

anoxia were a UbiD family decarboxylase, CAAX protease self-immunity, and the same TPR

repeat that was also upregulated in hypoxia. There were no significantly differentially expressed

genes between anoxia and hypoxia in this *Thaumarchaeota* (Table 1). The genes necessary for
 ammonia oxidation, i.e. subunits A, B and C of ammonia monooxygenase (Amo), were present
 in this *Thaumarchaeota*, but they were not differentially expressed under deoxygenation (Figure

683

2E).

684

685 In the *Thaumarchaeota* symbiont of *H. stellifera*, 17, 0 and 28 genes were significantly 686 differentially expressed in hypoxia, anoxia and hypoxia vs. anoxia, respectively. Similar to the 687 symbiont of Eurypon sp. 2, all 17 differentially expressed genes in the H. stellifera symbiont were upregulated in hypoxia. These genes included the stress response genes 'universal stress 688 protein, an HSP20 family protein, and two copies of UspA. HSP20 and UspA were also 689 upregulated in the Eurypon sp. 2 holobiont. Similarly, a PII signal transduction protein, CBS 690 691 domain, and two transcription regulators (one identical: TBP stabilizer, and one distinct to H. stellifera symbionts) were upregulated in both Thaumarchaeota species in hypoxia. Unlike in 692 693 Eurypon sp. 2, the Thaumarchaeota in H. stellifera upregulated a helix turn helix ASNC type 694 domain and a Lrp/AsnC ligand binding domain (Figure 2B). No genes were significantly

- 695 differentially expressed in anoxia in this *Thaumarchaeon*.
- 696

As in the other holobiont species, Thaumarchaeota genes for ammonia oxidation were not 697 significantly differentially expressed with H. stellifera symbionts (Figure 2F). Twenty-seven 698 699 genes were upregulated and one gene (K141 339108 19, unannotated) was downregulated in 700 anoxia vs. hypoxia in the Thaumarchaeota within H. stellifera (Figure 2D). Most of the 701 upregulated genes in anoxia vs. hypoxia were also upregulated in hypoxia. Upregulated genes 702 in anoxia vs. hypoxia unique to the *H. stellifera* holobiont included additional transcriptional 703 regulators, an integrase domain, and a putative nitroreductase. The phylogenies of this 704 upregulated nitroreductase and all nitroreductases from the studied holobionts as well as

publicly available nitroreductase sequences from other *Thaumarchaeota* are shown in
 Supplemental Figure 9.

707

For both *Thaumarchaeota*, only two COGs ('signal transduction mechanisms' and

'transcription') were significantly enriched ($P_{adj} < 0.05$, Supplemental Figure 7A). These two

- 710 COGs were both downregulated in hypoxia and anoxia vs. hypoxia within *H. stellifera*
- symbionts. All other COG classes were comparable in their expression levels regardless of
- holobiont species or oxygen concentration (Supplemental Figure 7A).
- 713

714 Gammaproteobacteria gene expression

715 The Gammaproteobacteria associated with Eurypon sp. 2 only exhibited significant differential

expression under hypoxia but not in any other oxygen condition (Table 1). In hypoxia, three

genes were upregulated: type 1 restriction-modification system, flavin adenine dinucleotide

- binding, and signal sequence binding (Figure 3A). The 29 downregulated genes in hypoxia
- included two genes involved in sulfur processing (k212078_2, k237243_12), cytochrome c
- 720 (class I), a gene associated with the assembly of the periplasmic c-type cytochromes





Figure 3. All significantly differentially expressed genes for the Gammaproteobacteria in both 727

sponge species (A: Eurypon sp 2, B: H. stellifera. For Gammaproteobacteria associated with 728

729 Eurypon sp. 2, there were only significant differences between hypoxia vs. normoxia (A). In

symbionts associated with H. stellifera, there were only significantly differentially expressed 730

genes in anoxia vs. hypoxia (B,C). All downregulated genes in anoxia vs. hypoxia are shown in B. 731

There were 402 genes that were downregulated in anoxia vs. hypoxia in *Gammaproteobacteria* 732

733 from *H. stellifera*, so only the top 10 based on p value are shown (C).

