**Response to Reviewers**

**>We highlighted our responses to the editor and reviewers in bold red text preceded by a “>” symbol.**

PONE-D-21-38235

Characterizing the secret diets of siphonophores (Cnidaria: Hydrozoa) using DNA metabarcoding

PLOS ONE

Dear Dr. Damian-Serrano,

Thank you for submitting your manuscript to PLOS ONE. After careful consideration, we feel that it has merit but does not fully meet PLOS ONE’s publication criteria as it currently stands.

I have now constructive reviews from two experts on studies of metabarcoding on marine organisms. Both reviewers see merit in the study and are enthusiastic about its novelty and usefulness. At the same time, both reviewers raise concerns that need to be addressed before the manuscript can be further considered for publication. Of **particular concern, in my view,** are the comments from reviewer 1 on the lack of statistical analysis and the poor description of the methods. In addition, both reviewers recommend some degree of rewriting to make the manuscript more useful and appealing. The many queries from reviewer 2 indicate points that readers might find confusing. Reviewer 1 also points concerns about the type and quality of graphs. I encourage you to consider whether other types of graphs may be more appropriate.

I encourage you to submit a revised manuscript by Mar 30 2022 11:59PM. If you will need more time than this to complete your revisions, please reply to this message or contact the journal office at plosone@plos.org. When you're ready to submit your revision, log on to [https://www.editorialmanager.com/pone/](https://urldefense.com/v3/__https://www.editorialmanager.com/pone/__;!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDVcZQpHWQ$) and select the 'Submissions Needing Revision' folder to locate your manuscript file.

Please include the following items when submitting your revised manuscript:

* A rebuttal letter that responds to each point raised by the academic editor and reviewer(s). You should upload this letter as a separate file labeled 'Response to Reviewers'.
* A marked-up copy of your manuscript that highlights changes made to the original version. You should upload this as a separate file labeled 'Revised Manuscript with Track Changes'.
* An unmarked version of your revised paper without tracked changes. You should upload this as a separate file labeled 'Manuscript'.

If you would like to make changes to your financial disclosure, please include your updated statement in your cover letter. Guidelines for resubmitting your figure files are available below the reviewer comments at the end of this letter.

If applicable, we recommend that you deposit your laboratory protocols in [protocols.io](http://protocols.io/) to enhance the reproducibility of your results. [Protocols.io](http://protocols.io/)assigns your protocol its own identifier (DOI) so that it can be cited independently in the future. For instructions see: [https://journals.plos.org/plosone/s/submission-guidelines#loc-laboratory-protocols](https://urldefense.com/v3/__https://journals.plos.org/plosone/s/submission-guidelines*loc-laboratory-protocols__;Iw!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDXqrlSpNg$). Additionally, PLOS ONE offers an option for publishing peer-reviewed Lab Protocol articles, which describe protocols hosted on [protocols.io](http://protocols.io/). Read more information on sharing protocols at [https://plos.org/protocols?utm\_medium=editorial-email&utm\_source=authorletters&utm\_campaign=protocols](https://urldefense.com/v3/__https://plos.org/protocols?utm_medium=editorial-email&utm_source=authorletters&utm_campaign=protocols__;!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDWwKFBI3g$).

We look forward to receiving your revised manuscript.

Kind regards,

Hans G. Dam, Ph. D.

Academic Editor

PLOS ONE

**>We thank the Academic Editor for the opportunity to resubmit our manuscript and for this helpful feedback. We have addressed the reviewers' feedback to the best of our ability. We expanded our descriptions of the methods, edited the writing to improve the clarity and appeal, and substituted the problematic graphs for supplementary data tables. However, we find that the request for more statistical analyses comparing the different species is not appropriate for the type and quantity of data we collected, the sampling design and limitations, and the scope of this study. We elaborate on this rationale on the responses to R1 and in the revised manuscript.**

Journal Requirements:

When submitting your revision, we need you to address these additional requirements.

