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

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Abstract

Deoxygenation can be fatal for many marine animals; however, some sponge species are tolerant of hypoxia and anoxia. Indeed, two sponge species, *Eurypon* sp. 2 and *Hymeraphia stellifera,* survive seasonal anoxia for months at a time. To understand their tolerance mechanisms, we performed differential gene expression analyses on the sponges, their mitochondria and their microbial symbionts under *in situ* conditions of normoxia, hypoxia and anoxia. Each species possessed a unique microbiome, but the microbiomes of each species were dominated by a species-specific *Thaumarchaeon* and a *Gammaproteobacterium*. Holobiont gene expression was species and oxygen level dependent, though there were some shared interspecific responses to deoxygenation. In general, few changes occurred in the expression of sponge metabolic genes as a function of oxygenation level, indicating that they may remain metabolically active under anoxia. However, ATP synthesis genes were significantly upregulated under hypoxia when compared to normoxia, and genes for DNA replication were downregulated. Mitochondrial gene expression was effectively unchanged under both hypoxia and anoxia. Nevertheless, both anoxia and hypoxia caused upregulation of heat shock proteins (HSPs), indicating cellular level adaptations to deoxygenation stress. A meta-analysis demonstrated that sponge transcriptional responses to anoxia were distinct from those displayed by other invertebrates while dormant, rejecting the hypothesis of sponge dormancy under anoxia. *Thaumarchaeota* symbionts also upregulated stress response genes in hypoxia, while maintaining expression of oxygen-dependent metabolic pathways under hypoxia and anoxia. *Gammaproteobacteria* symbionts showed relatively few noteworthy changes in gene expression in response to anoxia but decreased metabolic gene expression in hypoxia. There was no clear evidence of upregulated anaerobic respiration in the transcriptomes of the sponge holobionts under anoxia or hypoxia. The tolerance of some sponges to prolonged anoxia warrants further investigation and could give them an advantage in future oceans following climate change as well as in ancient oceans when oxygen concentrations were lower than at present.

Introduction

Ocean oxygen levels are predicted to decline by 1–7% over the next century (Keeling, Körtzinger, and Gruber 2010). Climate change is expanding marine oxygen-minimum zones, while enhanced nutrient runoff is causing coastal eutrophication and subsequent deoxygenation (Kirchman 2021). Deoxygenation is expected to negatively affect the physiology, metabolism and survival of many marine organisms (Diaz and Rosenberg 2008). As a result of deoxygenation and other anthropogenic stressors, we are facing large scale habitat and biodiversity losses, but the full effects of these stressors are still unknown.

Sessile organisms that cannot move or migrate in response to deoxygenation, are particularly vulnerable. Sponges (Phylum: Porifera Grant, 1836), sessile, ecologically important filter-feeding animals, are hypothesized to suffer from increasing hypoxic/anoxic areas worldwide (Breitburg et al. 2018; Levin 2018; Stramma et al. 2008), and some *in situ* mortality of sponges (along with other taxa) following deoxygenation has been reported (Altieri et al. 2017; Stachowitsch 1984). However, species specific responses to deoxygenation in Porifera are variable, and some species are tolerant of hypoxia. Lab experiments have shown that the demosponge *Halichondria (Halichondria) panicea* Pallas, 1766 can survive low oxygen levels down to 0.5–4% of air saturation (a.s.) (Mills et al. 2014), and *Tethya wilhelma* can survive down to 0.25–2% a.s. for 4 days and <0.05% a.s. for at least one hour (Mills et al. 2018). Additional demosponge species that tolerate similar levels of hypoxia include *Geodia barretti* Bowerbank, 1858 with 7% a.s. (20 µM O2, (Leys and Kahn 2018)) and *Haliclona pigmentifera* Dendy, 1905 (0.50-0.68% a.s., (Gunda and Janapala 2009)). In a recent experimental study, four taxonomically distinct sponge species (*Cliona celata* Grant, 1826, *Suberites carnosus* Johnston, 1842, *Polymastia crocea* Kelly-Borges and Bergquist, 1997 and *Suberites australiensis* Bergquist, 1968) were exposed to various levels of hypoxia (1.5–40% a.s., 0.13–3.3 mg O_2 L^{-1}) in aquaria. These sponges exhibited species-specific physiological, morphological and behavioral responses to deoxygenation. Sub-lethal oxygen thresholds for these sponges were in the range of 6–20% a.s. (0.48–1.56 mg O_2 L^{-1}) while mortality was only observed in one of the two species (*P. crocea*) exposed to severe hypoxia lower than 5% a.s. (0.13 mg O_2 L^{-1}) (Micaroni, Strano, et al. 2021). The sponge species most sensitive to deoxygenation in controlled experiments, *P. crocea*, had a median lethal time (time until death, LT50) of 286 h at ~1.5% a.s. (0.13 mg O_2 L^{-1}, (Micaroni, Strano, et al. 2021), which was still longer than most marine organisms (reviewed in Micaroni, Strano, et al. 2021 and Hughes et al. 2020).

Few studies, however, have investigated the deoxygenation tolerance of sponges *in situ*. Bayer et al. (2020) found that the glass sponge *Vazella pourtalesii* Schmidt, 1870 lives in low-oxygen conditions (< 4 mL/L) on the Scotian Shelf, Canada. Schuster et al. (2021) found at least nine encrusting demosponge species of the orders Axinellida Lévi, 1953 and Poecilosclerida Topsent, 1928 that tolerate seasonal anoxia in a Marine Lake in Lough Hyne, Ireland for ~three months at a time, and there are more sponge species at this seasonally anoxic site (Bell and Barnes 2000). The anoxia tolerant sponges of Lough Hyne therefore have LT50s that exceed their ca. three month exposure to anoxia, which are rivaled only by the anemone *Bunodosoma cavernatum* Bosc, 1802 (LT50 in anoxia >1.5 months, (Ellington 1982). Moreover, the abundance (percent cover) of encrusting sponges in the seasonally anoxic site of Lough Hyne

only slightly decreased over 30 years despite widespread sponge losses in the lough (Micaroni, McAllen, et al. 2021), indicating that these sponges are uniquely suited to their seasonally anoxic niche.

Many invertebrates exposed to harsh conditions like anoxia or hypoxia have evolved molecular response pathways to survive in these environments. For instance, under anoxia, marine mollusks shift from lipid, carbohydrate and amino acid metabolism to carbohydrate catabolism as the primary fuel for ATP generation (Larade and Storey 2009). Some mollusks also maximize anoxic survival time (mollusc LT50s at 0 mg L^{-1}: 360–672 h, (Micaroni, Strano, et al. 2021)) by building up a fixed reserve of internal fuels (primarily glycogen) to increase ATP output and to reduce acidity. A small suite of upregulated genes also plays a critical role in cell preservation, facilitating long-term viability under hypoxia (Larade and Storey 2009). Some anemones may have elements of a facultative anaerobic metabolism (Sassaman and Mangum 1972); however, even the anoxia-tolerant *B. cavernatum* requires more oxygen than normal to recover after exposure to anoxia (Ellington 1982).

Only one study has investigated the transcriptomic response of a sponge to hypoxia. After four days of exposure to hypoxia (0.25–2% a.s.), 128 genes within the sponge *T. wilhelma* were differentially expressed compared to controls, but none of these genes were clearly related to metabolic or stress responses (Mills et al. 2018). However, one hour exposure to near anoxia (<0.05% a.s., 105 nM O_2) elicited stress responses in *T. wilhelma's* transcriptome (Mills et al. 2018), demonstrating that transcriptomic responses were mostly observed under very low oxygen levels. Nevertheless, the underlying mechanisms of sponge deoxygenation tolerance and long-term anoxia tolerance are largely unknown.

Since parts of the hypoxia-inducible factor (HIF) pathway, which mediates the hypoxic responses in other animals, are lacking in sponges (Mills et al. 2018), we hypothesized that the sponge microbiome may play a role in their deoxygenation tolerance. Sponges can host diverse microbiomes that perform vital functions and can constitute large portions of their biomass (Wilkinson 1978). The host and microbiome together are referred to as a 'holobiont' (N. S. Webster and Taylor 2012). Some sponge symbionts have anaerobic capabilities, including sulfate reduction (Hoffmann et al. 2005), denitrification, and anaerobic ammonium oxidation (anammox) (Hoffmann et al. 2009). In *V. pourtalesii*, the microbial community is thought to be specialized for the low-oxygen conditions in which it lives (Bayer et al. 2020).

The microbiomes of anoxia-tolerant sponges from Lough Hyne were unchanged under anoxia, hypoxia and normoxia despite drastic changes in the microbial communities of the water and sediment, indicating that the holobiont as a whole is tolerant to deoxygenation. Furthermore, Schuster et al. (2021) hypothesized potential metabolic pathways within the microbiome that might function under deoxygenation. The microbiomes of the anoxia tolerant sponge species *Eurypon* sp. 2 and *Hymeraphia stellifera* Bowerbank, 1864 were both dominated by two microbes – one *Thaumarchaeon* and one *Gammaproteobacterium*, though each sponge hosted a different species from each clade. *Eurypon* sp. 2 harbors the *Thaumarchean* OTU1 (closest related isolate: *Nitrosopumilus adriaticus* strain NF5), and *H. stellifera* harbors OTU3 (closest related isolate: *Nitrosopumilus oxyclinae* strain HCE1). For the *Gammaproteobacteria*, *Eurypon*

135 sp. 2 contains OTU2 (closest related isolate: *Arenicella chitinivorans* strain ALEN 1); whereas *H.*
136 *stellifera* contains OTU7 (closest related isolate: *A. chitinivorans* strain ALEN 1, (Schuster et al.
137 2021).
138
139 To understand the mechanisms of sponge holobiont tolerance and survival under extended
140 anoxia, we performed differential gene expression analysis of the sponges *Eurypon* sp. 2 and *H.*
141 *stellefera* as well as their respective mitochondria and microbial symbionts including
142 *Thaumarchaeota* and *Gammaproteobacteria*. Sponges were collected from Lough Hyne under
143 *in situ* normoxia, hypoxia and anoxia. Since neither of these holobionts are established model
144 species, we generated reference transcriptomes for the sponges and assembled the genomes
145 of their symbionts and mitochondria from metagenomic sequences. Additionally, we performed
146 a meta-analysis of the sponge differential expression data to determine if the responses of
147 Lough Hyne sponges to deoxygenation are similar to those of *T. wilhelma* under deoxygenation,
148 other invertebrates under heat stress (Dixon et al. 2015), in a dauer state (Sinha, Sommer, and
149 Dieterich 2012), and during diapause (Gong et al. 2013). Finally, we considered the implications
150 of sponge deoxygenation tolerance for future oceans as well as early animal evolution under
151 low atmospheric oxygen concentrations.
152