734

735 In the Gammaproteobacteria associated with H. stellifera, genes were only significantly

differentially expressed between anoxia and hypoxia (436 genes, p <0.1, Table 1, Figure 3B,C). 736

737 Twenty genes were upregulated in anoxia vs. hypoxia and all were annotated (Figure 3B).

738 Upregulated genes in anoxia vs. hypoxia included five genes associated with the ribosome

(k141 198365 15, k141 198365 8, k141 198365 17, and k141 245017 3), various genes for 739

molecular transport, a transmembrane proton pump (k141_226857_1), succinyl-CoA 740

synthetase, and formate dehydrogenase (alpha subunit). There were also 416 genes that were 741

significantly downregulated in anoxia vs. hypoxia (Table 1). The top ten upregulated genes 742

(lowest p values) are exhibited in Figure 3C. 743

744

Gammaproteobacterial COG expression is shown in Supplemental Figure 7B. Only one COG 745

746 class, 'translation, ribosomal structure and biogenesis,' was significantly enriched in

Gammaproteobacteria under the different oxygen conditions (Padi < 0.05). This COG class was 747

downregulated in Gammaproteobacteria associated with Eurypon sp. 2 under hypoxia as well 748

749 as those of *H. stellifera* in anoxia vs. hypoxia (Figure 7B).

751 Discussion

752

Sponge holobiont gene expression was significantly changed by exposure to deoxygenation in situ, though responses were generally species-specific, dependent on oxygen level, and varied across holobiont members. The potential functions of this differential expression are discussed below for each member of the two holobionts: sponge, sponge mitochondria, *Thaumarchaeota* and *Gammaproteobacteria*. The implications of this study for past and future oceans are considered.

759

760 Differential expression in sponges during deoxygenation

761 Cellular stress responses were observed in the expression of HSPs, a group of stress response genes that have been widely studied in marine invertebrates. These proteins are molecular 762 763 chaperones involved in protein folding, unfolding, transport and the assembly of protein complexes, and they are present in nearly all organisms (Li and Srivastava 2004). Both Lough 764 Hyne sponges upregulated HSP genes in response to hypoxia and anoxia (vs normoxia), 765 though there was downregulation of some HSP genes in deoxygenation as well. Eurypon sp. 2 766 and *H. stellifera* both upregulated Hsp90 genes in hypoxia, but they upregulated different HSPs 767 in anoxia: HSPA8 and Hsp90 for Eurypon sp. 2 and H. stellifera, respectively. It should be noted 768 that Hsp90 upregulation in H. stellifera in anoxia was driven by only one replicate. However, in 769 770 the pairwise comparison with the highest replication in this study, hypoxia vs. normoxia in 771 Eurypon sp. 2, Hsp90 genes were significantly upregulated in hypoxia. Hsp90 expression was 772 also higher in anoxia than normoxia for Eurypon sp. 2, but this was not significant. Based on 773 these trends, it is possible that increased replication would result in a clearer upregulated signal 774 from Hsp90 genes, since false negatives in gene expression analyses decrease with more replication (Schurch et al., 2016). Eurypon sp. 2 also upregulated HSPA8 and Hsp70 in hypoxia 775 but downregulated Hsp40. In long term hypoxia, T. wilhelma exhibited a similar HSP expression 776 777 pattern, upregulating Hsp90 but downregulating Hsp70 (Mills et al. 2018). Sponges under heat 778 stress also upregulate Hsp70 (López-Legentil et al. 2008; Guzman and Conaco 2016; Webster 779 et al. 2013) and HSP90 (Guzman and Conaco 2016). Pollutants such as lead and zinc can also 780 stimulate the upregulation of HSPs in sponges (Efremova et al. 2002; Schröder et al. 2006). 781 HSPs are therefore crucial to sponge stress response. HSP expression patterns and other key 782 transcriptomic responses of the sponge holobionts are outlined in Figure 4.

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783

Figure 4. Summary of sponge holobiont responses to deoxygenation presented in a tabular 784 format with species on the left (Es: Eurypon sp. 2, Hs: H. stellifera) and oxygen condition on the 785 top (hypoxia, anoxia, and hypoxia vs. normoxia). The major components of the holobiont are 786 illustrated as follows: sponge cell (tan), mitochondria (red), Gammaproteobacteria (green), and 787 Thaumarchaeota (purple). Upregulated and downregulated genes and KOGs are indicated with 788 red and blue arrows, respectively. Unchanged gene expression (GE) is noted by the = sign. 789 Correlations (corr.) with external dormancy datasets are indicated along with the direction of 790 the correlation (+ or -). 791

792

In metazoans with a complete HIF pathway, Hsp90 also serves to stabilize HIFa and therefore
 plays an important role in hypoxia tolerance (Semenza 2007). In the absence of HIFa in

sponges, Hsp90 may play a greater role in oxygen homeostasis given that it is upregulated
under hypoxia in all sponges that have been examined transcriptomically to date, i.e. *Eurypon*sp. 2, *H. stellifera* and *T. wilhelma*, and that Hsp90 is upregulated in *H. stellifera* under anoxia.
Hsp90 could stabilize currently unknown proteins that are involved in sponge oxygen regulation
or modulate transcription factors other than HIFa. Further research is needed to determine the
exact role of Hsp90 in sponge oxygen homeostasis, but it presents a good candidate for a gene
underlying sponge tolerance to deoxygenation.

