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

--Manuscript Draft--

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Keywords:	Gelatinous zooplankton, trophic ecology, predator-prey interactions, pelagic food webs, siphonophores
Abstract:	Siphonophores (Cnidaria: Hydrozoa) are abundant and diverse gelatinous predators in open-ocean ecosystems. Due to limited access to the midwater, little is known about the diets of most deep-dwelling gelatinous species, which constrains our understanding of food-web structure and nutrient flow in these vast ecosystems. Visual gut-content methods can rarely identify soft-bodied rapidly-digested prey, while observations from submersibles often overlook small prey items. These methods have been differentially applied to shallow and deep siphonophore taxa, confounding habitat and methodological biases. DNA metabarcoding can be used to assess both shallow and deep species' diets under a common methodological framework, since it can detect both small and gelatinous prey. We (1) further characterized the diets of open-ocean siphonophores using DNA metabarcoding, (2) compared the prey detected by visual and molecular methods to evaluate their technical biases, and (3) evaluated tentacle-based predictions of diet. To do this, we performed DNA metabarcoding analyses on the gut contents of 39 siphonophore species across depths to describe their diets, using six barcode regions along the 18S gene. Taxonomic identifications were assigned using public databases combined with local zooplankton sequences. We identified 55 unique prey items, including crustaceans, gelatinous animals, and fish across 47 siphonophore specimens in 24 species. We reported 29 novel predator-prey interactions, among them the first insights into the diets of nine siphonophore species, many of which were congruent with the dietary predictions based on tentilla morphology. Our analyses detected both small and gelatinous prey taxa underrepresented by visual methods in species from both shallow and deep habitats, indicating that siphonophores play similar trophic roles across depth habitats. We also reveal hidden links between siphonophores and filter-feeders near the base of the food web. This study expands our understanding of the ecological roles of siphonophores in the open ocean, their trophic roles within the 'jelly-web', and the importance of their diversity for nutrient flow and ecosystem functioning. Understanding these inconspicuous yet ubiquitous predator-prey interactions is critical to predict the impacts of climate change, overfishing, and conservation policies on oceanic ecosystems.
Order of Authors:	Alejandro Damian-Serrano, Ph.D. Elizabeth D. Hetherington, Ph.D. C. Anela Choy, Ph.D. Steven H.D. Haddock, Ph.D. Alexandra Lapides, B.S. Casey W. Dunn, Ph.D.
Opposed Reviewers:	
Response to Reviewers:	Response to Reviewers >We highlighted our responses to the editor and reviewers in bold red text preceded by

a “>” symbol.

PONE-D-21-38235

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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/> and select the 'Submissions Needing Revision' folder to locate your manuscript file.

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- 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'.
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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.

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We look forward to receiving your revised manuscript.

Kind regards,

Hans G. Dam, Ph. D.

Academic Editor

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>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.

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>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>.

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>), 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>. 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 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).

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>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). Photos B & D were published under a CC BY license in another Figure from another paper - attached as Other to the submission. 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-posed 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 siphonophore 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, N.C., 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>

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>

>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

	<p>reliability (genus and species-level METAXA2 assignment scores: <i>Centropages</i> sp. 54.99% for barcode V5-V7L, <i>Acartia tonsa</i> 91.5% for barcode V5-V7S, and <i>Temora discaudata</i> 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).</p> <p>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.</p> <p>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).</p> <p>>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.</p> <p>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).</p> <p>DAPC appears first time in page 15. It stands for...</p> <p>>We added a definition for this acronym in the introduction where it was missing (lines 110-111).</p> <p>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...</p> <p>>We streamlined and pruned a few repetitive parts found in the Results & Discussion (lines 394-403, 410-417, 437-445, and 632-641).</p> <p>"at the Yale Peabody Museum of Natural History" are the museum numbers available/matching the other data?</p> <p>>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.</p> <p>"The primers were designed using Geneious v.x.x.x." Please remember to fill these before publication.</p> <p>>We modified the manuscript text to include the version number (line 172).</p> <p>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?</p> <p>>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).</p>
Additional Information:	
Question	Response
Financial Disclosure	This research was funded by the Yale Institute of Biospheric Studies through a Doctoral Dissertation Improvement Award to A.D.-S.
Enter a financial disclosure statement that describes the sources of funding for the	(https://yibs.yale.edu/research/yibs-small-grant-program/dissertation-improvement-grants), as well as by the National Science Foundation grants NSF-OCE 1829835 (to

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DAMIAN-SERRANO ET AL.

SIPHONOPHORE DIET METABARCODING

Cover letter:

Dear Editor,

I am writing on behalf of my co-authors to submit our manuscript titled "Characterizing the secret diets of siphonophores (Cnidaria:Hydrozoa) using DNA metabarcoding" to be considered for publication in PLOS One. All authors approve of the manuscript and its submission to PLOS One. The manuscript is original work and has not been simultaneously submitted to any other journal.

We believe that this manuscript will be of interest to a broad readership and is appropriate for publication in PLOS One, as it advances our understanding of marine trophic ecology and pelagic food web structure, and constitutes the first application of DNA metabarcoding to elucidate the diets of oceanic gelatinous predators. The structure of oceanic food webs is critical for the sustainability of marine ecosystems, commercial fisheries, and the biological carbon pump. Thus, it is essential to understand its structure and how changing the community composition will affect its architecture. This knowledge will allow scientists to better predict the impacts of climate change, overfishing, and conservation policies on the functioning of oceanic ecosystems. This work will draw attention from scientists in the fields of food web ecology, biological oceanography, and cnidarian zoology.

In this study, we apply DNA metabarcoding to reveal the diets of siphonophores, a group of understudied open-ocean gelatinous predators. In open-ocean food webs, gelatinous zooplankton constitute one of the most abundant and diverse animal groups, occupying central positions in the food web as predators and prey of each other. This gelatinous fraction is often labeled the 'jelly-web' (<https://doi.org/10.1002/lno.11605>), and comprises an understudied but large part of the trophic connectivity in these ecosystems. Our results provide the first insight into the diets of nine siphonophore species and compare our findings in another fifteen species with those from previous visual gut content inspections and remotely-operated vehicle observations across different pelagic depth habitats. We also use these results to evaluate the dietary predictions posited in Damian-Serrano et al. 2021 (<https://doi.org/10.1093/iob/obab019>) based on tentacle and stinging-capsule morphologies.

Our analyses detected both small and gelatinous prey taxa underrepresented by visual methods in species from both shallow and deep habitats, indicating that siphonophores play similar trophic roles across depth habitats. We also reveal hidden links between siphonophores and gelatinous filter-feeders such as salps and larvaceans, which transfer nutrients from herbivorous phytoplankton consumers to higher trophic levels in the food-web. Our results suggest a greater involvement of siphonophores in the 'jelly-web' than previously considered. These findings also confirm suspected biases against rapidly-digested gelatinous prey in visual gut-content inspection which had precluded a comprehensive understanding of the role of these animals in the food web. We also find a large prevalence of small prey in deep sea species which may have gone unnoticed in submersible observations, as suggested in (<https://doi.org/10.1098/rspb.2017.2116>). Finally, we found support for the predicted diets for seven siphonophore species, validating the eco-morphological link between tentacle morphology and diet.

Collecting these new data from siphonophore gut contents was a major undertaking. Siphonophores are fragile animals that live far offshore and in the deep sea, and thus are extremely hard to collect. Specimen collection required SCUBA diving and the deployment of remotely operated vehicles hundreds of kilometers from shore. We developed and optimized a custom metabarcoding protocol with six different barcode regions. In addition, we collected, identified, and sequenced several underrepresented pelagic species in order to enhance the accuracy of our taxonomic assignments.

Thank you for your consideration of our manuscript.

Yours sincerely,

Alejandro Damian-Serrano, Ph.D.

DAMIAN-SERRANO ET AL.

SIPHONOPHORE DIET METABARCODING

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4 Characterizing the secret diets of siphonophores (Cnidaria: Hydrozoa)

5 using DNA metabarcoding

6
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23

24 Abstract

25 Siphonophores (Cnidaria: Hydrozoa) are abundant and diverse gelatinous predators in
26 open-ocean ecosystems. Due to limited access to the midwater, little is known about the diets of
27 most deep-dwelling gelatinous species, which constrains our understanding of food-web structure
28 and nutrient flow in these vast ecosystems. Visual gut-content methods can rarely identify soft-
29 bodied rapidly-digested prey, while observations from submersibles often overlook small prey
30 items. These methods have been differentially applied to shallow and deep siphonophore taxa,
31 confounding habitat and methodological biases. DNA metabarcoding can be used to assess both
32 shallow and deep species' diets under a common methodological framework, since it can detect
33 both small and gelatinous prey. We (1) further characterized the diets of open-ocean
34 siphonophores using DNA metabarcoding, (2) compared the prey detected by visual and
35 molecular methods to evaluate their technical biases, and (3) evaluated tentacle-based
36 predictions of diet. To do this, we performed DNA metabarcoding analyses on the gut contents of
37 39 siphonophore species across depths to describe their diets, using six barcode regions along
38 the 18S gene. Taxonomic identifications were assigned using public databases combined with
39 local zooplankton sequences. We identified 55 unique prey items, including crustaceans,
40 gelatinous animals, and fish across 47 siphonophore specimens in 24 species. We reported 29
41 novel predator-prey interactions, among them the first insights into the diets of nine siphonophore
42 species, many of which were congruent with the dietary predictions based on tentilla morphology.
43 Our analyses detected both small and gelatinous prey taxa underrepresented by visual methods
44 in species from both shallow and deep habitats, indicating that siphonophores play similar trophic
45 roles across depth habitats. We also reveal hidden links between siphonophores and filter-feeders
46 near the base of the food web. This study expands our understanding of the ecological roles of
47 siphonophores in the open ocean, their trophic roles within the 'jelly-web', and the importance of
48 their diversity for nutrient flow and ecosystem functioning. Understanding these inconspicuous

49 yet ubiquitous predator-prey interactions is critical to predict the impacts of climate change,
50 overfishing, and conservation policies on oceanic ecosystems.

51 Introduction

52 The open-ocean is the largest volume of the biosphere habitable by animals [1]. This
53 environment hosts diverse communities and complex food webs [2]. Pelagic food webs sustain
54 manifold fisheries, top predators, and sustain the biological carbon pump [3]. Gelatinous animals
55 play fundamental roles in these food webs [4], acting as herbivores, detritivores, hosts, predators,
56 and prey. The subset of the pelagic food web involving gelatinous fauna has been referred to as
57 the “jelly web” [2]. Among the most abundant [5,6] and trophically-connected [4] gelatinous
58 predators are siphonophores — mid-trophic organisms that feed on a broad variety of prey such
59 as medusae, salps, crustaceans, molluscs, and fishes [4,7,8]. Siphonophores are sit-and-wait,
60 non-visual, ambush predators that rely on prey encountering their tentacles and tentilla [9]. They
61 are abundant and locally diverse colonial cnidarians in open-ocean communities, present in every
62 region of the ocean, with species ranging from above the surface (like the Portuguese man-o-
63 war) to the hadal region (>7000m deep) [10]. In addition, siphonophore aggregations can have
64 significant predatory impacts on larval fish stocks [11].

65 Progress in elucidating siphonophore diets has been slow due to the intrinsic challenges
66 of working with these animals. Observation and collection of open-ocean taxa requires expensive
67 research vessels and instrumentation to reach their habitat. In addition, siphonophores are
68 extremely fragile, requiring the use of blue water SCUBA divers and Remotely Operated Vehicles
69 (ROVs) to collect them alive and intact [12]. These techniques can be used to collect live
70 specimens for gut content inspection, and video recordings from ROVs allow scientists to observe
71 feeding events. Traditional collection methods such as plankton nets not only break up

72 siphonophore colonies, but can also lead to artifactual ingestions in the cod-end that confound
73 their natural diets.

74 The diets of some epipelagic siphonophores have been examined through gut content
75 analyses of SCUBA-collected colonies [7,13], and have been reviewed in Hetherington et al. [8].
76 Recent studies based on ROV observations have shed some light on the diets of deep midwater
77 siphonophores [4,8]. However, these approaches are limited by their biases. Visual gut content
78 inspection favors hard-bodied prey that digest slowly, leaving behind diagnostic body parts (i.e.
79 exoskeleton, shell, eyes, etc.). Therefore, soft-bodied, rapidly-digested taxa, such as gelatinous
80 zooplankton, are often underrepresented in dietary assessments. ROVs can observe feeding on
81 gelatinous prey before they become digested. However, ROV observations are skewed towards
82 large prey items that can be easily identified from the camera screen (such as large medusae,
83 ctenophores, crustaceans, or fishes), and can overlook important prey items such as copepods
84 and larvae [8]. In addition, prey are relatively scarce in the open ocean, especially in the deeper
85 regions [2], thus it is infrequent to find specimens capturing prey or carrying visually-identifiable
86 prey in their guts [7].

87 With the advent of DNA metabarcoding, the diets of many marine predators have been
88 established from gut content DNA [14-17]. These high-throughput amplicon sequencing
89 technologies have extremely high detection sensitivity and bypass the biases posed by visual
90 methods. Recently, the application of DNA metabarcoding to marine predator gut contents has
91 demonstrated the capacity of these methods to detect gelatinous prey [18-22]. In the study of
92 gelatinous zooplankton as consumers, this technology has only been applied to assess the
93 microbial diet of the tunicate *Salpa thompsoni* [23], the predatory diet of the scyphomedusa
94 *Aurelia coerulea* [24], and the diet of the lobate ctenophore *Mnemiopsis leidyi* [25]. However, this
95 technology has not yet been applied to study the diets of siphonophores..

96 A review of the literature on siphonophore diets observed significant differences between
97 the diets of epipelagic and deep-dwelling siphonophore species [8]. Gelatinous prey appeared to
98 be more prevalent in deep-sea observations while small crustaceans appeared to be the
99 predominant prey in shallow gut content samples. Since epipelagic species' diets were exclusively
100 assessed through microscopic gut content inspection and deep-sea species' diets through ROV
101 observations, it is not possible to determine whether these differences are due to ecological or
102 methodological reasons. To disentangle these confounding factors, it is critical to assess both
103 shallow and deep species' diets under the same methodological framework. In this case, DNA
104 metabarcoding is an ideal choice, since it can detect both small and gelatinous prey, thus being
105 able to bridge across the methodological shortcomings of visual methods. Here we aim to apply
106 a uniform method to describe diets across the water column as a single, interconnected, deep-
107 pelagic ecosystem.

108 Siphonophore tentillum and nematocyst morphology are directly linked to feeding guild
109 [26,27] used these relationships to generate feeding guild predictions for 45 siphonophore species
110 using their tentillum and nematocyst morphology as predictors in a discriminant analysis of
111 principal components (DAPC). The feeding guild categories comprise fish specialists (which feed
112 primarily on teleost fish prey), large crustacean specialists (which feed primarily on krill, decapod
113 shrimps, mysids, lophogastrids, amphipods, and other macro-planktonic crustaceans larger than
114 1cm), small crustacean specialists (which feed primarily on copepods, ostracods, cladocerans,
115 larvae, and other meso-planktonic crustaceans smaller than 1cm), gelatinous specialists (which
116 are able to feed on large gelatinous animals such as salps, ctenophores, or medusae in addition
117 to other zooplankton), and generalists (which feed on a variety of small and large, soft- and hard-
118 bodied prey not including gelatinous animals). These predictions were cast on siphonophore
119 species for which no dietary information was available, and thus remained to be tested with new
120 data on siphonophore diets.

121 Here we use DNA metabarcoding to identify the gut contents of several siphonophore
122 species to obtain more comprehensive insights into their diets. Our primary aims are: (1) Expand
123 the existing knowledge on the diets of open-ocean siphonophores using DNA metabarcoding, (2)
124 qualitatively compare the prey detected by visual and molecular methods to evaluate their
125 technical biases, (3) apply a uniform method to describe siphonophore diets across depth
126 habitats, and (4) evaluate the morphology-based predictions of feeding guilds.

127 Materials and Methods

128 Ethics statement

129 Our specimen collection and protocol were compliant with all local regulations and under the
130 marine collection permit SC-191140006, issued to Steven H.D. Haddock by the California
131 Department of Fish and Wildlife. Since no vertebrates or cephalopods were involved, we did not
132 need oversight from an animal care board.

133 Siphonophore collection

134 In order to sample a representative set of taxa across the siphonophore phylogeny, we
135 targeted a set of 41 species (aiming for 10 specimens per species) including cystonects,
136 apolemiids, pyrostephids, euphysonects, and calycophorans from shallow and deep waters. Most
137 species were sampled from the Offshore California Current Ecosystem (OCCE) except for the
138 Portuguese man-o-war *P. physalis*, which was collected off Bermuda in the Sargasso Sea;
139 *Sulculeolaria chuni* and some *Nanomia* spp. (labeled as “Atlantic”) which were collected off Rhode
140 Island in the Block Island sound; *Forskalia* sp. M123-SS8 and shallow *Nanomia* sp.
141 KiloMoana2018-BW7-4 which were collected off the coast of Hawaii. While all the *Nanomia*
142 populations sampled in this study have been referred to as *Nanomia bijuga*, we suspect that there

143 may be undescribed cryptic *Nanomia* species among the specimens sampled based on the
144 disparate tentillum morphologies we observed. Therefore, we decided to have them labeled at
145 the genus level. One *Nanomia* specimen (KiloMoana2018-BW7-4) was collected off the coast of
146 Kona, HI. The pleustonic (surface floating) *Physalia physalis* samples were collected manually
147 using a bucket from a small boat. Species found between the 0-20m deep were collected using
148 blue water diving techniques following the guidelines in Haddock & Heine [28]. Species from 200-
149 4000m were collected using ROVs. All animals were collected live and brought back to the ship
150 (or field station in Bermuda for *P. Physalis*) for dissection (Fig 1). Live colonies were photographed
151 (sometimes recorded on video), and zooids of diagnostic value (nectophores, bracts, tentacles)
152 were dissected, when possible, fixed in 4% formalin, and stored as vouchers at the Yale Peabody
153 Museum of Natural History (voucher catalog numbers provided in specimen metadata Table S15).

