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# 1 Transcriptomic responses of sponge 2 holobionts to in situ, seasonal anoxia 3 and hypoxia

4  
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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  
24 mitochondria and their microbial symbionts under *in situ* conditions of normoxia, hypoxia and  
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  
34 heat shock proteins (HSPs), indicating cellular level adaptations to deoxygenation stress. A  
35 meta-analysis demonstrated that sponge transcriptional responses to anoxia were distinct from  
36 those displayed by other invertebrates while dormant, and the hypothesis of sponge dormancy  
37 under anoxia was not supported. *Thaumarchaeota* symbionts also upregulated stress response  
38 genes in hypoxia, while maintaining expression of oxygen-dependent metabolic pathways under  
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  
41 hypoxia. There was no clear evidence of upregulated anaerobic respiration in the  
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  
57 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  
65 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,  
67 1858 with 7% a.s. (20  $\mu\text{M O}_2$ , 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*  
71 Bergquist, 1968) were exposed to various levels of hypoxia (1.5–40% a.s., 0.13–3.3  $\text{mg O}_2 \text{ L}^{-1}$ )  
72 in aquaria. These sponges exhibited species-specific physiological, morphological and  
73 behavioral responses to deoxygenation. Sub-lethal oxygen thresholds for these sponges were  
74 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  
75 two species (*P. crocea*) exposed to severe hypoxia lower than 5% a.s. (0.13  $\text{mg O}_2 \text{ L}^{-1}$ )  
76 (Micaroni et al. 2021a). The sponge species most sensitive to deoxygenation in controlled  
77 experiments, *P. crocea*, had a median lethal time (time until death, LT50) of 286 h at ~1.5% a.s.  
78 (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  
83 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  
85 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  
88 their ca. three month exposure to anoxia, and, compared to other animals, their tolerance is  
89 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

91 the seasonally anoxic site of Lough Hyne only slightly decreased over 30 years despite  
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<sup>-1</sup>: 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  
103 anemones may even have elements of a facultative anaerobic metabolism (Sassaman and  
104 Mangum 1972).

105  
106 Only one study has investigated the transcriptomic response of a sponge to hypoxia. After four  
107 days of exposure to hypoxia (0.25–2% a.s.), 128 genes within the sponge *T. wilhelma* were  
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  
110 (<0.05% a.s., 105 nM O<sub>2</sub>) elicited stress responses in *T. wilhelma*'s transcriptome (Mills et al.  
111 2018), demonstrating that transcriptomic responses were mostly observed under very low  
112 oxygen levels.

113  
114 In most metazoans, the hypoxia-inducible factor (HIF) pathway is essential for maintaining  
115 oxygen homeostasis in response to hypoxia (Samenza 2007). However, in sponges and  
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  
122 The sponge microbiome may play a role in sponge deoxygenation tolerance. Sponges can host  
123 diverse microbiomes that perform vital functions and can constitute large portions of their  
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  
126 sulfate reduction (Hoffmann et al. 2005), denitrification, and anaerobic ammonium oxidation  
127 (anammox) (Hoffmann et al. 2009). In *V. pourtalesii*, the microbial community is thought to be  
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  
132 communities of the water and sediment, indicating that the holobiont as a whole is tolerant to  
133 deoxygenation (Schuster et al., 2021). Specifically, the microbiomes of the anoxia-tolerant  
134 sponge species *Eurypon* sp. 2 and *Hymenaphia stellifera* Bowerbank, 1864 were both  
135 dominated by two microbes – one *Thaumarchaeon* and one *Gammaproteobacterium*, though

136 each sponge hosted a different species from each clade. *Eurypon* sp. 2 harbors the  
137 *Thaumarchaeon* OTU1 (closest related isolate: *Nitrosopumilus adriaticus* strain NF5), and *H.*  
138 *stellifera* harbors OTU3 (closest related isolate: *Nitrosopumilus oxycliniae* strain HCE1). For the  
139 *Gammaproteobacteria*, *Eurypon* sp. 2 contains OTU2 (closest related isolate: *Arenicella*  
140 *chitinivorans* strain ALEN 1); whereas *H. stellifera* contains OTU7 (closest related isolate: *A.*  
141 *chitinivorans* strain ALEN 1, (Schuster et al. 2021). Although their location within Lough Hyne  
142 sponges has not yet been determined, *Gammaproteobacteria* and *Thaumarchaeota* symbionts  
143 are abundant and widely distributed throughout the sponge tissue in other species (Moeller et  
144 al. 2019). These symbionts also participate in integrated metabolic processes with their host  
145 species (Moitinho-Silva et al. 2017).

146  
147 Schuster et al. (2021) hypothesized potential metabolic pathways within these two symbiont  
148 taxa that might function under deoxygenation. Based on the genomes of their close relatives,  
149 the *Thaumarchaeota* in these sponges were hypothesized to have components of a facultatively  
150 anaerobic metabolism, including fumarate, nitrite, and sulfite respiration and the ability to  
151 ferment (Bayer et al. 2020), which could aid in deoxygenation tolerance. A recent study  
152 demonstrated that another close *Thaumarchaeota* relative, *Nitrosopumilus maritimus*, can  
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,  
155 comparative proteomics suggested that proteins upregulated during oxygen production included  
156 some AsnC family proteins and nitroreductases (Kraft et al. 2021). We therefore hypothesized  
157 that nitroreductases and AsnC family proteins would be upregulated under hypoxia and anoxia  
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.*  
164 *stellifera* as well as their respective mitochondria and microbial symbionts including  
165 *Thaumarchaeota* and *Gammaproteobacteria*. Sponges were collected from Lough Hyne under  
166 *in situ* normoxia, hypoxia and anoxia. Since neither of these holobionts are established model  
167 species, we generated reference transcriptomes for the sponges and assembled the genomes  
168 of their symbionts and mitochondria from metagenomic sequences. Additionally, we performed  
169 a meta-analysis of the sponge differential expression data to determine if the responses of  
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),  
172 and during diapause (Gong et al. 2013). Finally, we considered the implications of sponge  
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  
183 categorized as follows: normoxic (5.3–12 mg L<sup>-1</sup>, 49.3–111% a.s.), hypoxic (1.30–3.56 mg L<sup>-1</sup>,  
184 12.1–33.1% a.s.), and anoxic (0.00–0.01 mg L<sup>-1</sup>, 0.00–0.93% a.s., i.e. instrument detection  
185 limit). All samples were frozen in liquid nitrogen within ~15 minutes of collection and kept at  
186 liquid nitrogen temperatures in a dry shipper until returned to the laboratory where samples  
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**

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

199

200 Additionally, high molecular weight (HMW) DNA was extracted from one individual of each  
201 species using the protocol from Vargas et al. (2021) for metagenomic sequencing. The *Eurypon*  
202 sp. 2 sample used for metagenomic sequencing was taken under anoxia (DC24), and RNA was  
203 also sequenced from this sample. Due to limited availability of samples and DNA, a sample of  
204 *H. stellifera* that was not included in the RNAseq (DC297, sampled under normoxia) was used  
205 for metagenome sequencing. DNA and RNA yields were determined using a Qubit™ (Thermo  
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