802 Patterns of species and oxygen-condition-dependent expression of GO terms were observed in 803 sponges in response to deoxygenation. GO terms such as 'proteasome binding' and 'Hsp70 binding' were upregulated in all cases of decreased oxygen in Eurypon sp. 2, suggesting that 804 the species-specific response to deoxygenation in Eurypon sp. 2 may center around repairing or 805 806 degrading damaged proteins. In H. stellifera, on the other hand, upregulated GO terms related 807 to DNA repair in anoxia, including 'damaged DNA binding', 'DNA binding,' and 'catalysis of DNA and RNA,' so DNA repair may be more central to the deoxygenation tolerance of this species. 808 Both protein and DNA repair upregulation might be a response to reactive oxygen species 809 810 (ROS), which can become more abundant under hypoxia due to imbalances in the

- mitochondrial electron transport chain (Görlach et al. 2015). If there is any oxygen present in the
- sponges under environmental anoxia, e.g. oxygen produced by *Thaumarchaeota* (see Kraft et
- al. 2022, <u>Thaumarchaeota gene expression</u> below), then ROSs could cause cellular stress in
- anoxia. Otherwise, the presence of sulfide in the water under anoxia (Schuster et al. 2021) or
- ROSs other than superoxide (O_2) could damage proteins and DNA in anoxic conditions.

816 The EPC KOG class was significantly downregulated in all oxygen conditions in both sponges 817 except for hypoxia and hypoxia vs. anoxia in *H. stellifera*, when it was significantly upregulated and unchanged, respectively (Figure 1, 4). Within the EPC class, however, there were species-818 819 specific responses to anoxia and hypoxia at the level of individual differentially expressed 820 genes. Most nuclear ATP synthase genes were upregulated in hypoxia in Eurypon sp. 2 but 821 were unchanged under anoxia. Similarly, two ATP synthase subunits (ATP5A1 and ATP6V0C) 822 were upregulated in hypoxia in *H. stellifera*. In anoxia, ATP5A1 was also upregulated, but ATP6V0C was downregulated. Additionally, downregulation of SLC25A27 in Eurypon sp. 2 in 823 824 hypoxia may increase the coupling across the mitochondrial membrane to improve ATP 825 synthesis. We note that the electron transport chain genes Ndufs8 and Surf1 were also downregulated in hypoxia Eurypon sp. 2. The NNT gene, also involved in electron transport, 826 was downregulated in anoxia vs. hypoxia in H. stellifera. Nevertheless, most of the genes 827 associated with the respiration electron transport chain were not differentially expressed, and 828 electron transport likely continued under deoxygenation. Sponges may therefore have increased 829 ATP production under hypoxia but not under anoxia. In a similar way, mollusks may upregulate 830 mitochondrial electron transport system activity and ATP synthesis capacity to improve 831 832 respiration efficiency under hypoxia (Hochachka and Lutz 2001).

Glycolysis, which can produce ATP and NADH without oxygen (Stryer 1995), was expected to
increase under anoxia. However, relatively few genes directly or indirectly involved in glycolysis
were differentially expressed in our sponges under deoxygenation. In *Eurypon* sp. 2,
pantothenate kinase 1 (PANK1), ATP-dependent 6-phosphofructokinase (PFKL) and
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only aldehyde dehydrogenase 3 family, member a2 (ALDH3A2) was significantly upregulated in 838 839 hypoxia (see Supplemental Reports, Supplemental Figure 5). In H. stellifera, acetyl-coenzyme A synthetase 1 (ACSS1) was significantly downregulated in hypoxia, but PCK1 and pyruvate 840 kinase (PKM) were upregulated in hypoxia. In anoxia for H. stellifera, beta-enolase (ENO3) and 841 PCK1 were upregulated. Based on these results, it is unlikely that glycolysis rates increased 842 under anoxia and hypoxia in either species. It is more probable that glycolysis continued under 843 844 anoxia and hypoxia at normal rates in both species, since gene expression in the other ~70 845 genes associated with glycolysis was not significantly changed under deoxygenation (Figure 4). 846 Either way, the sponges would have at least some access to anaerobically generated ATP