153 ## Materials and Methods

154

155 ### Sampling

156 Sponges were sampled between July 2018 and August 2019 at Labhra Cliff (Lough Hyne
157 Nature Reserve, Ireland) as described in Schuster et al. (2021) under permit no. R23-27/2018
158 issued by the Irish Department of Environment, Heritage, and Local Governments. Samples
159 were taken under various *in situ* oxygen conditions that were categorized as follows: normoxic
160 (5.3–12 mg L^{-1}, 49.3–111% a.s.), hypoxic (1.30–3.56 mg L^{-1}, 12.1–33.1% a.s.), and anoxic
161 (0.00–0.01 mg L^{-1}, 0.00–0.93% a.s., i.e. instrument detection limit). All samples were frozen in
162 liquid nitrogen within ~15 minutes of collection and kept at liquid nitrogen temperatures in a dry
163 shipper until returned to the laboratory where samples were stored in a -80°C freezer until
164 extraction. The sponges were identified using molecular barcodes (see Schuster et al. (2021))
165 as *Eurypon* sp. 2 and *H. stellifera*.
166

167 ### DNA/RNA extraction

168 Total RNA was isolated using TRIzol™ Reagent from Invitrogen (ThermoFisher Scientific,
169 Denmark). For RNA sequencing (RNAseq), eight samples of *H. stellifera* (anoxic = 2, hypoxic =
170 5, normoxic = 1) were taken, and sixteen samples of *Eurypon* sp. 2 (anoxic = 2, hypoxic = 7,
171 normoxic = 7) were collected (n = 24 in total). Since these two species are indistinguishable *in*
172 *situ*, the samples taken for each species were unequal in number. Remaining DNA in the RNA
173 extracts were removed using the DNase MAX kit (Qiagen).
174
175 Additionally, high molecular weight (HMW) DNA was extracted from one individual of each
176 species using the protocol from Vargas et al. (2021) for metagenomic sequencing. The *Eurypon*
177 sp. 2 sample used for metagenomic sequencing was taken under anoxia (DC24), and RNA was
178 also sequenced from this sample. Due to limited availability of samples and DNA, a sample of
179 *H. stellifera* that was not included in the RNAseq (DC297, sampled under normoxia) was used

for metagenome sequencing. DNA and RNA yields were determined using a Qubit™ (Thermo Fisher Scientific, USA) and a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, US). RNA quality was evaluated on an Agilent TapeStation using an RNA ScreenTape (Agilent Technologies).

Reference transcriptomes and metatranscriptomes

All RNA samples were sequenced twice with two different library preps - one for polyadenylated (poly A) messenger RNA (mRNA, from eukaryotes) and one for total RNA (to also capture prokaryotic RNA). Library preparation for the eukaryotic (sponge) RNA was done using NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England Biolabs, USA). For the prokaryotic RNAseq, the supernatant from the poly A enrichment was treated with NEBNext® rRNA Depletion Kit (Bacteria) and then NEBNext® rRNA Depletion Kit (Human/Mouse/Rat, New England Biolabs, USA) to eliminate ribosomal RNA (rRNA) that causes sequence biases. This combination of poly A exclusion and rRNA depletion eliminated the most rRNA compared to other combinations of methods (or single methods in isolation) in preliminary tests, yielding sequences with only ~25% rRNA. For the eukaryotic (n = 24) and prokaryotic (n = 24) RNA samples, library preparation was completed using the NEBNext Ultra II Directional RNA library preparation kit (New England Biolabs, Denmark). Libraries pooled in equimolar concentrations were sequenced (5M reads per sample, 2 x 50 bp) at the Center for Functional Genomics and Tissue Plasticity (ATLAS) at SDU, using a NovaSeq 6000 (Illumina, USA).

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

For the eukaryotic RNA, shallow (2 x 50 bp) sequencing generated 9.20 x 10^8 and 4.38 x 10^8 reads for *Eurypon* sp. 2 and *H. stellifera*, respectively, across all sampled sponges, and deeper sequences (2 x 150 bp) generated 2.05 x 10^8 and reads 3.45 x 10^8 for each sponge of the two species, respectively. For the prokaryotic RNA, shallow sequencing yielded 1.27 x 10^9 and 6.68 x 10^8 reads for *Eurypon* sp. 2 and *H. stellifera*, respectively. The deeper prokaryotic sequencing generated 1.52 x 10^8 and 1.20 x 10^8 for *Eurypon* sp. 2 and *H. stellifera*, respectively (totals are shown in Supplemental Table 1).

To obtain reference transcriptomes for each sponge species without prokaryotic symbionts, all eukaryotic RNA sequences were combined into a single file. These sequences were trimmed for quality control using public scripts (Matz 2015; Meyer 2016) and assembled with Trinity v 2.8.5 (Grabherr et al. 2011). This assembly was filtered for contaminants and annotated using egg-NOG mapper (Huerta-Cepas et al. 2019). The final sponge transcriptomes included only genes with metazoan annotations or no annotations. For a detailed description of all filtering and assembly steps, see Strehlow et al. (2020) and (https://zenodo.org/badge/latestdoi/573040118) for all scripts used in the current paper. Sponge reference transcriptomes fit within the expected range of size, gene number, and N50 (Supplemental Table 1) of published sponge transcriptomes (Strehlow et al. 2020; Riesgo et al. 2014). Additionally, sponge reference transcriptomes were effectively complete based on Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis (Supplemental Table 1, (Simão et al. 2015)) and were therefore suitable for use for subsequent differential expression analysis (see below). Sponge reference transcriptome assemblies are available through NCBI under BioProject number PRJNA89319.

Reference Metagenome

Two reference metagenomes were sequenced for *Eurypon* sp. 2 and *H. stellifera* at DNASense ApS (Aalborg, Denmark). DNA was fragmented to ~550 bp (M220 Focused-ultrasonicator, Covaris, USA) and used for library preparation following the NEBNext Ultra II DNA library preparation kit. The DNA library was paired-end sequenced (2 x 150 bp) on a NovaSeq platform (Ilumina, USA).

Sequences were assembled using Megahit v.1.2.9 (D. Li et al. 2016). The final contig fasta files were used to plot GC (%) vs. mean coverage of mapped reads to identify individual bins. For this, we used the custom python scripts spadescontigstocovgc.py (Francis 2020a) as well as the R script contig_gc_coverage.R (Francis 2020b) to extract bins for the *Thaumarchaeota* and *Gammaproteobacteria* symbiont of each sponge species. Final statistics for each genome are shown in Supplemental Table 1. Raw sequences are available from NCBI under BioProject number PRJNA893197. Metagenome assemblies are available on https://github.com/bstrehlow/Sponge-transcriptomic-responses-to-seasonal-anoxia.

Mitogenomes

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

For future utilization, single contigs that likely represent the mitochondrial genomes of *Eurypon* sp. 2 and *Hymeraphia stellifera* were retrieved from the reference metagenome in addition to the coding regions used for differential expression below. The assembled contigs for each metagenome were blasted against the mitochondrial genome of DC128 (*Eurypon* sp. 2) using blastn, with an e-value cutoff of $1x10^{-50}$ for *Eurypon* sp. 2 (DC97) and $1x10^{-30}$ for *Hymeraphia stellifera* (DC297). In general, there was only a single hit for each protein coding gene, confirming a single contig as the mitochondrial genome for each sample. Based on the blast, these two contigs contained all protein coding genes retrieved from the transcriptome that were

the focus of this analysis. These 'mitogenomes' can be accessed here: https://zenodo.org/badge/latestdoi/573040118.

Differential Expression Analysis

Trimmed reads from the shallow sequencing for each individual were mapped back to their respective reference transcriptome or genome using bowtie2 (version 2.3.5.1, (Langmead and Salzberg 2012) and mapped reads were counted for each contig using samcount.pl (see Matz 2015). Eukaryotic reads were mapped to the sponge reference transcriptome for each species. Prokaryotic reads were mapped to their respective metagenomic bins - metagenome assembled genomes (MAGs), i.e. *Thaumarchaeota* or *Gammaproteobacteria*, within each sponge species. The mitochondrial reference was broken into its 14 genes and prokaryotic (total RNA) reads from both species were mapped to their matching mitochondrial reference and counted as above.

Analyses were performed to compare the gene expression of each target (sponge species, symbiont species, and mitochondria; n=8) under the three different oxygen conditions (anoxia, hypoxia and normoxia). Differential gene expression analysis was performed with the package DESeq2 v. 1.22.2 (Love, Huber, and Anders 2016) in R (R Core Team 2016) with (RStudio Team 2020). Three pairwise comparisons of oxygen condition (normoxia vs. hypoxia, normoxia vs. anoxia, and anoxia vs. hypoxia) were computed from DESeq2 results. Significantly differentially expressed genes between each of these pairwise comparisons were identified using a false discovery rate (FDR) adjusted p-value threshold of 0.1. The ReportingTools software package (Huntley et al. 2013) was used to create HTML pages displaying4plots of filtered expressed genes and their annotations in various oxygen conditions (https://zenodo.org/badge/latestdoi/573040118). We note that comparisons to *H. stellifera* under normoxia were made with only one normoxic sample due to sampling restrictions. Despite this sampling limitation, the normoxic data for *H. stellifera* are still shown as a baseline and reference. For each sponge species, log fold changes (LFC, from DESeq2) in all genes were calculated so that positive LFCs were upregulated and negative values were downregulated in hypoxia or anoxia relative to normoxia. For the comparisons between hypoxia and anoxia, positive LFCs were upregulated in anoxia and vice versa.

KOG analysis

EuKaryotic Orthologous Groups (KOG class) assignments were used to compare large scale trends and potential correlations in differential gene expression between Lough Hyne sponges under varied oxygen conditions and external gene expression data.

Delta-ranks, a measure of each KOG class enrichment with up or downregulated genes, were calculated from LFC data using the KOGMWU package in R (Dixon et al. 2015), and delta-ranks were plotted using the package pheatmap (Kolde 2013). While it is also possible to calculate delta-ranks with signed p-values (see KOGMWU supporting documentation), LFC was used here to include changes between normoxia and other conditions for *H. stellifera*. Since there was only one normoxic replicant for this species, LFC was a more appropriate metric than signed p values. Within each pairwise comparison (i.e. hypoxic, anoxic, and anoxic vs hypoxic), significantly enriched KOGs were also calculated using KOGMWU (FDR-adjusted $p < 0.05$).

These KOG enrichments were also compared to those calculated from the sponge *Tethya wilhelma* after four days of exposure to hypoxia and one hour of exposure to anoxia (Mills et al. 2018). To test whether KOG enrichment in sponges exposed to variable oxygen conditions was correlated with gene expression in other invertebrate stress responses, delta rank data was included from adult *Acropora millepora* coral exposed to heat stress (31.5 °C for three days, (Dixon et al. 2015). Similarly, to test the similarity of the sponge data to noted decreases in metabolic activity in invertebrates, KOGs enriched in the dauer (dormant) state in *Caenorhabditis elegans* (Sinha, Sommer, and Dieterich 2012) and the diapause state of the midge *Sitodiplosis mosellana* (Gong et al. 2013) were added using delta rank tables calculated by Strader et al. (2016). Correlations between the KOG delta ranks in different datasets were performed using Pearson's Correlation in KOGMWU. Within the significantly enriched KOGs 'replication, recombination and repair' and 'energy production and conversion,' genes that were significantly differentially expressed genes were plotted in heatmaps to further investigate sponge transcriptomic responses to deoxygenation at the gene level in Lough Hyne sponges.