154 **Gut content metabarcoding**

155 Shortly after collection of the live specimens, we dissected and pooled several
156 gastrozooids from each colony, making sure that those with visible gut contents are included in
157 addition to several other without conspicuous prey, and also including visible egested food pellets
158 at the bottom of the sampling container. This non-random approach was aimed at increasing our
159 prey detection rate, but may have introduced sampling bias against inconspicuous prey items,
160 further precluding any meaningful quantitative analyses of the data. Nonetheless, since the
161 majority of gastrozooids sampled lacked conspicuous prey content, we gave inconspicuous prey
162 a broad chance of being represented. Pooling multiple gastrozooids as a single sample is
163 reasonable, since all gastrozooids in a colony share an actively-flowing, interconnected
164 gastrovascular cavity. Thus, we expect DNA from one prey capture in one gastrozooid to be
165 present within multiple gastrozooids. For small species (like *Sphaeronectes* spp.), we sampled

166 the whole siphosome as a pooled collection of all gastrozooids. Samples were frozen at -80°C
 167 until DNA extraction.

168 To extract DNA, we digested the samples with proteinase K at 56°C for 1-2h, used the
 169 DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), eluting twice at 56°C for 10min into a final
 170 volume of 100µl. We used a set of six primer pairs that amplify six barcode regions within the 18S
 171 gene ('V3', 'V5-V7S', 'V5-V7L', 'V7', 'V7p+V8', and 'V9'). The primers were designed using
 172 Geneious 11.1.5 [29], constraining the search to short (>300bp) amplicon products with a high
 173 chance of remaining uncleaved after digestion in the gastrozooid, flanked by priming sites
 174 conserved (to a maximum mismatch of 3bp) across metazoans. The search for conserved priming
 175 sites was conducted on an alignment of 18S genes from 975 species across all metazoan phyla
 176 downloaded from GenBank (available in
 177 github.com/dunnlab/siphweb_metabarcoding/Primer_design). The primer search was optimized
 178 to only retrieve non-degenerate primer pairs with compatible annealing temperatures and without
 179 problematic dimerization and hairpin temperatures. Primer sequences are shown in Table 1, and
 180 their properties can be found in Table T1 in the protocol
 181 ([dx.doi.org/10.17504/protocols.io.5qpvo57o7l4o/v2](https://doi.org/10.17504/protocols.io.5qpvo57o7l4o/v2)).

182 **Table 1. Barcodes used in this study.**

Barcode	18S region covered ^a	Forward primer	Reverse primer	Start position ^b	End position ^b
V3	Within V3	166F: AACGGCTACCAACATCCAAGG	166R: CACCAGACTTGCCTCCAAT	420	566
V5-V7S	Between V5 and the beginning of V7 (short amplicon)	152: TGACGGAAGGGCACCACAG	152R: TCCACCAACTAAGAACGGCC	1187	1339
V5-V7L	Between V5 and the beginning of V7 (long amplicon)	271F: AAACGATGCCGACTAGCGAT	272R: TCCACCAACTAAGAACGGCC	1067	1339
V7	Within V7	179F: GGCCGTTCTAGTTGGTGGA	179R: TGC GGCCCAGAACATCTAAG	1319	1489

V7p+V8	Part of V7 and most of V8	261F: AACAGGTCTGTGATGCCCTT	261R: TGTGTACAAAGGGCAGGGAC	1472	1687
V9	Within V9	134F: CTTTGTACACACCGCCCGTC	134R: CCTTGTTACGACTTTACTTC CTCT	1675	1790

183 ^aThe hypervariable region boundaries were annotated following the gene positions defined in
 184 Hadziavdic et al. [30].

185 ^bStart and end positions calculated on the 18S gene of *Lymnaea diaphana* (GenBank
 186 JF909497.1).

187 Using these primer pairs, we ran six parallel PCR reactions for each successful extraction,
 188 selecting only those which had yielded a DNA concentration above 10ng/l. For each 25µl reaction
 189 volume, we used 2 µl of extraction template, 0.5 µl of each primer (at a 10µM concentration), 1µl
 190 of BSA, 0.2 µl of GoTaq (Promega) polymerase and the standard reagents and proportions of the
 191 Promega GoTaq kit (Madison, WI, USA). The thermal cycles included: an initial denaturation at
 192 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing (variable), and
 193 elongation at 72°C for 1 min, followed by final elongation at 72°C for 5 min. For barcode V9, we
 194 used an annealing temperature of 48°C for 45s per cycle. For all other barcodes, we used an
 195 annealing temperature of 54°C for 60s per cycle. Each batch of reactions for each barcode
 196 included a positive and a negative control (the elution buffer used in extraction), and the products
 197 were visualized using gel electrophoresis (2% agarose gel dyed with SYBR Safe DNA Stain) to
 198 check for amplicon size and monitor the controls. The PCR products were purified using
 199 ExcelaPure UF PCR Purification Plates (EdgeBiosystems, Gaithersburg, Maryland, USA). The
 200 DNA yield of each purified product was assessed using a Qubit 2.0 fluorometer and the dsDNA
 201 High Sensitivity assay (Thermo Fisher Scientific, USA). Purified PCR products from each barcode
 202 region for each sample were combined in equimolar pools based on their DNA yield in order to
 203 have equal representation in the sequencing lane. Further details on the quality control, PCR mix,
 204 and amplicon pooling are fully described in the online protocol

205 (dx.doi.org/10.17504/protocols.io.5qpvo57o7l4o/v2). All molecular bench work was carried out at
206 the Yale DNA Analysis Facility. Amplicon pools were sequenced using Illumina MiSeq (Illumina,
207 San Diego, CA, USA) 250bp paired-end technology (except samples from specimens
208 KiloMoana2018-BW7-4 *Nanomia* sp., D1019-D5 undescribed physonect, D856-SS8
209 *Stephanomia amphyridis*, D861-D12 *Bargmannia amoena*, D858-D6 *Apolemia lanosa*, and
210 D860-D6 *Erenna sirena* which were sequenced using Illumina MiSeq 150bp) at the Yale Center
211 for Genomic Analysis.

212 Prey reference database

213 In order to enhance the accuracy of the taxonomic assignments of reads, we also built an
214 18S gene barcoding database. To do this, we collected 60 specimens of 30 species of
215 zooplankton and micronekton from the OCCE using a Tucker trawl. We targeted plausible prey
216 species from motile open-ocean taxa that cohabitatae with siphonophores and are
217 underrepresented in SILVA databases, including fishes, crustaceans, jellyfishes, urochordates,
218 chaetognaths, polychaetes, and mollusks. Specimens were photographed live, tissue was
219 sampled and frozen, and the rest of the animal was fixed in formalin as a voucher to be identified
220 and preserved at the Yale Peabody Museum of Natural History. DNA extraction, quality control,
221 PCR, and amplicon cleanup was carried out in a similar fashion as the metabarcoding protocol
222 described above (and detailed in [dx.doi.org/10.17504/protocols.io.5qpvo57o7l4o/v2](https://doi.org/10.17504/protocols.io.5qpvo57o7l4o/v2)), using the
223 PCR program with an annealing temperature of 54°C, and a single pair of primers (166F and
224 134R), spanning the full extent of the sequence containing all barcode regions used in the gut
225 content metabarcoding (from V3 to V9). Purified amplicons were sent in plates with the forward
226 and reverse primer separately for Sanger sequencing from both ends at the Yale DNA Analysis
227 Facility. A total of 89 newly-submitted sequences were then assembled and trimmed at a 95%
228 quality cutoff in Geneious and concatenated with the latest SILVA database

229 (SILVA_138_SSURef_NR99 downloaded on February 23, 2021) pruned to remove non-
230 eukaryotic sequences.

231 **Bioinformatic pipeline**

232 Amplicon libraries were demultiplexed by primer sequence using custom bash code.
233 Primer sequences were removed using *cutadapt* [31]. The forward and reverse reads were
234 matched and repaired using *bbtools* [32], then denoised and de-replicated using the DADA2 [33]
235 plugin in QIIME2 [34] with a truncation quality threshold of 28. We *de novo* clustered the unique
236 features into operational taxonomic units (OTUs) using the VSEARCH [35] plugin in QIIME2 with
237 a similarity threshold of 95%. To reduce computational load, only the top 100 most abundant
238 features among the clustered OTUs were selected for taxonomic assignment. Taxonomic
239 identifications were assigned using the assignment software METAXA2 [36] with a 70% reliability
240 cutoff, comparing the sequences against the SILVA123.1 reference library [37], and our custom-
241 built library built using SILVA138 as a foundation. SILVA123.1 contains 61383 eukaryotic
242 reference sequences, while our custom database built off SILVA138.1 contains 79044. Animals
243 in the SILVA123.1 taxonomy are annotated to the ranks of superphylum, phylum, subphylum,
244 class, subclass, order, family, genus, and species. However, SILVA138.1 animal taxonomy was
245 annotated at the levels of clade (e.g. Bilateria, Protostomia, Deuterostomia, Ecdysozoa,
246 Lophotrochozoa), phylum, class, subclass, order, suborder, and species. All bioinformatics
247 analyses were carried out in the Yale High Performance Computing Cluster. The taxonomic
248 assignments and read count data were merged, then parsed to match the sample of origin and
249 the DNA sequence they derived from. Sequence post-processing scripts can be found in the
250 GitHub repository (https://github.com/dunnlab/siphweb_metabarcoding/Scripts).

251 **Assignment interpretation**

252 Different barcode regions and reference databases displayed different assignment
253 sensitivities for different taxa. Moreover, the two reference databases were annotated at different
254 taxonomic levels, thus revealing unequal assignment reliabilities at different phylogenetic depths.
255 Therefore, the assignment information from different barcodes and reference databases was
256 integrated to interpret the source and taxon of the detected reads. When the assignments from
257 the two databases disagreed or reported suspicious (e.g. non-marine) taxa, we manually checked
258 the sequences in NCBI BLAST. In summary, a combination of annotation database consensus,
259 barcode region consensus, number of reads, manual BLAST checks, and natural history informed
260 priors were used to assign these interpretations.

261 Taxonomic assignments were manually inspected and annotated with the interpreted
262 consensus taxon and interpreted source (predator, prey, secondary predation, parasite,
263 environmental, unrecognizable sequence, contamination, or cross contamination). Predator
264 sources correspond to the siphonophore DNA from the gastrozooid. These annotations were
265 given to OTUs with typically high read abundances, often taxonomically-assigned as
266 siphonophores, hydrozoans, or unculturable eukaryotes. Prey sources were annotated when
267 plausible prey taxa were assigned. We know that siphonophores can only capture prey that
268 actively swims to trigger tentilla discharge [9,27]. Therefore, we interpreted that DNA from non-
269 pelagic and/or non-motile organisms cannot be sourced from dietary contributions from
270 microorganisms, marine snow, eggs, or microscopic ciliated larvae. Secondary predation sources
271 correspond to OTUs assigned to animals that were more likely consumed by the co-detected prey
272 than by the siphonophore. Crustacean, gastropod, and larvacean sequences in *P. physalis*
273 samples were interpreted as secondary predation (prey of their fish prey) given our knowledge on
274 the prey-capture limitations of these animals and the feeding habits of their fish prey.

275 Parasite interpretations were annotated onto OTUs assigned as trematodes, cestodes,
276 ichthyophonids, and myxozoans, since the most likely explanation for their presence in the

samples is due to parasitism in the siphonophores or their prey. We used the environmental category to annotate OTUs likely originated from the microbial community (such as diatoms, dinoflagellates, uncultured eukaryotes) or eDNA (such as rotifers, sharks, ascidians, sponges, bivalves, anemones, echiurids, gastrotrichs, echinoderms, or bryozoans), given their taxonomic assignment and read abundance. OTUs assigned as ‘uncultured eukaryotes’ were BLAST-checked to differentiate between environmental microbes and failed assignments of siphonophore sequences. We used the contamination category to interpret OTUs assigned to tetrapods (likely humans), pollen, brachiopods, nematodes, mites, and insects. Amplification experiments on negative controls indicated that these contaminants originated from specimen manipulation in the field and not from the lab bench. The cross-contamination interpretation was used to annotate some suspicious OTUs with low reads in some samples that matched other taxa that were being extracted and amplified at the lab bench. Reads suspected of cross-contamination (assigned to taxa present in the potential sources of contamination, present across multiple samples in the same run with very low read abundances) were conservatively annotated as such.

When all barcode regions except ‘V5-V7S’ indicate mysid prey but ‘V5-V7S’ identifies a similar number of reads as stomatopod prey, we interpret those reads as mysid prey. Assignments of shark identities by barcode region ‘V5-V7S’ in one of the *P. physalis* samples (specimen BIOS19-D1-P5) were identified as ray-finned fish prey using BLAST searches and interpreted as such, in agreement with the other barcode regions. Assignments of decapod crustacean identities by barcode region ‘V5-V7S’ (in samples D1137-D7 *Forskalia* sp., D1243-BW25 *Diphyes dispar*, and D1244-SS8 *Nanomia* sp.) were interpreted as euphausiid prey in agreement with the assignments on the rest of the barcode regions. The taxonomic composition of the samples was analyzed and visualized in the R programming environment. Scripts and data available in our GitHub repository.

302 Prey field characterization

303 In order to compare the observed diet to the environmental abundances of potential prey
304 taxa, we collected zooplankton and micronekton samples on the same day and station location
305 as the relevant siphonophore gut content samples. The plankton samples paired with epipelagic
306 siphonophore specimens were collected using a weighted hand-held plankton net (ring diameter
307 of 1m for the Bermuda samples, 0.5m for the OCCE and Block Island sound samples, mesh size
308 of 250 μ m) towed for ~10min at a few meters depth at a speed of ~1kt. Paired with the ROV-
309 collected mesopelagic siphonophore specimens, we collected zooplankton and micronekton
310 samples using a Tucker trawl (frame area: 2m², mesh size: 500 μ m) towed for ~2h between 900m
311 and the surface at night. Environmental community samples were visually examined live to collect
312 specimens to sequence for the 18S reference library and other purposes, which were annotated
313 as removed. Samples were concentrated using metal sieves and fixed in 4% formalin. Back in
314 the Yale Peabody Museum of Natural History, these samples were visually identified and
315 quantified from a splitter aliquot. Identifications were carried out to the lowest taxonomic level as
316 well as to a broad group level (e.g., copepods, decapods, krill, fish, hydromedusae, chaetognaths,
317 polychaetes etc.). A few individual specimens were removed from the haul before preservation to
318 serve other scientific goals during fieldwork, and therefore these samples may be imperfect
319 representations of the community. In order to estimate how selective siphonophore species are
320 for different prey types in the environment, we calculated Strauss Linear Index (LI) [38] at the
321 broad taxonomic group level.

322
$$LI = ri - pi \quad (1)$$

323 We used this index to capture the difference between the fraction of each prey type in the
324 environment (p_i) and the observed frequencies of prey types in the gut contents (r_i).

325 Comparisons to published sources

326 We aimed to compare and expand previous predation results from submersible
327 observations and visual gut content inspections with the new results of DNA metabarcoding of
328 gut contents. Therefore, we used the dietary data compiled in Damian-Serrano et al. [26] from 11
329 published sources divided into those that used gut content inspections and those that used
330 human- and remotely-operated submersible observations. Many of the submersible observations
331 correspond to ROV observations carried out in the Offshore California Current Ecosystem,
332 spatially overlapping with the location where the majority of our metabarcoding samples were
333 collected. Salps, ctenophores, and medusae were merged into a gelatinous prey type for
334 comparative purposes. Published records for *Apolemia uvaria* were considered equivalent to
335 *Apolemia* sp. for genus level comparisons. Records of all *Forskalia* species were considered
336 equivalent to *Forskalia* sp. In order to test the morphology-based dietary predictions generated in
337 Damian-Serrano et al. [27], we used the Bayesian posterior probabilities for each feeding guild
338 for each species. Small-crustacean guild predictions were mapped to copepod, ostracod, and
339 cladoceran prey. Large-crustacean guild predictions were mapped to decapod, euphausiid,
340 mysid, lophogastrid, stomatopod, and amphipod prey. Generalist guild predictions were mapped
341 to all prey types except gelatinous prey (following the intended distinction with gelatinous
342 specialists used in Damian-Serrano et al. [26]).

343 Results and Discussion

344 We extracted, amplified, and sequenced the gut contents of 159 specimens from 41
345 siphonophore species (Fig 2). We obtained a total of 4148 unique sequences, including 758 from
346 region “V3”, , 614 from region “V5-V7S”, 442 from region “V5-V7L”, 497 from region “V7”, and
347 341 from region “V7p+V8”, and 1502 sequences from region “V9” (Tables S4, S8, S13, and S14).