### 209 **Reference transcriptomes and metatranscriptomes**

210 All RNA samples were sequenced twice with two different library preps - one for polyadenylated  
211 (poly A) messenger RNA (mRNA, from eukaryotes) and one for total RNA (to also capture  
212 prokaryotic RNA). Library preparation for the eukaryotic (sponge) RNA was done using  
213 NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England Biolabs, USA).  
214 For the prokaryotic RNAseq, the supernatant from the poly A enrichment was treated with  
215 NEBNext® rRNA Depletion Kit (Bacteria) and then NEBNext® rRNA Depletion Kit  
216 (Human/Mouse/Rat, New England Biolabs, USA) to eliminate ribosomal RNA (rRNA) that  
217 causes sequence biases. This combination of poly A exclusion and rRNA depletion eliminated  
218 the most rRNA compared to other combinations of methods (or single methods in isolation) in  
219 preliminary tests, yielding sequences with only ~25% rRNA. For the eukaryotic (n = 24) and  
220 prokaryotic (n = 24) RNA samples, library preparation was completed using the NEBNext Ultra II  
221 Directional RNA library preparation kit (New England Biolabs, Denmark). Libraries pooled in  
222 equimolar concentrations were sequenced (5M reads per sample, 2 x 50 bp) at the Center for  
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  
230 availability of sample tissue. As in the sequencing above, two libraries were created and  
231 sequenced for each sample - one with eukaryotic RNA and one with prokaryotic RNA. For this  
232 deeper sequencing, mRNA in the sponge transcriptomes was purified using the NucleoTrap  
233 mRNA kit (Macherey-Nagel, Germany). Prokaryotic RNA was rRNA depleted using the Ribo-  
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  
238 sequenced (2 x 150 bp) on a HiSeq X platform (Illumina, USA) by DNASense ApS (Aalborg,  
239 Denmark).

240  
241 For the eukaryotic RNA, shallow (2 x 50 bp) sequencing generated  $9.20 \times 10^8$  and  $4.38 \times 10^8$   
242 reads for *Eurypon* sp. 2 and *H. stellifera*, respectively, across all sampled sponges, and deeper  
243 sequences (2 x 150 bp) generated  $2.05 \times 10^8$  and reads  $3.45 \times 10^8$  for each sponge of the two  
244 species, respectively. For the prokaryotic RNA, shallow sequencing yielded  $1.27 \times 10^9$  and  $6.68$   
245  $\times 10^8$  reads for *Eurypon* sp. 2 and *H. stellifera*, respectively. The deeper prokaryotic sequencing  
246 generated  $1.52 \times 10^8$  and  $1.20 \times 10^8$  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  
250 eukaryotic RNA sequences were combined into a single file. These sequences were trimmed for  
251 quality 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-  
253 NOG mapper (Huerta-Cepas et al. 2019). The final sponge transcriptomes included only genes  
254 with metazoan annotations or no annotations. For a detailed description of all filtering and  
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  
257 range of size, gene number, and N50 (Supplemental Table 2) of published sponge  
258 transcriptomes (Strehlow et al. 2020; Riesgo et al. 2014). Additionally, sponge reference  
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.

## 263 264 **Reference Metagenomes**

265 Two reference metagenomes were sequenced for *Eurypon* sp. 2 and *H. stellifera* at DNASense  
266 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  
268 preparation kit. The DNA library was paired-end sequenced (2 x 150 bp) on a NovaSeq platform  
269 (Illumina, USA).

270



271 Sequences were assembled using Megahit v.1.2.9 (Li et al. 2016). The final contig fasta files  
272 were used to plot GC (%) vs. mean coverage of mapped reads to identify individual bins. For  
273 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  
276 shown in Supplemental Table 2. Raw sequences are available from NCBI under BioProject  
277 number PRJNA893197. Metagenome assemblies are available on  
278 <https://github.com/bstrehlow/Sponge-transcriptomic-responses-to-seasonal-anoxia>.

279

### 280 **Mitogenomes**

281 Using only the deep sequences of total ('prokaryotic') RNA, the mitochondrial genomes of single  
282 individuals of *Eurypon* sp. 2 (DC128) and *H. stellifera* (DC129) were assembled following the  
283 pipeline in Plese et al. (2019).

284

285 For future utilization, single contigs that likely represent the mitochondrial genomes of *Eurypon*  
286 sp. 2 and *Hymeraphia stellifera* were retrieved from the reference metagenome in addition to  
287 the coding regions used for differential expression below. The assembled contigs for each  
288 metagenome were blasted against the mitochondrial genome of DC128 (*Eurypon* sp. 2) using  
289 blastn, with an e-value cutoff of  $1 \times 10^{-50}$  for *Eurypon* sp. 2 (DC97) and  $1 \times 10^{-30}$  for *Hymeraphia*  
290 *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,  
292 these two contigs contained all protein coding genes retrieved from the transcriptome that were  
293 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.  
301 Prokaryotic reads were mapped to their respective metagenomic bins - metagenome assembled  
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,  
309 hypoxia and normoxia). Differential gene expression analysis was performed with the package  
310 DESeq2 v. 1.22.2 (Love et al. 2016) in R (R Core Team 2016) with (RStudio Team 2020). Three  
311 pairwise comparisons of oxygen condition (normoxia vs. hypoxia, normoxia vs. anoxia, and  
312 anoxia vs. hypoxia) were computed from DESeq2 results. Significantly differentially expressed  
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.  
315 2013) was used to create PDF pages displaying 4 plots of significantly differentially expressed

316 genes and their annotations in various oxygen conditions, as well as box plots, p-values, and  
317 LFCs (<https://zenodo.org/badge/latestdoi/573040118>). These files are searchable in case  
318 particular genes are of interest to future studies. We note that comparisons to *H. stellifera* under  
319 normoxia were made with only one normoxic sample due to sampling restrictions. Despite this  
320 sampling limitation, the normoxic data for *H. stellifera* are still shown as a baseline and  
321 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  
323 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),  
333 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  
335 documentation), LFC was used here to include changes between normoxia and other conditions  
336 for *H. stellifera*. Since there was only one normoxic replicant for this species, LFC was a more  
337 appropriate metric than signed p values. Within each pairwise comparison (i.e. hypoxic, anoxic,  
338 and anoxic vs hypoxic), significantly enriched KOGs were also calculated using KOGMWU  
339 (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.  
343 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,  
352 recombination and repair' and 'energy production and conversion,' genes that were significantly  
353 differentially expressed were plotted in heatmaps to further investigate sponge transcriptomic  
354 responses to deoxygenation at the gene level in Lough Hyne sponges.

355

356 KOG enrichment analysis was performed as above for each of the two major symbionts within  
357 the two sponge species; however, Clusters of Orthologous groups (COGs) were used since  
358 they are designed for prokaryotes rather than eukaryotic KOGs and are fewer in number.

