

Dear Dr. MICHEL,

We are very grateful to the reviewers for their insights and assessments. We are particularly pleased that Reviewer 1 has decided that the manuscript is ready to be recommended in PCI Zoology. We are also grateful to Reviewer 3 for comments and for highlighting the novelty and importance of the study.

We have made most of the requested changes from Reviewer 3, with the exception being the creation of an additional synthesis table/figure. We experimented with an additional table/figure as suggested, but it quickly became too large and repetitive, while not adequately reflecting the subtleties of the data with regard to species and oxygen level (see detailed response below). In order to clarify these differences as well as elaborate on hypothetical functional changes under deoxygenation as requested by Reviewer 3, we have expanded discussion of the functionality of key genes and processes in the discussion section. We think that these changes address the reviewer's concerns and improve the clarity of the conclusions of the manuscript. Our responses to reviewers are shown in blue below.

We think that our manuscript is now suitable for recommendation in PCI Zoology.

All the best on behalf of the authors,

Brian Strehlow, PhD

Reviewer 1

The authors made the requested changes: their revisions have answered/addressed all of my questions and concerns from the original submission. I consider the paper ready to be recommended in PCI Zoology.

[Many thanks to reviewer 1 for their previous comments and this recommendation.](#)

Reviewer 3

Review of the paper "Transcriptomic responses of sponge holobionts to in situ, seasonal anoxia and hypoxia" by Brian Strehlow et al.

This manuscript presents a study of the transcriptomic response of two sponge species to hypoxic and anoxic conditions in situ. The study focuses not only on the transcriptomic response of the sponges but also (very novel!) on that of their mitochondria and dominant symbionts. The manuscript is well written and the metadata, data analysis pipelines and scripts are available in an open repository, following good open-science practices.

This study deals with a very important and timely subject in the current context of climate change, in which benthic communities and ocean ecosystems in general are predicted to suffer oxygen limited. The authors found very interesting results, such as some potential gene functions for oxygen homeostasis in sponges (e.g., Hsp90) or the potential role of Thaumarchaeota symbionts in O₂ limiting conditions, being a source of O₂ for the sponge when O₂ is limiting.

This work has already undergone a review stage in which the authors have made an effort to simplify the presentation of their results and help the reader to get the take-home messages of their work. I still believe that the manuscript could be improved to highlight the most relevant results (see below my comment on this subject in the General comments).

Please find below some comments on general aspects of the manuscript and a series of detailed remarks on specific points.

General comments

- In the Introduction, the authors cite a number of studies on the effect of hypoxia/anoxia on sponges. I suggest avoiding the use of zoological nomenclature to refer to each sponge species mentioned to improve the readability of the article. Ex. *Polymastia crocea* rather than *Polymastia crocea* Kelly-Borges and Bergquist, 1997

We believe that it is important that taxonomists are cited for their work, but recognize that this hinders readability. Since PCI Zoology has no clear rules about citing authorities for species, we have therefore added “taxonomic authorities” as footnotes. This has improved readability while still recognizing taxonomic work.

- Experimental design is complex (2 species, 3 O₂ conditions, ...) as is the data analysis (transcriptomics on sponge, mitochondria, symbionts) and the results explanation (pairwise conditions comparison, etc.). Replication also varies between species and conditions, limiting some comparatives (and eventually limiting data interpretation, which authors have been cautious in discussing, which I appreciate). I suggest adding a section or paragraph on the sampling strategy to help readers understand the experimental design and the results interpretation.

A paragraph has been added to describe the sampling strategy. The sampling section now reads (lines 179-203):

‘Sponges were sampled between July 2018 and August 2019 at Labhra Cliff (Lough Hyne Nature Reserve, Ireland; N51°30.05309 W9°18.17679) as described in Schuster et al. (2021) under permit no. R23-27/2018 issued by the Irish Department of Environment, Heritage, and Local Governments. Dissolved oxygen profiles with depth were determined using a Pro20 dissolved oxygen meter (YSI, USA). Profiles for different sampling times are shown in Schuster et al. (2021). Sponge samples were taken using SCUBA (depth range: 15-29 m) under various in situ oxygen conditions that were categorized as follows: normoxic (5.3–12 mg L⁻¹, 49.3–111% a.s.), hypoxic (1.30–3.56 mg L⁻¹, 12.1–33.1% a.s.), and anoxic (0.00–0.01 mg L⁻¹, 0.00–0.93% a.s., i.e. instrument detection limit). Oxygen concentration at each sampling point was verified using a HOBO dissolved oxygen logger (U26-001; Onset, USA). All samples were frozen in liquid nitrogen within ~15 minutes of collection and kept in a dry shipper until returned to the laboratory where samples were stored in a -80°C freezer until RNA and DNA extraction.