1. Please ensure that your manuscript meets PLOS ONE's style requirements, including those for file naming. The PLOS ONE style templates can be found at

[https://journals.plos.org/plosone/s/file?id=wjVg/PLOSOne\_formatting\_sample\_main\_body.pdf](https://urldefense.com/v3/__https://journals.plos.org/plosone/s/file?id=wjVg*PLOSOne_formatting_sample_main_body.pdf__;Lw!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDWhrIsULQ$) and

[https://journals.plos.org/plosone/s/file?id=ba62/PLOSOne\_formatting\_sample\_title\_authors\_affiliations.pdf](https://urldefense.com/v3/__https://journals.plos.org/plosone/s/file?id=ba62*PLOSOne_formatting_sample_title_authors_affiliations.pdf__;Lw!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDWHw2chGQ$)

**>We have adjusted the formatting of the manuscript document and figures to match PLOS ONE’s requirements.**

2. Please update your submission to use the PLOS LaTeX template. The template and more information on our requirements for LaTeX submissions can be found at [http://journals.plos.org/plosone/s/latex](https://urldefense.com/v3/__http://journals.plos.org/plosone/s/latex__;!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDWVEp4gZw$).

3. Thank you for stating the following in the Acknowledgments Section of your manuscript:

"We thank Gisella Caccone, Carol Mariani, and T.J. Johnson for the Yale DNA Analysis Facility for their invaluable training and their assistance on this study, as well as the staff of the Yale Center for Genomic Analyses for helping us design the sequencing strategy for this study, and the Yale Center for Research Computing for providing assistance with high-performance computing. We thank Bianca R. Brown for her assistance designing the read processing pipeline and Johan Bengtsson-Palme for his help troubleshooting our usage of METAXA2. We are grateful to the crews of the R/V Western Flyer and R/V Kilo Moana, the Bermuda Institute of Ocean Sciences, and Jeff Godfrey for making the collection of these samples possible. This research was funded by the Yale Institute of Biospheric Studies through a Doctoral Dissertation Improvement Award to A.D.-S., as well as by NSF-OCE 1829835 (to C.W.D.), OCE-1829805 (to S.H.D.H.), and OCE-1829812 (to C.A.C.)"

We note that you have provided funding information. However, funding information should not appear in the Acknowledgments section or other areas of your manuscript. We will only publish funding information present in the Funding Statement section of the online submission form.

Please remove any funding-related text from the manuscript and let us know how you would like to update your Funding Statement.

**>We have removed the funding-related text from the manuscript acknowledgements.**

Currently, your Funding Statement reads as follows: "This research was funded by the Yale Institute of Biospheric Studies through a Doctoral Dissertation Improvement Award to A.D.-S. ([https://yibs.yale.edu/research/yibs-small-grant-program/dissertation-improvement-grants](https://urldefense.com/v3/__https://yibs.yale.edu/research/yibs-small-grant-program/dissertation-improvement-grants__;!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDWkVCtTSA$)), as well as by the National Science Foundation grants NSF-OCE 1829835 (to C.W.D.), OCE-1829805 (to S.H.D.H.), and OCE-1829812 (to C.A.C.) [https://www.nsf.gov/geo/oce/programs/biores.jsp](https://urldefense.com/v3/__https://www.nsf.gov/geo/oce/programs/biores.jsp__;!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDVS4BVucQ$). The funders did not play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript."

Please include your amended statements within your cover letter; we will change the online submission form on your behalf.

**>The Funding Statement remains accurate.**

4. Please note that in order to use the direct billing option the corresponding author must be affiliated with the chosen institute. Please either amend your manuscript to change the affiliation or corresponding author, or email us at [plosone@plos.org](mailto:plosone@plos.org) with a request to remove this option.

**>We have changed the affiliation of A.D.S. to Yale University where he carried out this research.**

5. Please include your full ethics statement in the ‘Methods’ section of your manuscript file. In your statement, please include the full name of the IRB or ethics committee who approved or waived your study, as well as whether or not you obtained informed written or verbal consent. If consent was waived for your study, please include this information in your statement as well.