359

## 360 **Gene ontology (GO) term enrichment analysis**

361 Log fold changes calculated above were used for rank-based gene ontology (GO) analysis with  
362 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  
364 each pairwise comparison of oxygen condition in both sponge species. These analyses were  
365 not completed for the two symbiont types because GO terms were not available for these  
366 microbes.

367

## 368 **Phylogenetic positions of nitroreductase proteins**

369 To determine if nitroreductases in sponge symbionts are related to genes that may be involved  
370 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.  
372 Phylogenetic position of nitroreductase proteins from *H. stellifera* and *E. sp. 2* were investigated  
373 by aligning them to other known nitroreductase proteins from Genbank using Mafft v7 (Kato  
374 and Standley 2013). The ModelTest-NG v.0.1.7 function (Edler et al. 2020) implemented within  
375 RaxmlGUI 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  
377 using raxmlGUI 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 (<http://tree.bio.ed.ac.uk/software/figtree/>).

379

## 380 **Results**

381

### 382 **Sponge differential expression**

383 There were three pairwise comparisons for significantly differentially expressed genes - hypoxia  
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  
387 genes were significantly differentially expressed under hypoxia (vs. normoxia), anoxia (vs.  
388 normoxia), and anoxia vs. hypoxia, respectively ( $p < 0.1$ , Table 1). The differentially expressed  
389 genes under each oxygen condition ranged between 1.5–2.8% of the genes of the *Eurypon sp.*  
390 *2* reference transcriptome, and 41–48% of these genes are annotated, which reflects the overall  
391 annotation percentage of 39.7% for the whole transcriptome (Supplemental Table 2). In  
392 hypoxia, the majority of these differentially expressed genes were upregulated (68%). Under  
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).

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

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

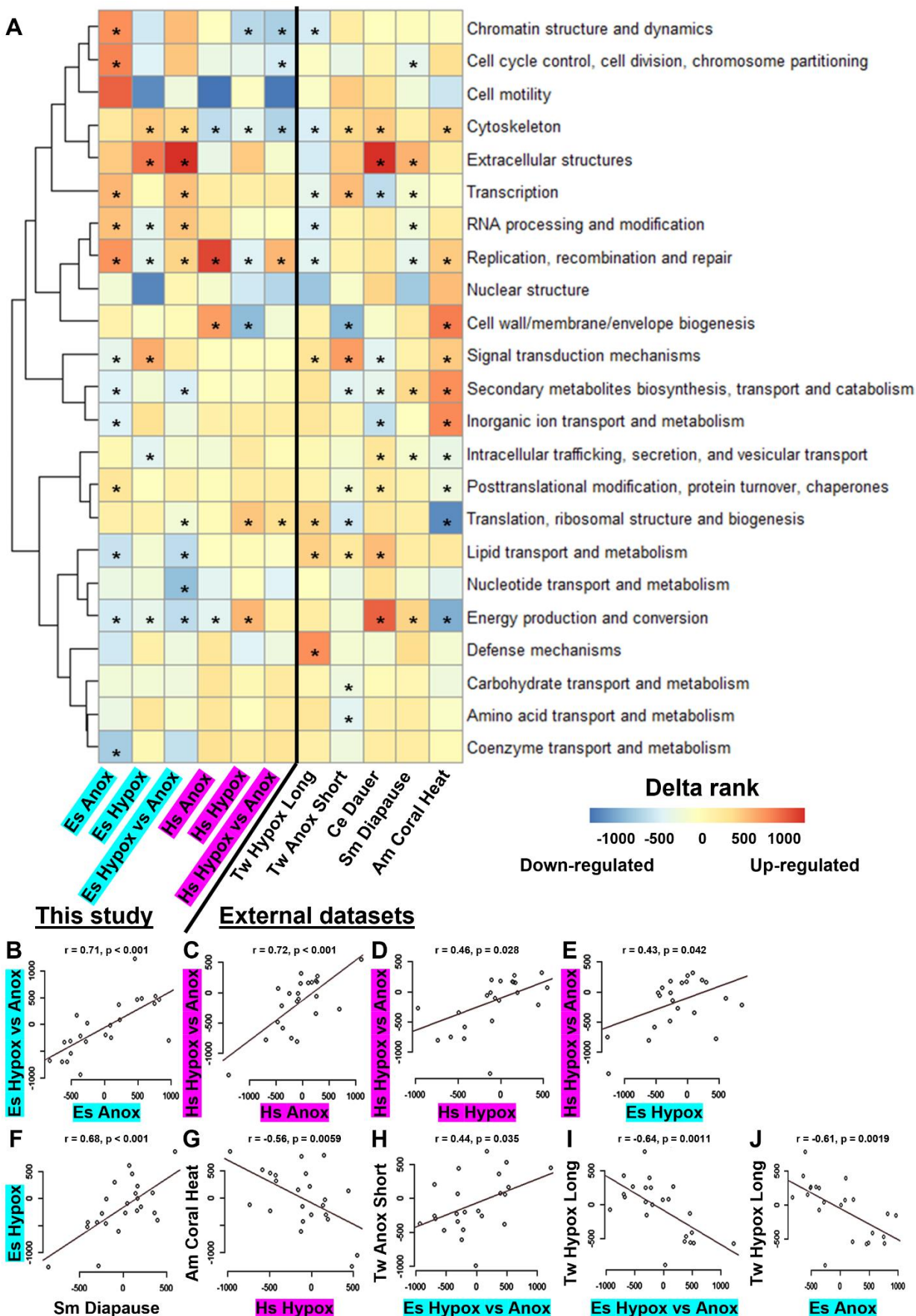
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In the sponge *H. stellifera*, 523, 284, and 2,154 genes were significantly differentially expressed in hypoxia, anoxia, and in anoxia vs. hypoxia, respectively ( $p < 0.1$ , Table 1). Differentially expressed genes in the different oxygen conditions corresponded to 0.42-3.4% of the total number of genes in the *H. stellifera* transcriptome. Approximately 25-37% of the differentially expressed genes were annotated, which, as in the other sponge species, roughly corresponded to the global percentage (37.0%) of genes annotated within the *H. stellifera* reference 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 (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).

#### KOG enrichments in sponges

418 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  
422 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  
447 Within *Eurypon* sp. 2, KOG class expression patterns in hypoxia vs. anoxia were significantly  
448 positively correlated to those of hypoxia ( $r = 0.71$ ,  $p < 0.01$ , Figure 1B). Similarly, in *H. stellifera*,  
449 KOG enrichment in hypoxia vs. anoxia was positively correlated to that of hypoxia ( $r = 0.72$ ,  $p <$   
450  $0.001$ , Figure 1C) and anoxia ( $r = 0.46$ ,  $p < 0.05$ , Figure 1D). All significant correlations between  
451 datasets are shown in (Figure 1B-J), and all relationships that were not significantly correlated  
452 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  
459 In *Eurypon* sp. 2, five genes with RRR KOG annotations were significantly upregulated in  
460 hypoxia, e.g. HELZ and HELZ2 transcription associated helicases, and 11 genes were  
461 significantly downregulated ( $p < 0.1$ , Supplemental Figure 4A), including five genes from the  
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  
464 DNA polymerase epsilon catalytic subunit ( $p < 0.1$ , Supplemental Figure 4B). The majority of  
465 significantly differentially expressed RRR genes (five) were downregulated in anoxia ( $p < 0.1$ ,  
466 Supplemental Figure 4B). The majority of RRR genes (nine) were also downregulated in anoxia  
467 vs. hypoxia, with only two significantly upregulated in anoxia (Supplemental Figure 4B). The  
468 genes NBN and RFC1 were downregulated in anoxia compared to both normoxia and hypoxia  
469 (Supplemental Figure 4B,C). Similarly, the gene 'siz regulation of ARF protein signal  
470 transduction' was upregulated in anoxia compared to both normoxia and hypoxia (Supplemental  
471 Figure 4B,C).