348 A total of 337 unique sequences were interpreted as prey items, 36 as secondary predation, 292
349 as contamination from extrinsic sources, 2857 as natural environmental DNA sources, 791 as
350 siphonophore sequences, 85 as parasites (myxozoans, trematodes, and other helminths), and 14
351 unrecognizable sequences (Tables S13 and S14). We identified prey items in 47 specimens
352 (~30%) from 24 siphonophore species (Fig 2, Tables S1 and S3). This prevalence of empty guts
353 is consistent with the feeding habits of sit-and-wait ambush predators in oligotrophic
354 environments, with scarce feeding events separated by periods of starvation [39]. We identified
355 55 unique prey items, 42 of which were crustaceans (25 of which were copepods), three of them
356 were fishes, four of them were urochordates, five corresponded to other gelatinous predators
357 (ctenophores and a medusa), and one matching to a bivalve mollusc (Fig 2 and Fig S1). Most
358 (112 out of 159) siphonophore specimens collected did not yield any putative prey taxa concepts
359 (Table S3). Among the 47 specimens with prey, 40 of them had DNA from a single prey item,
360 while only six had two prey items, and one *Apolequia* sp. specimen had three prey items (Fig S1).
361 The use of six different barcode regions with different priming sites and taxonomic specificity
362 allowed us to detect a broader taxonomic range of prey and to validate dubious annotations (Fig
363 3, Table S12).

364 Dietary findings by taxon

365 *Physalia physalis*

366 The Portuguese man-o-war is the only pleustonic (surface floating) member of the
367 siphonophores, and the most encountered by beachgoers. Man-o-wars are well-known to feed
368 exclusively on relatively large and motile soft-bodied prey such as fish, chaetognaths, or pelagic
369 gastropods [40]. In our gut content samples of the Portuguese man-o-war from Bermuda, we
370 found three specimens with ray-finned fish sequences (Fig S1), some of which had visually
371 recognizable fish in the gastrozooids when collected. Fish prey is congruent with published visual

372 inspections of their gut contents [40,41]. In all three specimens with fish prey we also found
373 benthic and hard-bodied taxa (mysid, alpheid shrimp, spider crab, copepod, benthic gastropod,
374 and a sipunculid worm), as well as larvacean prey sequences (Table S12). Their nematocysts are
375 not able to subdue crustacean prey, and their feeding reflex would not be triggered by a prey as
376 small as a larvacean [42]. Therefore, we interpreted the presence of these taxa in the gut contents
377 as secondary predation in the gut contents of the fish prey (Tables S2 and S6). In addition, we
378 also detected ctenophore prey in one specimen. This could be also a case of secondary predation,
379 but we suspect a ctenophore could be large enough to be prey of the man-o-war. If that is the
380 case, this would be the first record of *P. physalis* consuming gelatinous zooplankton, which would
381 place the man-o-war as a central species in the epipelagic ‘jelly-web’ [43]. Comparisons with their
382 surrounding prey field show these specimens were strongly selective for fish and strongly
383 exclusive of copepods (Fig 4).

384 ***Apolemia* spp.**

385 These are among the longest siphonophores, with colonies attaining lengths as long as
386 30m [9]. Their tentacles are different from other siphonophores since they have no tentilla and
387 carry birhopaloid nematocysts directly on the tentacles [27]. *Apolemia* species are known to
388 consume diverse prey including crustaceans, molluscs, polychaetes, chaetognaths, fish, and
389 gelatinous zooplankton [4,7]. While this may suggest these species are generalists, Damian-
390 Serrano et al. [26] hypothesized that they may be gelatinous zooplankton specialists, since they
391 consume a larger proportion of this prey type than other siphonophores. In addition, the
392 nematocysts of *Apolemia* have similar traits to those in other gelativorous cnidarians [27], and
393 their apparent generality could be explained by the sheer number of fine tentacles deployed for
394 prey capture per colony, which would inevitably entangle almost anything that swims by. We found
395 copepod and salp prey sequences in *Apolemia rubriversa* (Figs 2 and 4). The salp prey found in
396 *A. rubriversa* is congruent with its characterization as a gelatinous specialist [26], and may

397 indicate a direct trophic pathway between siphonophores and consumers of microbial production.
398 While the morphology-based predictions indicate that *A. lanosa* is likely a gelatinous prey
399 specialist [27], we only found copepod prey in our sample. However, it is possible that the doliolid
400 and hydromedusa reads we conservatively labelled as potential cross-contamination could
401 correspond to real prey. We also analyzed samples from an undescribed *Apolemia* species,
402 where we found a combination of gelatinous (ctenophore), soft-bodied (larvacean), and
403 crustacean (mysid and euphausiid) prey, congruent with a generalist diet (Fig 2). Considering the
404 differences we found between species, it seems possible that these coexisting species of
405 midwater *Apolemia* are partitioning their trophic niche by differentiating the relative proportion of
406 crustacean and gelatinous prey in their diets.

407 ***Bargmannia* spp.**

408 The three *Bargmannia* species considered here are frequently-observed in the midwaters
409 off Monterey Bay, and have relatively simple tentilla with large stenotele nematocysts and an
410 undifferentiated terminal filament [27]. ROVs have recorded *Bargmannia elongata* consuming
411 crustaceans and cephalopods. One *B. elongata* specimen had euphausiid and ostracod prey, in
412 agreement with the DAPC prediction for *B. elongata* to feed mainly on large crustaceans, but also
413 marginally on small crustaceans (Fig 5). During specimen collection we observed a mysid prey in
414 a specimen *B. amoena*. Our DNA metabarcoding identified this mysid as *Boreomysis* (METAXA
415 assignment score 54.68% for V7 on SILVA123.1), and found a copepod in another specimen.
416 Nothing was previously known, however, about the diet of *Bargmannia lata*. The two *B. lata*
417 specimens we sequenced consumed a ctenophore and a copepod, respectively.

418 The diets of these three closely-related, coexisting species appear to be non-overlapping,
419 which could be a consequence of competitive trophic niche partitioning. The findings for *B. lata*
420 are not congruent with the morphology-based prediction to be a large-crustacean specialist (Fig

421 5). We suspect that the lack of taxon sampling among the pyrostephids in [26] could have led to
422 overfitting in the DAPC. Finding ctenophore prey also supports the involvement of deep-sea
423 siphonophores in the midwater ‘jelly web’.

424 **Other deep-sea physonects**

425 Undescribed physonect sp. L was predicted to be a fish specialist with a secondary affinity
426 for large crustacean prey (Fig 5). However, we found this specimen consuming a ctenophore.
427 Other morphologically-similar deep-sea undescribed physonects (G and Zigzag) have been
428 observed consuming fish and squid prey [4], thus it is possible that they are specialized in
429 capturing and digesting soft-bodied prey more generally. The rarely-observed *Resomia dunnii* was
430 predicted to be a generalist (consumer of all types of prey except gelatinous taxa), which is
431 consistent with the copepod prey we found in its gut contents.

432 *Forskalia* species are frequently found in both shallow and deep waters, and have been
433 observed to consume various crustaceans, molluscs, worms and fish [7]. However, morphology
434 predicts *Forskalia* species to be large crustacean specialists [27]. We found three midwater
435 *Forskalia* specimens with copepod prey in the guts, one of them also had consumed a sergestid
436 shrimp. These results are fully congruent with those derived from visual methods, and partly
437 congruent with the morphological predictions. *Halistemma rubrum* tentilla closely resemble those
438 of *Forskalia*, and thus they are also predicted to be large-crustacean specialists [27]. This
439 prediction is congruent with our identification of a lophogastrid in the gut contents (Fig 4). On the
440 other hand, *Lychnagalma utricularia* is unique among the physonects for bearing a medusa-
441 shaped floating vesicle at the end of their large, coiled tentilla [27]. Nonetheless, they have been
442 also observed consuming large crustaceans through ROVs, such as sergestid shrimp. We found
443 two specimens both with sergestid shrimp prey DNA, yet one of them was also digesting a

444 euphausiid (Fig S1). This is consistent with their hypothesized large-crustacean specialization
445 (Fig 2 and Fig 5).

446 ***Nanomia* spp.**

447 These are among the most common siphonophores in both Atlantic and Pacific waters,
448 both in epipelagic and midwater environments. We have observed that epipelagic *Nanomia* tend
449 to have smaller tentilla than their mesopelagic counterparts, which may explain their tendency to
450 capture smaller crustaceans such as copepods [7] instead of larger crustaceans such as krill.
451 Midwater ROV observations of deep-dwelling *Nanomia* have predominantly reported interactions
452 with krill prey, as well as with the occasional chaetognath or sergestid shrimp [4]. We identified
453 one specimen of mesopelagic *Nanomia* with krill and stomatopod DNA in its gut contents,
454 congruent with its hypothesized large-crustacean specialist characterization (Fig 5). However,
455 epipelagic *Nanomia* seems to be less specialized on large crustacean prey, since the literature
456 reports a combination of copepod, decapod, mysid, and chaetognath prey [7]. In the North Pacific
457 Ocean, our metabarcoding identified copepod prey in an epipelagic *Nanomia* off California, a
458 hyperiid amphipod prey in an epipelagic *Nanomia* off Hawaii (Fig S1). The hyperiid amphipod
459 could have been a commensal or parasite on the *Nanomia* instead of prey, though this is unlikely
460 since only the gastrozoids were dissected while amphipods tend to colonize the nectophores or
461 bracts. In the North Atlantic Ocean, we sampled 14 specimens of epipelagic *Nanomia*, seven of
462 which contained copepod prey (Fig 2). Upon visual inspection of the sampled gastrozoids we
463 could identify *Temora*, *Centropages*, and *Acartia* copepods, the most abundant genera in the
464 plankton sample, whose identity was also supported by the metabarcoding results (genus and
465 species-level assignment scores: *Centropages* sp. 54.99% for barcode V5-V7L, *Acartia tonsa*
466 91.5% for barcode V5-V7S, and *Temora discaudata* 91.81% for barcode V7p+V8, using the
467 SILVA123.1 database). The corresponding environmental plankton samples showed that these
468 waters were dominated by cladocerans, and thus these *Nanomia* were positively selecting for

469 copepod prey (LI values between 0.69 and 0.72) and selecting against cladoceran prey, which
470 was not detected in the guts (Fig 4 and Fig S1). The exclusion of the overabundant cladocerans
471 from the diet of Atlantic *Nanomia* suggests that their specialization, if any, could be copepod-
472 specific.

473 **Calycophorans**

474 These siphonophores are characterized by their lack of a pneumatophore (gas-filled apical
475 vesicle) and their structurally-homogeneous tentilla [27]. However, these tentilla present a great
476 variation in nematocyst number and size, which may translate into dietary differences [26]. We
477 provided the first insights into the diets of two highly abundant deep-sea calycophorans, *Lensia*
478 *conoidea* and *Chuniphyes multidentata*, which morphology predicted as small-crustacean
479 specialists. Both sequenced specimens contained copepod DNA (Fig S1), supporting these
480 predictions (Fig 5). While gelatinous prey has been reported for *Desmophyes annectens* from
481 ROV observations [4], we found only copepod prey sequences. We did find gelatinous prey,
482 however, in *D. dispar* (salp prey), and *Sphaeronectes christiansonae* (nausithoid medusa). The
483 latter constitutes the first record of *S. christiansonae* feeding. While these medusae can be very
484 small, the minute size of this siphonophore may render this interaction dubious. The far more
485 common epipelagic *Sphaeronectes* species, *Sphaeronectes koellikeri*, appears to be a copepod
486 specialist according to visual gut content analysis, since they appear to feed exclusively on
487 copepods [7]. We sequenced the gut contents of two specimens of this species, one of them
488 indeed was consuming a copepod, yet the other was consuming a crab larva. The latter
489 constitutes a novel prey type for this species, yet still within the expected range of a small-
490 crustacean specialist. Another dietary hypothesis was supported for *S. chuni*. Their visually-
491 assessed diet appears comprised exclusively of copepods [7], and we detected copepod prey in
492 a specimen from the Atlantic ocean. On the other hand, while *Muggiaeaa atlantica* has also been
493 observed feeding exclusively on copepods [7], our specimen had only larvacean sequences that

494 could correspond to prey. While the read abundance for this OTU was low, the small size of this
495 prey type is reasonable given the small tentilla and gastrozoooids of *M. atlantica* [27,42].

496 The calycophoran *Vogtia* is the closest relative to *Hippopodius*, the only siphonophore
497 known to be an ostracod specialist [7]. Like many other hard-to-access mesopelagic taxa, the diet
498 of *Vogtia* has remained unknown, though tentillum morphology predicted them to be generalists
499 [27]. An oceanographic study [44] found spatial correlations between ostracods and *Vogtia*
500 species, and even mentions a *Vogtia* sp. specimen which had the exoskeleton of an ostracod in
501 its gut contents. Our DNA metabarcoding on *Vogtia serrata* has revealed one specimen feeding
502 on an ostracod, and a specimen feeding on a sergestid shrimp and a bivalve (with high selectivity,
503 LI = 0.5). These results are consistent with the generalist morphological prediction, and congruent
504 with the single visual finding of an ostracod in a congener [44]. The presence in the gut contents
505 of one of our specimens of an ostracod and a bivalve (likely a pediveliger larva), which has a very
506 similar shape to an ostracod (with two hard valves), indicates phylogenetic conservatism of prey
507 traits within Hippopodiidae.

508 Comparisons with visual methods

509 We report the first insights into the diets of nine siphonophore species and reveal 29 novel
510 predator-prey interactions (Fig 2 and Fig 5). When comparing our metabarcoding findings with
511 the published visual observations from gut content inspections and submersible dives, we found
512 five interactions congruent with ROV observations, and eight interactions (six of them involving
513 copepods) congruent with visual gut content inspections of SCUBA-collected colonies (Fig 5).

514 The published records on the diets of siphonophores appear to differ in prey-type
515 composition between epi- and deep-pelagic habitats. However, the different methodological
516 limitations inherent to each visual method (small prey underestimated by submersibles, soft-
517 bodied prey underestimated by gut content inspections) are hypothesized to be responsible for

such differences [8]. Our approach has detected prey types, such as larvaceans, ctenophores, bivalves, and ostracods previously missed by visual methods. The gelatinous animals (i.e. ctenophores, medusae, salps) identified by submersibles as prey of deep-pelagic siphonophores were found present in the gut contents of several deep species (*Apolemia* sp., *B. lata*, undescribed physonect L, and *S. christiansonae*), supporting the validity of these observations. However, the gelatinous prey recorded by submersibles in prayids such as *Praya dubia* and *D. annectens* [4] were not recovered in our *D. annectens* samples (Fig 5), suggesting that either our sample sizes were not large enough, or that ROVs had observed accidental entanglement of jellies on their tentacle nets which did not end in ingestion. In addition, we found several small crustaceans in the gut contents of epipelagic species (*Forskalia* sp., *Nanomia* sp., *S. koellikeri*, *S. chuni*, and *D. dispar*) in agreement with visual gut contents observations in shallow habitats. On the other hand, we also found gelatinous and soft-bodied invertebrate prey in shallow-dwelling species (*P. physalis*, *D. dispar*, and *M. atlantica*); as well as small-bodied animals among the prey of deep-pelagic siphonophores (*Apolemia* spp., *Bargmannia* spp., *R. dunnii*, *V. serrata*, *D. annectens*, *C. multidentata*, and *L. conoidea*) (Fig 2). Copepods and ctenophores were the most frequent prey among bathypelagic siphonophores, while other crustaceans (such as ostracods, decapods, and krill) appeared as prey more frequently among the mesopelagic taxa. While these findings are consistent with the hypothesis that small prey is underestimated in submersible observations and rapidly-digested, soft-bodied prey is underestimated by gut content inspections, our sample sizes are insufficient to determine whether the relative contribution of these prey differs between habitats.

DNA metabarcoding was able to detect prey both small and large, gelatinous and hard-bodied, for both deep and shallow-dwelling siphonophore species. These results show that the trophic roles of siphonophores in epi- and deep-pelagic food webs could be more similar than previously-published records may indicate, due to the biases brought by the different diet-

543 assessment methods applied in each habitat. Vertical migration is an important driver of pelagic
544 food web structure [45,46]. We found copepods, decapods, and euphausiids in the gut contents
545 of both meso- and epipelagic siphonophores. These prey taxa are well-known vertical migrators
546 [47-49], suggesting that there might be some vertical trophic connectivity between these habitats
547 as prey migrates between them. In addition, a few siphonophore species (including *V. serrata* and
548 *L. conoidea* in this study) are also known diel vertical migrators [50], but their patterns of feeding
549 with depth remain unknown. Finally, our selectivity estimates (for four epipelagic and two
550 mesopelagic species) indicate that siphonophores may play a similar role as selective predators
551 across all depths in the water column.

552 Comparisons with prey field

553 We examined 8 prey-field samples that corresponded to the colocalized ambient prey of
554 15 out of 47 specimens (some trawls correspond to more than one sampled specimen). The
555 epipelagic plankton samples from Bermuda (colocalized with the *P. physalis* specimens) were
556 dominated by copepods, followed by decapod larvae and chaetognaths. While fish larvae were
557 scarce in these samples, they were still far more abundant than in any other sampled location.
558 The Atlantic epipelagic plankton samples (colocalized with the *S. chuni* and Atlantic shallow
559 *Nanomia* specimens) were dominated by cladocerans, followed by copepods, larvaceans and
560 salps. The Pacific epipelagic plankton sample from California (colocalized with the *D. dispar*
561 specimens) was also dominated by copepods, followed by cladocerans and larvaceans. The
562 quantified midwater tucker trawl from California (colocalized with *V. serrata* specimen D1137-D8
563 and *Forskalia* sp. specimen D1137-D9) was also dominated by copepods (albeit larger species),
564 followed by euphausiids (both adult and larval), chaetognaths, and ostracods.