**>We have added an Ethics Statement to the Materials & Methods section (lines 129-132).**

6. We note that Figure 2 in your submission contain copyrighted images. All PLOS content is published under the Creative Commons Attribution License (CC BY 4.0), which means that the manuscript, images, and Supporting Information files will be freely available online, and any third party is permitted to access, download, copy, distribute, and use these materials in any way, even commercially, with proper attribution. For more information, see our copyright guidelines: [http://journals.plos.org/plosone/s/licenses-and-copyright](https://urldefense.com/v3/__http://journals.plos.org/plosone/s/licenses-and-copyright__;!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDUCI4xlUQ$).

We require you to either (1) present written permission from the copyright holder to publish these figures specifically under the CC BY 4.0 license, or (2) remove the figures from your submission:

a. You may seek permission from the original copyright holder of Figure 2 to publish the content specifically under the CC BY 4.0 license.

We recommend that you contact the original copyright holder with the Content Permission Form ([http://journals.plos.org/plosone/s/file?id=7c09/content-permission-form.pdf](https://urldefense.com/v3/__http://journals.plos.org/plosone/s/file?id=7c09*content-permission-form.pdf__;Lw!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDUl00yb4g$)) and the following text:

“I request permission for the open-access journal PLOS ONE to publish XXX under the Creative Commons Attribution License (CCAL) CC BY 4.0 ([http://creativecommons.org/licenses/by/4.0/](https://urldefense.com/v3/__http://creativecommons.org/licenses/by/4.0/__;!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDVDaxdeag$)). Please be aware that this license allows unrestricted use and distribution, even commercially, by third parties. Please reply and provide explicit written permission to publish XXX under a CC BY license and complete the attached form.” Please upload the completed Content Permission Form or other proof of granted permissions as an ""Other"" file with your submission.

In the figure caption of the copyrighted figure, please include the following text: “Reprinted from [ref] under a CC BY license, with permission from [name of publisher], original copyright [original copyright year].”

b. If you are unable to obtain permission from the original copyright holder to publish these figures under the CC BY 4.0 license or if the copyright holder’s requirements are incompatible with the CC BY 4.0 license, please either i) remove the figure or ii) supply a replacement figure that complies with the CC BY 4.0 license. Please check copyright information on all replacement figures and update the figure caption with source information. If applicable, please specify in the figure caption text when a figure is similar but not identical to the original image and is therefore for illustrative purposes only.

**>We have substituted the copyrighted photos in Fig 2 (original labeling: D,E,F,G,H,I) for alternative photos. We submitted a replacement figure that reflects this change. We obtained signed Content Permission Forms for the photos C, F, and J from the copyright holders (forms uploaded as Other). Photo H is published under a CC BY license and not copyrighted. Photos B & D are reprinted from a figure in Munro et al. 2018 (uploaded as Other), also published separately under a CC BY license. Finally, we modified the figure caption to reflect these changes and include the reprinting, permissions, and original copyrights (lines 917-924).**

**Reviewer #1:**

GENERAL COMMENTS

The is a well-designed study that uses diverse approaches to examine the diet numerous species of a fascinating – and challenging – group of pelagic marine predators: siphonophores. Comparisons between siphonophore species allows an initial view of possible prey specialization. The approaches are all appropriate, including: DNA metabarcoding and morphological analysis of gut contents, in situ field observations of the living organisms, and characterization of the prey field based on plankton net tows. The strengths and weaknesses of each approach are clearly explained. Overall, this study will advance our understanding of pelagic food web dynamics and provide a useful foundation for continued research in the field.

**>We thank Reviewer 1 for their generous commentary and constructive feedback.**

Despite my overall very positive view of the design of the study, I have several serious concerns. Primary is that the results are explained in very general terms, summarizing patterns, but not giving specifics for each predator species.

**>In the “Dietary findings by taxon” subsection of the Results & Discussion we address specific findings for each predator species in context with their natural history. These species-specific results are synthesized in lines 365-507.**

Most importantly, there is no evidence of statistical analysis of metabarcoding results for the different predator species, which is needed to test the hypothesis of prey specialization.