472

473 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*.  
478 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  
482 Like RRR, the KOG class 'energy production and conversion' (EPC) was significantly enriched  
483 ( $P_{\text{adj}} < 0.05$ ) between all conditions except for anoxia vs. hypoxia in *H. stellifera* ( $P_{\text{adj}} = 0.07$ ).  
484 EPC was significantly upregulated in hypoxia in *H. stellifera* but downregulated in all remaining  
485 conditions in both sponge species (Figure 1A).

486  
487 Within *Eurypon* sp. 2 specifically, four genes were significantly downregulated and twelve genes  
488 were upregulated within the EPC class in hypoxia ( $p < 0.1$ , Supplemental Figure 5A). Various  
489 nuclear genes for processes in the mitochondria were differentially expressed in hypoxia.  
490 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  
493 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  
500 sulfite oxidase (SUOX), malate dehydrogenase (MDH1), and two genes belonging to the ATP  
501 gaunido phosphatase family (Supplemental Figure 5B). Conversely, four genes involved in  
502 phosphoenolpyruvate carboxykinase activity were significantly upregulated in anoxia ( $p < 0.1$ ,  
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).  
511 It is noteworthy, however, that all of the upregulated genes in anoxia vs. normoxia and hypoxia  
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

518 upregulated in *H. stellifera* in hypoxia ( $p < 0.1$ , Supplemental Figure 5D). Mitochondrial  
519 sulfide:quinone oxidoreductase (SQRDL), three genes associated with phosphoenolpyruvate  
520 carboxykinase, and a histone demethylase (KDM6A) were also significantly upregulated in  
521 hypoxia in *H. stellifera*. Conversely, PCK1 (also associated with phosphoenolpyruvate  
522 carboxykinase) and MDH1 were significantly downregulated in hypoxia ( $p < 0.1$ , Supplemental  
523 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  
526 (Supplemental Figure 5F).

527  
528 As in hypoxia, SQRDL, four genes associated with phosphoenolpyruvate carboxykinase activity,  
529 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 ( $P_{\text{adj}} < 0.01$ ) in all but one oxygen  
539 condition – anoxia in *Eurypon* sp. 2 ( $P_{\text{adj}} = 0.1$ ). 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  
545 condition and species-specific (Figure 1A). With the exception of EPC, KOGs pertaining to  
546 metabolic processes were rarely significantly enriched. Expression of the KOG classes  
547 'carbohydrate metabolism' and 'amino acid transport and metabolism' were not significantly  
548 changed under any oxygen condition ( $P_{\text{adj}} > 0.05$ ). There was some significant enrichment of  
549 metabolic KOGs, but only in *Eurypon* sp. 2. 'Coenzyme transport and metabolism' and  
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  
554 transcription of the majority of genes labeled with metabolic KOGs remained unchanged under  
555 anoxia and hypoxia.

556  
557 Comparing the Lough Hyne dataset with external datasets of invertebrate KOG expression in  
558 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  
560 anoxia ( $r = 0.44$ ,  $p < 0.05$ , Figure 1H). The hypoxic response of *Eurypon* sp. 2 resembled that of  
561 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

563 under anoxia (versus normoxia) were not positively correlated to any other external datasets for  
564 either sponge species.

565  
566 Three significant negative correlations, which corresponded to opposing KOG expression  
567 patterns, were observed between Lough Hyne sponges and external datasets. Hypoxia  
568 responses in *H. stellifera* were negatively correlated with those of *A. millepora* under heat stress  
569 ( $r = -0.56$ ,  $p < 0.01$ , Figure 1G). The long exposure of *T. wilhelma* to hypoxia was negatively  
570 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  
576 between the two sponge species (Supplemental Figures 1-3). The strongest upregulated GO  
577 enrichments ( $p < 0.01$ ) in hypoxia in *Eurypon* sp. 2 were cellular signaling and 'lipoprotein  
578 particle receptor binding' (Supplemental Figure 1A). For *H. stellifera*, on the other hand, the  
579 strongest upregulated enrichments ( $p < 0.01$ ) in hypoxia included the terms 'protein tyrosine  
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  
594 downregulated in anoxia versus hypoxia ( $p < 0.01$ , Supplemental Figure 3A). The former term  
595 was also upregulated under anoxia vs. normoxia. Conversely, the strongest enrichments in *H.*  
596 *stellifera* in anoxia vs. hypoxia were downregulated in anoxia and were composed of three of  
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 ( $0.01 < p < 0.1$ ), 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 albeit  
604 at varied significance levels ( $p < 0.05$ , 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

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  
614 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  
617 downregulated in hypoxia but upregulated in anoxia ( $p < 0.1$ , Supplemental Figures 1B, 2B  
618 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  
622 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  
624 ribosome' were upregulated in both species in hypoxia ( $p < 0.1$ , Supplemental Figure 1),  
625 although they were enriched to different degrees of significance. No significantly enriched GO  
626 terms were shared between species in normoxia vs. anoxia or hypoxia vs. anoxia.

627  
628 At the gene level, significantly differentially expressed heat shock proteins (Hsps) in both  
629 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  
631 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  
633 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).  
635 In *H. stellifera*, HSP90A1 was upregulated in hypoxia and anoxia ( $p < 0.1$ , Supplemental Figure  
636 8B). Two additional Hsp genes (both for HSP90B1) were also upregulated in anoxia. Finally, the  
637 two HSP90B1 genes were upregulated in anoxia vs. hypoxia, but HSPA5 was downregulated in  
638 anoxia vs. hypoxia ( $p < 0.05$ , Supplemental Figure 8B).

639

#### 640 **Mitochondrial gene expression**

641 All 14 coding genes from the sponge mitochondria were transcribed in both species and in all  
642 oxygen conditions. There was only one mitochondrial gene (*nad2*) that was significantly  
643 differentially expressed but only in *Eurypon* sp. 2. This gene was significantly upregulated ( $p <$   
644  $0.1$ ) in anoxia vs hypoxia (Supplemental Figure 6A, Table 1). Similar counts for each individual  
645 mitochondrial gene across both sponge species and all oxygen conditions were found. For  
646 instance, counts for *atp6* were consistently twice as high as counts for *atp8* (Supplemental  
647 Figure 6).