565 We found both positive and negative selectivity when comparing identified siphonophore
566 prey to quantified co-localized prey fields. We found strong negative (<-0.5) selectivity for

567 copepods in *P. physalis* specimens and in one specimen of *V. serrata*. However, in 11
568 siphonophore specimens from 4 species (out of the 6 species that were quantitatively assessed),
569 we found strong positive selectivity (>0.5) for a specific prey type (Fig S1). These cases include:
570 selectivity for fish in *P. physalis*; selectivity for copepods in *S. chuni*, and Atlantic *Nanomia* sp.,
571 selectivity for ostracods in *V. serrata*, and selectivity for salps in *D. dispar* (Fig 4). These selectivity
572 values suggest a strong influence of predator-specific differences in prey capture capabilities for
573 different prey types. However, more replication is necessary in order to test for prey-type
574 specialization.

575 Epipelagic siphonophores are known to be highly selective and specialized carnivores
576 [7,26,51,52]. ROV observations have revealed that some deep-sea siphonophores are also highly
577 specialized [4]. However, the lack of paired diet and planktonic community samples has limited
578 an assessment of their feeding selectivity. For both the shallow- and deep-dwelling siphonophore
579 species assessed here, we found their prey belonged to the less-abundant components of the
580 co-localized planktonic community, demonstrating high prey-type selectivity. However, the
581 selectivity index values presented in this study should be interpreted with care, since the prey
582 field data is quantitative (abundance-based) but the gut content values are only binary at the
583 specimen level, and frequency-based at the predator species level. Overall, crustaceans
584 (especially copepods) were identified as the most frequent prey type among siphonophore diets.
585 Copepods are typically the most abundant prey type in planktonic communities, thus being able
586 to feed on them is likely an advantageous strategy for any planktivorous predator [53]. Fish prey
587 were detected only in the Portuguese man-o-war samples, in agreement with published
588 observations of man-o-war feeding [40,41].

589 Our findings are congruent with the idea that siphonophores span multiple trophic
590 positions, consuming prey across low (salps, larvaceans, copepods, ostracods) and high (fish,
591 ctenophores, medusae) trophic levels. We found larvaceans and salps as prey of shallow- and

592 deep-dwelling siphonophores. These urochordates have an important role in the biological carbon
593 pump, sequestering carbon from phytoplanktonic producers into the deep sea by means of fecal
594 matter production and carcass depositions [54,55]. The role of predation on gelatinous herbivores
595 is often underestimated in oceanic food-web models, or primarily attributed to vertebrate
596 predators [56]. Our results show that some siphonophores like *Apolemia* sp., *A. lanosa*, *M.*
597 *atlantica*, and *D. dispar* may play an important mid-trophic role incorporating this soft-bodied
598 herbivore productivity into the food web, and providing an alternative avenue to transfer carbon
599 into the deep sea.

600 Comparisons with morphology predictions

601 Comparing our metabarcoding findings with morphology-based predictions [27], we found
602 support for 10 of the 16 predicted interactions between siphonophores and prey. Among the
603 physonects, our results supported the predictions of *B. elongata* eating krill and ostracods, *R.*
604 *dunni* eating copepods, *Forskalia* sp. eating decapods, and *H. rubrum* eating lophogastrids.
605 Among the calycophorans, we found support for the predictions of *V. serrata* eating decapods,
606 ostracods, and molluscs; also *C. multidentata* and *L. conoidea* eating copepods. Among the
607 siphonophore species studied there were 70 predicted interactions that were not found among
608 the metabarcoding results (Fig 5). Out of the 10 taxa with both morphology-based predictions and
609 metabarcoding results, six had all prey congruent with the predictions, three had all prey
610 incongruent with the predictions, and *Forskalia* sp. presented both cases.

611 Food-web structure is determined largely by community composition and its patterns in
612 time and space, as the organismal assemblages determine what predators are present and what
613 prey is available to them [57-59]. However, organismal traits constrain which predators can eat
614 which prey [60,61]. The most commonly-studied trait to predict oceanic food web structure has
615 been size [62,63]. This is due to the importance of gape size in most predators (i.e. fish, squids,

616 crustaceans etc.) with singular and rigid buccal openings [64,65]. Siphonophores differ from most
617 predators by having many gastrozooid mouths along their length, all capable of stretching out
618 significantly to ingest prey [66], sometimes utilizing multiple zooids to wrap around large prey [67].
619 While prey size is still an important constraint for siphonophore-prey interactions [42],
620 siphonophore size is far less relevant. Moreover, some studies have found that phylogenetically-
621 conserved predator traits other than size may also be important predictors of food web structure
622 [68,69]. Diet is a strong predictor of both extant and ancestral siphonophore tentilla morphology,
623 as well as of its evolutionary dynamics [26]. These relationships were utilized predict the diets of
624 understudied siphonophore species based on the morphology of their tentilla and nematocysts
625 [27]. Here, we were able to test these predictions for ten species and found that most of the prey
626 items found were congruent with these predictions, indicating that tentilla morphology is a strong
627 predictor of siphonophore diets. This finding suggests that some components of the open-ocean
628 food web may be structured by variation in complex morphological traits exclusive to specific
629 predator groups.

630 Siphonophores are hypothesized to easily evolve between feeding specializations and
631 into a generalist diet due to their modular body plan and their functionally-specialized tentilla [26].
632 Our results show that closely-related species, such as those within the genera *Bargmannia*,
633 *Apolemia*, and *Nanomia*, appear to feed on different prey. We hypothesize that these species
634 could be further subspecializing to avoid competition or adapt to different prey fields at different
635 depth habitats. This hypothesis is congruent with the conclusions in Damian-Serrano et al. [26]
636 that siphonophore dietary evolution can drive rapid morphological shifts. Moreover, we find that
637 *Apolemia* sp., as well as *V. serrata*, could be generalists feeding on a variety of crustacean and
638 soft-bodied prey. If a more extensive and quantitative sampling of these taxa was to validate this
639 trophic reclassification, that would suggest that a generalist diet had evolved not just three (as

640 proposed in Damian-Serrano et al. [26]), but up to five times independently, further reinforcing
641 their conclusions.

642 **Methodological considerations**

643 While DNA-based tools can detect prey unrecognized by visual methods, they are not free
644 of shortcomings. Since all life stages of an animal have the same genetic signature,
645 metabarcoding tools are unable to distinguish between larval, juvenile, or adult prey. These
646 ontogenetic stages can have vastly different ecological implications and pose different challenges
647 during prey capture. In addition, the application of metabarcoding to predator diets is usually not
648 quantitative, since too many sources of variation may lead to differences in read abundance. For
649 example, different animal clades have different sizes, cell densities (due to variable acellular
650 mesoglea content), digestion rates, number of copies of the target gene, or primer affinities during
651 the PCR [70-72]. Due to the difficulties inherent to locating and sampling the species examined
652 in this study, frequency-based quantitative comparisons were not possible for most species either.
653 In addition, the sample size limitations of this study may have biased the results towards higher
654 apparent specialization, and may have missed some important components of the diets of some
655 target species. This caveat is also common in submersible observation data and limits the
656 reliability of comparisons across these methods.

657 Siphonophores differ from other consumers in several ways which impose further
658 limitations to the value of gut content metabarcoding. The most important aspect is their feeding
659 mode and feeding rate, especially as deep-sea ambush predators, which typically consume one
660 prey at a time and do not get a chance to capture another until far after the former has been
661 digested [9]. Therefore, most siphonophores are found with empty guts or digesting one or few
662 prey items at a time. Thus, the sample size required for frequency-based analyses is much higher
663 than for other consumers which feed more frequently. Moreover, except for a couple species such

664 as *Rhizophysa eysenhardtii* and *Rosacea cymbiformis* which are diurnal feeders [7], most species
665 also feed during the night. In the open ocean, diel vertical migration drastically changes the prey
666 field composition for siphonophores at night [45]. Given the fieldwork limitations in this study, we
667 were only able to collect siphonophore gut contents during the day, thus likely biasing their diet
668 towards their diurnal prey captures. Finally, secondary predation (the prey of the prey) cannot be
669 empirically distinguished from direct predation, and thus we must rely on natural-history based
670 assumptions.

671 Conclusions

672 This study uses DNA metabarcoding technology to investigate the diets of a diverse range
673 of siphonophores. We identified 55 unique prey items in the gut contents of 24 siphonophore
674 species, the majority of which were crustaceans (most of which were copepods), in addition to
675 fishes, molluscs, and gelatinous taxa (Figs 2 and 4). Our results expand the existing knowledge
676 on siphonophore diets, detecting prey types previously missed by visual methods, and providing
677 insights into the diets of several understudied siphonophore species. We show that whole
678 gastrozooids can be utilized for DNA metabarcoding of diets without need for further dissection
679 or the use of predator-blocking primers. We identified representatives from diverse animals (Fig
680 3, Tables S5-S11), which demonstrates the phylogenetic range of taxa that can be amplified with
681 our primer pairs. By comparing the taxonomic composition of the gut contents to that of the
682 environmental planktonic community, we find support for the idea that both shallow and deep-
683 dwelling siphonophore species selectively prey on distinct components of zooplankton and
684 micronekton communities (Fig 4). Many of the prey types found in both shallow and deep-dwelling
685 species match published records based on visual methods, but some prey types appear
686 underrepresented by those methods. Moreover, we find that many of the tentillum morphology-
687 based dietary predictions for these species were supported by the metabarcoding results (Fig 5).

688 Overall, we provide novel insights into the ecology and natural history of several
689 siphonophore species, revealing that siphonophores across all depths are selective predators
690 which have diversified their feeding habits to consume fish, crustaceans, gelatinous predators,
691 gelatinous filter-feeders, meroplanktonic larvae, and other pelagic invertebrates. Our results
692 reveal a significant involvement of deep- and shallow-dwelling siphonophores in the open-ocean
693 ‘jelly web’, highlight suspected biases from visual methods, and support the hypothesized value
694 of tentilla morphology to predict their diets. This study also demonstrates the suitability and
695 effectiveness of DNA metabarcoding to identify the prey consumed by gelatinous predators.

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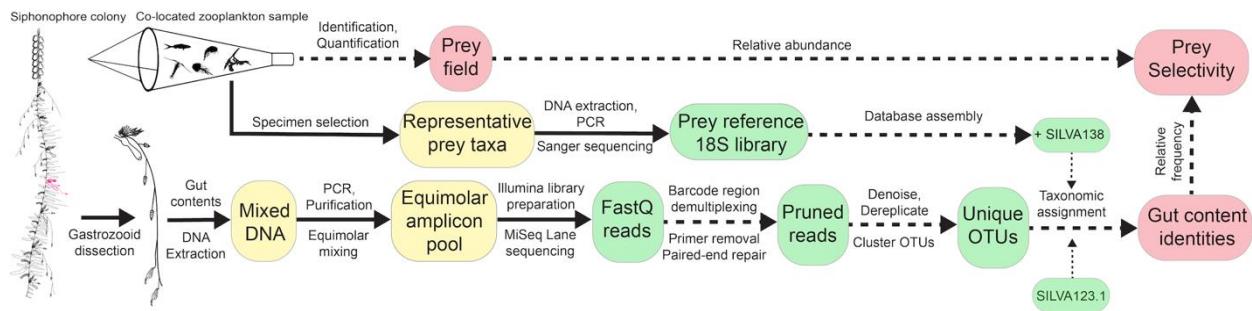
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901

902 **Figures**

903
 904 **Fig 1. Gut content metabarcoding workflow used in this study.** Siphonophore colony
 905 illustrated by Freya Goetz. Silhouettes in the plankton net downloaded from phylopic.org. Solid
 906 arrows indicate physical material transfer and processing, dashed lines indicate information
 907 transfers and processing. Yellow islands indicate elements processed in the laboratory bench,
 908 green islands represent bioinformatic datasets processed in the high-performance computing
 909 cluster, and red islands represent curated data products.

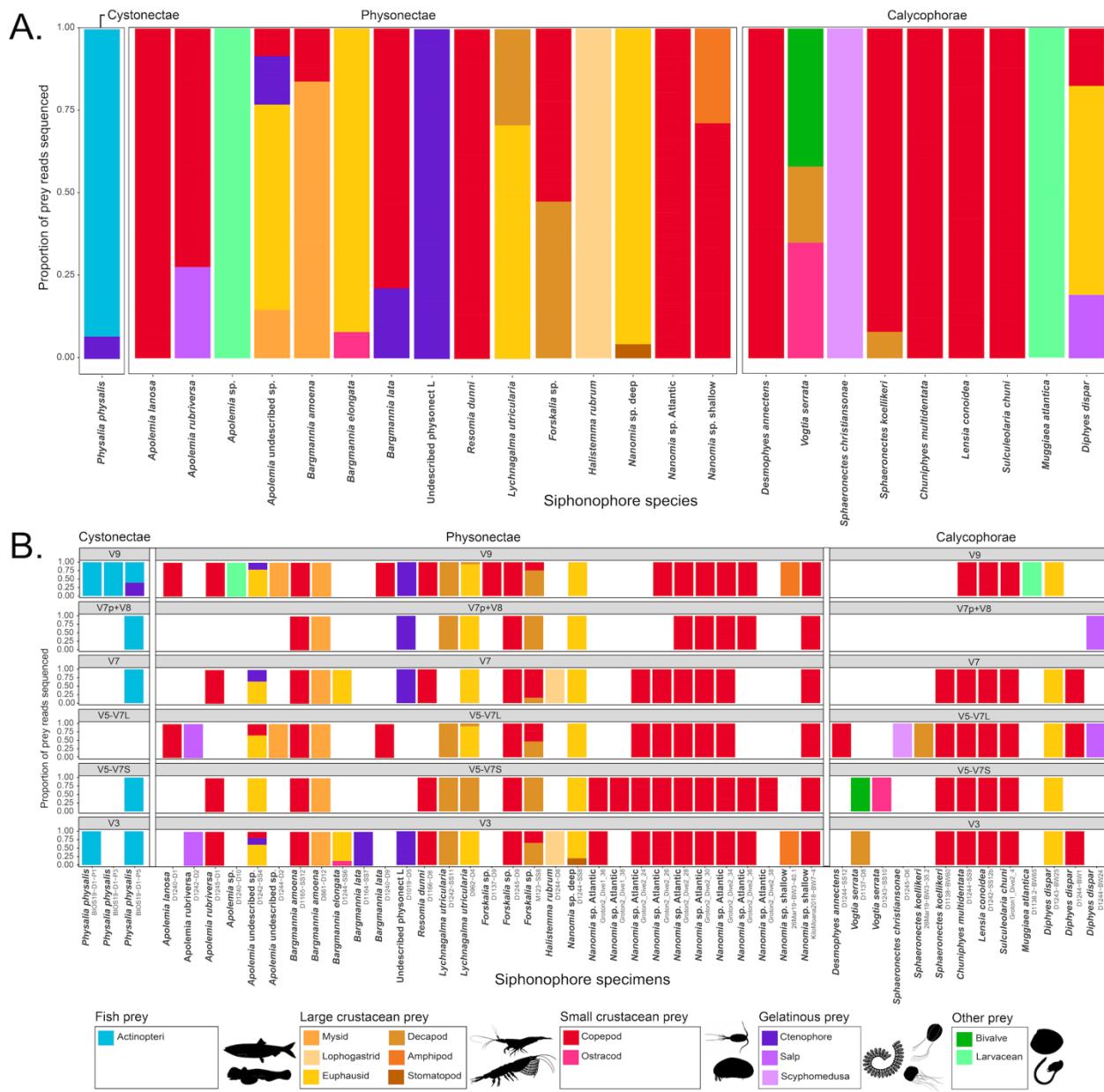
910

Species	Vertical habitat	N sampled	N with prey	Literature-based guild	Tentilla-based prediction of guild	Metabarcoding prey	Photo
<i>Physalia physalis</i>	Pleustonic	5	3	Fish		Fish, ctenophore	A
Undescribed physonect ZigZag	Mesopelagic	1	0				
Undescribed physonect L	Bathypelagic	2	1		Fish	Ctenophore	
<i>Erenna sirena</i>	Bathypelagic	2	0	Fish			
<i>Erenna cornuta</i>	Bathypelagic	1	0				
<i>Stephanomia amphitrichis</i>	Bathypelagic	3	0				
<i>Apolémia rubriversa</i>	Mesopelagic	7	2	Gelatinous		Copepod, salp	
<i>Apolémia</i> sp.	Mesopelagic	3	3	Gelatinous		Copepod, mysid, euphausid, ctenophore, larvacean	
<i>Apolémia lanosa</i>	Bathypelagic	3	1		Gelatinous	Copepod	B
<i>Nanomia</i> sp. shallow Pacific	Epipelagic	12	2	Large crustacean		Copepod, amphipod	
<i>Nanomia</i> sp. shallow Atlantic	Epipelagic	14	9			Copepod	
<i>Halistemma rubrum</i>	Epipelagic	1	1		Large crustacean	Lophogastrid	
<i>Nanomia</i> sp. deep	Mesopelagic	3	1	Large crustacean		Euphausid, stomatopod	
<i>Lynchagalma utricularia</i>	Mesopelagic	4	2	Large crustacean		Decapod, euphausid	
<i>Resomia ornicephala</i>	Mesopelagic	1	0				
<i>Craspedo latethica</i>	Mesopelagic	2	0				
<i>Lilyopsis fluorocantha</i>	Mesopelagic	1	0				
<i>Praya dubia</i>	Mesopelagic	2	0	Large crustacean			
<i>Desmophyes annectens</i>	Mesopelagic	1	1				
<i>Bargmannia elongata</i>	Mesopelagic	2	1		Large crustacean	Copepod	E
<i>Bargmannia amoena</i>	Bathypelagic	3	2	Large crustacean		Ostracod, euphausid	F
<i>Bargmannia lata</i>	Bathypelagic	3	2			Copepod, mysid	
<i>Murru claudanielis</i>	Bathypelagic	2	0		Large crustacean	Copepod, ctenophore	
<i>Forskalia</i> sp.	Epipelagic	3	3	Generalist		Copepod, decapod	
<i>Agalma okenii</i>	Epipelagic	3	0	Generalist			
<i>Frillaglma vityazi</i>	Mesopelagic	3	0		Generalist		
<i>Vogtia serrata</i>	Mesopelagic	3	2		Generalist	Ostracod, decapod, bivalve	G
<i>Resomia dunnii</i>	Bathypelagic	3	1		Generalist	Copepod	H
<i>Sulculeolaria chuni</i>	Epipelagic	1	1	Small crustacean		Larvacean	
<i>Mugilaea atlantica</i>	Epipelagic	5	1	Small crustacean		Copepod, euphausid, salp	I
<i>Diphyes dispar</i>	Epipelagic	15	3	Small crustacean		Copepod, decapod	J
<i>Cordigalgma ordinatum</i>	Epipelagic	1	0	Small crustacean		Scyphomedusa	
<i>Sphaeronectes koellikeri</i>	Epipelagic	10	2	Small crustacean			
<i>Sphaeronectes christiansonnei</i>	Mesopelagic	1	1				
<i>Lensa conoidea</i>	Mesopelagic	1	1				
<i>Chuniphyes multidentata</i>	Mesopelagic	5	1				
<i>Gymnophraea lapislazula</i>	Mesopelagic	3	0				
<i>Kephys ovata</i>	Mesopelagic	2	0				
<i>Chuniphyes moserae</i>	Bathypelagic	1	0				