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

Gene ontology (GO) term enrichment analysis

Log fold changes calculated above were used for rank-based gene ontology (GO) analysis with adaptive clustering (GO_MWU, (Wright et al. 2015)) to determine what GO terms were significantly enriched under various oxygen conditions. These analyses were run individually for each pairwise comparison of oxygen condition in both sponge species. These analyses were not completed for the two symbiont types because GO terms were not available for these microbes.

Phylogenetic positions of nitroreductase proteins

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

Results

Sponge differential expression

There were three potential pairwise comparisons for significantly differentially expressed genes - hypoxia vs. normoxia, anoxia vs. normoxia, and anoxia vs. hypoxia. The number of significantly differentially expressed genes in these comparisons for sponges, their mitochondria

358 and their symbionts are summarized in Table 1. In the sponge *Eurypon* sp. 2, 737, 1,376 and
359 1,090 genes were significantly differentially expressed under hypoxia (vs. normoxia), anoxia (vs.
360 normoxia), and anoxia vs. hypoxia, respectively ($p < 0.1$, Table 1). The differentially expressed
361 genes under each oxygen condition ranged between 1.5–2.8% of the genes of the *Eurypon* sp.
362 2 reference transcriptome, and 41-48% of these genes are annotated, which reflects the overall
363 annotation percentage of 39.7% for the whole transcriptome (Supplemental Table 1). In
364 hypoxia, the majority of these differentially expressed genes were upregulated (68%). Under
365 anoxia, on the other hand, the majority of genes were downregulated (60%). In anoxia vs.
366 hypoxia, 57% of genes were upregulated in anoxia compared to hypoxia in *Eurypon* sp. 2
367 (Table 1).
368
369 **Table 1.** Number of significantly differentially expressed genes (DEGs) under deoxygenation in
370 the sponge holobionts summarized per reference genome or transcriptome. The direction (up or
371 down) within each paired oxygen condition comparison is relative to the lower oxygen condition.
372 The number of annotated genes is shown in parentheses.
373
374

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

375
376 In the sponge *H. stellifera*, 523, 284, and 2,154 genes were significantly differentially expressed
377 in hypoxia, anoxia, and in anoxia vs. hypoxia, respectively ($p < 0.1$, Table 1). Differentially
378 expressed genes in the different oxygen conditions corresponded to 0.42 - 3.4% of the total

379 number of genes in the *H. stellifera* transcriptome. Approximately 25-37% of the differentially
380 expressed genes were annotated, which, as in the other sponge species, roughly corresponded
381 to the global percentage (37.0%) of genes annotated within the *H. stellifera* reference
382 transcriptome. Under hypoxia in this species, the vast majority of differentially expressed genes
383 (89%) were upregulated. As in *Eurypon* sp. 2, the majority of differentially expressed genes
384 (84%) were downregulated in anoxia in *H. stellifera*. The majority of differentially expressed
385 genes between anoxia and hypoxia were upregulated in anoxia (71%, Table 1).
386
387 <u>KOG enrichments in sponges</u>
388
389 The global gene expression patterns of Lough Hyne sponges in response to deoxygenation
390 were compared across the two species by assessing up and down regulation of KOG classes
391 (broad functional annotations assigned to genes). Within these sponges, KOG expression in
392 response to hypoxia and anoxia (versus normoxia) were largely distinct in both species (Figure
393 1A). However, KOG expression in *H. stellifera* in hypoxia vs. anoxia was significantly positively
394 correlated, i.e. similar, to that of *Eurypon* sp. 2 under hypoxia ($r = 0.43$, $p < 0.05$, Figure 1J), but
395 global expression patterns were only similar between the two species in this single case.
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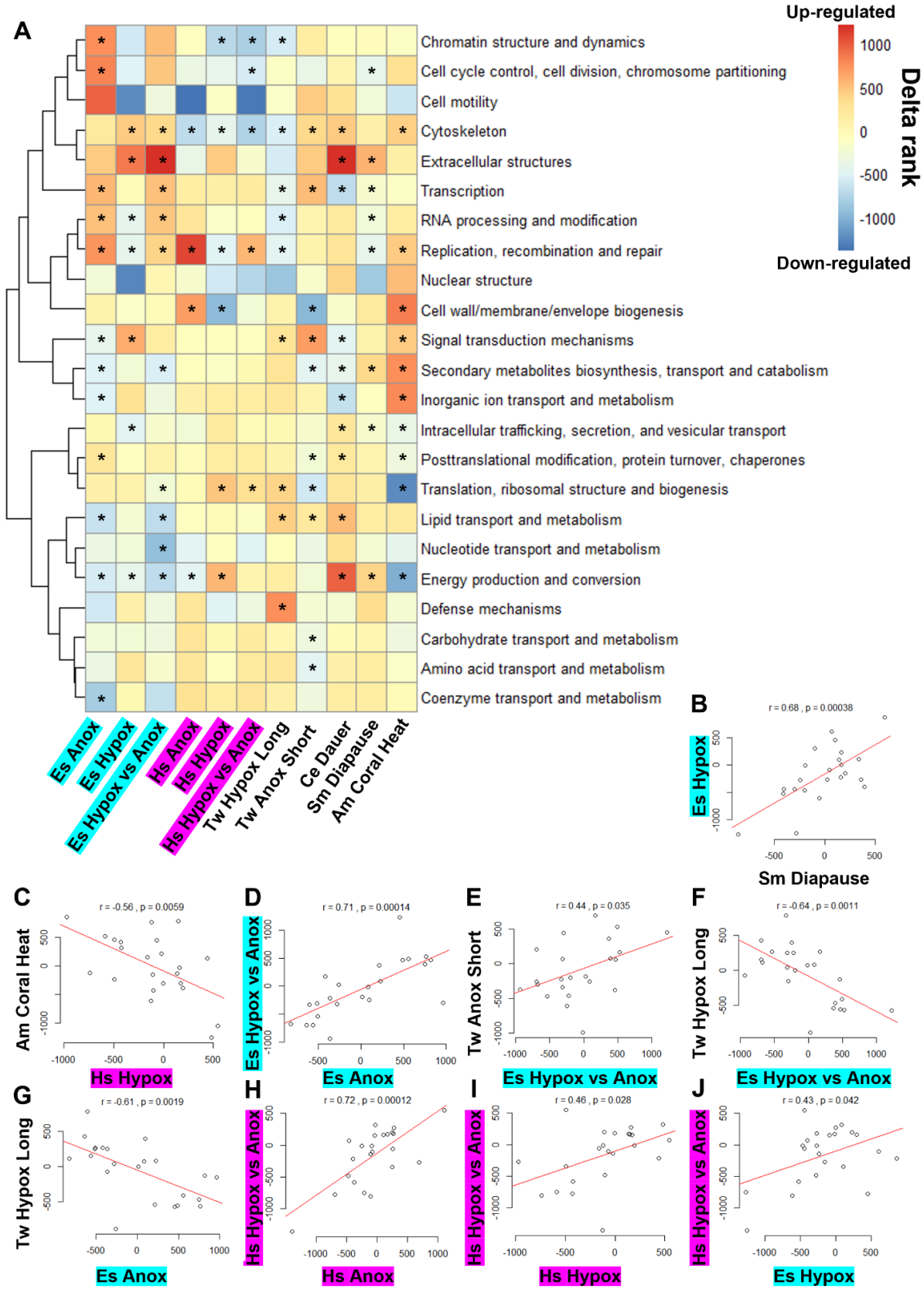
399


A
Up-regulated
Delta rank
Down-regulated
1000
500
0
-500
-1000
Chromatin structure and dynamics
Cell cycle control, cell division, chromosome partitioning
Cell motility
Cytoskeleton
Extracellular structures
Transcription
RNA processing and modification
Replication, recombination and repair
Nuclear structure
Cell wall/membrane/envelope biogenesis
Signal transduction mechanisms
Secondary metabolites biosynthesis, transport and catabolism
Inorganic ion transport and metabolism
Intracellular trafficking, secretion, and vesicular transport
Posttranslational modification, protein turnover, chaperones
Translation, ribosomal structure and biogenesis
Lipid transport and metabolism
Nucleotide transport and metabolism
Energy production and conversion
Defense mechanisms
Carbohydrate transport and metabolism
Amino acid transport and metabolism
Coenzyme transport and metabolism
Es Anox
Es Hypox
Es Hypox vs Anox
Hs Anox
Hs Hypox
Hs Hypox vs Anox
Tw Hypox Long
Tw Anox Short
Ce Dauer
Sm Diapause
Am Coral Heat
B
r = 0.68 , p = 0.00038
Es Hypox
Sm Diapause
C
r = -0.56 , p = 0.0059
Am Coral Heat
Hs Hypox
D
r = 0.71 , p = 0.00014
Es Hypox vs Anox
Es Anox
E
r = 0.44 , p = 0.035
Tw Anox Short
Es Hypox vs Anox
F
r = -0.64 , p = 0.0011
Tw Hypox Long
Es Hypox vs Anox
G
r = -0.61 , p = 0.0019
Tw Hypox Long
Es Anox
H
r = 0.72 , p = 0.00012
Hs Hypox vs Anox
Hs Anox
I
r = 0.46 , p = 0.028
Hs Hypox vs Anox
Hs Hypox
J
r = 0.43 , p = 0.042
Hs Hypox vs Anox
Es Hypox

400

Figure 1. KOG expression profiles of sponges under varied oxygen concentrations compared to each other and external datasets. A. Clustered heatmap (based on correlation) of KOG class (rows) enrichments for upregulated or downregulated genes. Highlighted columns correspond to data generated in this study (Purple: *Hymeraphia stellifera* [Hs], Cyan: *Eurypon* sp. 2 [Es]). Up or down regulation with respect to normoxia is indicated in 'Hypox' (hypoxia) and 'Anox' (anoxia) columns, and differential regulation with respect to hypoxia is shown in 'Hypox vs. Anox' columns, i.e. upregulated KOGs (red delta ranks) are upregulated in anoxia with respect to hypoxia. Within each column on the heatmap, KOGs that are significantly enriched (FDR-adjusted $p < .05$) are marked with asterisks. KOG enrichments from the sponge *Tethya wilhelma* after four days exposure to hypoxia (Tw Hypox Long) and one hour exposure to anoxia (Tw Anox Short), the coral *Acropora millepora* after three days of exposure to heat stress (Am Coral Heat), *Caenorhabditis elegans* in a dauer state (Ce Dauer), and the midge *Sitodiplosis mosellana* in diapause (Sm Diapause) from external studies were included for comparative purposes. B-J. Significant ($p < 0.05$) correlations between the KOG enrichments of different treatments or datasets (columns in A). All other possible comparisons between the various datasets were not significant.