648

649

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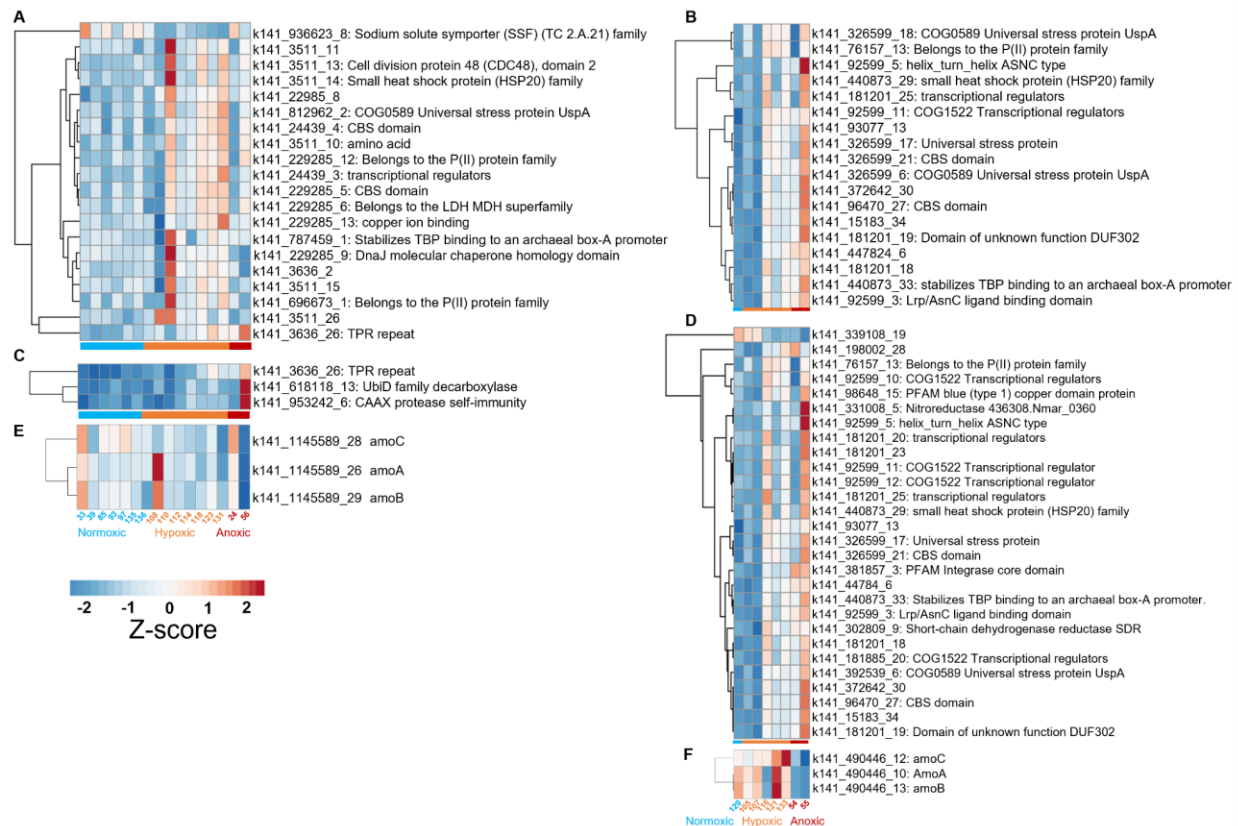
## 652 Prokaryotic differential expression

653

### 654 Thaumarchaeota gene expression

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

666



667

668 **Figure 2.** A-D. All significantly differentially expressed genes for the *Thaumarchaeota* in both sponge  
 669 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  
 673 three genes involved in ammonia oxidation (AmoA, B and C) in either symbiont, but their heatmaps are  
 674 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  
678 anoxia were a UbiD family decarboxylase, CAAX protease self-immunity, and the same TPR  
679 repeat that was also upregulated in hypoxia. There were no significantly differentially expressed  
680 genes between anoxia and hypoxia in this *Thaumarchaeota* (Table 1). The genes necessary for  
681 ammonia oxidation, i.e. subunits A, B and C of ammonia monooxygenase (Amo), were present  
682 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  
688 were upregulated in hypoxia. These genes included the stress response genes 'universal stress  
689 protein, an HSP20 family protein, and two copies of UspA. HSP20 and UspA were also  
690 upregulated in the *Eurypon* sp. 2 holobiont. Similarly, a PII signal transduction protein, CBS  
691 domain, and two transcription regulators (one identical: TBP stabilizer, and one distinct to *H.*  
692 *stellifera* symbionts) were upregulated in both *Thaumarchaeota* species in hypoxia. Unlike in  
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*.

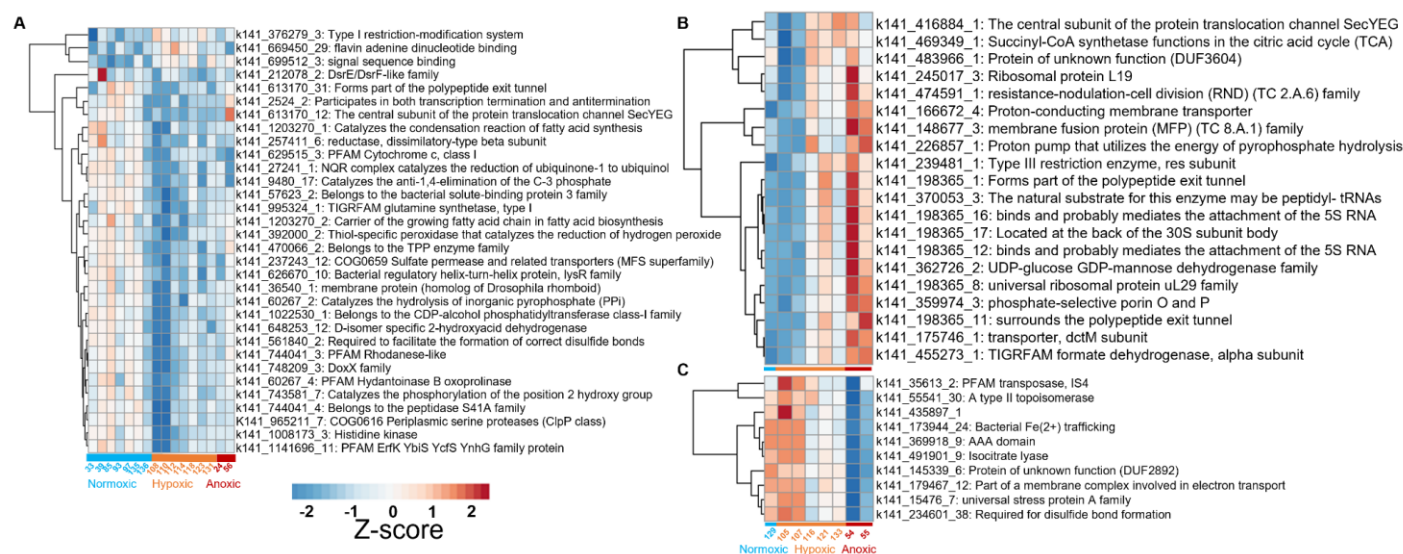
696  
697 As in the other holobiont species, *Thaumarchaeota* genes for ammonia oxidation were not  
698 significantly differentially expressed with *H. stellifera* symbionts (Figure 2F). Twenty-seven  
699 genes were upregulated and one gene (K141\_339108\_19, unannotated) was downregulated in  
700 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  
705 publicly available nitroreductase sequences from other *Thaumarchaeota* are shown in  
706 Supplemental Figure 9.