911

912 **Fig 2. Summary table of the siphonophore species sampled for this study indicating their**
 913 **vertical habitat, the number of specimens sampled, the number of specimens with**
 914 **recognizable prey sequences, and hypothesized feeding guild.** Guilds are based on
 915 published feeding records used in Damian-Serrano et al. [26], predicted feeding guild from the
 916 DAPC analysis in Damian-Serrano et al. [27] based on tentilla morphology, and prey found in this
 917 study. Photo credits: (A) Casey Dunn, (B, D,) Stephan Siebert, CC BY licensed and reprinted
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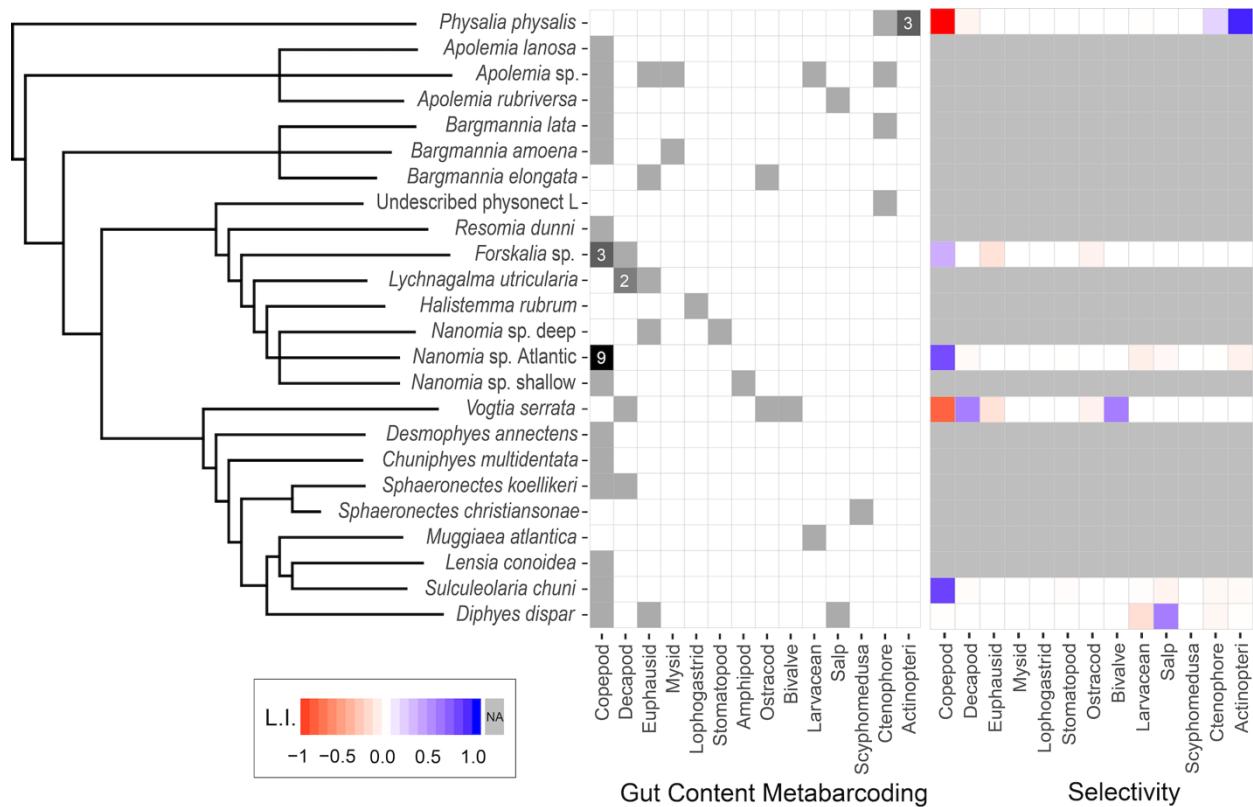
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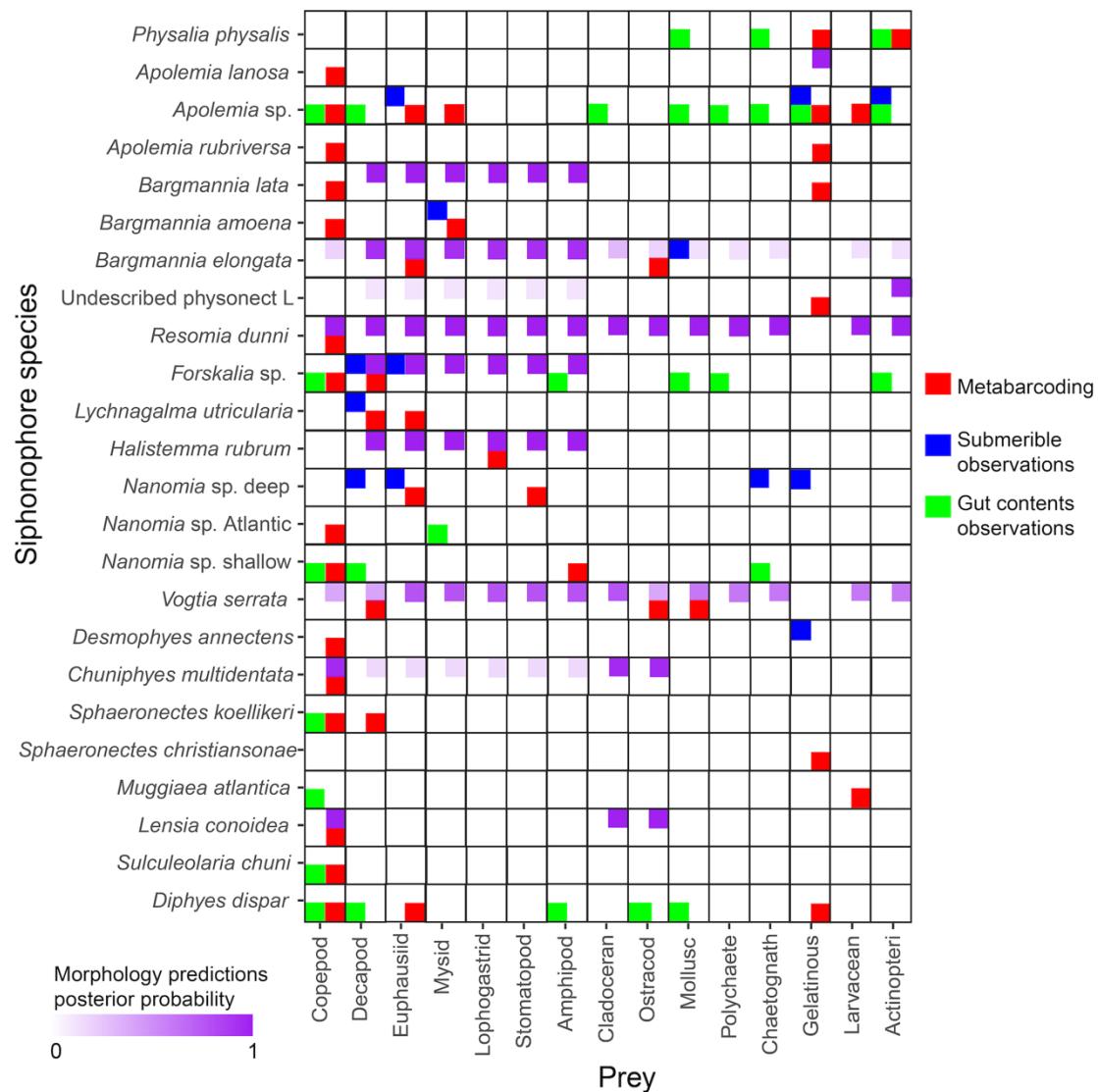
927 **Fig 3. Relative log-abundances of prey reads colored by taxon.** (A) For each siphonophore
928 species, and (B) for each siphonophore specimen and barcode.

929



930

931 **Fig 4. Species-wise grid with the frequency of the major prey types identified from the**
 932 **metabarcoding data and the average prey-type selectivity.** Gut content cells in white indicate
 933 absence, and cells in grey indicate presence in one specimen, or more than one specimen if
 934 labeled with a number. Selectivity colors mapped to Strauss' L.I. values. The siphonophore
 935 cladogram (left) is a simplified version of the phylogenetic tree published in Damian-Serrano et
 936 al. [26].

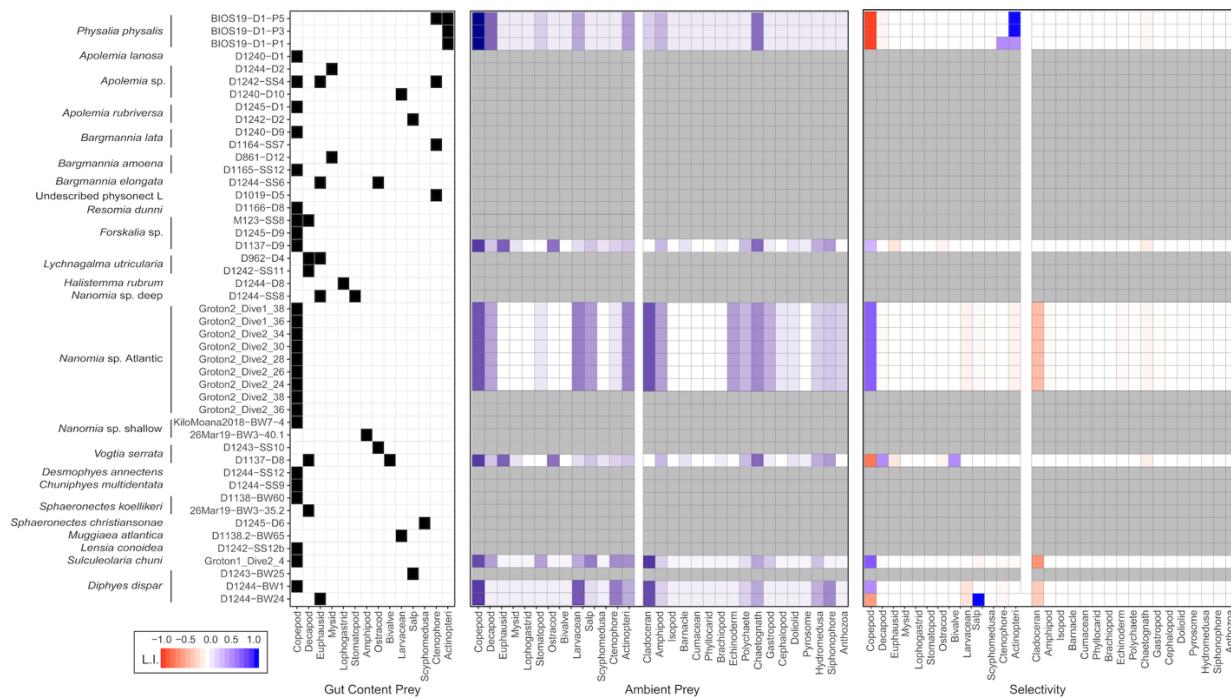


937

938 **Fig 5. Feeding interactions between siphonophores and their prey from different data**

939 **sources.** Including prey identified by our metabarcoding results (red), observations published
 940 submersible observations (blue), observations published visual gut content analyses (green),
 941 and prey types predicted by the morphology-based DAPC model in Damian-Serrano et al. [27].
 942 Gelatinous prey refers to ctenophores, medusae, and salps. Larvaceans were excluded as their
 943 own category since they are not gelatinous when swimming freely outside their mucous
 944 ‘houses’, which would be the only times they would be able to trigger a prey-capture response in
 945 siphonophore tentacles.

946 **Supporting information**



947

948 **Fig S1. Species-wise grid with the frequency of the major prey types identified from the**
 949 **metabarcoding data and the average prey-type selectivity.** Gut content cells in white
 950 indicate absence, and cells in grey indicate presence in one specimen, or more than one
 951 specimen if labeled with a number. Selectivity colors mapped to Strauss' L.I. values.

952 **Table S1. Read abundances assigned to each DNA source interpretation category for**
 953 **each siphonophore species.**

954 **Table S2. Read abundances assigned to each DNA source interpretation category for**
 955 **each siphonophore species by barcode.**

956 **Table S3. Read abundances assigned to each DNA source interpretation category for**
 957 **each siphonophore specimen.**

958 **Table S4. Read abundances assigned to each DNA source interpretation category for**
 959 **each siphonophore specimen by barcode.**

960 **Table S5. Read abundances assigned to each OTU broad taxon for each siphonophore**
 961 **species.**

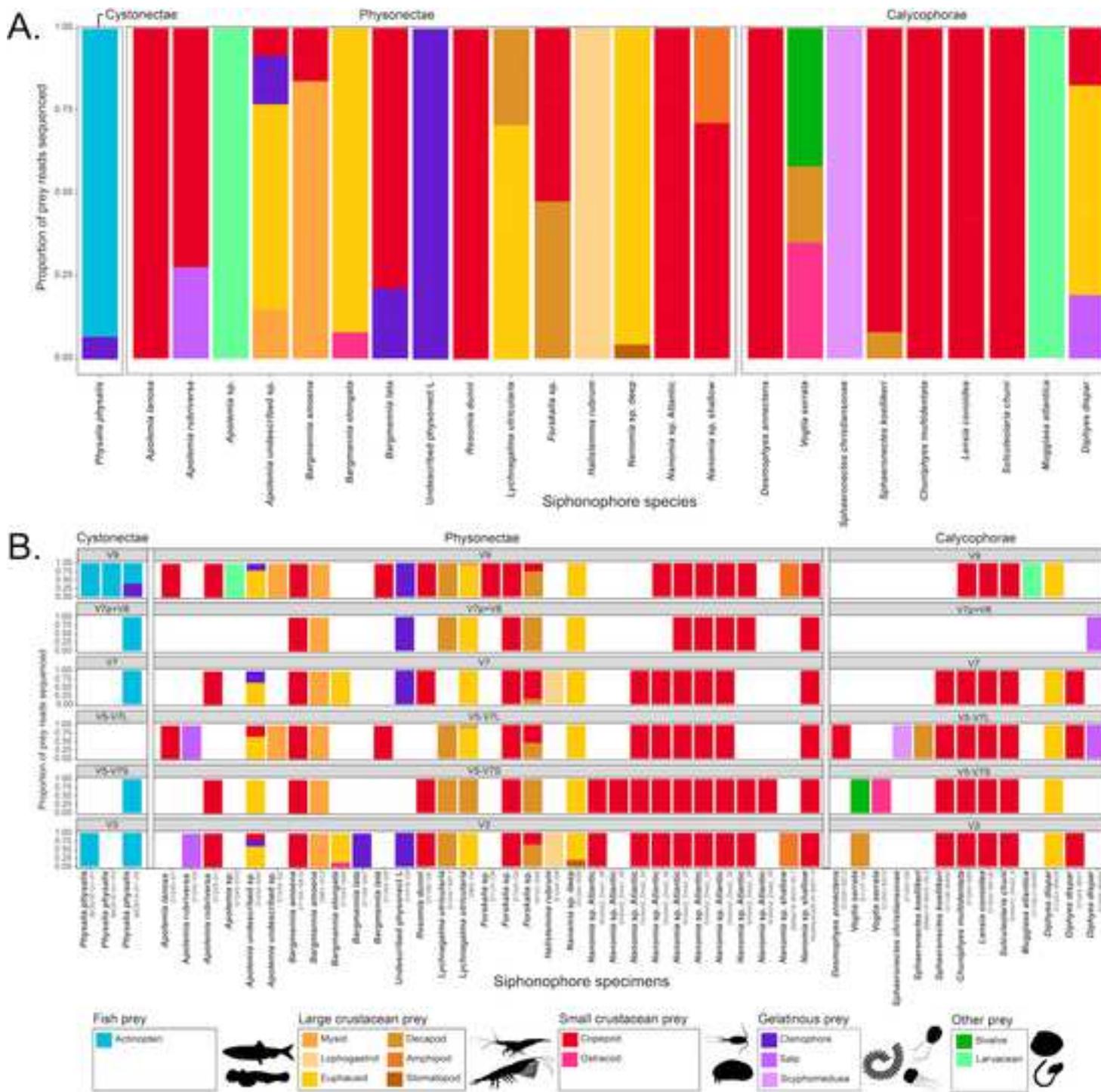
962 **Table S6. Read abundances assigned to each OTU broad taxon for each siphonophore**
 963 **species by barcode.**