- through glycolysis during environmental deoxygenation.
- 848 In the KOG class 'replication, recombination and repair' (RRR), there was significant
- 849 upregulation in anoxia and anoxia vs. hypoxia, but downregulation in hypoxia (vs. normoxia) for
- both sponge species. In *Eurypon* sp. 2, many MCM genes involved in the initiation of DNA
- replication were downregulated in hypoxia but remained unchanged in anoxia. Certain aspects
- of DNA replication, e.g. the DNA polymerase epsilon catalytic subunit, may even be upregulated
- in anoxia. The pattern was less clear in *H. stellifera*, but MCM genes were downregulated in
- anoxia compared to hypoxia (Figure 4). These data indicated that DNA replication may
- decrease under hypoxia but remain stable during anoxia, possibly due to increased oxidative
- stress under hypoxia compared to normoxia (see above). This pattern of a 'stronger' response
- to hypoxia than anoxia mirrored that of nuclear ATP synthesis genes.
- 858 Between the two study species, there was only one significant, positive correlation of KOG class
- expression between hypoxia in *Eurypon* sp. 2 and hypoxia vs. anoxia in *H. stellifera*,
- 860 demonstrating the general species-specific responses of most KOGs to deoxygenation. Within a
- single sponge species, no correlations were seen between KOG enrichments in anoxia or
- 862 hypoxia (vs. normoxia), highlighting the transcriptomic differences between responses to
- 863 hypoxia and anoxia. KOG enrichments in hypoxia *versus* anoxia, on the other hand, were
- positively correlated with those of anoxia in *Eurypon* sp. 2 and both anoxia and hypoxia in *H.*
- 865 stellifera.
- When quantitatively compared to transcriptomic datasets from the sponge T, wilhelma using 866 KOGMWU, transcriptomic responses in Lough Hyne sponges were only positively correlated 867 868 between *T. wilhelma* under short-term anoxia and *Eurypon* sp. 2 in hypoxia vs. anoxia (Figure 869 1H). This positive correlation could indicate similar transcription patterns demonstrated by T. wilhelma and Eurypon sp. 2 transitioning from hypoxia to anoxia. However, no further 870 871 similarities between the responses of *T. wilhelma* and Lough Hyne sponges to deoxygenation were observed. In fact, the long-term exposure of T. wilhelma to hypoxia was negatively 872 correlated to KOG enrichments for both anoxia vs. hypoxia and normoxia vs. anoxia in Eurypon 873 sp. 2 (Figure 1I, J), meaning that KOG expression was generally opposite between these two 874 species for these conditions in terms of up and down regulation. The physiological responses of 875 876 T. wilhelma and Eurypon sp. 2 to deoxygenation are presumably very different. These results 877 demonstrate that large-scale transcriptomic responses to deoxygenation depend on both the 878 studied species and oxygen concentration.
- 879

880 Based on KOG correlation analyses, the hypothesis that sponges are dormant, i.e. temporarily 881 ceasing growth, development, and physical activity, under anoxia was not supported (Figure 4). 882 Sponge gene expression under anoxia did not correlate to either diapause or a dauer state in C. 883 elegans (Sinha et al. 2012) or S. mosellana (Gong et al. 2013), respectively. Although these dormancy datasets come from distantly related invertebrates, Strader et al. (2016) 884 885 demonstrated a correlation between the KOG expression patterns of a specific phenotype of 886 coral larvae and diapause in S. mosellana, hypothesizing that this potential functional analog of 887 dormancy could serve to increase larval dispersal. Therefore, the sponges of Lough Hyne could only be considered dormant under anoxia if their transcriptional method of dormancy was 888 889 distinct from that of an insect, nematode and coral, which is possible but not parsimonious given 890 that animals are monophyletic (Feuda et al. 2017). Nevertheless, it was inconclusive whether or 891 not sponges are pumping water or feeding during anoxia, but sponges were definitively pumping under hypoxia (Schuster et al. 2021). Sponge pumping and filtering activity remain to be 892

893 quantified under anoxia and hypoxia in situ.

894 Paradoxically, Eurypon sp. 2 responses to hypoxia were positively correlated to diapause in S. 895 mosellana (Figure 1F, 5). Therefore, the hypoxic response in Eurypon sp. 2 species, rather than the anoxic response, was similar to diapause in terms of global KOG expression patterns. The 896 897 RRR kog was also downregulated in *Eurypon* sp. 2 under hypoxia as well as in *S. mosellana* 898 under diapause, which may partially explain the correlation between these datasets. Despite the 899 correlation, the EPC expression patterns between the two species were opposing. EPC was significantly downregulated in hypoxia in Eurypon sp. 2 and upregulated in diapause in S. 900 901 Mosselana. These KOG enrichments are calculated from thousands of genes, so any individual 902 gene within a KOG class might still be upregulated even if the KOG class is significantly 903 downregulated. Therefore, it is possible that ATP production increased in hypoxia based on the 904 upregulation of ATP synthase genes, in spite of the downregulation of the EPC KOG. Diapause 905 can be associated with the reduction of cell division (MacRae 2010), which may occur in sponges under hypoxia but not anoxia based on MCM gene expression. In comparison, KOG 906 907 enrichments under anoxia were not positively correlated to any external datasets for either 908 sponge species (Figure 1), indicating that sponge transcriptomic responses to anoxia are 909 relatively novel compared to those of other taxa in response to various factors in the existing literature (Dixon et al. 2015; Strader et al. 2016). 910