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

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

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

In *H. stellifera*, three genes were significantly differentially expressed in the RRR KOG class in hypoxia, and all were upregulated ($p < 0.1$, Supplemental Figure 4D). No genes within this class

were significantly differentially expressed in anoxia (vs. normoxia). Eleven genes were upregulated and eight genes were downregulated in anoxia vs. hypoxia (Supplemental Figure 4E). Similar to *Eurypon* sp. 2, HELZ2 was downregulated in anoxia vs. hypoxia in *H. stellifera*. In contrast to *Eurypon* sp. 2 a distinct MCM gene (MCM6) was downregulated in anoxia vs. hypoxia. Additionally, four genes from the structural maintenance of chromosomes protein 6 (SMC6) complex were downregulated in anoxia vs. hypoxia.

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

Within *Eurypon* sp. 2 specifically, four genes were significantly downregulated and twelve genes were upregulated within the EPC class in hypoxia ($p < 0.1$, Supplemental Figure 5A). Various nuclear genes for processes in the mitochondria were differentially expressed in hypoxia. SLC25A27 (a mitochondrial membrane uncoupling protein), ndufs8 (a NADH dehydrogenase core subunit in the mitochondrial membrane - complex I), and Surf1 (part of the complex that regulates cytochrome c oxidase assembly) were downregulated in hypoxia (Supplemental Figure 5A). However, other nuclear mitochondrial genes were upregulated, including four subunits of ATP synthase and two additional genes with annotations corresponding to ATP synthesis (Supplemental Figure 5A). In anoxia, fourteen genes were upregulated and five genes were downregulated in the EPC class (Supplemental Figure 5B).

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

In *H. stellifera*, eight genes were upregulated and two genes were downregulated in hypoxia. As in *Eurypon* sp. 2, the ATP5A1 ATP synthase subunit was upregulated in hypoxia. An additional ATP synthase subunit (ATP6V0C) that was not significantly upregulated in *Eurypon* sp. 2 was upregulated in *H. stellifera* in hypoxia ($p < 0.1$, Supplemental Figure 5D). Mitochondrial sulfide:quinone oxidoreductase (SQRDL), three genes associated with phosphoenolpyruvate

491 carboxykinase, and a histone demethylase (KDM6A) were also significantly upregulated in
492 hypoxia in *H. stellifera*. Conversely, PCK1 (also associated with phosphoenolpyruvate
493 carboxykinase) and MDH1 were significantly downregulated in hypoxia ($p < 0.1$, Supplemental
494 Figure 5D). In anoxia in *H. stellifera*, ATP5A1 was significantly upregulated, and ATP6V0C,
495 PCK1, and MDH1 were significantly downregulated (Supplemental Figure 5E). Comparing
496 anoxia vs. hypoxia, eight genes were upregulated and five genes were downregulated
497 (Supplemental Figure 5F).
498
499 As in hypoxia, SQRDL, four genes associated with phosphoenolpyruvate carboxykinase activity,
500 and KDM6A were upregulated in anoxia vs. hypoxia. Additionally, two unshared genes,
501 including the mitochondrial Succinyl-CoA:3-ketoacid coenzyme A transferase 1 (OXCT1) and a
502 mitochondrial carrier protein, were upregulated in anoxia vs. hypoxia (Supplemental Figure 5F).
503 Five genes were downregulated in anoxia vs. hypoxia in *H. stellifera*. Two of these four only
504 exhibited high expression levels in one hypoxic sample (DC105) that drove the observed
505 difference (Supplemental Figure 5F). The other three downregulated genes in anoxia vs.
506 hypoxia were mitochondrial creatine kinase (CKMT2), NAD P transhydrogenase (NNT), and
507 proto-oncogene tyrosine-protein kinase (Src).
508
509 The 'cytoskeleton' KOG class was also significantly enriched ($P_{adj} < 0.01$) in all but one oxygen
510 condition – anoxia in *Eurypon* sp. 2 ($P_{adj} = 0.1$). Interestingly, enrichment patterns in this KOG
511 were species specific, being significantly upregulated in hypoxia and anoxia vs. hypoxia in
512 *Eurypon* sp. 2 and downregulated in all conditions for *H. stellifera*.
513
514 The other twenty KOG classes were only significantly enriched in Lough Hyne sponges under a
515 few (or no) different oxygen conditions and patterns of significant enrichment were generally
516 condition and species-specific (Figure 1A). With the exception of EPC, KOGs pertaining to
517 metabolic processes were rarely significantly enriched. Expression of the KOG classes
518 'carbohydrate metabolism' and 'amino acid transport and metabolism' were not significantly
519 changed under any oxygen condition ($P_{adj} > 0.05$). There was some significant enrichment of
520 metabolic KOGs, but only in *Eurypon* sp. 2. 'Coenzyme transport and metabolism' and
521 'inorganic ion transport and metabolism' were both downregulated in anoxia, and 'nucleic acid
522 transport metabolism' was downregulated in hypoxia vs. anoxia. For both these conditions
523 (anoxia and hypoxia vs. anoxia), 'Lipid transport and metabolism' and 'Secondary metabolites
524 biosynthesis, transport and catabolism' were significantly downregulated. However, the
525 transcription of the majority of genes labeled with metabolic KOGs remained unchanged under
526 anoxia and hypoxia.
527
528 Comparing the Lough Hyne dataset with external datasets of invertebrate KOG expression in
529 response to stress, only two positive correlations were found. In *Eurypon* sp. 2, expression
530 patterns under Anoxia vs. Hypoxia were comparable to those of *T. wilhelma* under short-term
531 anoxia ($r = 0.44$, $p < 0.05$, Figure 1E). The hypoxic response of *Eurypon* sp. 2 resembled that of
532 diapause in *S. mosellana* ($r = 0.68$, $p < 0.001$, Figure 1B), but this was the only correlation found
533 between Lough Hyne sponge data and diapause or dauer states. Importantly, KOG enrichments
534 under anoxia (versus normoxia) were not positively correlated to any other external datasets for
535 either sponge species.

536
537 Three significant negative correlations, which corresponded to opposing KOG expression
538 patterns, were observed between Lough Hyne sponges and external datasets. Hypoxia
539 responses in *H. stellifera* were negatively correlated with those of *A. millepora* under heat stress
540 (r = -0.56, p < 0.01, Figure 1C). The long exposure of *T. wilhelma* to hypoxia was negatively
541 correlated to both anoxia (r = -0.64, p < 0.01, Figure 1G) and hypoxia vs. anoxia (r = -0.61, p <
542 0.01, Figure 1F) in *Eurypon* sp. 2.
543
544 <u>GO term enrichments in sponges</u>
545
546 Generally, significantly enriched GO terms between the different treatments were not shared
547 between the two sponge species (Supplemental Figures 1-3). The strongest upregulated GO
548 enrichments (p < 0.01) in hypoxia in *Eurypon* sp. 2 were cellular signaling and 'lipoprotein
549 particle receptor binding' (Supplemental Figure 1A). For *H. stellifera*, on the other hand, the
550 strongest upregulated enrichments (p < 0.01) in hypoxia included the terms 'protein tyrosine
551 kinase binding,' 'molecular adaptor,' 'structural molecule,' and 'structural constituent of
552 ribosome'; whereas the downregulation of the term 'motor' was strong (p < 0.01, Supplemental
553 Figure 1B).
554
555 Under anoxia, strong upregulation of the 'proteasome binding' term was observed in *Eurypon*
556 sp. 2 (p < 0.01), and the terms 'phosphoenolpyruvate carboxykinase', 'carbohydrate kinase,'
557 monovalent inorganic cation transmembrane transporter,' and 'sodium ion transmembrane
558 transporter' were highly downregulated (p < 0.01, Supplemental Figure 2A). In *H. stellifera*
559 under anoxia, the terms 'damaged DNA binding' and 'calcium-dependent phospholipid binding'
560 were highly upregulated, and four terms associated with various aspects of the cytoskeleton
561 were downregulated (p < 0.01, Supplemental Figure 2B).
562
563 In *Eurypon* sp. 2 under anoxia vs. hypoxia, the terms 'lipoprotein receptor binding' and
564 'proteasome binding' were strongly upregulated in anoxia, but no terms were strongly
565 downregulated in anoxia versus hypoxia (p < 0.01, Supplemental Figure 3A). The former term
566 was also upregulated under anoxia vs. normoxia. Conversely, the strongest enrichments in *H.*
567 *stellifera* in anoxia vs. hypoxia were downregulated in anoxia and were composed of three of
568 the four cytoskeletal binding GO terms that were also downregulated in anoxia vs. normoxia
569 (see above, p < 0.01, Supplemental Figure 3B), but no strong upregulation of GO terms was
570 observed.
571
572 Considering GO enrichments at lower significance levels (0.01 < p < 0.1), within *Eurypon* sp. 2,
573 the GO terms 'proteasome binding', 'lipoprotein particle receptor binding,' and 'Hsp70 binding'
574 (HSP: Heat shock protein) were significantly upregulated in all cases of decreased oxygen albeit
575 at varied significance levels (p < 0.05, Supplemental Figures 1–3A). Between only two levels,
576 the terms 'transmembrane signaling receptor,' 'secondary active transmembrane transporter,'
577 and 'molecular transducer' were upregulated in hypoxia but downregulated in anoxia. However,
578 the term lipoprotein particle receptor binding was downregulated in anoxia and upregulated in
579 hypoxia (p < 0.1, Supplemental Figures 1,2A). Additionally, the term 'structural constituent of
580 ribosome' was upregulated in anoxia but downregulated in anoxia vs. hypoxia (p < 0.1,