**>We agree with Reviewer 1 that meaningful statistical comparisons are required to test hypotheses on prey specialization. Our data are not adequate for this goal, but they can be used to generate new specialization hypotheses for understudied species, and to challenge long-standing assumptions based on visual gut assessments as well as morphology-derived predictions of trophic guild. In the Results & Discussion, we refer to our findings as congruent or incongruent with previously-posited specialization hypotheses generated from quantitative visual observations and morphological analyses. We do not use our findings to make strong claims on the degree of prey type specialization, but rather to evaluate current specialization hypotheses under the light of novel prey types detected. We have modified the text in a few spots where comments on specialization based on our results were worded too strongly (lines 444, 449-450, 454-455, 471, and 550). We also added a disclaimer on the interpretation of the selectivity results given our low sample sizes (lines 573-574).**

Another concern is that the writing is overall quite disappointing. Most especially, the metabarcoding methods are not explained at all,

**>We have rewritten and reorganized some sections to improve clarity and readability (lines 158-165, 285-287, 360-361, 393-403, 409-415, and 632-640). In addition, we have fleshed out the metabarcoding methods, including more details about the DNA extraction (lines 168-170), primer design (lines 171-179 and Table 1), PCR reagents, thermocycler program, amplicon purification, and quality control (lines 187-203) into the Materials & Methods section. We have also expanded the section on assignment interpretation to more explicitly describe the criteria used for downstream data annotation (lines 252-291).**

and this paper uses unique (and meaningless) names for the target regions of the 18S rRNA gene used for analysis. This is very unfortunate and extremely unwise; in all cases that I am familiar with, authors refer to the 18S rRNA regions used for metabarcoding using names for the hypervariable regions (V1-V2, V4, V7, V9), and also provide primer names based on both regions and nucleotide sequence positions. The use of standardized and meaningful names allows and encourages comparison with the relevant literature in this fast-moving field possible.

**>We agree with Reviewer 1 and decided to rename the target regions to reflect their position relative to the hypervariable regions of the 18S gene. We identified the nucleotide positions and hypervariable region names using Table S3 from “Hadziavdic, K., Lekang, K., Lanzen, A., Jonassen, I., Thompson, E. M., & Troedsson, C. (2014). Characterization of the 18S rRNA gene for designing universal eukaryote specific primers. *PloS one*, *9*(2), e87624”, and renamed the barcoding regions consequently. These were double checked with published V3, V4, V5, V7, V8, and V9 animal sequences from GenBank to confirm the position of our primers within or between these regions. We added a table (Table 1, lines 182-186) with region names for the barcodes, start-end positions, and sequences for the primers in the Materials & Methods section. We also renamed references to these barcode regions throughout the manuscript (lines 171, 346-347, and 225), figures (Fig 3B), and supplements (Tables S2, S4, S6, S8, and S11-S14).**

Not only does this paper NOT provide any details about the gene regions and molecular protocols used, the citation for specific methods (Damian-Serrano et al., 2020) is not in the reference list.

**>We added more details specifying the gene regions (lines 171-180 , Table 1) and the molecular protocols (lines 168-170 and 187-203). The floating citation occurred as a copyediting error, as it refers to the protocols.io DOI link which was removed from the references but should have been provided, replacing each one of those citations. We fixed those instances in the revised manuscript text (lines 181, 205, and 222).**

Methods

Metabarcoding protocols: some information should be included in the paper itself, not cited to other sources. Most especially, the regions of 18S rRNA should be named to be consistent with usual terminology (i.e., designation of hypervariable regions) and primer sequences should be provided in the text, a table or perhaps SM. Statistical analyses – if any – need to be clearly explained.

**>We added Table 1 (lines 182-186) with names and sequences for the primers in the Materials & Methods section and renamed references to these barcode regions throughout the manuscript, figures, and supplements.**

Results and Discussion

The results from the different methods (metabarcoding, morphology, in situ observation) for each species are summarized in very general terms, sometimes with lists of taxonomic groups of prey. The text occasionally mentions of “prey species”, but species cannot reliably or accurately be identified based on metabarcoding using regions of 18S rRNA, which lack the variation to identify species reliably.