911

912 Sponge mitochondrial gene expression during deoxygenation

913

A high degree of transcriptional stability under deoxygenation was observed in sponge mitochondria. Only one of the 14 mitochondrial protein-coding genes, nad2, was significantly differentially expressed in the different oxygen conditions, where it was upregulated in anoxia vs. hypoxia in *Eurypon* sp 2 (Figure 4). Both of the sponge species had higher expression of nad2 in anoxia than in normoxia or hypoxia, but this difference was only significant in *Eurypon* sp. 2.

- 921 Surprisingly little is known about differential gene expression in mitochondria, even within
- 922 humans. In the limited studies available, mitochondrial gene expression was significantly
- 923 different between human cancer cells and normal cells (Reznik et al. 2017) as well as between
- different populations of healthy pancreas tissue (Medini et al. 2021). In these studies, nad2 was

925 significantly downregulated in most studied cancers, though it was still significantly upregulated 926 in a few cancer types, and nad2 was also significantly differentially expressed between 927 pancreas cell populations. Based on the general upregulation of nuclear genes associated with 928 respiration in hypoxia, mitochondrial gene upregulation was expected in hypoxia when compared to normoxia and anoxia, but the opposite was true for nad2 in Eurypon sp. 2 in the 929 current study. The other six nad genes, which code for parts of the same membrane respiratory 930 931 chain complex (complex 1, NADH dehydrogenase), however, were not significantly differentially 932 expressed in either species. In both human studies, when one mitochondrial gene was 933 significantly differentially expressed, most other mitochondrial genes were significantly 934 differentially expressed in the same direction (Reznik et al. 2017; Medini et al. 2021). The physiological meaning (if any) of this single, significantly differentially expressed mitochondrial 935 936 gene in sponges remains to be determined.

937

938 Compared to that of humans, even less is known about mitochondrial gene expression in 939 marine invertebrates. Mitochondrial gene expression is usually excluded from differential 940 expression analyses due to the differences in library preparation potentially needed for sequencing mitochondrial RNA and/or the exclusion of mitochondrial RNA from reference 941 942 transcriptomes (sensu Meyer 2016). This dearth is partly due to the nature of mitochondrial 943 transcription, which generates a single continuous transcript. Following transcription, however, mitochondrial transcripts are broken up into individual coding mRNA, tRNA or rRNA (D'Souza 944 945 and Minczuk 2018). After the breakup, the levels of mRNAs from specific genes are controlled post-transcriptionally (D'Souza and Minczuk 2018). It is therefore possible to have different 946 counts between the different mitochondrial genes despite their single transcript origin 947 948 (Supplemental Figure 6). The consistent 'expression' of transcripts across oxygen conditions for 949 each mitochondrial protein-coding gene following continuous transcription are likely to be 950 necessary for the maintenance of mitochondrial function.

951

It remains unclear how sponge mitochondria maintain this transcription from an energetic 952 953 standpoint under anoxia. Within the mitochondrial electron transport chain, oxygen is used as 954 the terminal electron acceptor and is crucial for the generation of ATP through oxidative phosphorylation. Theoretically, anoxia should cause electron transport and oxidative 955 956 phosphorylation in the mitochondria to cease (Chandel and Schumacker 2000). If the 957 mitochondria are transcribing solely to maintain oxidative phosphorylation machinery until 958 oxygen returns, then they would need to use at least enough energy (~2 ATP per base) to 959 transcribe for months at a time without oxygen (Lynch and Marinov 2015), and this energetic 960 requirement would apply to the transcription of sponge nuclear genes as well. Glycolysis may provide some anaerobic ATP production, even if it is not upregulated in anoxia or hypoxia. 961 962 Alternatively, to maintain ATP production through aerobic respiration, the sponges of Lough Hyne might have access to oxygen produced by their *Thaumarchaeota* symbionts, a topic 963 explored in more detail below. 964

966 Microbiome gene expression during deoxygenation

967 <u>Thaumarchaeota gene expression</u>

74 Thaumarchaeota symbionts from both sponge species exhibited significant differential gene 75 expression under hypoxia. Only the *Thaumarchaeota* from *Eurypon* sp. 2 (OTU1) exhibited 75 differential gene expression under anoxia, and only those of *H. stellifera* (OTU3) differentially 75 expressed genes under anoxia vs. hypoxia. Though there were some species-specific 75 responses, overall gene expression in both *Thaumarchaeota* was similar, supporting the 76 hypothesis from Schuster et al. (2021) that these taxonomically similar symbionts between two 77 sponge species might have similar functional roles.