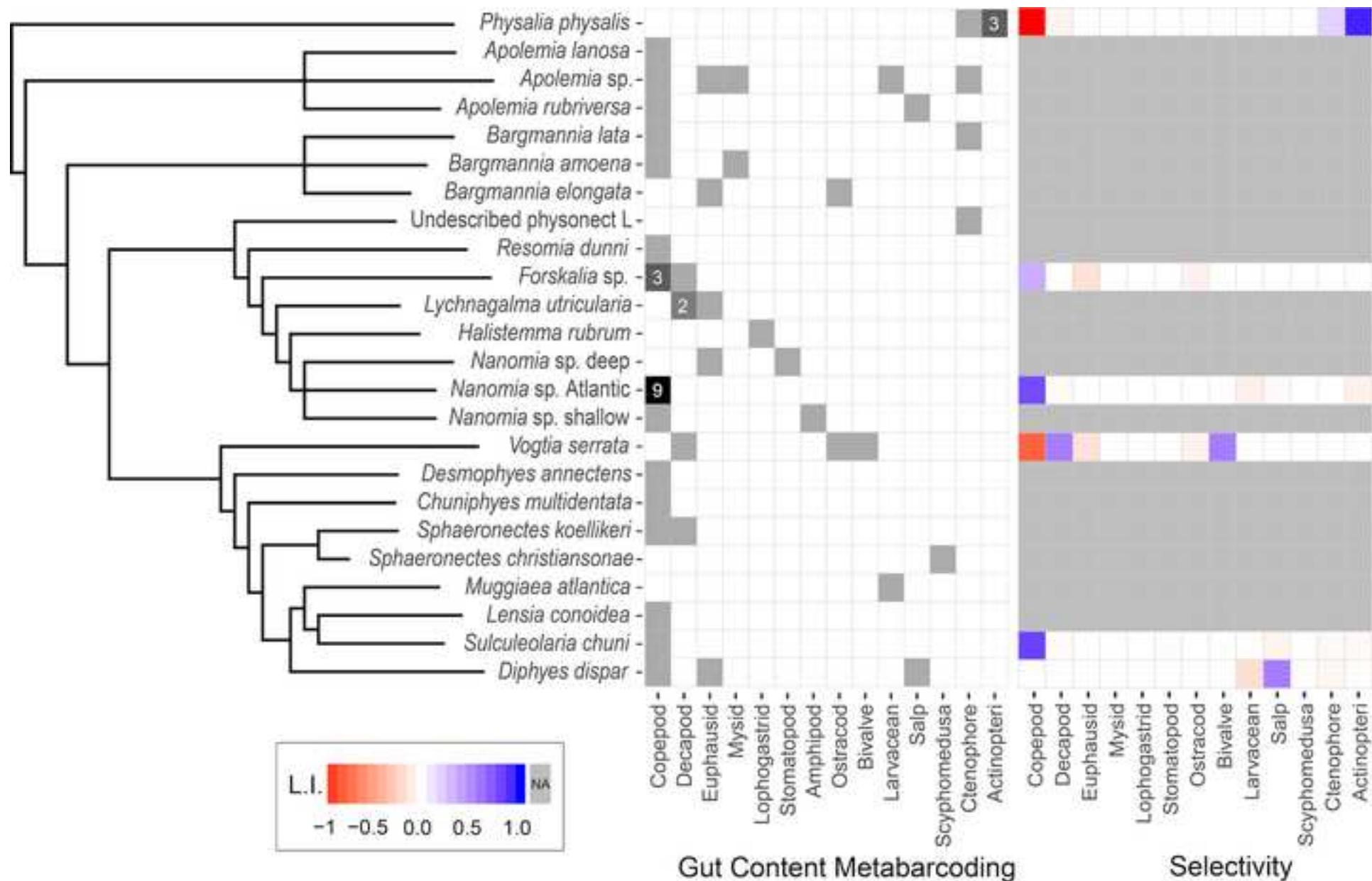
964 **Table S7. Read abundances assigned to each OTU broad taxon for each siphonophore**
 965 **specimen.**

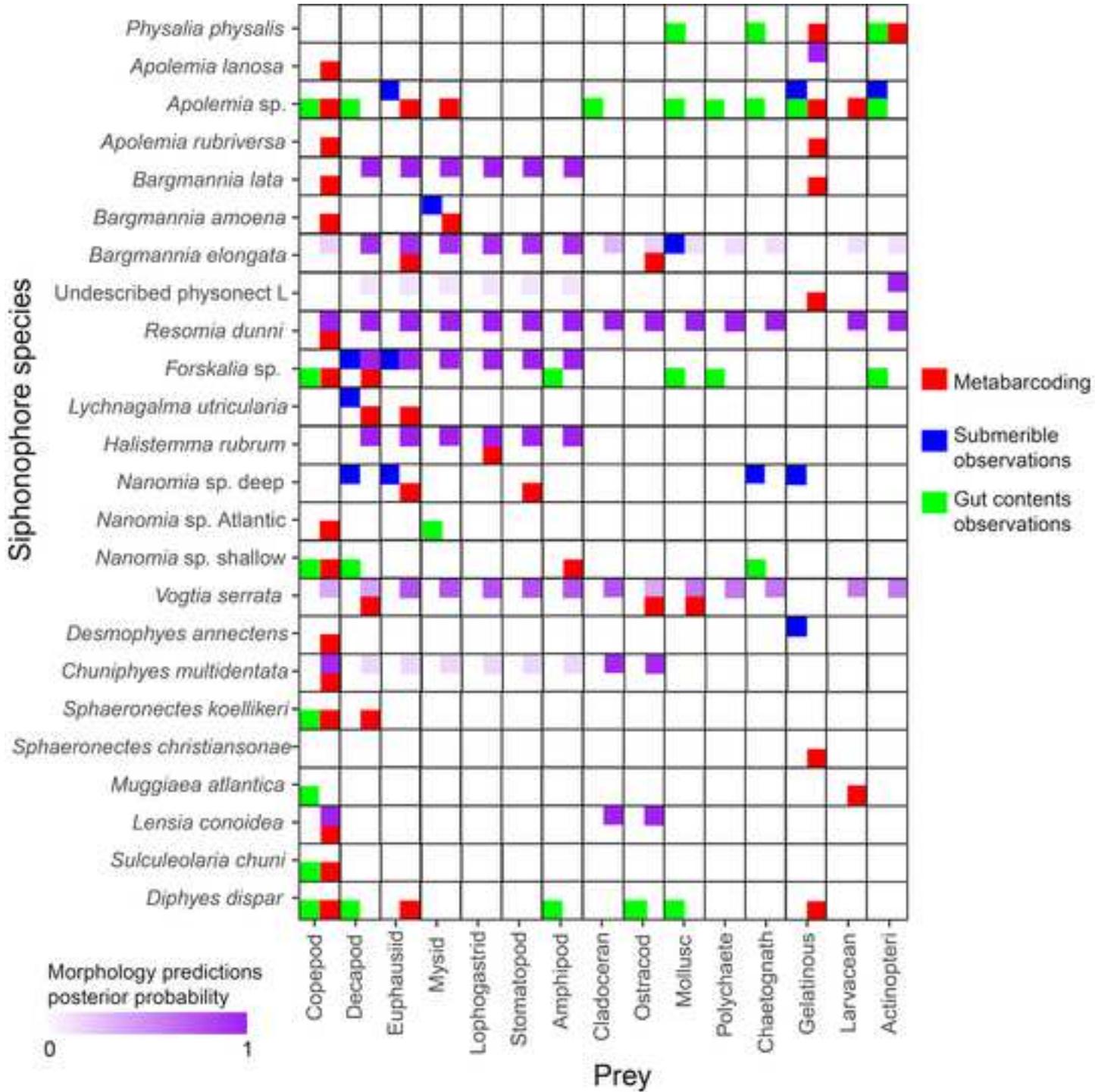
- 966 **Table S8.** Read abundances assigned to each OTU broad taxon for each siphonophore
967 specimen by barcode.
- 968 **Table S9.** Read abundances assigned to each prey OTU broad taxon for each
969 siphonophore species.
- 970 **Table S10.** Read abundances assigned to each prey OTU broad taxon for each
971 siphonophore specimen.
- 972 **Table S11.** Read abundances assigned to each prey OTU broad taxon for each
973 siphonophore species by barcode.
- 974 **Table S12.** Read abundances assigned to each prey OTU broad taxon for each
975 siphonophore specimen by barcode.
- 976 **Table S13.** Number of unique sequences assigned to each barcode in each DNA source
977 interpretation category.
- 978 **Table S14.** Number of unique sequences assigned to each barcode in each OTU broad
979 taxon.
- 980 **Table S15.** Specimen collection metadata and Yale Peabody Museum catalog numbers
981 for voucher specimens.

Fig 3

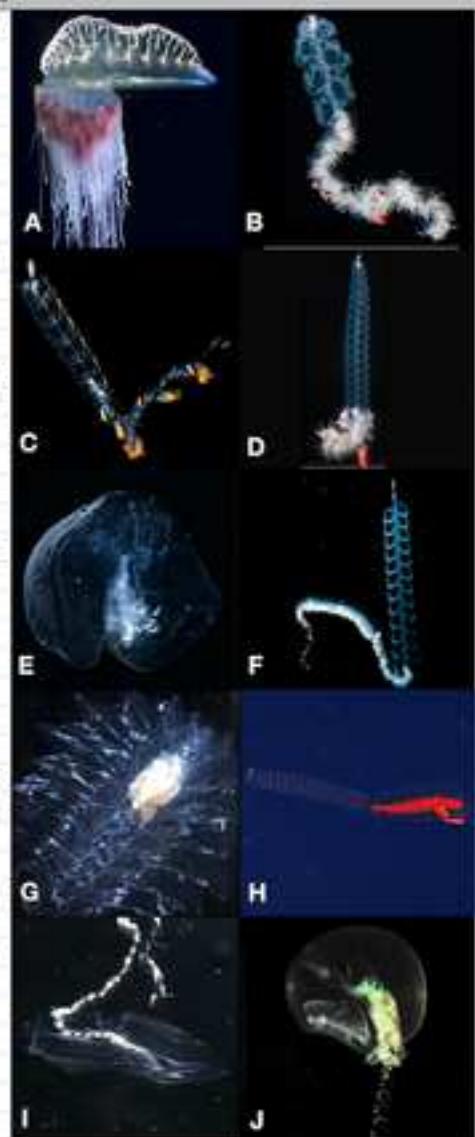
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Species	Vertical habitat	N sampled	N with prey	Literature-based guild	Tentilla-based prediction of guild	Metabarcoding prey	Photo
<i>Physalia physalis</i>	Pleustonic	5	3	Fish		Fish, copephore	A
Undescribed physonect ZigZag	Mesopelagic	1	0				B
Undescribed physonect L	Bathypelagic	2	1		Fish	Copepod	C
<i>Erema siren</i>	Bathypelagic	2	0	Fish			D
<i>Erema cornuta</i>	Bathypelagic	1	0				E
<i>Stephanomia amphitricha</i>	Bathypelagic	3	0				F
<i>Apolemia rubrivirsa</i>	Mesopelagic	7	2	Gelatinous		Copepod, salp	G
<i>Apolemia</i> sp.	Mesopelagic	3	3	Gelatinous		Copepod, mysid, euphausiid, copephore, larvacean	H
<i>Apolemia lanosa</i>	Bathypelagic	3	1		Gelatinous	Copepod	I
<i>Nomia</i> sp. shallow Pacific	Epipelagic	12	2	Large crustacean		Copepod, amphipod	J
<i>Nomia</i> sp. shallow Atlantic	Epipelagic	14	9			Copepod	
<i>Histiophryne rubra</i>	Epipelagic	1	1		Large crustacean	Lophognathid	
<i>Nomia</i> sp. deep	Mesopelagic	3	1	Large crustacean		Euphausiid, stomatopod	
<i>Lycophagia umbicularia</i>	Mesopelagic	4	2	Large crustacean		Decapod, euphausiid	
<i>Resomia ornicephala</i>	Mesopelagic	1	0	Large crustacean			
<i>Crassrea latethica</i>	Mesopelagic	2	0		Large crustacean		
<i>Lilyopsis floracantha</i>	Mesopelagic	1	0		Large crustacean		
<i>Preya dubia</i>	Mesopelagic	2	0	Large crustacean			
<i>Dermophyes annectens</i>	Mesopelagic	1	1			Copepod	
<i>Bergmannia elongata</i>	Mesopelagic	2	1		Large crustacean	Decapod, euphausiid	
<i>Bergmannia amoena</i>	Bathypelagic	3	2	Large crustacean		Copepod, mysid	
<i>Bergmannia lata</i>	Bathypelagic	3	2		Large crustacean	Copepod, copephore	
<i>Marius claudianus</i>	Bathypelagic	2	0		Large crustacean		
<i>Forskalia</i> sp.	Epipelagic	3	3	Generalist		Copepod, illicipod	
<i>Agalma okinai</i>	Epipelagic	3	0	Generalist			
<i>Frillaglma riyazi</i>	Mesopelagic	3	0		Generalist		
<i>Vogtia serrata</i>	Mesopelagic	3	2		Generalist	Decapod, hyperiid, bivalve	G
<i>Resomia dutini</i>	Bathypelagic	3	1		Generalist	Copepod	H
<i>Sulculeolana chuni</i>	Epipelagic	1	1	Small crustacean		Copepod	
<i>Maggiella atlantica</i>	Epipelagic	5	1	Small crustacean		Larvacean	
<i>Diphyes dispar</i>	Epipelagic	15	3	Small crustacean		Copepod, euphausiid, salp	I
<i>Cordagalma ordinatum</i>	Epipelagic	1	0	Small crustacean			
<i>Sphaeronectes koellikeri</i>	Epipelagic	10	2	Small crustacean		Copepod, illicipod	J
<i>Sphaeronectes christiansenae</i>	Mesopelagic	1	1			Synpodocidae	
<i>Lensa conoidea</i>	Mesopelagic	1	1		Small crustacean	Copepod	
<i>Chuniphyes multidentata</i>	Mesopelagic	5	1		Small crustacean	Copepod	
<i>Gymnophraea lapidaria</i>	Mesopelagic	3	0		Small crustacean		
<i>Kophyes ovata</i>	Mesopelagic	2	0		Small crustacean		
<i>Chuniphyes modesta</i>	Bathypelagic	1	0		Small crustacean		



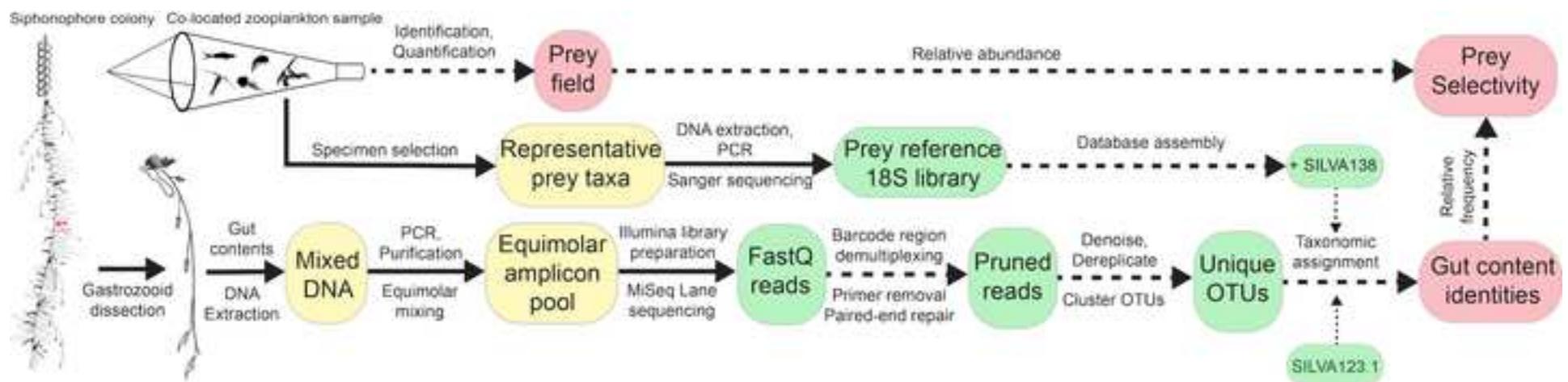


Table S1. Read abundances assigned to each DNA source interpretation category for each siphonophore species.



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Table S3. Read abundances assigned to each DNA source interpretation category for each siphonophore specimen.