581 Supplemental Figures 1,3A). No additional GO terms other than the three present in all oxygen
582 levels for *Eurypon* sp. 2 were enriched in both the pairwise comparison of anoxia and anoxia vs.
583 hypoxia.
584
585 No GO terms were significantly up or downregulated in all three levels of oxygen conditions for
586 *H. stellifera*. However, some GO terms were significantly enriched in two levels. The term
587 'ATPase, coupled to transmembrane movement of ions, rotational mechanism' was
588 downregulated in hypoxia but upregulated in anoxia ($p < 0.1$, Supplemental Figures 1B, 2B
589 respectively). The GO terms 'I-SMAD binding', thioesterase binding,' and 'ubiquitin-like protein
590 ligase binding' were all upregulated in hypoxia (vs. normoxia) and anoxia vs. hypoxia, while the
591 'motor' term was downregulated ($p < 0.1$, Supplemental Figures 1B, 3B).
592
593 There were only two shared GO enrichments between both sponge species for any given
594 oxygen condition. The terms 'protein tyrosine kinase binding' and 'structural constituent of the
595 ribosome' were upregulated in both species in hypoxia ($p < 0.1$, Supplemental Figure 1),
596 although they were enriched to different degrees of significance. No significantly enriched GO
597 terms were shared between species in normoxia vs. anoxia or hypoxia vs. anoxia.
598
599 At the gene level, significantly differentially expressed heat shock proteins (Hsps) in both
600 sponges are shown in Supplemental Figure 8. In *Eurypon* sp. 2, one Hsp gene
601 (DNAJC8/Hsp40) was downregulated in hypoxia ($p < 0.01$), but four Hsp genes (HSPA8, two
602 genes for HSP90B1, and Hsp70) were significantly upregulated in hypoxia ($p < 0.1$,
603 Supplemental Figure 8A). Additionally, HSPA8 and C11orf73 (Hsp70 binding protein) were
604 upregulated in anoxia vs normoxia ($p < 0.05$, Supplemental Figure 8A). Another Hsp70 gene
605 was also significantly downregulated in anoxia vs. hypoxia ($p < 0.05$, Supplemental Figure 8A).
606 In *H. stellifera*, HSP90A1 was upregulated in hypoxia and anoxia ($p < 0.1$, Supplemental Figure
607 8B). Two additional Hsp genes (both for HSP90B1) were also upregulated in anoxia. Finally, the
608 two HSP90B1 genes were upregulated in anoxia vs. hypoxia, but HSPA5 was downregulated in
609 anoxia vs. hypoxia ($p < 0.05$, Supplemental Figure 8B).
610
611 **Mitochondrial gene expression**
612 All 14 coding genes from the sponge mitochondria were transcribed in both species and in all
613 oxygen conditions. There was only one mitochondrial gene (nad2) that was significantly
614 differentially expressed but only in *Eurypon* sp. 2. This gene was significantly upregulated ($p <$
615 0.1) in anoxia vs hypoxia (Supplemental Figure 6A, Table 1). Similar counts for each individual
616 mitochondrial gene across both sponge species and all oxygen conditions were found. For
617 instance, counts for atp6 were consistently twice as high as counts for atp8 (Supplemental
618 Figure 6).
619
620 **Prokaryotic differential expression**
621
622 <u>*Thaumarchaeota* gene expression</u>
623 For the *Thaumarchaeota* associated with *Eurypon* sp. 2, 20, 3 and 0 genes were differentially
624 expressed in hypoxia, anoxia, and hypoxia vs. anoxia, respectively ($p < 0.1$, Table 1). These
625 genes accounted for 0-1.3% of this *Thaumarchaeota* genome, and 75-100% of the differentially

expressed genes were annotated (Table 1), compared to 69.2% annotated in the entire reference genome. All but one of the differentially expressed genes in hypoxia were upregulated (Table 1, Figure 2A). The downregulated gene belonged to the sodium solute symporter family (SSF, TC 2.A.21, Figure 2A). The upregulated genes in hypoxia included three stress response genes (COG0589 Universal stress protein [UspA], small heat shock protein [HSP20], and DnaJ molecular chaperone homology domain), two CBS domains, a member of the lactate/malate dehydrogenase (LDH MDH) superfamily, a tetratricopeptide (TPR) repeat, two transcription regulators, and a PII signal transduction protein.

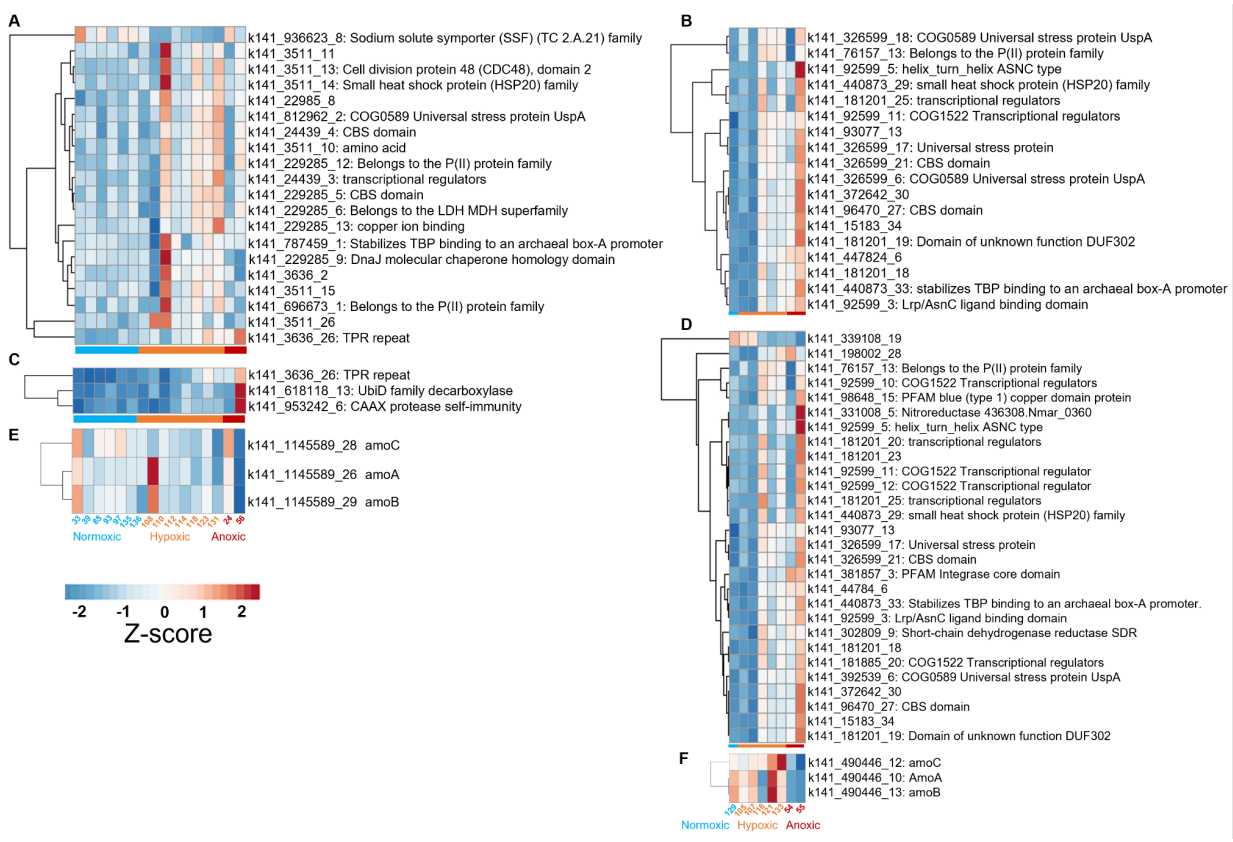


Figure 2. A-D. All significantly differentially expressed genes for the *Thaumarchaeota* in both sponge species (left: *E.* sp 2, right: *H. stellifera*. For *Thaumarchaeota* associated with *E.* sp. 2, only two pairwise comparisons of oxygen conditions had significant differences: A. hypoxia vs. normoxia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

Only three genes were significantly differentially expressed in anoxia in *Eurypon* sp. 2's *Thaumarchaeota*, and all were upregulated (Table 1, Figure 2C). The upregulated genes in anoxia were a UbiD family decarboxylase, CAAX protease self-immunity, and the same TPR repeat that was also upregulated in hypoxia. There were no significantly differentially expressed genes between anoxia and hypoxia in this *Thaumarchaeota* (Table 1). The genes necessary for ammonia oxidation, i.e. subunits A, B and C of ammonia monooxygenase (Amo), were present

650 in this *Thaumarchaeota*, but they were not differentially expressed under deoxygenation (Figure
651 2E).
652
653 In the *Thaumarchaeota* symbiont of *H. stellifera*, 17, 0 and 28 genes were significantly
654 differentially expressed in hypoxia, anoxia and hypoxia vs. anoxia, respectively. Similar to the
655 symbiont of *Eurypon* sp. 2, all 17 differentially expressed genes in the *H. stellifera* symbiont
656 were upregulated in hypoxia. These genes included the stress response genes 'universal stress
657 protein, an HSP20 family protein, and two copies of UspA. HSP20 and UspA were also
658 upregulated in the *Eurypon* sp. 2 holobiont. Similarly, a PII signal transduction protein, CBS
659 domain, and two transcription regulators (one identical: TBP stabilizer, and one distinct to *H.*
660 *stellifera* symbionts) were upregulated in both *Thaumarchaeota* species in hypoxia. Unlike in
661 *Eurypon* sp. 2, the *Thaumarchaeota* in *H. stellifera* upregulated a helix turn helix ASNC type
662 domain and a Lrp/AsnC ligand binding domain (Figure 2B). No genes were significantly
663 differentially expressed in anoxia in this *Thaumarchaeon*.
664
665 Twenty-seven genes were upregulated and one gene (K141_339108_19, unannotated) was
666 downregulated in anoxia vs. hypoxia in the *Thaumarchaeota* within *H. stellifera* (Figure 2D).
667 Most of the upregulated genes in anoxia vs. hypoxia were also upregulated in hypoxia. Some
668 unshared genes included additional transcriptional regulators, an integrase domain, and a
669 putative nitroreductase.
670
671 The phylogenies of this nitroreductase and all nitroreductases from the studied holobionts as
672 well as publicly available nitroreductase sequences from other *Thaumarchaeota* are shown in
673 Supplemental Figure 9. As in the other holobiont species, *Thaumarchaeota* genes for ammonia
674 oxidation were not significantly differentially expressed with *H. stellifera* symbionts (Figure 2F).
675
676 For both *Thaumarchaeota*, only two COGs ('signal transduction mechanisms' and
677 'transcription') were significantly enriched ($P_{adj} < 0.05$, Supplemental Figure 7A). These two
678 COGs were both downregulated in hypoxia and anoxia vs. hypoxia within *H. stellifera*
679 symbionts. All other COG classes were comparable in their expression levels regardless of
680 holobiont species or oxygen concentration (Supplemental Figure 7A).
681
682 <u>*Gammaproteobacteria* gene expression</u>
683 The *Gammaproteobacteria* associated with *Eurypon* sp. 2 only exhibited significant differential
684 expression under hypoxia but not in any other oxygen condition (Table 1). In hypoxia, three
685 genes were upregulated: type 1 restriction-modification system, flavin adenine dinucleotide
686 binding, and signal sequence binding (Figure 3A). The 29 downregulated genes in hypoxia
687 included two genes involved in sulfur processing (k212078_2, k237243_12), cytochrome c
688 (class I), a gene associated with the assembly of the periplasmic c-type cytochromes
689 (k561840_2), a NQR complex gene, various genes involved in pore formation, a Rhodanese-
690 like gene, glutamine synthetase (type I), and YbiS YcfS YnhG family protein.
691
692
693