**>Throughout the text we have mainly referred to prey as ‘taxa’ or ‘items’. There was one mention of ‘species’ in the Conclusions which we have now changed to ‘taxa’ (line 675). While we agree (and find in our analyses) that normally 18S barcodes are unable to distinguish taxa beyond the family level, this specificity is also taxon-specific. While most taxa are species-invariant across the gene, others may have unique mutations that the assigner software can reliably identify. We leveraged a couple of such exceptions when discussing findings with high species-level assignment reliability, which were further confirmed by identifying those species visually in the guts during collection in the field (and in the neighboring planktonic community samples). We have now included the % reliability from the METAXA2 analysis for those specific mentions in the text (lines 414-415 and 465-467).**

The metabarcoding results for the various species of Siphonophores must be statistically evaluated and compared, in order to make conclusions about prey specialization. Stacked-bar graphs do not count as statistical analysis!

**>In the ‘Methodological considerations’ subsection of the Results & Discussion we further explain why given the phylogenetic diversity of prey (variable 18S gene copy number and PCR affinity) and the unknown digestion rates, statistical analyses based on read abundance would be meaningless (lines 647-663). In other words, there is no meaningful quantitative information held in the read abundances measured for each sample. Therefore, our conclusions are based on the presence and absence of specific prey types in the gut contents. Moreover, siphonophores are hard-to-collect, fragile, open-ocean, and deep-sea ambush predators which feed infrequently, and thus usually only one or two prey items if any. This limited sampling replication for most species, on top of the high rates of empty guts in most species, led to insufficient intraspecific data to enable meaningful quantitative analyses to be conducted based on prey frequency across specimens. For these reasons, we decided to keep our assessments descriptive and qualitative, where they can serve as novel primary observations to generate and question ecological and evolutionary hypotheses on siphonophore feeding.**

One topic that might be described in more detail is the comparison of reference databases for classification and identification of prey taxa. The Methods says that sequences were compared “…against the standard GenBank reference library, the SILVA123.1 reference library (Quast et al. 2012), and our custom-built library (based on SILVA138)”. But there did not seem to be any summary of important differences from different databases.

**>We removed the mention of the ‘standard GenBank reference library’ since we ended up not using those assignments in the study. Assignments derived from the SILVA 123 and 138-custom databases were not compared but integrated, since they provided complementary information. For example, their taxonomy metadata was encoded at different taxonomic ranks, thus providing complementary assignments. Moreover, in many cases, depending on taxon, one database would have detailed taxonomic specificity while the other would only reliably assign phylum. Neither database alone would have been able to provide the complete picture, thus we did not use the raw assignment data directly in any of our downstream analyses or figures. The manually-curated, summarized taxonomic and sequence-source interpretations were based on the integration of the assignments for each barcode and database within a sample. We expanded the Materials & Methods section to clarify these points (lines 252-258), and added some more specifics on the size, taxonomic rankings, and curation of these databases (lines 241-246). In any case, we believe that a comparison of the taxonomic assignment efficiencies of these databases is beyond the scope of the paper.**

Figures and Tables

The extensive use of stacked-bar graphs is not informative or useful. Some figures are unlikely to reproduce well. The PDF file of the manuscript provided for review did not provide sufficient resolution to allow reading the axis labels for most of the SM Figures (SM Figures 3,4,5,7,8, 9, 12). All of these figures are completely unreadable! The reliance on color – or shades of colors – is also not a best practice for publication. Alternatives would be summary tables with all samples or specimens, with stacked bar graphs for summaries by species (with means and ranges, or similar).

**>This is a great suggestion. We agree that many of the supplementary figures would be better presented as data tables. We have removed these supplementary figures and have included the data tables in the supplement as Tables S1-S14.**

**Reviewer #2:**

The manuscript by Damian-Serrano et al. applies visual and metabarcoding approaches to assign prey preference to number of shiphonophore species. The manuscript is well written, and provides a novel view on the diet of these key components of the pelagic ecosystem.

**>We thank Reviewer 2 for their kind comments and attentive feedback.**

I have however a few questions and comments that would improve the present manuscript or correct some points that I feel deserve attention.