975 Relatively few genes involved in anaerobic metabolism were expressed in Thaumarchaeota 976 symbionts under any oxygen condition. Lactate/malate dehydrogenase, which could be involved 977 in fermentation (Bayer et al. 2020), was upregulated in *Eurypon* sp. 2 *Thaumarchaeota*, but only in hypoxia. Genes for the other anaerobic pathways suggested by Bayer et al. 2020, i.e. 978 979 succinate dehydrogenase/ fumarate reductase, nitrite reductase and sulfite reductase, were 980 present in both *Thaumarchaeota* genomes in the present study but were not significantly 981 differentially expressed under different oxygen conditions. The genes for nitrite reductase 982 exhibited low expression across all samples (0-20 counts) in both Thaumarcheaota, and so did 983 the dehydrogenase/ fumarate reductase gene in the Thaumarcheaota of H. stellifera. Therefore, 984 these genes with low expression may not be used under the conditions of this study. Only three 985 genes in the *Thaumarchaeota* of *Eurypon* sp. 2 under anoxia were significantly differentially 986 expressed, and none of these were involved in anaerobic metabolism. No genes were 987 differentially expressed in H. stellifera Thaumarchaeota under anoxia. Therefore, there was no 988 transcriptomic evidence for the upregulation of anaerobic metabolism within Thaumarchaeota in 989 anoxia.

990 Transcription of ammonium monooxygenase (AMO) genes did not significantly change under 991 anoxia or hypoxia, even though the ammonia oxidation pathway is oxygen dependent. Like the sponge mitochondria, the Thaumarchaeota are therefore either transcribing AMO genes to be 992 993 ready when oxygen returns, or they are obtaining trace oxygen for this pathway by synthesizing 994 oxygen themselves (Kraft et al. 2022). If sponge Thaumarchaeota symbionts are also able to produce oxygen under anoxic conditions like their relative N. maritimus, then they should 995 996 express amo genes under anoxia as well as normoxia. The proposed pathway for oxygen 997 production in *N. maritimus* involves the reduction of nitrite to nitric oxide that is dismutated into 998 oxygen and nitrous oxide (Kraft et al. 2022). Water sampled for nitrite concentrations in Lough 999 Hyne using established methods (Grasshoff et al. 1999) revealed that nitrite concentrations 1000 under anoxia ranged from 0.2–1.2 µM (Supplemental Table 3), increasing with depth below the 1001 thermocline. Therefore, the conditions in Lough Hyne would theoretically allow for 1002 Thaumarchaeota to produce oxygen under anoxia.

AsnC family proteins and nitroreductase expression was upregulated during oxygen production
in *N. maritimus* (Kraft et al. 2021). In *Archaea*, AsnC proteins can regulate metabolic processes,
including the switch between aerobic and anaerobic metabolisms (Lemmens et al. 2019).
Similarly, nitroreductases can have a wide range of metabolic functions (Akiva et al. 2017).

1007 *Thaumarchaeota* within *H. stellifera* upregulated a Lrp/AsnC ligand binding domain under 1008 hypoxia and in anoxia vs. hypoxia. Another AsnC type protein was also upregulated under 1009 hypoxia, and a nitroreductase was upregulated under anoxia vs. hypoxia. However, these 1010 proteins did not directly correspond to those upregulated in *N. maritimus* during oxygen 1011 production (Supplemental Figure 9), and the latter proteins are not definitively known to be 1012 involved in oxygen production, since the pathway remains unknown.

1013 In hypoxia, both *Thaumarchaeota* upregulated the stress response genes USpA and a protein 1014 from the HSP20 family (Figure 4). It was surprising that *Thaumarchaeota* may be stressed by 1015 hypoxia since free-living thaumarchaeal Nitrosopumilales can be found in high abundances in oxygen-minimum zones (OMZs) where oxygen is either at low concentrations or below 1016 1017 detection (Löscher et al. 2012), though some Thaumarchaeota OTUs decrease in abundance in 1018 suboxic and anoxic zones (Sollai et al. 2019). Members of the genus Nitrosopumilus, however, increase their absolute abundance in suboxic regions, but then decrease in abundance with 1019 depth in the anoxic zone (Sollai et al. 2019), indicating a preference for suboxic environments. If 1020 suboxia is also the preferred niche of sponge Thaumarchaeota symbionts, the upregulation of 1021 1022 stress response genes in response to hypoxia may correspond to a rise in intracellular oxidative 1023 stress following an increase in metabolic activity in hypoxia, like their sponge hosts.

