Dear Dr. MICHEL,

We are grateful to you and the reviewers for the helpful and detailed assessments. We have addressed the reviewers' comments and now believe that our manuscript is suitable for recommendation by PCI Zoology. Notably, we have now included a new summary figure (Figure 4) and clarified the major findings of the manuscript in lines 1053-1094, as requested by the reviewers. This greatly adds to the clarity of the manuscript. Reviewer reports are addressed point-by-point below in blue. Please note that line numbers refer to the bioRxiv version (v3) unless otherwise indicated.

Best wishes on behalf of all authors,

Brian Strehlow, PhD

Responses to reviewers

Reviewer 1

General comments

The article by Strehlow et al., aims to investigate the functional effect of *in situ* deoxygenation on two sponge holobionts (*Eurypon* sp. 2 and *Hymeraphia stellifera* and their microbiome) using a transcriptomics approach. This study provides unique outcomes for the research field on sponge holobionts, helping to better understand their metabolic adaptation under hypoxia and anoxia, especially in an environmental context which could be relevant in for future oceanic conditions. The research conducted in this paper constitutes a logical complementary approach following Schuster et al. (2021), dedicated to the study of the microbiome composition from the same sampling set from Lough Huyne (Ireland). Authors provided informations on the up- and down-regulated functions changing from normoxia to hypoxia, normoxia to anoxia, and hypoxia to anoxia, not only for the sponge transcriptome, but also from transcriptomes of the sponge mitochondria, and two symbionts members discussed in Schuster et al., (2021) affiliated to *Thaumarchaeota* and *Gammaproteobacteria.* Through an extensive effort to describe all the functional differences observed, and integrating other studies (as a meta-analysis), the authors also highlighted the species-specific nature of the response to the deoxygenation. In overall the article is well written and the discussion is nicely structured.

Considering these reasons, I think that the paper has a great potential to have a good impact in the field of the sponge holobiont. However I have two major comments that should be considered, followed by minor remarks being more specific.

Major comments

1/ My main comment is about the sample size used, which is the most limiting factor of this study. 3 of the 6 sample groups have 2 replicates or less (Hs norm : n=1 ; Hs anox : n = 2, Es anox : n = 2) which appear to be limiting for proper statistical analyses, especially for differential transcriptomics analyses (Schurch et al., 2016). I believe that despite this small sample size, the informations obtained by the authors are still valuable, and authors did wise choices with the adapted statistical methods used. However I recommend to show more caution in the discussion (as done in the result section) to highlight that some comparisons (especially those involving anoxia for both sponge holobionts) needs to be considered in a study context with a limited number of replicates.

We have endeavored to qualify and contextualize our results within the limited replication in the discussion (see lines 768-775).

2/ The readability of the article could be improved especially considering the high amount of informations presented in the results section, and followed by the discussion. Here are some suggestions :

- A lot of information is grouped in Figure 1, and the whole figure is actually analyzed all along from L. 387 to 597 and further discussed. As the figure represent an important part of the whole manuscript, it might help the readability to split the figure in two separate ones, for example with one dedicated for the internal comparison with the two sponge species, and another dedicated to the comparisons with the external datasets.

We have made several changes to Figure 1 to improve clarity and readability. In Figure 1A, we have made the heat map larger and divided the internal comparisons and external comparisons with a line and the labels ‘this study’ and ‘external datasets,’ respectively. The correlation plots that compare within the dataset from this study are now all shown on one line (B-E), and the correlation plots that compare internal data to external data are shown on the line below (F-J). This new delineation is explained in the legend (Lines 430-445). Upon reflection, we decided not to split the heatmap in Figure 1A in two to compare external and internal datasets because that would involve having two figures depicting the same data, i.e. the internal datasets. We believe that our changes represent the best balance between readability and avoiding repetition.

- As many results from the differential analyses were presented, I think that a final figure schematizing all the functional processes discussed for both sponge holobionts (including

mitochondria and symbionts) is necessary. I know that it might be a lot of work, but in overall, I believe that this paper constitute a nice work that deserve a schematic figure to gather all the informations presented. This figure could help the reader to easily get the bigger picture summarizing the main metabolic pathways affected by the hypoxic and anoxic conditions mentioned in the paper, especially considering the HSP, the ATP / glycolysis, and nad2. Moreover, this figure could be presented as a complementary addition to the figure 8 from Schuster et al., (2021).

A schematic figure (Figure 4) has now been provided (lines 784-791). We agree that this aids the reader substantially.

- Finally, the article suffers from a lack of proper conclusion at the end (after the part of relevance for animal evolution). This conclusion could easily help the reader to get the take home message with the help of the final figure suggested above.

We have added/reformatted the summary section 'Summary of potential adaptations of sponge holobionts to low oxygen' to better summarize the main points of the paper and offer conclusions and directions for further research (lines 1053-1094). This section is also substantially aided by the addition of Figure 4.