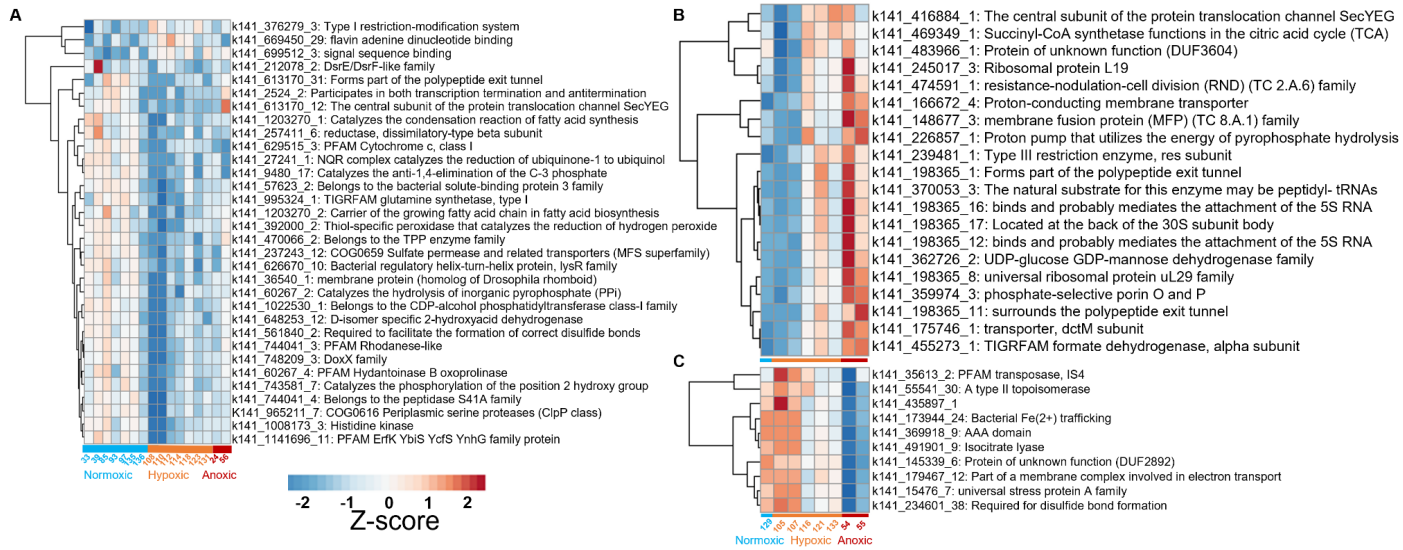


Figure 3. All significantly differentially expressed genes for the *Gammaproteobacteria* in both sponge species (A: *Eurypon* sp 2, B: *H. stellifera*. For *Gammaproteobacteria* associated with *Eurypon* sp. 2, there were only significant differences between hypoxia vs. normoxia (A). In symbionts associated with *H. stellifera*, there were only significantly differentially expressed genes in anoxia vs. hypoxia (B,C). All downregulated genes in anoxia vs. hypoxia are shown in B. There were 402 genes that were downregulated in anoxia vs. hypoxia in *Gammaproteobacteria* from *H. stellifera*, so only the top 10 based on p value are shown (C).

In the *Gammaproteobacteria* associated with *H. stellifera*, genes were only significantly differentially expressed between anoxia and hypoxia (436 genes, $p < 0.1$, Table 1, Figure 3B,C). Twenty genes were upregulated in anoxia vs. hypoxia and all were annotated (Figure 3B). Upregulated genes in anoxia vs. hypoxia included five genes associated with the ribosome (k141_198365_15, k141_198365_8, k141_198365_17, and k141_245017_3), various genes for molecular transport, a transmembrane proton pump (k141_226857_1), succinyl-CoA synthetase, and formate dehydrogenase (alpha subunit). There were also 416 genes that were significantly downregulated in anoxia vs. hypoxia (Table 1). The top ten upregulated genes (lowest p values) are exhibited in Figure 3C.

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

Discussion

Sponge holobiont gene expression was significantly changed by exposure to deoxygenation in situ, though responses were generally species-specific, dependent on oxygen level, and varied across holobiont members. The potential functions of this differential expression are discussed

724 below for each member of the two holobionts: sponge, sponge mitochondria, *Thaumarchaeota*
725 and *Gammaproteobacteria*. The implications of this study for past and future oceans are
726 considered.
727
728 **Differential expression in sponges during deoxygenation**

729 There were clear differences in gene expression of the sponges in response to normoxia,
730 hypoxia, and anoxia. In both sponge species, the majority of significantly differentially expressed
731 genes were upregulated in hypoxia but downregulated in anoxia, when compared to normoxia.
732 Despite this general similarity in differential expression in response to oxygen conditions
733 between species, there were also noteworthy, species-specific responses to deoxygenation.
734 Thus, while both species significantly upregulated genes for responding to cellular stress in
735 hypoxia and anoxia, the specific genes depended on the species. Therefore, gene expression
736 depended on both oxygen condition and sponge species. Significantly enriched GO terms were
737 largely species specific and may indicate general physiological differences between sponge
738 species. KOG expression in response to deoxygenation was also largely species specific,
739 except for two key KOGs: 'energy production and conversion' (EPC) and 'replication,
740 recombination and repair' (RRR). Significant enrichment patterns of these KOGs were nearly
741 identical for both species, suggesting that differential expression of genes with EPC and RRR
742 functions is likely crucial to deoxygenation tolerance regardless of species. Interestingly, KOG
743 expression associated with metabolism, other than in the EPC class, was largely unchanged in
744 both studied species across all oxygen conditions, suggesting consistency in most metabolic
745 processes under deoxygenation.

746 Cellular stress responses were observed in the expression of HSPs, a group of stress response
747 genes that have been widely studied in marine invertebrates. These proteins are molecular
748 chaperones involved in protein folding, unfolding, transport and the assembly of protein
749 complexes, and they are present in nearly all organisms (Z. Li and Srivastava 2004). Both
750 Lough Hyne sponges upregulated HSP genes in response to hypoxia and anoxia (vs normoxia),
751 though there was downregulation of some HSP genes in deoxygenation as well. *Eurypon* sp. 2
752 and *H. stellifera* both upregulated HSP90 genes in hypoxia, but they upregulated different HSPs
753 in anoxia: HSPA8 and Hsp90 for *Eurypon* sp. 2 and *H. stellifera*, respectively. *Eurypon* sp. 2
754 also upregulated HSPA8 and Hsp70 in hypoxia but downregulated Hsp40. In long term hypoxia,
755 *T. wilhelma* exhibited a similar but inverse HSP expression pattern, upregulating Hsp90 but
756 downregulating Hsp70 (Mills et al. 2018). Sponges under heat stress also upregulate Hsp70
757 (López-Legentil et al. 2008; Guzman and Conaco 2016; N. Webster et al. 2013) and HSP90
758 (Guzman and Conaco 2016; N. Webster et al. 2013). Pollutants such as lead and zinc can also
759 stimulate the upregulation of HSPs in sponges (Efremova et al. 2002; Schröder et al. 2006).
760 These proteins are likely crucial to sponge stress response, and more work is necessary to fully
761 understand sponge stress response in general.

762 Patterns of species and oxygen-condition-dependent expression of GO terms were observed in
763 sponges in response to deoxygenation. In hypoxia, only one GO term was significantly
764 upregulated in both species - 'protein tyrosine kinase binding'. Tyrosine kinases are involved in
765 regulating a wide range of cellular functions, so extrapolating functionality from this GO term is
766 difficult. However, GO terms with more explicit functions were differentially enriched in both

767 species. GO terms such as 'proteasome binding' and 'Hsp70 binding' were upregulated in all
768 cases of decreased oxygen in *Eurypon* sp. 2, suggesting that the species-specific response to
769 deoxygenation in *Eurypon* sp. 2 may center around repairing or degrading damaged proteins. In
770 *H. stellifera*, on the other hand, upregulated GO terms related to DNA repair in anoxia, including
771 'damaged DNA binding', 'DNA binding,' and 'catalysis of DNA and RNA,' so DNA repair may be
772 more central to the deoxygenation tolerance of this species.

773 The EPC KOG class was significantly downregulated in all oxygen conditions in both sponges
774 except for hypoxia and hypoxia vs. anoxia in *H. stellifera*, when it was significantly upregulated
775 and unchanged, respectively (Figure 1). Within the EPC class, however, there were species-
776 specific responses to anoxia and hypoxia at the level of individual differentially expressed
777 genes. Most nuclear ATP synthase genes were upregulated in hypoxia in *Eurypon* sp. 2 but
778 were unchanged under anoxia. Similarly, two ATP synthase subunits (ATP5A1 and ATP6V0C)
779 were upregulated in hypoxia in *H. stellifera*. In anoxia, ATP5A1 was also upregulated, but
780 ATP6V0C was downregulated. Additionally, downregulation of SLC25A27 in *Eurypon* sp. 2 in
781 hypoxia may increase the coupling across the mitochondrial membrane to improve ATP
782 synthesis. Interestingly, the electron transport chain genes Ndufs8 and Surf1 were also
783 downregulated in hypoxia *Eurypon* sp. 2. The NNT gene, also involved in electron transport,
784 was downregulated in anoxia vs. hypoxia in *H. stellifera*. Nevertheless, most of the genes
785 associated with the respiration electron transport chain were not differentially expressed, and
786 electron transport likely continued under deoxygenation. Sponges may therefore have increased
787 ATP production under hypoxia but not under anoxia. Similarly, mollusks may upregulate the
788 mitochondrial electron transport system activity and ATP synthesis capacity to improve
789 respiration efficiency under hypoxia (Hochachka and Lutz 2001).

790 Glycolysis, which can produce ATP and NADH without oxygen (Stryer 1995), was expected to
791 increase under anoxia. However, relatively few genes directly or indirectly involved in glycolysis
792 were differentially expressed in our sponges under deoxygenation. In *Eurypon* sp. 2,
793 pantothenate kinase 1 (PANK1), ATP-dependent 6-phosphofructokinase (PFKL) and
794 Phosphoenolpyruvate carboxykinase 2 (PCK2) were significantly upregulated in anoxia, and
795 only aldehyde dehydrogenase 3 family, member a2 (ALDH3A2) was significantly upregulated in
796 hypoxia (see Supplemental Reports, Supplemental Figure 5). In *H. stellifera*, acetyl-coenzyme A
797 synthetase 1 (ACSS1) was significantly downregulated in hypoxia, but PCK1 and pyruvate
798 kinase (PKM) were upregulated in hypoxia. In anoxia for *H. stellifera*, beta-enolase (ENO3) and
799 PCK1 were upregulated. These results might indicate that glycolysis rates increased under
800 anoxia and hypoxia in both species. Due to the consistency in gene expression of the other ~70
801 genes associated with glycolysis; however, it is more likely that glycolysis continued under
802 anoxia and hypoxia at normal rates in both species. Either way, the sponges would have at
803 least some access to anaerobically generated ATP through glycolysis during environmental
804 deoxygenation. Similarly, gluconeogenesis, the production of glucose for glycolysis during
805 periods of fasting(Chandramouli et al. 1997), may have increased with deoxygenation. PCK
806 upregulation in anoxia in both sponge species and hypoxia in *H. stellifera* may indicate
807 increased gluconeogenesis. It is unclear if sponges are pumping water or feeding during anoxia,
808 since assays with fluorescein dye did not show obvious pumping in anoxia, but sponges were
809 definitively pumping under hypoxia (Schuster et al. 2021). However, increased gluconeogenesis
810 under anoxia might serve to boost energy levels regardless of pumping activity.