I missed having numbered lines for the comments - it makes very difficult to track later my comments into the manuscript…

**>We apologize for the oversight. We have now added line numbering to the resubmission.**

IN the introductions, I am not sure how any organism can be herbivore in the midwater, where there is no primary production. I assume Choy et al. talked about the marine food webs, but the phrase in the intro refers to the midwater. Please modify one way or the other (marine food webs / removing herbivory from midwater).

**>We were referring to the many salp species are diel vertical migrators moving up from the midwater to the epipelagic to graze on phytoplankton at night, and are prey to midwater predators like siphonophores and narcomedusae (Choy et al. 2017), thus being part of the midwater food web. However, this comment reminded us that the scope of our study is the pelagic food web more broadly, including epipelagic and pleustonic predator-prey interactions. Therefore, we have modified the introductory mentions of ‘midwater’ to the pelagic ecosystem more broadly (lines 53 and 56).**

“However, this technology has not yet been applied to study the diets of gelatinous animals”. These below are recent, published before submission, but maybe not published when the draft of this manuscript was written. So, please do a small search on this topic and add the corresponding references before publication. I think I have seen at least another one from ctenophora, but I might be wrong (couldn't find it in a very brief google search)

Pauli, NC., Metfies, K., Pakhomov, E.A. et al. Selective feeding in Southern Ocean key grazers—diet composition of krill and salps. Commun Biol 4, 1061 (2021). [https://doi.org/10.1038/s42003-021-02581-5](https://urldefense.com/v3/__https://doi.org/10.1038/s42003-021-02581-5__;!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDXUAG6K1A$)

Sun, T., Wang, L., Zhao, J. et al. Application of DNA metabarcoding to characterize the diet of the moon jellyfish Aurelia coerulea polyps and ephyrae. Acta Oceanol. Sin. 40, 160–167 (2021). [https://doi.org/10.1007/s13131-021-1800-8](https://urldefense.com/v3/__https://doi.org/10.1007/s13131-021-1800-8__;!!C5qS4YX3!XYvSJG9XDPDkEeIzWFK8fjhLrGz8et6NuukoKw1512ZU5ycOTnQ0JE0sYDVYaxDocg$)

**>We have now corrected this statement in the text to “siphonophores” instead of “gelatinous animals”, and included the two references provided by the reviewer on the use of this technology on other gelatinous consumers. After searching the web again, we also found and included this preprint reference: “Schroeder, A., Camatti, E., Pansera, M. and Pallavicini, A., (2022). Applying DNA Metabarcoding for The Diet Investigation of The Invasive Ctenophore *Mnemiopsis leidyi* in A Transitional Environment” (lines 91-94).**

“2857 as natural environmental DNA sources” what was the criteria for this?

**>The “Environmental” category for source interpretations collected everything that could not be explained as predator (siphonophore), prey, secondary predation, parasite, or contamination, and thus would be likely a part of the microbial community (such as diatoms, dinoflagellates, uncultured eukaryotes) or eDNA (such as rotifers, sharks, ascidians, sponges, bivalves, anemones, echiurids, gastrotrichs, echinoderms, or bryozoans). This discrimination was based on the natural history of siphonophores (as we explain below, they cannot feed on marine snow, eggs, or microscopic ciliated larvae) and read abundance for each unique sequence in each sample (some trace sequences with a few reads are likely sourced from eDNA). We added an explanation in the Materials & Methods section to clarify the criteria for the all source interpretation categories (lines 266-291).**

Marine snow is known to be a preferred “prey” item for many mesopelagic animals, both invertebrate and fish, due to its great organic compounds, easy to digest and very energetic. Removing that link in the trophic web would have a significant effect in the carbon and energy transfer. Unless there is a reason from the tentilla limitation? Do they have to be alive to trigger feeding response?

**>Yes and yes. Siphonophores are ambush predators that rely on the swimming behavior of prey to trigger tentilla discharge and prey capture, and thus cannot feed on marine snow, eggs, microbes, or microscopic ciliated larvae. For this reason, we did not consider DNA from taxa that could have only been present in the environment by those means as “Prey”, but rather as ”Environmental”. We added a clarification of this rationale in the Materials & Methods (lines 267-270).**

The 95% for 18S would go almost to order level, but I see that the assignments in morphological ID also go to that level. So, OK. But I find difficult after to accept the IDs given to species or genus level such as Acartia, Temora or Centropages. I suppose that, at 95%, the whole family is there collapsed.