707  
708 For both *Thaumarchaeota*, only two COGs ('signal transduction mechanisms' and  
709 'transcription') were significantly enriched ( $P_{\text{adj}} < 0.05$ , Supplemental Figure 7A). These two  
710 COGs were both downregulated in hypoxia and anoxia vs. hypoxia within *H. stellifera*  
711 symbionts. All other COG classes were comparable in their expression levels regardless of  
712 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  
716 expression under hypoxia but not in any other oxygen condition (Table 1). In hypoxia, three  
717 genes were upregulated: type 1 restriction-modification system, flavin adenine dinucleotide  
718 binding, and signal sequence binding (Figure 3A). The 29 downregulated genes in hypoxia  
719 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

721 (k561840\_2), a NQR complex gene, various genes involved in pore formation, a Rhodanese-  
 722 like gene, glutamine synthetase (type I), and YbiS YcfS YnhG family protein.  
 723  
 724  
 725



726  
 727 **Figure 3.** All significantly differentially expressed genes for the *Gammaproteobacteria* in both  
 728 sponge species (A: *Eurypon* sp 2, B: *H. stellifera*. For *Gammaproteobacteria* associated with  
 729 *Eurypon* sp. 2, there were only significant differences between hypoxia vs. normoxia (A). In  
 730 symbionts associated with *H. stellifera*, there were only significantly differentially expressed  
 731 genes in anoxia vs. hypoxia (B,C). All downregulated genes in anoxia vs. hypoxia are shown in B.  
 732 There were 402 genes that were downregulated in anoxia vs. hypoxia in *Gammaproteobacteria*  
 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  
 736 differentially expressed between anoxia and hypoxia (436 genes,  $p < 0.1$ , Table 1, Figure 3B,C).  
 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  
 739 (k141\_198365\_15, k141\_198365\_8, k141\_198365\_17, and k141\_245017\_3), various genes for  
 740 molecular transport, a transmembrane proton pump (k141\_226857\_1), succinyl-CoA  
 741 synthetase, and formate dehydrogenase (alpha subunit). There were also 416 genes that were  
 742 significantly downregulated in anoxia vs. hypoxia (Table 1). The top ten upregulated genes  
 743 (lowest p values) are exhibited in Figure 3C.

744  
 745 *Gammaproteobacterial* COG expression is shown in Supplemental Figure 7B. Only one COG  
 746 class, 'translation, ribosomal structure and biogenesis,' was significantly enriched in  
 747 *Gammaproteobacteria* under the different oxygen conditions ( $P_{adj} < 0.05$ ). This COG class was  
 748 downregulated in *Gammaproteobacteria* associated with *Eurypon* sp. 2 under hypoxia as well  
 749 as those of *H. stellifera* in anoxia vs. hypoxia (Figure 7B).