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Table S5. Read abundances assigned to each OTU broad taxon
for each siphonophore species.



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Table S7. Read abundances assigned to each OTU broad taxon for each siphonophore specimen.



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Table S9. Read abundances assigned to each prey OTU broad taxon for each siphonophore species.

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Table S10. Read abundances assigned to each prey OTU broad taxon for each siphonophore specimen.

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Table S15. Specimen collection metadata and Yale Peabody
Museum catalog numbers for voucher specimens.



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Table S2. Read abundances assigned to each DNA source interpretation category for each siphonophore species by barcode.



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Table S4. Read abundances assigned to each DNA source interpretation category for each siphonophore specimen by



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Table S6. Read abundances assigned to each OTU broad taxon
for each siphonophore species by barcode.



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Table S8. Read abundances assigned to each OTU broad taxon for each siphonophore specimen by barcode.

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Table S11. Read abundances assigned to each prey OTU broad taxon for each siphonophore species by barcode.



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Table S12. Read abundances assigned to each prey OTU broad taxon for each siphonophore specimen by barcode.

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Table S13. Number of unique sequences assigned to each barcode in each DNA source interpretation category.

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Table S14. Number of unique sequences assigned to each OTU
broad taxon.

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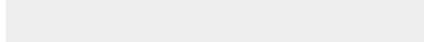




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DAMIAN-SERRANO ET AL.

SIPHONOPHORE DIET METABARCODING

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

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Short title: Siphonophore diets using DNA metabarcoding

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38 **Abstract**

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39 Siphonophores (Cnidaria: Hydrozoa) are abundant and diverse gelatinous predators in
40 open-ocean ecosystems. Due to limited access to the midwater, little is known about the diets of
41 most deep-dwelling gelatinous species, which constrains our understanding of food-web structure
42 and nutrient flow in these vast ecosystems. Visual gut-content methods can rarely identify soft-
43 bodied rapidly-digested prey, while observations from submersibles often overlook small prey
44 items. These methods have been differentially applied to shallow and deep siphonophore taxa,
45 confounding habitat and methodological biases. DNA metabarcoding can be used to assess both

shallow and deep species' diets under a common methodological framework, since it can detect both small and gelatinous prey. We (1) further characterized the diets of open-ocean siphonophores using DNA metabarcoding, (2) compared the prey detected by visual and molecular methods to evaluate their technical biases, and (3) evaluated tentacle-based predictions of diet. To do this, we performed DNA metabarcoding analyses on the gut contents of 39 siphonophore species across depths to describe their diets, using six barcode regions along the 18S gene. Taxonomic identifications were assigned using public databases combined with local zooplankton sequences. We identified 55 unique prey items, including crustaceans, gelatinous animals, and fish across 47 siphonophore specimens in 24 species. We reported 29 novel predator-prey interactions, among them the first insights into the diets of nine siphonophore species, many of which were congruent with the dietary predictions based on tentilla morphology. Our analyses detected both small and gelatinous prey taxa underrepresented by visual methods in species from both shallow and deep habitats, indicating that siphonophores play similar trophic roles across depth habitats. We also reveal hidden links between siphonophores and filter-feeders near the base of the food web. This study expands our understanding of the ecological roles of siphonophores in the open ocean, their trophic roles within the 'jelly-web', and the importance of their diversity for nutrient flow and ecosystem functioning. Understanding these inconspicuous yet ubiquitous predator-prey interactions is critical to predict the impacts of climate change, overfishing, and conservation policies on oceanic ecosystems.

65 **Keywords**

66 ~~Gelatinous zooplankton, trophic ecology, predator-prey interactions, pelagic food webs,~~
67 ~~siphonophores~~

68 **Introduction**

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69 The open-ocean midwater is the largest volume of the biosphere habitable by animals
70 (Harbison 1992) [1]. This environment hosts diverse communities and complex food webs
71 (Robison 2004) [2]. Midwater Pelagic food webs sustain manifold fisheries, top predators, and
72 sustain the biological carbon pump (Falkowski et al. 1998) [3]. Gelatinous animals play
73 fundamental roles in these food webs (Choy et al. 2017) [4], acting as herbivores, detrivores,
74 hosts, predators, and prey. The subset of the midwater pelagic food web involving gelatinous
75 fauna has been referred to as the “jelly web” (Robison 2004) [2]. Among the most abundant [5,6]
76 (O'Brien 2007, Grossman et al. 2015) and trophically-connected [4] (Choy et al. 2017) gelatinous
77 predators are siphonophores — mid-trophic organisms that feed on a broad variety of prey such
78 as medusae, salps, crustaceans, molluscs, and fishes [4,7,8] (Purcell 1981a, Choy et al. 2017,
79 Hetherington et al. in review). Siphonophores are sit-and-wait, non-visual, ambush predators that
80 rely on prey encountering their tentacles and tentilla (Mackie et al. 1988) [9]. They are abundant
81 and locally diverse colonial cnidarians in open-ocean communities, present in every region of the
82 ocean, with species ranging from above the surface (like the Portuguese man-o-war) to the hadal
83 region (>7000m deep) (Jamieson and Linley 2021) [10]. In addition, siphonophore aggregations
84 can have significant predatory impacts on larval fish stocks (Purcell 1981b) [11].

85 Progress in elucidating siphonophore diets has been slow due to the intrinsic challenges
86 of working with these animals. Observation and collection of open-ocean taxa requires expensive
87 research vessels and instrumentation to reach their habitat. In addition, siphonophores are
88 extremely fragile, requiring the use of blue water SCUBA divers and Remotely Operated Vehicles
89 (ROVs) to collect them alive and intact (Haddock 2004) [12]. These techniques can be used to
90 collect live specimens for gut content inspection, and video recordings from ROVs allow scientists
91 to observe feeding events. Traditional collection methods such as plankton nets not only break
92 up siphonophore colonies, but can also lead to artifactual ingestions in the cod-end that confound
93 their natural diets.

94 The diets of some epipelagic siphonophores have been examined through gut content
95 analyses of SCUBA-collected colonies [7,13], and have been reviewed in Hetherington et al. [8]
96 (Biggs 1977, Purcell 1981a, reviewed in Hetherington et al. in review). Recent studies based on
97 ROV observations have shed some light on the diets of deep midwater siphonophores [4,8] (Chey
98 et al. 2017, Hetherington et al. in review). However, these approaches are limited by their biases.
99 Visual gut content inspection favors hard-bodied prey that digest slowly, leaving behind diagnostic
100 body parts (i.e. exoskeleton, shell, eyes, etc.). Therefore, soft-bodied, rapidly-digested taxa, such
101 as gelatinous zooplankton, are often underrepresented in dietary assessments. ROVs can
102 observe feeding on gelatinous prey before they become digested. However, ROV observations
103 are skewed towards large prey items that can be easily identified from the camera screen (such
104 as large medusae, ctenophores, crustaceans, or fishes), and can overlook important prey items
105 such as copepods and larvae (Hetherington et al. in review)[8]. In addition, prey are relatively
106 scarce in the open ocean, especially in the deeper regions (Robison 2004)[2], thus it is infrequent
107 to find specimens capturing prey or carrying visually-identifiable prey in their guts (Purcell, 1981a)
108 [7].

109 With the advent of DNA metabarcoding, the diets of many marine predators have been
110 established from gut content DNA [14-17] (Leray et al. 2013, Harms Tuohy et al. 2016,
111 Fernández Álvarez et al. 2018, Reis et al. 2018). These high-throughput amplicon sequencing
112 technologies have extremely high detection sensitivity and bypass the biases posed by visual
113 methods. Recently, the application of DNA metabarcoding to marine predator gut contents has
114 demonstrated the capacity of these methods to detect gelatinous prey [18-22] (Connell et al. 2014,
115 McInnes et al. 2017, Clarke et al. 2018, Jensen et al. 2018, Marques et al. 2019). In the study of
116 gelatinous zooplankton as consumers, this technology has only been applied to assess the
117 microbial diet of the tunicate *Salpa thompsoni* [23], the predatory diet of the scyphomedusa

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118 *Aurelia coerulea* [24], and the diet of the lobate ctenophore *Mnemiopsis leidy* [25]. However, this
119 technology has not yet been applied to study the diets of siphonophores, gelatinous animals.

120 In Hetherington et al. (in review), we A reviewed and summarized of the literature on
121 siphonophore diets, and observed significant differences between the diets of epipelagic and
122 deep-dwelling siphonophore species [8]. Gelatinous prey appeared to be more prevalent in deep-
123 sea observations while small crustaceans appeared to be the predominant prey in shallow gut
124 content samples. Since epipelagic species' diets were exclusively assessed through microscopic
125 gut content inspection and deep-sea species' diets through ROV observations, it is not possible
126 to determine whether these differences are due to ecological or methodological reasons. To
127 disentangle these confounding factors, it is critical to assess both shallow and deep species' diets
128 under the same methodological framework. In this case, DNA metabarcoding is an ideal choice,
129 since it can detect both small and gelatinous prey, thus being able to bridge across the
130 methodological shortcomings of visual methods. Here we aim to apply a uniform method to
131 describe diets across the water column as a single, interconnected, deep-pelagic ecosystem.

132 Siphonophore tentillum and nematocyst morphology are directly linked to feeding guild
133 [26,27] (Damian Serrano et al. 2021a). Damian Serrano et al. (2021b) used these relationships
134 to generate feeding guild predictions for 45 siphonophore species using their tentillum and
135 nematocyst morphology as predictors in a discriminant analysis of principal components (DAPC).
136 The feeding guild categories comprise fish specialists (which feed primarily on teleost fish prey),
137 large crustacean specialists (which feed primarily on krill, decapod shrimps, mysids,
138 lophogastrids, amphipods, and other macro-planktonic crustaceans larger than 1cm), small
139 crustacean specialists (which feed primarily on copepods, ostracods, cladocerans, larvae, and
140 other meso-planktonic crustaceans smaller than 1cm), gelatinous specialists (which are able to
141 feed on large gelatinous animals such as salps, ctenophores, or medusae in addition to other
142 zooplankton), and generalists (which feed on a variety of small and large, soft- and hard-bodied

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143 prey not including gelatinous animals). These predictions were cast on siphonophore species for
144 which no dietary information was available, and thus remained to be tested with new data on
145 siphonophore diets.

146 Here we use DNA metabarcoding to identify the gut contents of several siphonophore
147 species to obtain more comprehensive insights into their diets. Our primary aims are: (1) Expand
148 the existing knowledge on the diets of open-ocean siphonophores using DNA metabarcoding, (2)
149 qualitatively compare the prey detected by visual and molecular methods to evaluate their
150 technical biases, (3) apply a uniform method to describe siphonophore diets across depth
151 habitats, and (4) evaluate the morphology-based predictions of feeding guilds.

152 Materials and Methods

153 Ethics statement

154 Our specimen collection and protocol were compliant with all local regulations and under the
155 marine collection permit SC-191140006, issued to Steven H.D. Haddock by the California
156 Department of Fish and Wildlife. Since no vertebrates or cephalopods were involved, we did not
157 need oversight from an animal care board.

158 Siphonophore collections

159 —In order to sample a representative set of taxa across the siphonophore phylogeny,
160 we targeted a set of 41 species (aiming for 10 specimens per species) including cystonects,
161 apolemids, pyrostephids, euphysonects, and calycocephorans from shallow and deep waters (Fig.
162 2). Most species were sampled from the Offshore California Current Ecosystem (OCCE) except
163 for the Portuguese man-o-war *P. physalis*, which was collected off Bermuda in the Sargasso Sea;
164 *Sulculeolaria chuni* and some *Nanomia* spp. (labeled as “Atlantic”) which were collected off

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165 Rhode Island in the Block Island sound; *Forskalia* sp. M123-SS8 and shallow *Nanomia* sp.
166 KiloMoana2018-BW7-4 which were collected off the coast of Hawaii. While all the *Nanomia*
167 populations sampled in this study have been referred to as *Nanomia*- *bijuga*, we suspect that
168 there may be undescribed cryptic *Nanomia* species among the specimens sampled based on the
169 disparate tentillum morphologies we observed (pers. obs.). Therefore, we decided to have them
170 labeled at the genus level. One *Nanomia* specimen (KiloMoana2018-BW7-4) was collected off
171 the coast of Kona, HI. The pleustonic (surface floating) *Physalia physalis* samples were collected
172 manually using a bucket from a small boat. Species found between the 0-20m deep were collected
173 using blue water diving techniques following the guidelines in Haddock & Heine [28] Haddock &
174 Heine (2005). Species from 200-4000m were collected using ROVs. All animals were collected
175 live and brought back to the ship (or field station in Bermuda for *P. Physalis*) for dissection (Fig.
176 Fig. 1). Live colonies were photographed (sometimes recorded on video), and zooids of diagnostic
177 value (nectophores, bracts, tentacles) were dissected, when possible, fixed in 4% formalin, and
178 stored as vouchers at the Yale Peabody Museum of Natural History (voucher catalog numbers
179 provided in specimen metadata Table S15).

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180 **Gut content metabarcoding**

181 — Shortly after collection of the live specimens, we dissected and pooled several
182 gastrozooids from each colony, prioritizing making sure that those with visible gut contents are
183 included in addition to several other without conspicuous prey, in addition to and also including
184 any visible egested food pellets at the bottom of the sampling container. This non-random
185 approach was aimed at increasing our prey detection rate, but may have introduced sampling
186 bias against inconspicuous prey items, further precluding any meaningful quantitative analyses
187 of the data. Nonetheless, since the majority of gastrozooids sampled lacked conspicuous prey
188 content, we gave inconspicuous prey a broad chance of being represented. Pooling multiple

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189 gastrozooids as a single sample is reasonable, since all gastrozooids in a colony share an
 190 actively-flowing, interconnected gastrovascular cavity. Thus, we expect DNA from one prey
 191 capture in one gastrozooid to be present within multiple gastrozooids. For small species (like
 192 *Sphaeronectes* spp.), we sampled the whole siphosome as a pooled collection of all gastrozooids.

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193 Samples were frozen at -80°C until DNA extraction.

194 To extract DNA, we digested the samples with proteinase K at 56°C for 1-2h, used the
 195 DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), eluting twice at 56°C for 10min into a final
 196 volume of 100µl. We used a set of six primer pairs that amplify six barcode regions within the 18S
 197 gene ('V3', 'V5-V7S', 'V5-V7L', 'V7', 'V7p+V8', and 'V9'). The primers were designed using
 198 Geneious 11.1.5 [29], constraining the search to short (>300bp) amplicon products with a high
 199 chance of remaining uncleaved after digestion in the gastrozooid, flanked by priming sites
 200 conserved (to a maximum mismatch of 3bp) across metazoans. The search for conserved priming
 201 sites was conducted on an alignment of 18S genes from 975 species across all metazoan phyla
 202 downloaded from GenBank (available in
 203 github.com/dunnlab/siphweb_metabarcoding/Primer_design). The primer search was optimized
 204 to only retrieve non-degenerate primer pairs with compatible annealing temperatures and without
 205 problematic dimerization and hairpin temperatures. Primer sequences are shown in Table 1, and
 206 their properties can be found in Table T1 in the protocol
 207 (dx.doi.org/10.17504/protocols.io.5qpvo57o7l4o/v2).