In total 70 sponge samples were taken under anoxia (n = 6), hypoxia (n = 25), and normoxia (n = 39). Although all these red-orange encrusting sponges were indistinguishable in situ, species identification using molecular barcodes revealed that there were nine species (see Schuster et al. 2021). This level of cryptic diversity is common in sponges, and necessitates care in post-sampling species identification. Two species, *Eurypon* sp. 2 and *H. stellifera*, comprised the majority of the samples and were represented across the oxygen conditions; therefore, these two species were selected for the transcriptomic analysis of sponge, symbionts, and mitochondria in this study. For RNA sequencing (RNAseq), eight samples of *H. stellifera* (anoxic = 2, hypoxic = 5, normoxic = 1) were taken, and sixteen samples of *Eurypon* sp. 2 (anoxic = 2, hypoxic = 7, normoxic = 7) were sequenced (n = 24 in total). However, certain oxygen levels within each species, i.e. anoxia in both species and normoxia in *H. stellifera*, had limited replicates (n < 3). The implications of this limited replication are considered in the analysis methods and discussion. Metadata for individual samples, including oxygen concentration, season, depth, collection date, and individual sample code (e.g. DC##) are included in Supplemental Table 1.’

- State in the Methods which O₂ conditions have limited replicates (i.e., n < 3). This limitation in replicates may be responsible of some of your non-significant results.

This is now specified in lines 198-200 (see excerpt above as well).

- As above mention, the complex experimental design and the pairwise comparison of the conditions evaluated make complicated to identify the functional genes affected by O₂ limitation. In the Results, please not only indicate the up- or downregulated genes but also the functions affected. See my comment below as well.

Since one gene can have multiple functions, and gene functions in sponges are based on orthology with genes from model organisms and therefore hypothetical to a degree, considerations of gene function necessitate consulting the literature extensively. Discussion of function outside the name of GO terms or KOG classes is generally left to the discussion section (e.g. Kenkel et al. 2020). Therefore, gene function, and in particular gene function in deoxygenation tolerance (which is largely hypothetical), belongs in the discussion. Moreover, if function is discussed for every gene in both results and discussion, the manuscript becomes very bulky, and we have already cut out the discussion of many significantly differentially expressed genes based on previous comments from reviewer 2 (see below).

“Reviewer 2: The discussion is very long and sometimes hard to . It’s not easy to know what’s important in the long list of genes/groups of genes up- or down-regulated in the different compartments or the different treatments. The publication would be clearer if only the take home messages were kept and not the differences observed at the margins, which in the final analysis are not significant in answering the question of tolerance.

Response: The following lines were removed for the sake of clarity: 817-827, 1010-1013, 767-783, 822-888, 891-892, 906-910, 926-928, 930-943, and 1050-1052 (see tracked changes version for these lines).”

That being said, we have left significantly differentially expressed genes in the results that were not part of the main takeaways in the discussion in the hope that they may aid future researchers when considering specific genes of interest. We have also provided lists of all of the hundreds of significantly differentially expressed genes in the supplemental material to this end.

Reference

Kenkel, Carly D., Veronique J. L. Mocellin, and Line K. Bay. 2020. “Global Gene Expression Patterns in Porites White Patch Syndrome: Disentangling Symbiont Loss from the Thermal Stress Response in Reef-Building Coral.” *Molecular Ecology* 29 (20): 3907–20.

- I know the authors have already done a big job making Figure 4, but I still think a figure or summary table showing the biological processes (e.g., energy metabolism, DNA repair, etc) affected by hypoxia, anoxia and deoxygenation for each species is necessary. Figure 4 illustrates differences between species (including mitochondria and symbionts) and O₂ conditions, but it does not show the differences of functions expressed to cope to or as consequence of limitation of O₂. In this new figure/table, you could specify if the function has changed and if it is provided by the symbiont or it happened in the sponge itself.

We attempted to generate a table to discuss all potential functions, but it quickly became unwieldy and illegible with two species and three oxygen levels, and there are many qualifications that need to be made about functionality and differences between species. For example, discussion of the functions of HSP genes in cellular stress and in response to deoxygenation comprises 22 lines (777-799), and the function of Hsp90 in deoxygenation tolerance comprises 8 lines (811-819). In a table, the majority of this information would be repeated for each species and oxygen level, preventing the reader from understanding the nuances between species and oxygen level. Therefore, we believe that potential functions of the genes are better described in the discussion.