750

## 751 Discussion

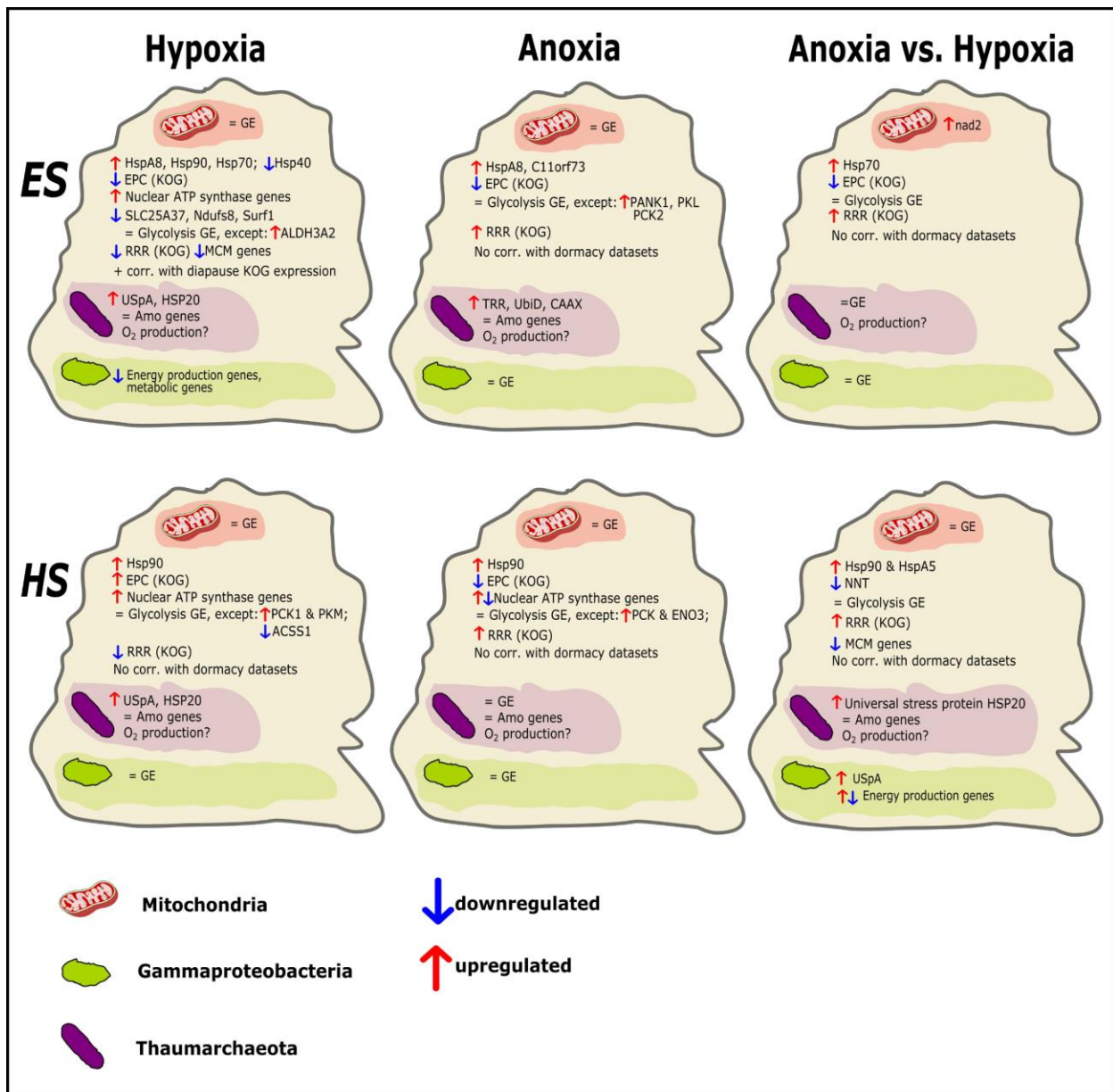
752

753 Sponge holobiont gene expression was significantly changed by exposure to deoxygenation in  
754 situ, though responses were generally species-specific, dependent on oxygen level, and varied  
755 across holobiont members. The potential functions of this differential expression are discussed  
756 below for each member of the two holobionts: sponge, sponge mitochondria, *Thaumarchaeota*  
757 and *Gammaproteobacteria*. The implications of this study for past and future oceans are  
758 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  
762 genes that have been widely studied in marine invertebrates. These proteins are molecular  
763 chaperones involved in protein folding, unfolding, transport and the assembly of protein  
764 complexes, and they are present in nearly all organisms (Li and Srivastava 2004). Both Lough  
765 Hyne sponges upregulated HSP genes in response to hypoxia and anoxia (vs normoxia),  
766 though there was downregulation of some HSP genes in deoxygenation as well. *Eurypon* sp. 2  
767 and *H. stellifera* both upregulated Hsp90 genes in hypoxia, but they upregulated different HSPs  
768 in anoxia: HSPA8 and Hsp90 for *Eurypon* sp. 2 and *H. stellifera*, respectively. It should be noted  
769 that Hsp90 upregulation in *H. stellifera* in anoxia was driven by only one replicate. However, in  
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  
775 replication (Schurch et al., 2016). *Eurypon* sp. 2 also upregulated HSPA8 and Hsp70 in hypoxia  
776 but downregulated Hsp40. In long term hypoxia, *T. wilhelma* exhibited a similar HSP expression  
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.



783  
 784 **Figure 4.** Summary of sponge holobiont responses to deoxygenation presented in a tabular  
 785 format with species on the left (*Es*: *Euryyon* sp. 2, *Hs*: *H. stellifera*) and oxygen condition on the  
 786 top (hypoxia, anoxia, and hypoxia vs. normoxia). The major components of the holobiont are  
 787 illustrated as follows: sponge cell (tan), mitochondria (red), *Gammaproteobacteria* (green), and  
 788 *Thaumarchaeota* (purple). Upregulated and downregulated genes and KOGs are indicated with  
 789 red and blue arrows, respectively. Unchanged gene expression (GE) is noted by the = sign.  
 790 Correlations (corr.) with external dormancy datasets are indicated along with the direction of  
 791 the correlation (+ or -).

792

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



795 sponges, Hsp90 may play a greater role in oxygen homeostasis given that it is upregulated  
796 under hypoxia in all sponges that have been examined transcriptomically to date, i.e. *Eurypon*  
797 sp. 2, *H. stellifera* and *T. wilhelma*, and that Hsp90 is upregulated in *H. stellifera* under anoxia.  
798 Hsp90 could stabilize currently unknown proteins that are involved in sponge oxygen regulation  
799 or modulate transcription factors other than HIFa. Further research is needed to determine the  
800 exact role of Hsp90 in sponge oxygen homeostasis, but it presents a good candidate for a gene  
801 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  
804 binding' were upregulated in all cases of decreased oxygen in *Eurypon* sp. 2, suggesting that  
805 the species-specific response to deoxygenation in *Eurypon* sp. 2 may center around repairing or  
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  
808 and RNA,' so DNA repair may be more central to the deoxygenation tolerance of this species.  
809 Both protein and DNA repair upregulation might be a response to reactive oxygen species  
810 (ROS), which can become more abundant under hypoxia due to imbalances in the  
811 mitochondrial electron transport chain (Görlach et al. 2015). If there is any oxygen present in the  
812 sponges under environmental anoxia, e.g. oxygen produced by *Thaumarchaeota* (see Kraft et  
813 al. 2022, [Thaumarchaeota gene expression](#) below), then ROSs could cause cellular stress in  
814 anoxia. Otherwise, the presence of sulfide in the water under anoxia (Schuster et al. 2021) or  
815 ROSs other than superoxide (O<sub>2</sub><sup>-</sup>) 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  
818 and unchanged, respectively (Figure 1, 4). Within the EPC class, however, there were species-  
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  
823 ATP6V0C was downregulated. Additionally, downregulation of SLC25A27 in *Eurypon* sp. 2 in  
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  
826 downregulated in hypoxia *Eurypon* sp. 2. The NNT gene, also involved in electron transport,  
827 was downregulated in anoxia vs. hypoxia in *H. stellifera*. Nevertheless, most of the genes  
828 associated with the respiration electron transport chain were not differentially expressed, and  
829 electron transport likely continued under deoxygenation. Sponges may therefore have increased  
830 ATP production under hypoxia but not under anoxia. In a similar way, mollusks may upregulate  
831 mitochondrial electron transport system activity and ATP synthesis capacity to improve  
832 respiration efficiency under hypoxia (Hochachka and Lutz 2001).

833 Glycolysis, which can produce ATP and NADH without oxygen (Stryer 1995), was expected to  
834 increase under anoxia. However, relatively few genes directly or indirectly involved in glycolysis  
835 were differentially expressed in our sponges under deoxygenation. In *Eurypon* sp. 2,  
836 pantothenate kinase 1 (PANK1), ATP-dependent 6-phosphofructokinase (PFKL) and  
837 Phosphoenolpyruvate carboxykinase 2 (PCK2) were significantly upregulated in anoxia, and

838 only aldehyde dehydrogenase 3 family, member a2 (ALDH3A2) was significantly upregulated in  
839 hypoxia (see Supplemental Reports, Supplemental Figure 5). In *H. stellifera*, acetyl-coenzyme A  
840 synthetase 1 (ACSS1) was significantly downregulated in hypoxia, but PCK1 and pyruvate  
841 kinase (PKM) were upregulated in hypoxia. In anoxia for *H. stellifera*, beta-enolase (ENO3) and  
842 PCK1 were upregulated. Based on these results, it is unlikely that glycolysis rates increased  
843 under anoxia and hypoxia in either species. It is more probable that glycolysis continued under  
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  
847 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  
850 both sponge species. In *Eurypon* sp. 2, many MCM genes involved in the initiation of DNA  
851 replication were downregulated in hypoxia but remained unchanged in anoxia. Certain aspects  
852 of DNA replication, e.g. the DNA polymerase epsilon catalytic subunit, may even be upregulated  
853 in anoxia. The pattern was less clear in *H. stellifera*, but MCM genes were downregulated in  
854 anoxia compared to hypoxia (Figure 4). These data indicated that DNA replication may  
855 decrease under hypoxia but remain stable during anoxia, possibly due to increased oxidative  
856 stress under hypoxia compared to normoxia (see above). This pattern of a 'stronger' response  
857 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  
859 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  
861 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  
864 positively correlated with those of anoxia in *Eurypon* sp. 2 and both anoxia and hypoxia in *H.*  
865 *stellifera*.