**>We agree (and find in our analyses) that normally 18S barcodes are unable to distinguish taxa beyond the family level, but this specificity is also taxon-specific. While most taxa are species-invariant across the gene, others may have unique mutations that the assigner software can reliably identify. When we discuss the finding of copepod species in Atlantic *Nanomia*, we found high species-level assignment reliability (genus and species-level METAXA2 assignment scores: *Centropages* sp. 54.99% for barcode V5-V7L, *Acartia tonsa* 91.5% for barcode V5-V7S, and *Temora discaudata* 91.81% for barcode V7p+V8, using the SILVA123 database), which were further confirmed by identifying those species visually in the guts and as abundant members of the immediate planktonic community. We have now included these details in the text (lines 465-467).**

In your results-discussion: “We identified prey items in 47 specimens” Does this mean you sequenced de 159 gut contents, and only 47 had prey sequences? This part is important since, if you just sequences those in which you “saw” a prey, you would be still biasing against small and gelatinous. Please read this as a plain question – I am NOT saying I think you did the later, just asking for clarification.

Linked to next question:In methods: “prioritizing those with visible gut contents”. The authors are still biasing towards what they were trying to avoid. Please be careful then interpreting the results, since they small or gelatinous preys might be underrepresented (since preference was given to the ones with macroscopic preys).

**>We sequenced all 159 gastrozooid samples (with and without visible prey content) and only 47 of them had prey sequences. A siphonophore colony can have hundreds of gastrozooids, and we only sampled and pooled a few, normally ~10. The “prioritizing” mentioned in the Materials & Methods section refers to making sure that if any of the gastrozooids has visible swelling or discoloration (indicating the presence of prey), it was included in the cryotube sample together with several other seemingly-empty gastrozooids. This approach could bias quantitative assessments of the data, and is one reason why statistical analyses were not appropriate for our dataset. However, it helped compensate for the already pervasive rate of empty guts, enriching the sampling success. We added clarification of this strategy, together with a justification on the pooling of gastrozooids given the expected gastrovascular mixing within the same colony (lines 155-166).**

DAPC appears first time in page 15. It stands for…

**>We added a definition for this acronym in the introduction where it was missing (lines 110-111).**

Everything else in discussion reads well, although I found it a bit long and somewhat repetitive between sections. Not sure if condensing could be done, although since PLoS has no page limit due to its digital nature…

**>We streamlined and pruned a few repetitive parts found in the Results & Discussion (lines 394-403, 410-417, 437-445, and 632-641).**

“at the Yale Peabody Museum of Natural History” are the museum numbers available/matching the other data?

**>Some of the samples we collected were vouchered with a photograph, and others with a physical voucher specimen housed at the Yale Peabody Museum of Natural History. We added these numbers to Table S15.**

“The primers were designed using Geneious v.x.x.x.” Please remember to fill these before publication.

**>We modified the manuscript text to include the version number (line 172).**

In figure 5, prey section, what is "gelatinous" detected by metabarcoding? I mean, there are other gelatinous plankton on the list. Is that Cnidaria + Ctenophora?

**>Gelatinous in Fig. 5 refers to ctenophores, medusae, and salps. Larvaceans were excluded as their own category or small soft-bodied prey, represented as an expectation of prey only for Generalist predators, not for Gelatinous specialists, given the feeding guild definitions in Damian-Serrano et al (2021). Our rationale here is that larvaceans are not gelatinous-bodied animals, but rather muscular tadpole-like swimmers. They do produce a gelatinous-like external mucous filter, so they are commonly categorized within the gelatinous fraction of zooplankton. However, the only way they could interact with siphonophore tentilla is when the gelatinous filter is abandoned and the animal is swimming freely. We added a clarification of this rationale in the caption of Figure 5 (lines 942-945).**