To facilitate the discussion of the complex experimental design and what changes occur where, we now outlined the structure of the discussion in the first paragraph as follows:

'The potential functions of this differential expression are discussed below for each member of the two holobionts: sponge, sponge mitochondria, *Thaumarchaeota* and *Gammaproteobacteria*. Changes in gene expression within each member of the holobiont is discussed in a separate section below, and then the potential functional adaptations to deoxygenation at the level of the holobiont are summarized.'

Additionally, we have endeavored to clarify hypothetical functions under deoxygenation more explicitly in the following lines: 780-781, 799-801, 835-837, 838-839, 853-854, 879-881, 960-961, 1048-1050, and 1094.

For the biological processes, we have referenced relative GO terms and KOG classes in the discussion that are pertinent to our main takeaways, and all significantly enriched GO terms and KOG classes are displayed in Supplemental Figures 1-3 and Figure 1, respectively. However, discussing the function of every significantly enriched process (10-30 per species, per treatment level) in the context of deoxygenation tolerance is too speculative and cumbersome. Moreover, although many processes are enriched, that does not mean that all genes involved in this process are differentially expressed. The same is true for KOG classes. Additionally, many processes are functionally ambiguous, e.g. 'structural molecule' or 'molecule adaptor.' We therefore focused on pathways and functionalities that we could confirm on the KOG, GO and gene level to boost the reliability of our conclusions in the discussion.

Detailed comments

L 158: change "...in sponge Thaumarchaeota" to "...in sponges with Thaumarchaeota", to "...in sponge symbionts Thaumarchaeota" or an alternative to make it clear.

Clarified to 'sponge associated *Thaumarchaeota*.'

L173-174: change "low atmospheric oxygen concentrations" to "low environmental oxygen concentrations".

Changed (line 174).

L 182-185: indicate how you measured in situ oxygen conditions

This is now specified in line 179-180 and 185.

L 194: add "Metadata for individual samples, including sample code, oxygen concentration, ... are included in Supplemental Table 1". These codes are used afterwards (e.g., L202) and it is not clear where they came from

This has now been clarified.

L 195-197: were these species identified based on their skeletons? Please, specify.

Species were identified using molecular barcodes following the genetic species concept as specified in lines 187-188. Full taxonomic descriptions, including skeletal elements such as SEM and spicule measurements, are currently in the making and will be published in a separate MS.

L 202: add "sample" before DC24, otherwise this code may not make sense to readers who do not read the Supplemental Information.

Done.

L 223: specify what the acronym SDU means

Done.

L 243: correct to "... generated 2.05 x 10⁸ and reads 3.45 x 10⁸ reads for..."

Done.

L 371 & 372: write the full name of Eurypon sp. 2 rather than E. sp. 2.

Done.

L 422-424: suggestion to simplify as "Expression patterns of both species were only similar in one case. KOG expression in *H. stellifera* under hypoxia versus anoxia significantly positively correlated with that of Eurypon sp. 2 under the same conditions ($r = 0.43$, $p < 0.05$, Figure 1E)."

Done.

L 450-452: I suggest deleting this sentence as it is repetitive with figure legend and does not provide new relevant information.

Done.

L 456: "...depending on oxygen availability" or "... on the oxygen level".

Changed to the latter option.

L 498: given the nature of the study, the text is full of acronyms and abbreviations, so I suggest avoiding those not absolutely necessary, as for example DEGs.

We have removed the following acronyms - DEG, RRR, and EPC.

L 507: delete "of the same", so sentence read "Upregulated genes included all genes that were significantly upregulated in anoxia"

Done.

L 511: change to "It is noteworthy
, however, that all genes upregulated genes in anoxia..."

Done.

Figure 2 & 3: I suggest to write above the heatmaps the sponge species to which they belong, that is, Eurypon sp 2 for left heatmaps and *H. stellifera* for right ones.

Done.

L 669-674: write the full name of Eurypon sp. 2 rather than E. sp. 2. Italicize *H. stellifera* in L 671.

Done.

L 686: cite Table 1

Done.

L 777-779: change to "Sponges under heat stress also upregulate Hsp70 (López-Legentil et al. 2008; Guzman and Conaco 2016; Webster et al. 2013) and Hsp90 (Guzman and Conaco 2016), as it occurred in both Lough Hyne sponges under hypoxia"

This sentence has been clarified.

L 786: anoxia vs hypoxia, doesn't it?

Yes, this has been changed.

L 815: correct superoxide formula to O₂⁻²

Corrected, but to O₂⁻, which is the accepted formula.

L 901: correct the typo to *S. mosellana*
Done.

L 996: change to “AMO genes” rather than “amo genes”
Done.

L 1037: no need of italics
Removed.

L 1076: write the full name of *Eurypon* sp. 2 rather than *E. sp. 2*.
Done.