Minor / specific comments

L. 125. to 127 While mentioning the results of Schuster et al., (2021), I would suggest to also indicate that the sampling for the anoxic, hypoxic and normoxic conditions were performed at different sampling times. It helps to consider how this difference of condition goes with seasonal differences (since it's mentioned in the title of the article).

We have added in the introduction that the anoxia occurs in the summer (line 86). We have also added a supplemental table (Supplemental Table 1) that includes all sampling metadata (see response to next comment).

L. 156 to 161. It is still unclear for me to understand exactly when the samples were exactly collected for each oxygen condition, as I cannot open the eps formatted Figure S3 from Schuster et al., (2021) which probably summarizes this information. I think it would be good to also have this information again in this manuscript to avoid to many "round trips" between this paper and Schuster et al., (2021).

We have clarified the sampling condition by adding a supplemental table (Supplemental Table 1) and including the following lines in the text: 'Metadata for individual samples, including oxygen concentration, season, depth and collection date are included in Supplemental Table 1.' (Lines 194-195).

L. 292 to 294. (see main comment) Authors pointed the insufficient number of replicates for *H. stellifera* under normoxia (n = 1). I would also suggest to make the same remark for the anoxic condition for both sponge species (n = 2). Does this low number of replicates justify the choice of having a FDR adjusted p-val threshold of 0.1 ?

We used 0.1 as a threshold for two reasons: 1) it was decided before we analyzed the data and is becoming the standard practice for these types of analysis (e.g. Kenkel et al., 2020; Strader et al., 2016) and 2) this study was exploratory in nature and we were not sure what genes would be involved so we wanted to work within a broad scope. Moreover, all p values and fold changes for significantly differentially expressed genes are reported in files in the zenodo data repository. We have highlighted this more in the text lines 314-318. These files also allow future researchers to search for any genes of interest they have, and the reporting of all the p-values and box plots allows them to consider stricter FDR thresholds if desired.

References

Strader, Marie E., Galina V. Aglyamova, and Mikhail V. Matz. 2016. “Red Fluorescence in Coral Larvae Is Associated with a Diapause-like State.” Molecular Ecology 25 (2): 559–69.

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.

L. 341 to 350 : Authors mention the methods associated to the phylogenetic positions of nitroreductase proteins. If I am not missing anything, perhaps it would be wise to shortly describe what are the aims / hypothesis associated to this specific part, in the introduction.

The hypothesis of this section as well as the background (see comments from Reviewer 2) was added in lines 154-158. The aims of the phylogenetic aspect are further specified in lines 369-370.

Here are below some of my suggestions/questions that could be considered by the authors to enrich their discussion :

L. 758 to 761. Does the oxidative stress induced by lead and zinc concentrations (or other trace metals) could be connected to an inhibition of the water pumping and consequently to an anoxic state, explaining also the upregulation of HSPs ?

We believe that it is possible that lead and zinc exposure could cause sponges to stop pumping, resulting in internal anoxia. However, pumping was not measured in studies that exposed sponges to these metals (Efremova et al. 2002; Schröder et al. 2006). While many stressors can cause arrests or decreased pumping rates in sponges (Massaro et al., 2012; Strehlow et al.

2016), the nature and degree to which internal oxygen concentration changes based on stressors and pumping rates is understudied and likely species-specific. Moreover, some species can arrest pumping and deplete internal oxygen stores in the absence of stress or stimulus (Kumala et al., 2021). We have therefore elected not to speculate on the role of internal anoxia in sponge stress response in this paper.

References

Efremova, Sofia M., Boris A. Margulis, Irina V. Guzhova, Valeria B. Itskovich, Stephanie Lauenroth, Werner E. G. Müller, and Heinz C. Schröder. 2002. “Heat Shock Protein Hsp70 Expression and DNA Damage in Baikalian Sponges Exposed to Model Pollutants and Wastewater from Baikalsk Pulp and Paper Plant.” Aquatic Toxicology 57 (4): 267–80.

Kumala, Lars, Morten Larsen, Ronnie N. Glud, and Donald E. Canfield. 2021. “Spatial and Temporal Anoxia in Single-Osculum Halichondria Panicea Demosponge Explants Studied with Planar Optodes.” Marine Biology 168 (12).

Massaro, A. J., Weisz, J. B., Hill, M. S., & Webster, N. S. (2012). Behavioral and morphological changes caused by thermal stress in the Great Barrier Reef sponge Rhopaloeides odorabile. Journal of Experimental Marine Biology and Ecology, 416–417, 55–60. https://doi.org/10.1016/j.jembe.2012.02.008

Schröder, H. C., S. M. Efremova, B. A. Margulis, I. V. Guzhova, V. B. Itskovich, and W. E. G. Müller. 2006. “Stress Response in Baikalian Sponges Exposed to Pollutants.” Hydrobiologia 568 (1): 277–87.