811 For the KOG class ‘replication, recombination and repair’ (RRR), there was significant
812 upregulation in anoxia and anoxia vs. hypoxia but down regulation in hypoxia for both sponge
813 species. In *Eurypon* sp. 2, many MCM genes involved in the initiation of DNA replication were
814 downregulated in hypoxia but remained unchanged in anoxia. Certain aspects of DNA
815 replication, e.g. the DNA polymerase epsilon catalytic subunit, may even be upregulated in
816 anoxia. The pattern was less clear in *H. stellifera*, but MCM genes were downregulated in
817 anoxia compared to hypoxia. These data indicated that DNA replication may decrease under
818 hypoxia but remain stable during anoxia. This pattern of a ‘stronger’ response to hypoxia than
819 anoxia mirrored that of nuclear ATP synthesis genes.

820 Between the two study species, there was only one significant, positive correlation of KOG class
821 expression between hypoxia (and normoxia) in *Eurypon* sp. 2 and hypoxia vs. anoxia in *H.*
822 *stellifera*, demonstrating the general species-specific responses of most KOGs to
823 deoxygenation. Species-specific responses between *Eurypon* sp. 2 and *H. stellifera* may reflect
824 the distinct microbial communities harbored by each species (Schuster et al. 2021). In contrast,
825 different coral taxa generally exhibit more correlations in KOG expression in response to
826 different stressors than sponge taxa do in response to a single stressor, although stress
827 responses in corals can still differ widely between species and stressor (Kenkel, Mocellin, and
828 Bay 2020). Within a single sponge species, no correlations were seen between KOG
829 enrichments in anoxia or hypoxia (vs. normoxia), highlighting the transcriptomic differences
830 between responses to hypoxia and anoxia. KOG enrichments in hypoxia *versus* anoxia, on the
831 other hand, were positively correlated with those of anoxia in *Eurypon* sp. 2 and both anoxia
832 and hypoxia in *H. stellifera*.

833 When quantitatively compared to transcriptomic datasets from the sponge *T. wilhelma* using
834 KOGMWU, KOG enrichments in Lough Hyne sponges were only positively correlated between
835 the sponge *T. wilhelma* under short-term anoxia and *Eurypon* sp. 2 in hypoxia vs. anoxia
836 (Figure 1E). This positive correlation could indicate similar transcription patterns demonstrated
837 by these two sponge species transitioning from hypoxia to anoxia. However, no further
838 similarities between the responses of *T. wilhelma* and Lough Hyne sponges to deoxygenation
839 were observed. In fact, the long-term exposure of *T. wilhelma* to hypoxia was negatively
840 correlated to KOG enrichments for both anoxia vs. hypoxia and normoxia vs. anoxia in *Eurypon*
841 sp. 2 (Figure 1F, G), meaning that KOG expression was generally opposite between these two
842 species for these conditions in terms of up and down regulation. The physiological responses of
843 these two species to deoxygenation are presumably very different. As indicated by analyses in
844 Mills et al.(2018), there was also no correlation between the KOG expression for the exposure
845 of *T. wilhelma* to anoxia and hypoxia (Figure 1A). These results demonstrate that the large-
846 scale transcriptomic responses to deoxygenation depend on both the studied species and
847 oxygen concentration.

848 Physiological and behavioral responses of sponges to various levels of hypoxia also depend on
849 sponge species, oxygen level, and exposure duration (Micaroni, Strano, et al. 2021). In their
850 study (Micaroni, Strano, et al. 2021) found that only one of four tested species, *S. carnosus,*
851 exhibited a significant change in respiration rate in response to hypoxic (0.5 mg L^{-1}---) exposure,
852 halving its respiration rate. This decrease occurred only after seven days of exposure,
853 demonstrating the effects of exposure duration on metabolism. *S. australiensis* expanded its

854 body and aquiferous system and temporarily increased its pumping rate following hypoxic (0.4
855 mg L^{-1}---) exposure, but these behavioral changes were not observed under more severe
856 hypoxia (0.13 mg L^{-1}---). A decrease in behavioral response to more severe hypoxia was
857 mirrored in gene expression of ATP synthase genes in the current study. There may be an
858 oxygen threshold, below which increased production of ATP is no longer advantageous. We
859 hypothesize that these different physiological and behavioral responses underpin tolerance to
860 hypoxia and may be controlled by differential transcriptomic responses, which warrant further
861 investigation.

862 Based on KOG correlation analyses, we can reject the hypothesis that sponges are dormant,
863 i.e. temporarily ceasing growth, development, and physical activity, under anoxia. Sponge gene
864 expression under anoxia did not correlate to either diapause or a dauer state in *C. elegans*
865 *(Sinha, Sommer, and Dieterich 2012)* or *S. mosellana (Gong et al. 2013)*, respectively. Although
866 these dormancy datasets come from distantly related invertebrates, Strader et al. (2016)
867 demonstrated a correlation between the KOG expression patterns of a specific phenotype of
868 coral larvae and diapause in *S. mosellana*, hypothesizing that this potential functional analog of
869 dormancy could serve to increase larval dispersal. Therefore, the sponges of Lough Hyne could
870 only be considered dormant under anoxia if their transcriptional method of dormancy was
871 distinct from that of an insect, nematode and coral, which is possible but not parsimonious given
872 that animals are monophyletic (Feuda et al. 2017).

873 Paradoxically, *Eurypon* sp. 2 responses to hypoxia were positively correlated to diapause in *S.*
874 *mosellana* (Figure 1B). Therefore, the hypoxic response in *Eurypon* sp. 2 species, rather than
875 the anoxic response, was similar to diapause in terms of global KOG expression patterns. The
876 RRR kog was also downregulated in *Eurypon* sp. 2 under hypoxia as well as in *S. mosellana*
877 under diapause, which may partially explain the correlation between these datasets. Moreover,
878 diapause can be associated with the reduction of cell division (MacRae 2010), which may occur
879 in sponges under hypoxia but not anoxia based on MCM gene expression. In comparison, KOG
880 enrichments under anoxia were not positively correlated to any external datasets for either
881 sponge species (Figure 1), indicating that sponge transcriptomic responses to anoxia are
882 relatively novel compared to those of other taxa in response to various factors in the existing
883 literature (Dixon et al. 2015; Strader, Aglyamova, and Matz 2016).
884
885 **Sponge mitochondrial gene expression during deoxygenation**
886
887 A high degree of transcriptional stability under deoxygenation was observed in sponge
888 mitochondria. Only one of the 14 mitochondrial, protein coding genes, nad2, was significantly
889 differentially expressed in the different oxygen conditions, where it was significantly upregulated
890 in anoxia vs. hypoxia in *Eurypon* sp 2. Both of the sponge species had higher expressions of
891 nad2 in anoxia than in normoxia or hypoxia, but this difference was only significant in *Eurypon*
892 sp. 2. Both species also demonstrated remarkably similar and stable patterns of expression for
893 each mitochondrial gene, including nad2.
894
895 Surprisingly little is known about differential gene expression in mitochondria, even within
896 humans. In the limited studies available, mitochondrial gene expression has significantly differed
897 between human cancer cells (Reznik et al. 2017) and normal cells as well as between different

898 populations of healthy pancreas tissue (Medini, Cohen, and Mishmar 2021). In these studies
899 nad2 was significantly downregulated in most studied cancers, though it was still significantly
900 upregulated in a few cancer types, and nad2 was also significantly differentially expressed
901 between pancreas cell populations. Based on general upregulation of nuclear genes associated
902 with respiration in hypoxia, mitochondrial genes were expected to be upregulated in hypoxia
903 when compared to normoxia and anoxia, but the opposite was true for nad2 in *Eurypon* sp. 2.
904 The other six nad genes, which code for parts of the same membrane respiratory chain complex
905 (complex 1, NADH dehydrogenase), however, were not significantly differentially expressed in
906 either species. In both human studies, when one mitochondrial gene was significantly
907 differentially expressed, most mitochondrial genes were significantly differentially expressed in
908 the same direction (Reznik et al. 2017; Medini, Cohen, and Mishmar 2021). The physiological
909 meaning (if any) of this single, significantly differentially expressed mitochondrial gene in
910 sponges remains to be determined.
911
912 Compared to that of humans, even less is known about mitochondrial gene expression in
913 marine invertebrates. Mitochondrial gene expression is usually excluded from differential
914 expression analyses due to the differences in library preparation potentially needed for
915 sequencing mitochondrial RNA and/or the exclusion of mitochondrial RNA from reference
916 transcriptomes (*sensu* Meyer (2016). This dearth is partly due to the nature of mitochondrial
917 transcription, which generates a single continuous transcript. Following transcription, however,
918 mitochondrial transcripts are broken up into individual coding mRNA, tRNA or rRNA (D'Souza
919 and Minczuk 2018). After the breakup, the levels of mRNAs from specific genes are controlled
920 post-transcriptionally (D'Souza and Minczuk 2018). It is therefore possible to have different
921 counts between the different mitochondrial genes despite their single transcript origin
922 (Supplemental Figure 6). These consistent 'expression' levels of transcripts across oxygen
923 conditions for each mitochondrial protein-coding gene following continuous transcription are
924 likely to be necessary for the maintenance of mitochondrial function.
925
926 It remains unclear how sponge mitochondria maintain this transcription from an energetic
927 standpoint under anoxia. Within the mitochondrial electron transport chain, oxygen is used as
928 the terminal electron acceptor and is crucial for the generation of ATP through oxidative
929 phosphorylation. Theoretically, anoxia should cause electron transport and oxidative
930 phosphorylation in the mitochondria to cease (Chandel and Schumacker 2000). If the
931 mitochondria are transcribing solely to maintain oxidative phosphorylation machinery until
932 oxygen returns, then they would need to use at least enough energy (~2 ATP per base) to
933 transcribe for months at a time without oxygen (Lynch and Marinov 2015), and this energetic
934 requirement would apply to the transcription of sponge nuclear genes as well. Glycolysis may
935 provide some anaerobic ATP production, even if it is not upregulated in anoxia or hypoxia.
936 Alternatively, to maintain ATP production through aerobic respiration, the sponges of Lough
937 Hyne might have access to oxygen produced by their *Thaumarchaeota* symbionts, a topic
938 explored in more detail below.
939

940 **Microbiome gene expression during deoxygenation**

941 *Thaumarchaeota* gene expression

942 *Thaumarchaeota* symbionts from both sponge species exhibited significantly differentially
943 expressed genes under hypoxia. Only the *Thaumarchaeota* from *Eurypon* sp. 2 (OTU1)
944 exhibited differential gene expression under anoxia, and only those of *H. stellifera* (OTU3)
945 differentially expressed genes under anoxia vs. hypoxia. These species-specific responses
946 within the symbionts could result from phylogenetic differences between the two symbionts.
947 OTU3 is more closely related to the free-living *Nitrosopumilus maritimus* than OTU1 (Schuster
948 et al. 2021). Though there were some species-specific responses, overall gene expression in
949 both *Thaumarchaeota* was similar, supporting the hypothesis from Schuster et al. (2021) that
950 these taxonomically similar symbionts between two sponge species might have similar
951 functional roles.