1024

1025 *Gammaproteobacteria* gene expression

1026 Gene expression in the Gammaproteobacteria was species and oxygen-condition dependent. In 1027 Eurypon sp. 2, Gammaproteobacteria downregulated genes in hypoxia that were associated 1028 with respiration and energy production, including cytochrome c and associated genes (Welchen 1029 et al. 2016). The Gammaproteobacteria also downregulated key metabolic genes in hypoxia 1030 such as glutamine synthetase and structural genes like YbiS YcfS YnhG family protein, which is involved in cell wall production (Magnet et al. 2008). So unlike sponges or Thaumarchaeota, 1031 1032 Gammaproteobacteria in Eurypon sp. 2 (OTU2) may decrease metabolic activity under hypoxia, 1033 though this is likely not the case in *H. stellifera* symbionts (OTU7).

1034 Gammaproteobacteria in H. stellifera downregulated hundreds of genes in anoxia vs. hypoxia,

- 1035 including USpA, so, like other members of the holobiont, hypoxia requires a stronger
- 1036 transcriptomic response and, therefore, may be more stressful than anoxia for these
- 1037 *Gammaproteobacterial symbionts*. The few upregulated genes in anoxia vs. hypoxia included
- 1038 genes for energy production, e.g. a transmembrane proton pump, succinyl-CoA synthetase (part
- 1039 of the citric acid cycle), and formate dehydrogenase (alpha subunit), although other genes
- associated with energy production were downregulated. Formate dehydrogenase can function
- in both aerobic respiration and in anaerobic respiration (Jormakka, et al. 2003), but this enzyme
- 1042 was not significantly upregulated in anoxia compared to normoxia or in the
- 1043 *Gammaproteobacteria* of *Eurypon* sp. 2 under any condition. So, it is unlikely that this anaerobic
- 1044 pathway is active in sponge-associated *Gammaproteobacteria* under anoxia.

1045 *Gammaproteobacteria* symbionts were previously hypothesized to oxidize sulfide under anoxia 1046 (Schuster et al. 2021). Although they are not present in OTU2, OTU7 from *H. stellifera* has the

- 1047 sulfur oxidation genes soxA, soxY, and soxZ, which could oxidize sulfide, as well as the nitrate
- 1048 reductase gene NAP. However, the sox genes were not expressed in any samples in the

1049 current study, and NAP was expressed at very low levels across all samples (2-28 counts).

1050 Moreover, neither symbiont exhibited significant differential gene expression in anoxia versus

1051 normoxia. Consequently, there was very little transcriptomic evidence that the anaerobic

1052 pathways in these *Gammaproteobacteria* were activated during environmental anoxia.

1053 Summary of potential adaptations of sponge holobionts to low oxygen

1054 Metabolically, sponge transcription under anoxia was surprisingly similar to normoxia throughout the holobiont members, and there was no evidence of dormancy within the holobionts in anoxia 1055 1056 (Figure 4). No clear anaerobic respiration responses were observed in the transcriptomes of sponges, their mitochondria or their two microbial symbionts under environmental anoxia. The 1057 limited significant differential expression of glycolysis genes could indicate that this metabolic 1058 1059 process continues to comparable degrees under normoxia, hypoxia, and anoxia. Therefore, in the sponges and their microbes, glycolysis could potentially provide ATP without oxygen (Figure 1060 1061 4).

1062 Hypoxia, rather than anoxia, elicited upregulation in energy production machinery such as ATP 1063 synthase (Figure 4). These potential metabolic increases suggest that within the hypoxic limits 1064 studied here (1.30-3.56 mg L⁻¹), sponges perform optimally. Indeed, these limits are above 1065 those at which mortality or substantial physiological changes have been observed for sponges 1066 (Micaroni et al. 2021; Kumala et al. 2021; Mills et al. 2014). This increased energy production 1067 could be stressful, however, and the diversion of resources could limit cell division in hypoxia 1068 (Figure 4). Somewhere between the lower limit of hypoxia (1.37 mg L^{-1}) and the full anoxia 1069 studied here, sponges may no longer attempt to increase energy efficiency, and therefore 1070 elevated transcription of ATP synthase genes was not observed in anoxia.