Strehlow, B. W., Jorgensen, D., Webster, N. S., Pineda, M.-C., & Duckworth, A. (2016). Using a thermistor flowmeter with attached video camera for monitoring sponge excurrent speed and oscular behaviour. *PeerJ*, *4*, e2761. https://doi.org/10.7717/peerj.2761

L770 to 772. DNA damages are generally linked to an oxidative stress. Hypoxia and anoxia are known to induce such oxidative stress in other models which might affect the integrity oF the DNA structure. An upregulation of the DNA repair functions might be explained by the promoted ROS induced by the low oxygen levels (?)

We have added lines 809 to 815 to address this comment.

L817 to 818. This might be explained by the following reasons mentionned above : the important DNA damages that could be caused by the oxidative stress during hypoxia could disturb the DNA replication

We agree, and we have added this potential explanation to line 855-856.

Reviewer 2

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

This article reports on a study of the transcriptomic response of two sponge species to hypoxic and anoxic conditions. The study focused on the transcriptome of the sponges themselves, but also of their mitochondria and the associated microbiome (mainly the two dominant microbes: an archaea and a gammaproteobacterium).

This article deals with a very interesting subject and poses particularly interesting questions on the mechanisms that enable these organisms to tolerate a reduction in the quantity of oxygen in the environment, particularly in the current context of possible deoxygenation of the oceans.

The title clearly reflects the content of the article.

The introduction seems (as far as I can judge, not being a specialist in the subject) to correctly put the study into context and the literature on the effect of hypoxia or anoxia on sponges or other invertebrates on which data are available. I think it would be interesting to develop the paragraph on the missing HIF (hypoxia inducible factor) pathways in sponges and the vital functions that the microbiome can play, which remains very evasive (lines 115 to 118).

We have added a paragraph in the missing HIF pathway components in sponges (lines 114-120). We also moved hypotheses about the potential roles of the microbiome in sponge oxygen tolerance from the discussion and expanded the context around them (lines 147-160). These additions were valuable to the framing of the manuscript.

It would also be interesting to describe the host-microbiome relationship (i.e. location of microbes in/on host tissues, quantity, diversity, interactions with the host) if this information is known.

The potential host-microbiome relationships for the targets of this study were further described in lines 147-151 (see previous response). The location of these microbes has not yet been determined, so the following line was added: 'Although their location within Lough Hyne sponges has not yet been determined, Gammaproteobacteria and Thaumarchaeota symbionts are abundant and widely distributed throughout the sponge tissue in other species (Moeller et al. 2019).' Lines 141-145.

The stated aim of the study is to understand the mechanisms that enable tolerance to hypoxia and anoxia, and survival in prolonged anoxia. While a number of clues are given, overall we are left wanting to know what mechanisms are at work to explain such tolerance (no doubt due to the sheer volume of data, which is a bit confusing, see below).

Regarding Materials and methods, I lack expertise in molecular approaches and am not in a position to judge the choice of techniques and analyses used. On the other hand, I would suggest indicating the depths and temperatures of the sampling sites, if available.

We have clarified the sampling conditions by adding a supplemental table (Supplemental Table 1) and including the following lines in the text: 'Metadata for individual samples, including oxygen concentration, season, depth and collection date are included in Supplemental Table 1.'

(Line 195). Samples were all taken from the same site, and the latitude and longitude are now specified in line 180.

My main concern with this article is that the study provides an enormous amount of data, which dilutes and obscures the important messages. The summary also reflects the complexity of having so much data to summarise.

For example, some phrases that can be a bit confusing, such as:

- Line 799: These results might indicate that glycolysis rates increased under anoxia and hypoxia in both species. Due to the consistency in gene expression of the other ~70 genes associated with glycolysis; however, it is more likely that glycolysis continued under anoxia and hypoxia at normal rates in both species.

This sentence has been reworded for clarity (lines 843-847).

- Line 946: These species-specific responses within the symbionts could result from phylogenetic differences between the two symbionts. And in the next sentence: these taxonomically similar symbionts between two sponge species might have similar functional roles.

Since the vast majority of responses were shared between both symbionts, we have removed the sentences highlighting species-specific responses for the sake of clarity (lines 1035-1038, tracked changes version).

The discussion is very long and sometimes hard to follow. 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.

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).

A summary paragraph clearly indicating what the study explains (or can not explain) about the mechanisms involved in resistance to hypoxia would be very useful.

We agree and have now added a summary figure (figure 4) and paragraphs (lines 1053-1094).

Links between the different data would also be interesting: for example, if I've understood correctly, there could be dormancy in hypoxia in Eurypon sp. 2, how could that be correlated with the increase in ATP production?

We have addressed this question in lines 898-904. Furthermore, we believe that the incorporation of the summary figure, conclusion paragraphs, and associated text provide additional, interesting links between the different data.