952 In hypoxia, both *Thaumarchaeota* upregulated the stress response genes USpA and a protein
953 from the HSP20 family. It was surprising that *Thaumarchaeota* may be stressed by hypoxia.
954 free-living thaumarchaeal Nitrosopumilales can be found in high abundances in oxygen-
955 minimum zones (OMZs) where oxygen is either at low concentrations or below detection
956 (Löscher et al. 2012), though some *Thaumarchaeota* OTUs decrease in abundance in suboxic
957 and anoxic zones (Sollai et al. 2019). Members of the genus *Nitrosopumilus*, however, increase
958 their absolute abundance in suboxic regions, but then decrease in abundance with depth in the
959 anoxic zone (Sollai et al. 2019), indicating a preference for suboxic environments. If suboxia is
960 also the preferred niche of sponge *Thaumarchaeota* symbionts, the upregulation of stress
961 response genes in response to hypoxia may correspond to a rise in intracellular oxidative stress
962 following an increase in metabolic activity in hypoxia.

963 Based on the genomes of their close relatives, the *Thaumarchaeota* in these sponges were
964 hypothesized to have components of a facultatively anaerobic metabolism, including fumarate,
965 nitrite, and sulfite respiration and the ability to ferment (Bayer et al. 2020), which could aid in
966 deoxygenation tolerance. Lactate/malate dehydrogenase, which could be involved in
967 fermentation (Bayer et al. 2020), was upregulated in *Eurypon* sp. 2 *Thaumarchaeota*, but only in
968 hypoxia. Genes for the other anaerobic pathways suggested by Bayer et al. 2020, i.e. succinate
969 dehydrogenase/ fumarate reductase, nitrite reductase and sulfite reductase, were present in
970 both *Thaumarchaeota* in the present study but were not significantly differentially expressed
971 under different oxygen conditions. The genes for nitrite reductase exhibited low expression
972 across all samples (0–20 counts) in both *Thaumarcheaota,* and so did the dehydrogenase/
973 fumarate reductase gene in the *Thaumarcheaota* of *H. stellifera*. Therefore, these genes with
974 low expression may not be used under the conditions of this study. Only three genes in the
975 *Thaumarchaeota* of *Eurypon* sp. 2 under anoxia were significantly differentially expressed, and
976 none of these were involved in anaerobic metabolism. No genes were differentially expressed in
977 *H. stellifera Thaumarchaeota* under anoxia. Therefore, there was no transcriptomic evidence for
978 the upregulation of anaerobic metabolism within *Thaumarchaeota* in anoxia.

979 Transcription of ammonium monooxygenase (amo) genes did not significantly change under
980 anoxia or hypoxia, even though the ammonia oxidation pathway is oxygen dependent. Like the

981 sponge mitochondria, the *Thaumarchaeota* are therefore either transcribing amo genes to be
982 ready when oxygen returns, or they are obtaining trace oxygen for this pathway from another
983 source. A recent study demonstrated that *N. maritimus* produces trace levels of oxygen (100–
984 200 nM) under environmental anoxia, and it uses this oxygen to oxidize ammonia (Kraft et al.
985 2022). If the closely related sponge *Thaumarchaeota* symbionts are also able to produce
986 oxygen under anoxic conditions, then they should express amo genes under anoxia as well as
987 normoxia.

988 In comparing the proteome during oxygen production under oxygen depleted conditions versus
989 periods when no oxygen was produced under 100% a.s. by *N. maritimus*, Kraft et al. (2021)
990 found that proteins upregulated during oxygen production included some AsnC family proteins
991 and nitroreductases. In *Archaea*, AsnC proteins can regulate metabolic processes, including the
992 switch between aerobic and anaerobic metabolisms (Lemmens et al. 2019). Similarly,
993 nitroreductases can have a wide range of metabolic functions (Akiva et al. 2017).
994 *Thaumarchaeota* within *H. stellifera* upregulated a Lrp/AsnC ligand binding domain under
995 hypoxia and in anoxia vs. hypoxia. Another AsnC type protein was also upregulated under
996 hypoxia, and a nitroreductase under anoxia vs. hypoxia. However, these proteins did not directly
997 correspond to those upregulated in *N. maritimus* during oxygen production (Supplemental
998 Figure 9), and the latter proteins are not definitively known to be involved in oxygen production,
999 since the pathway remains unknown.
1000
1001 <u>*Gammaproteobacteria* gene expression</u>

1002 Gene expression in the *Gammaproteobacteria* was species and oxygen-condition dependent. In
1003 *Eurypon* sp. 2, *Gammaproteobacteria* downregulated genes in hypoxia that were associated
1004 with respiration and energy production, including cytochrome c and associated genes (Welchen
1005 et al. 2016). The *Gammaproteobacteria* also downregulated key metabolic genes in hypoxia
1006 such as glutamine synthetase and structural genes like YbiS YcfS YnhG family protein, which is
1007 involved in cell wall production (Magnet et al. 2008). So unlike sponges or *Thaumarchaeota*,
1008 *Gammaproteobacteria* in *Eurypon* sp. 2 (OTU2) may decrease metabolic activity under hypoxia,
1009 though this is likely not the case in *H. stellifera* symbionts (OTU7).

1010 *Gammaproteobacteria* in *H. stellifera* downregulated hundreds of genes in anoxia vs. hypoxia,
1011 including USpA, so, like other members of the holobiont, hypoxia requires a stronger
1012 transcriptomic response and, therefore, may be more stressful than anoxia for these
1013 *Gammaproteobacterial symbionts*. The few upregulated genes in anoxia vs. hypoxia included
1014 genes for energy production, e.g. a transmembrane proton pump, succinyl-CoA synthetase (part
1015 of the citric acid cycle), and formate dehydrogenase (alpha subunit), though other genes
1016 associated with energy production were downregulated. Formate dehydrogenase can function
1017 in both aerobic respiration or in anaerobic respiration(Jormakka, Byrne, and Iwata 2003), but
1018 this enzyme was not significantly upregulated in anoxia compared to normoxia or in the
1019 *Gammaproteobacteria* of *Eurypon* sp. 2 under any condition. So, it is unlikely that this anaerobic
1020 pathway is active in sponge-associated *Gammaproteobacteria* under anoxia.

1021 *Gammaproteobacteria* symbionts were previously hypothesized to use sulfide or thiosulfate as
1022 an electron donor anaerobically coupled to nitrate reduction, removing exogenous, toxic sulfide

1023 from the holobiont under anoxia (Schuster et al. 2021). Although they are not present in OTU2,
1024 OTU7 from *H. stellifera* has the sulfur oxidation genes soxA, soxY, and soxZ, which could
1025 oxidize sulfide and the nitrate reductase genes NAP. However, the sox genes were not
1026 expressed in any samples in the current study, and NAP was expressed at very low levels
1027 across all samples (2-28 counts). Moreover, neither symbiont exhibited significant differential
1028 gene expression in anoxia versus normoxia. Consequently, there was very little transcriptomic
1029 evidence that the anaerobic pathways in these *Gammaproteobacteria* were activated during
1030 environmental anoxia.

1031 **Adaptations of sponge holobionts to low oxygen**

1032 No clear anaerobic respiration responses were observed in the transcriptomes of sponges, their
1033 mitochondria or their two microbial symbionts under environmental anoxia. In the sponges and
1034 their microbes, glycolysis could potentially provide ATP without oxygen. If so, the limited
1035 significant differential expression of glycolysis genes could indicate that this metabolic process
1036 continues to comparable degrees under normoxia, hypoxia and anoxia. Metabolically, sponge
1037 transcription under anoxia was surprisingly similar to normoxia throughout the holobiont
1038 members, and there was no evidence of dormancy within the holobionts. Instead, in the
1039 sponges, hypoxia might elicit increases in energy production machinery such as ATP synthase.
1040 These potential metabolic increases may indicate that within the hypoxic limits studied here
1041 (1.30-3.56 mg L^{-1}), sponges perform optimally. Indeed, these limits are above those at which
1042 mortality or substantial physiological changes have been observed for sponges (Micaroni,
1043 Strano, et al. 2021; Kumala et al. 2021; Mills et al. 2014). This increased energy production
1044 could be stressful, however, and the diversion of resources could limit cell division in hypoxia.
1045 Somewhere between the lower limit of hypoxia (1.37 mg L^{-1}) and the full anoxia studied here,
1046 sponges may no longer attempt to increase energy efficiency, and therefore elevated
1047 transcription of ATP synthase genes was not observed in anoxia. For both the sponges and
1048 their *Thaumarcheaota* symbionts, cellular stress responses were evident under deoxygenation,
1049 indicating that the holobionts respond to these conditions at a cellular level. The gene
1050 expression associated with anoxia could either be a direct response, via currently unknown
1051 mechanisms, to deoxygenation itself or to the presence of hydrogen sulfide in anoxia.

1052 Given the deoxygenation tolerance of Lough Hyne sponges and their largely unchanged
1053 metabolic gene expression patterns under deoxygenation, the production of oxygen by
1054 *Thaumarchaeota* could also be crucial to holobiont survival. Although any oxygen produced
1055 would be at extremely low concentrations, it would have a short path of diffusion to the sponge
1056 cells, as *Thaumarchaeota* symbionts maintain high cell densities within sponges (Moeller et al.
1057 2019). In addition, mitochondria in general can utilize very low concentrations of oxygen (500
1058 nM) without changing their oxygen consumption rate (Chance 1965; Longmuir 1957; Wilson et
1059 al. 1979). It is plausible that, on a small spatial scale, oxygen generated by *Thaumarchaeota*
1060 could reach these concentrations under anoxia, allowing mitochondrial function to continue in
1061 sponges under anoxia. However, given that the genes responsible are still unknown, this
1062 pathway is still speculative in sponge *Thaumarchaeota*.

1063

1064 **Relevance to future ocean deoxygenation**

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

Relevance for animal evolution

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

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