For both the sponges and their Thaumarcheaota symbionts, cellular stress responses were 1071 1072 evident under deoxygenation. Both sponge species upregulated HSP genes under anoxia and 1073 hypoxia. These genes may be crucial for sponge deoxygenation tolerance in these species 1074 (Figure 4). We hypothesize that Hsp90 plays a role in sponge oxygen homeostasis, but this needs to be tested. The Thaumarchaeota symbionts upregulated HSP20 and other stress 1075 1076 response genes in anoxia and hypoxia for *H. stellifera* and hypoxia within *E.* sp. 2 (Figure 4). In 1077 hypoxia, archaeal and host upregulation of stress response genes may have been a response to oxidative stress following increased metabolic activities. In anoxia, cellular stress response 1078 1079 might result from the presence of sulfide. Additionally, post-transcriptional, metabolic changes may play a role in deoxygenation tolerance, and proteomic and metabolomic changes should be 1080 1081 examined in future studies.

1082 Given the largely unchanged metabolic gene expression patterns under hypoxia or anoxia in 1083 these sponges, the production of oxygen by *Thaumarchaeota* could also be crucial to holobiont 1084 survival (noted with a question mark in Figure 4). Although any oxygen produced would be at extremely low concentrations, it would have a short path of diffusion to the sponge cells, as 1085 1086 Thaumarchaeota symbionts maintain high cell densities within sponges (Moeller et al. 2019). In addition, mitochondria in general can utilize very low concentrations of oxygen (500 nM) without 1087 1088 changing their oxygen consumption rate, and cells can still respire below that threshold albeit at 1089 a lower rate (Chance 1965; Longmuir 1957; Wilson et al. 1979). In culture, oxygen generated by 1090 Thaumarchaeota reached 100-200 nM (Kraft et al., 2022). If sponge Thaumarchaeota produce 1091 oxygen at similar levels under anoxia, cellular respiration could continue in sponges under 1092 anoxia, though likely at lower respiration rates when compared to normoxia. However, given 1093 that the genes responsible are still unknown, the production of oxygen by sponge 1094 Thaumarchaeota needs to be verified experimentally.

1095 Relevance to future ocean deoxygenation

1096

1097 Sponges are predicted to be the 'winners' over other benthic organisms in the future due to their tolerance of ocean warming and acidification associated with climate change (Bell et al. 2018). 1098 Indeed, sponges have already increased in abundance in some locations while corals have 1099 decreased due to altered carbon and nutrient cycling (Pawlik et al. 2016). The additional 1100 1101 stressor of deoxygenation that comes with climate change and coastal eutrophication has been 1102 relatively overlooked compared to ocean warming and acidification. In other marine taxa, a 1103 meta-analysis demonstrated that the incorporation of deoxygenation as a factor along with 1104 warming could substantially decrease survival times of marine organisms compared to warming 1105 alone (Vaguer-Sunyer and Duarte 2011). Since sponges may be more tolerant than other taxa 1106 to changes in all three stressors of temperature, pH and oxygen, they could indeed become 1107 more abundant in a changing ocean. However, even sponges can be lethally affected by warming, acidification (Bennett et al. 2017) and deoxygenation (Micaroni et al. 2021) in 1108 1109 scenarios that might occur with climate change, since tolerance to these stressors is speciesspecific. So, surviving sponge species in a warmed, acidified, and deoxygenated ocean might 1110 1111 be abundant, but their abundance, and diversity would likely be lower than at present. The 1112 effects of all three of these stressors together need to be understood on a much wider range of organisms to accurately predict the composition of future oceans. More importantly, the causes 1113 1114 and effects of anthropogenic climate change need to be ameliorated as soon as possible to

avoid such diversity loss. 1115

1116 **Relevance for animal evolution**

Assuming that the first metazoans were more like an adult sponge than a ctenophore, based on 1117 1118 morphology (Nielsen 2008) and phylogeny (Feuda et al. 2017), then some species of early 1119 sponges or proto-animals could have tolerated low environmental oxygen levels and at least 1120 some periods of benthic anoxia and euxinia in the Neoproterozoic Era (~538.8–1,000 million years ago) when early animals, including sponges, first evolved (Dohrmann and Wörheide 1121 1122 2017). Lough Hyne sponges are a valuable model system for understanding early animal 1123 evolution. Early sponges could have survived under low and variable oxygen levels using 1124 mechanisms like those of Lough Hyne sponges. Therefore, contrary to longstanding 1125 hypotheses, the evolution of animals probably did not require high and stable levels of 1126 environmental oxygen.

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1135 **Conflict of interest disclosure**

1136 The authors declare that they comply with the PCI rule of having no financial conflicts of interest 1137 in relation to the content of the article.

1138 Data, scripts, code, and supplementary information availability

- 1139 Raw sequence data as well as genome and transcriptome assemblies are available at NCBI
- 1140 under BioProject number PRJNA893197
- 1141 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA893197). Scripts, code, and additional data
- 1142 are available here: <u>https://zenodo.org/badge/latestdoi/573040118</u>. Supplemental figures and
- 1143 tables are available on BioRxiv under 'Supplementary material.'

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