866 When quantitatively compared to transcriptomic datasets from the sponge *T. wilhelma* using  
867 KOGMWU, transcriptomic responses in Lough Hyne sponges were only positively correlated  
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.*  
870 *wilhelma* and *Eurypon* sp. 2 transitioning from hypoxia to anoxia. However, no further  
871 similarities between the responses of *T. wilhelma* and Lough Hyne sponges to deoxygenation  
872 were observed. In fact, the long-term exposure of *T. wilhelma* to hypoxia was negatively  
873 correlated to KOG enrichments for both anoxia vs. hypoxia and normoxia vs. anoxia in *Eurypon*  
874 sp. 2 (Figure 1I, J), meaning that KOG expression was generally opposite between these two  
875 species for these conditions in terms of up and down regulation. The physiological responses of  
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  
884 dormancy datasets come from distantly related invertebrates, Strader et al. (2016)  
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  
888 only be considered dormant under anoxia if their transcriptional method of dormancy was  
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  
892 under hypoxia (Schuster et al. 2021). Sponge pumping and filtering activity remain to be  
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  
896 the anoxic response, was similar to diapause in terms of global KOG expression patterns. The  
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  
900 significantly downregulated in hypoxia in *Eurypon* sp. 2 and upregulated in diapause in *S.*  
901 *Mosellana*. 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  
906 sponges under hypoxia but not anoxia based on MCM gene expression. In comparison, KOG  
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  
910 literature (Dixon et al. 2015; Strader et al. 2016).

911

## 912 **Sponge mitochondrial gene expression during deoxygenation**

913

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

920

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  
924 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  
929 compared to normoxia and anoxia, but the opposite was true for *nad2* in *Eurypon* sp. 2 in the  
930 current study. The other six *nad* genes, which code for parts of the same membrane respiratory  
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  
935 physiological meaning (if any) of this single, significantly differentially expressed mitochondrial  
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  
941 sequencing mitochondrial RNA and/or the exclusion of mitochondrial RNA from reference  
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,  
944 mitochondrial transcripts are broken up into individual coding mRNA, tRNA or rRNA (D'Souza  
945 and Minczuk 2018). After the breakup, the levels of mRNAs from specific genes are controlled  
946 post-transcriptionally (D'Souza and Minczuk 2018). It is therefore possible to have different  
947 counts between the different mitochondrial genes despite their single transcript origin  
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  
952 It remains unclear how sponge mitochondria maintain this transcription from an energetic  
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  
955 phosphorylation. Theoretically, anoxia should cause electron transport and oxidative  
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  
961 provide some anaerobic ATP production, even if it is not upregulated in anoxia or hypoxia.  
962 Alternatively, to maintain ATP production through aerobic respiration, the sponges of Lough  
963 Hyne might have access to oxygen produced by their *Thaumarchaeota* symbionts, a topic  
964 explored in more detail below.  
965

## 966 **Microbiome gene expression during deoxygenation**

### 967 *Thaumarchaeota* gene expression

968 *Thaumarchaeota* symbionts from both sponge species exhibited significant differential gene  
969 expression under hypoxia. Only the *Thaumarchaeota* from *Eurypon* sp. 2 (OTU1) exhibited  
970 differential gene expression under anoxia, and only those of *H. stellifera* (OTU3) differentially  
971 expressed genes under anoxia vs. hypoxia. Though there were some species-specific  
972 responses, overall gene expression in both *Thaumarchaeota* was similar, supporting the  
973 hypothesis from Schuster et al. (2021) that these taxonomically similar symbionts between two  
974 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  
978 in hypoxia. Genes for the other anaerobic pathways suggested by Bayer et al. 2020, i.e.  
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 *Thaumarchaeota*, and so did  
983 the dehydrogenase/ fumarate reductase gene in the *Thaumarchaeota* 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  
992 sponge mitochondria, the *Thaumarchaeota* are therefore either transcribing AMO genes to be  
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  
995 produce oxygen under anoxic conditions like their relative *N. maritimus*, then they should  
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  $\mu\text{M}$  (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.

1003 AsnC family proteins and nitroreductase expression was upregulated during oxygen production  
1004 in *N. maritimus* (Kraft et al. 2021). In *Archaea*, AsnC proteins can regulate metabolic processes,  
1005 including the switch between aerobic and anaerobic metabolisms (Lemmens et al. 2019).  
1006 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  
1016 oxygen-minimum zones (OMZs) where oxygen is either at low concentrations or below  
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,  
1019 increase their absolute abundance in suboxic regions, but then decrease in abundance with  
1020 depth in the anoxic zone (Sollai et al. 2019), indicating a preference for suboxic environments. If  
1021 suboxia is also the preferred niche of sponge *Thaumarchaeota* symbionts, the upregulation of  
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  
1031 involved in cell wall production (Magnet et al. 2008). So unlike sponges or *Thaumarchaeota*,  
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  
1040 associated with energy production were downregulated. Formate dehydrogenase can function  
1041 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  
1055 the holobiont members, and there was no evidence of dormancy within the holobionts in anoxia  
1056 (Figure 4). No clear anaerobic respiration responses were observed in the transcriptomes of  
1057 sponges, their mitochondria or their two microbial symbionts under environmental anoxia. The  
1058 limited significant differential expression of glycolysis genes could indicate that this metabolic  
1059 process continues to comparable degrees under normoxia, hypoxia, and anoxia. Therefore, in  
1060 the sponges and their microbes, glycolysis could potentially provide ATP without oxygen (Figure  
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<sup>-1</sup>), 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<sup>-1</sup>) 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.

1071 For both the sponges and their *Thaumarchaeota* symbionts, cellular stress responses were  
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  
1075 needs to be tested. The *Thaumarchaeota* symbionts upregulated HSP20 and other stress  
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  
1078 to oxidative stress following increased metabolic activities. In anoxia, cellular stress response  
1079 might result from the presence of sulfide. Additionally, post-transcriptional, metabolic changes  
1080 may play a role in deoxygenation tolerance, and proteomic and metabolomic changes should be  
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  
1085 extremely low concentrations, it would have a short path of diffusion to the sponge cells, as  
1086 *Thaumarchaeota* symbionts maintain high cell densities within sponges (Moeller et al. 2019). In  
1087 addition, mitochondria in general can utilize very low concentrations of oxygen (500 nM) without  
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  
1098 tolerance of ocean warming and acidification associated with climate change (Bell et al. 2018).  
1099 Indeed, sponges have already increased in abundance in some locations while corals have  
1100 decreased due to altered carbon and nutrient cycling (Pawlik et al. 2016). The additional  
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 (Vaquer-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  
1108 warming, acidification (Bennett et al. 2017) and deoxygenation (Micaroni et al. 2021) in  
1109 scenarios that might occur with climate change, since tolerance to these stressors is species-  
1110 specific. So, surviving sponge species in a warmed, acidified, and deoxygenated ocean might  
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  
1113 organisms to accurately predict the composition of future oceans. More importantly, the causes  
1114 and effects of anthropogenic climate change need to be ameliorated as soon as possible to  
1115 avoid such diversity loss.

### 1116 **Relevance for animal evolution**

1117 Assuming that the first metazoans were more like an adult sponge than a ctenophore, based on  
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  
1121 years ago) when early animals, including sponges, first evolved (Dohrmann and Wörheide  
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: <https://zenodo.org/badge/latestdoi/573040118>. Supplemental figures and  
1143 tables are available on BioRxiv under 'Supplementary material.'

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