208 **Table 1. Barcodes used in this study.**

Barcode	18S region covered ^a	Forward primer	Reverse primer	Start position ^b	End position ^b
V3	Within V3	166F: AACGGCTACCACATCCAAGG	166R: CACCAGACTTGCCTCCAAAT	420	566
V5-V7S	Between V5 and the beginning of V7 (short amplicon)	152: TGACGGAAGGGCACCAAGG	152R: TCCACCAACTAAGAACGGCC	1187	1339

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V5-V7L	Between V5 and the beginning of V7 (long amplicon)	271F: <u>AAACGATGCCGACTAGCGAT</u>	272R: <u>TCCACCAACTAAGAACGGCC</u>	1067	1339
V7	Within V7	179F: <u>GCGCGTTCTTAGTTGGTGGGA</u>	179R: <u>TGC GGCCAGAACATCTAAG</u>	1319	1489
V7p+V8	Part of V7 and most of V8	261F: <u>AACAGGTCTGTGATGCCCTT</u>	261R: <u>TGTGTACAAAGGGCAGGGAC</u>	1472	1687
V9	Within V9	134F: <u>CTTGTACACACCGCCCCGTC</u>	134R: <u>CCTTGTACGACTTTACTTCCTCT</u>	1675	1790

Formatted: Font: 8 pt**Formatted:** Superscript209 ^aThe hypervariable region boundaries were annotated following the gene positions defined in

210 Hadziavdic et al. [30].

211 ^bStart and end positions calculated on the 18S gene of *Lymnaea diaphana* (GenBank-**Formatted:** Indent: First line: 0"

212 JF909497.1).

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213 Using these primer pairs, we ran six parallel PCR reactions for each successful extraction,
 214 selecting only those which had yielded a DNA concentration above 10ng/l. For each 25µl reaction
 215 volume, we used 2 µl of extraction template, 0.5 µl of each primer (at a 10µM concentration), 1µl
 216 of BSA, 0.2 µl of GoTaq (Promega) polymerase and the standard reagents and proportions of the
 217 Promega GoTaq kit (Madison, WI, USA). The thermal cycles included: an initial denaturation at
 218 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing (variable), and
 219 elongation at 72°C for 1 min, followed by final elongation at 72°C for 5 min. For barcode V9, we
 220 used an annealing temperature of 48°C for 45s per cycle. For all other barcodes, we used an
 221 annealing temperature of 54°C for 60s per cycle.- Each batch of reactions for each barcode
 222 included a positive and a negative control (the elution buffer used in extraction), and the products
 223 were visualized using gel electrophoresis (2% agarose gel dyed with SYBR Safe DNA Stain) to
 224 check for amplicon size and monitor the controls. The PCR products were purified using
 225 ExcelaPure UF PCR Purification Plates (EdgeBiosystems, Gaithersburg, Maryland, USA). The
 226 DNA yield of each purified product was assessed using a Qubit 2.0 fluorometer and the dsDNA
 227 High Sensitivity assay (Thermo Fisher Scientific, USA). Purified PCR products from each barcode

228 region for each sample were combined in equimolar pools based on their DNA yield in order to
229 have equal representation in the sequencing lane. Further details on the DNA extraction, quality
230 control, PCR mix, amplicon purification, and amplicon pooling are fully described in the online
231 protocol
232 (dx.doi.org/10.17504/protocols.io.5qpvo57o7l4o/v2)dx.doi.org/10.17504/protocols.io.bd8ci9sw).
233 All molecular bench work was carried out at the Yale DNA Analysis Facility. We used a set of six
234 primer pairs that amplify six regions within the 18S gene (and part of the ITS1) named after their
235 expected amplicon length ('134', '152', '166', '179', '261', and '272'). The primers were designed
236 using Geneious v.x.x.x (Kearse et al. 2012), seeking short (>300bp) amplicon products with a
237 high chance of remaining uncleaved after digestion in the gastrozooid, flanked by priming sites
238 conserved (to a maximum mismatch of 3bp) across metazoans. The search for conserved priming
239 sites was conducted on an alignment of 18S genes from 975 species across all metazoan phyla
240 downloaded from GenBank. The primer search was optimized to only retrieve primer pairs with
241 compatible annealing temperatures and without problematic dimerization and hairpin
242 temperatures. Primer sequences and properties can be found in Table T1 in Damian Serrano
243 (2020). Amplicon pools were sequenced using Illumina MiSeq (Illumina, San Diego, CA, USA)
244 250bp paired-end technology (except samples in run Q from specimens KiloMoana2018-BW7-4
245 *Nanomia* sp., D1019-D5 undescribed physonect, D856-SS8 *Stephanomia amphytridis*, D861-
246 D12 *Bargmannia amoena*, D858-D6 *Apolemia lanosa*, and D860-D6 *Erenna sirena* which was
247 were sequenced using Illumina MiSeq 150bp) at the Yale Center for Genomic Analysis.

248 Prey reference database

249 —In order to enhance the accuracy of the taxonomic assignments of reads, we also built
250 an 18S gene barcoding database. To do this, we collected 60 specimens of 30 species of
251 zooplankton and microneuston from the OCCE using a Tucker trawl. We targeted plausible prey

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252 species from motile open-ocean taxa that cohabit with siphonophores and are
253 underrepresented in SILVA databases, including fishes, crustaceans, jellyfishes, urochordates,
254 chaetognaths, polychaetes, and mollusks. Specimens were photographed live, tissue was
255 sampled and frozen, and the rest of the animal was fixed in formalin as a voucher to be identified
256 and preserved at the Yale Peabody Museum of Natural History. DNA extraction, quality control,
257 PCR, and amplicon cleanup was carried out in a similar fashion as the metabarcoding protocol
258 described above in Damian Serrano (and detailed in
259 dx.doi.org/10.17504/protocols.io.5qpvo57o7l4o/v2.2020), except that only one using the PCR
260 program with an annealing temperature of 54°C
261 (<https://dx.doi.org/10.17504/protocols.io.bd8ci9sw>, Table T5A), and only one single pair of
262 primers were used (166F and 134R), spanning the full extent of the sequence containing all
263 barcode regions used in the gut content metabarcoding (from V3 to V9-1800bp). Purified
264 amplicons were sent in plates with the forward and reverse primer separately for Sanger
265 sequencing from both ends at the Yale DNA Analysis Facility. These A total of 89 newly-submitted
266 sequences were then assembled and trimmed at a 95% quality cutoff in Geneious and
267 concatenated with the latest SILVA database (SILVA_138_SSURef_NR99 downloaded on
268 February 23, 2021) pruned to remove bacterial non-eukaryotic sequences downloaded on
269 February 23, 2021 to generate our custom built database.

270 Bioinformatic pipeline

271 Amplicon libraries were demultiplexed by primer sequence using custom bash code.
272 Primer sequences were removed using cutadapt (Martin 2011) [31]. The forward and reverse
273 reads were matched and repaired using bbtools (Bushnell et al. 2017) [32], then denoised and
274 de-replicated using the DADA2 [33] (Callahan et al. 2016) plugin in QIIME2 [34] (Bolyen et al.
275 2019) with a truncation quality threshold of 28. We de novo clustered the unique features into

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276 operational taxonomic units (OTUs) using the VSEARCH [35] (Rognes et al. 2016) plugin in
277 QIIME2 with a similarity threshold of 95%. Using QIIME2, we computed sample composition and
278 diversity metrics and aligned the feature sequences with MAFFT (Katoh et al. 2009) to build a
279 phylogenetic tree with Fasttree (Price et al. 2009). To reduce computational load, only the top 100
280 most abundant features among the clustered OTUs were selected for taxonomic assignment.
281 Taxonomic identifications were assigned using the assignment software METAXA2 [36]
282 (Bengtsson-Palme et al. 2015) with a 70% reliability cutoff, comparing the sequences against ~~the~~
283 standard GenBank reference library, the SILVA123.1 reference library [37] (Quast et al. 2012),
284 and our custom-built library ~~(based built using~~ SILVA138 ~~as a foundation.)—~~ SILVA123.1
285 ~~contains~~ 61383 eukaryotic reference sequences, while our custom database built off SILVA138.1
286 ~~contains~~ 79044. Animals in the SILVA123.1 taxonomy are annotated to the ranks of superphylum,
287 phylum, subphylum, class, subclass, order, family, genus, and species. However, SILVA138.1
288 animal taxonomy was annotated at the levels of clade (e.g. Bilateria, Protostomia, Deuterostomia,
289 Ecdysozoa, Lophotrochozoa), phylum, class, subclass, order, suborder, and species. All
290 bioinformatics analyses were carried out in the Yale High Performance Computing Cluster. The
291 taxonomic assignments and read count data were merged, then parsed to match the sample of
292 origin and the DNA sequence they derived from. Sequence post-processing scripts can be found
293 in the GitHub repository (https://github.com/dunnlab/siphweb_metabarcoding/Scripts).

294 Assignment interpretation

295 ~~Taxonomic assignments were manually inspected and annotated with the interpreted~~
296 ~~consensus taxon and interpreted source (predator, prey, secondary predation, parasite,~~
297 ~~environmental eukaryote, unrecognizable sequence, contamination, or cross~~
298 ~~contamination). Different barcode regions and reference databases displayed different~~
299 ~~assignment sensitivities for different taxa. Moreover, the two reference databases were annotated~~

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300 at different taxonomic levels, thus revealing unequal assignment reliabilities at different
301 phylogenetic depths. Therefore, the assignment information from different barcodes and
302 reference databases was integrated to interpret the source and taxon of the detected reads. When
303 the assignments from the two databases disagreed or reported suspicious (e.g. non-marine) taxa,
304 we manually checked the sequences in NCBI BLAST. In summary, aA combination of annotation
305 database consensus, barcode region consensus, number of reads, manual BLAST checks, and
306 natural history informed priors were used to assign these interpretations.

307 Taxonomic assignments were manually inspected and annotated with the interpreted
308 consensus taxon and interpreted source (predator, prey, secondary predation, parasite,
309 environmental, unrecognizable sequence, contamination, or cross contamination). Predator
310 sources correspond to the siphonophore DNA from the gastrozooid. These annotations were
311 given to OTUs with typically high read abundances, often taxonomically-assigned as
312 siphonophores, hydrozoans, or unculturable eukaryotes. Prey sources were annotated when
313 plausible prey taxa were assigned. We know that siphonophores can only capture prey that
314 actively swims to trigger tentilla discharge [9,27]. Therefore, we interpreted that DNA from non-
315 pelagic and/or non-motile organisms cannot be sourced from dietary contributions from
316 microorganisms, marine snow, eggs, or microscopic ciliated larvae. Secondary predation sources
317 correspond to OTUs assigned to animals that were more likely consumed by the co-detected prey
318 than by the siphonophore. Crustacean, gastropod, and larvacean sequences in *P. physalisa*
319 samples were interpreted as secondary predation (prey of their fish prey) given our knowledge on
320 the prey-capture limitations of these animals and the feeding habits of their fish prey.

321 Parasite interpretations were annotated onto OTUs assigned as trematodes, cestodes,
322 ichthyophonids, and myxozoans, since the most likely explanation for their presence in the
323 samples is due to parasitism in the siphonophores or their prey. We used the environmental
324 category to annotate OTUs likely originated from the microbial community (such as diatoms,

325 dinoflagellates, uncultured eukaryotes) or eDNA (such as rotifers, sharks, ascidians, sponges,
326 bivalves, anemones, echinurids, gastrotrichs, echinoderms, or bryozoans), given their taxonomic
327 assignment and read abundance. OTUs assigned as 'uncultured eukaryotes' were BLAST-
328 checked to differentiate between environmental microbes and failed assignments of
329 siphonophore sequences. We used the contamination category to interpret OTUs assigned to
330 tetrapods (likely humans), pollen, brachiopods, nematodes, mites, and insects. Amplification
331 experiments on negative controls indicated that the human, mite, and insectthese contaminants
332 originated from specimen manipulation in the field and not from the lab bench. The cGross-
333 contamination interpretation was used to annotate some suspicious OTUs with low reads in some
334 samples that matched other taxa that were being extracted and amplified at the lab bench. -was
335 suspected for some samples in runs 0 and 5 due to simultaneous DNA extractions of reference
336 prey samples. Reads suspected of cross-contamination (assigned to taxa present in the potential
337 sources of contamination, present across multiple samples in the same run with very low read
338 abundances) were conservatively labelled annotated as such. Crustacean, gastropod, and
339 larvacean sequences in *P. physalia* samples were interpreted as secondary predation (prey of their
340 fish prey) given our knowledge on the prey capture limitations of these animals and the feeding
341 habits of their fish prey.

342 When all barcode regions except '152-V5-V7S' indicate mysid prey but 'V5-V7S152'
343 identifies a similar number of reads as stomatopod prey, we interpret those reads as mysid prey.
344 Assignments of shark identities by barcode region 'V5-V7S152' in one of the *P. physalia* samples
345 (extraction 169specimen BIOS19-D1-P5) were identified as ray-finned fish prey using BLAST
346 searches and interpreted as such, in agreement with the other barcode regions. Assignments of
347 decapod crustacean identities by barcode region 'V5-V7S152' (in extractions 111samples D1137-
348 D7_Forskalia sp., D1243-BW25_Diphyes dispar218, and D1244-SS8_Nanomia sp.225) were
349 interpreted as euphausiid prey in agreement with the assignments on the rest of the barcode

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350 regions. The taxonomic composition of the samples was analyzed and visualized in the R
351 programming environment. Scripts and data available in our GitHub repository.

352 Prey field characterization

353 In order to compare the observed diet to the environmental abundances of potential prey
354 taxa, we collected zooplankton and micronekton samples on the same day and station location
355 as the relevant siphonophore gut content samples. The plankton samples paired with epipelagic
356 siphonophore specimens were collected using a weighted hand-held plankton net (ring diameter
357 of 1m for the Bermuda samples, 0.5m for the OCCE and Block Island sound samples, mesh size
358 of 250µm) towed for ~10min at a few meters depth at a speed of ~1kt. Paired with the ROV-
359 collected mesopelagic siphonophore specimens, we collected zooplankton and micronekton
360 samples using a Tucker trawl (frame area: 2m², mesh size: 500µm) towed for ~2h between 900m
361 and the surface at night. Environmental community samples were visually examined live to collect
362 specimens to sequence for the 18S reference library and other purposes, which were annotated
363 as removed. Samples were concentrated using metal sieves and fixed in 4% formalin. Back in
364 the Yale Peabody Museum of Natural History, these samples were visually identified and
365 quantified from a splitter aliquot. Identifications were carried out to the lowest taxonomic level as
366 well as to a broad group level (e.g., copepods, decapods, krill, fish, hydromedusae, chaetognaths,
367 polychaetes etc.). A few individual specimens were removed from the haul before preservation to
368 serve other scientific goals during fieldwork, and therefore these samples may be imperfect
369 representations of the community. In order to estimate how selective siphonophore species are
370 for different prey types in the environment, we calculated Strauss ([1979](#))-Linear Index (LI) [\[38\]](#) at
371 the broad taxonomic group level.

372

$$LI = ri - pi \quad (1)$$

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373 We used this index to capture the difference between the fraction of each prey type in the
374 environment (p_i) and the observed frequencies of prey types in the gut contents (r_i).

375 Comparisons to published sources

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376 —We aimed to compare and expand previous predation results from submersible
377 observations and visual gut content inspections with the new results of DNA metabarcoding of
378 gut contents. Therefore, we used the dietary data compiled in [Damian-Serrano et al. \[26\]](#) [Damian-](#)
379 [Serrano et al. \(2021a\)](#) from 11 published sources divided into those that used gut content
380 inspections and those that used human- and remotely-operated submersible observations. Many
381 of the submersible observations correspond to ROV observations carried out in the Offshore
382 California Current Ecosystem, spatially overlapping with the location where the majority of our
383 metabarcoding samples were collected. Salps, ctenophores, and medusae were merged into a
384 gelatinous prey type for comparative purposes. Published records for *Apolemia uvaria* were
385 considered equivalent to *Apolemia* sp. for genus level comparisons. Records of all *Forskalia*
386 species were considered equivalent to *Forskalia* sp. In order to test the morphology-based dietary
387 predictions generated in [Damian-Serrano et al. \(2021b\) \[27\]](#), we used the Bayesian posterior
388 probabilities for each feeding guild for each species. Small-crustacean guild predictions were
389 mapped to copepod, ostracod, and cladoceran prey. Large-crustacean guild predictions were
390 mapped to decapod, euphausiid, mysid, lophogastrid, stomatopod, and amphipod prey.
391 Generalist guild predictions were mapped to all prey types except gelatinous prey (following the
392 intended distinction with gelatinous specialists used in [Damian-Serrano et al. \[26\]](#) [Damian-](#)
393 [Serrano et al. 2021a](#)).

394 Results and Discussion

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395 We extracted, amplified, and sequenced the gut contents of 159 specimens from 41
396 siphonophore species ([Fig 2](#)). We obtained a total of 4148 unique sequences, including [758 from](#)
397 [region "V3"](#), [1502 sequences from region "134"](#), 614 from region "[152V5-V7S](#)", [442 from region](#)
398 ["V5-V7L"](#), [758 from region "166"](#), 497 from region "[479V7](#)", and 341 from region "[261V7p+V8](#)"
399 and [1502 sequences from region "V9"](#) [442 from region "272"](#) ([Fig. 3, SM Figures](#)[Tables S4, S8, 5](#)
400 [and S13, and S149](#)). A total of 337 unique sequences were interpreted as prey items, 36 as
401 secondary predation, 292 as contamination from extrinsic sources, 2857 as natural environmental
402 DNA sources, 791 as siphonophore sequences, 85 as parasites (myxozoans, trematodes, and
403 other helminths), and 14 unrecognizable sequences ([Tables S13 and S14](#)). We identified prey
404 items in 47 specimens (~30%) from 24 siphonophore species ([Fig. 2, Fig. 3, SM Figures](#)[Tables](#)
405 [S1 and S340-12](#)). This prevalence of empty guts is consistent with the feeding habits of
406 [macrophagous](#) sit-and-wait ambush predators in oligotrophic environments, with scarce feeding
407 events separated by periods of starvation [[39](#)] ([Griffiths 1975](#)). We identified 55 unique prey items,
408 42 of which were crustaceans (25 of which were copepods), three of them were fishes, four of
409 them were [thaliaceans](#)[urochordates](#), five corresponded to other gelatinous predators
410 (ctenophores and a medusa), and one matching to a bivalve mollusc ([Fig 2 and SM Figure S1](#)).
411 Most (112 out of 159) siphonophore specimens collected did not yield any putative prey taxa
412 concepts- ([Table S3](#)) ([Fig. 2](#)). Among the 47 specimens with prey, 40 of them had DNA from a
413 single prey item, while only six had two prey items, and one *Apolebia* sp. specimen had three
414 prey items ([SM Figure](#)-[Fig S1](#)). The use of six different barcode regions [with different priming sites](#)
415 [and taxonomic specificity](#) ([within the 18S gene](#)) allowed us to detect a broader taxonomic range
416 of prey and to validate dubious annotations ([Fig. Fig 3, Table S12](#)).

417 **Dietary findings by taxon**

418 ***Physalia physalis***

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419 —The Portuguese man-o-war is the only pleustonic (surface floating) member of the
420 siphonophores, and the most encountered by beachgoers. Man-o-wars are well-known to feed
421 exclusively on relatively large and motile soft-bodied prey such as fish, chaetognaths, or pelagic
422 gastropods (Purcell 1984a [40]). In our gut content samples of the Portuguese man-o-war from
423 Bermuda, we found three specimens with ray-finned fish sequences (Fig S1), some of which had
424 visually recognizable fish in the gastrozooids when collected. Fish prey is congruent with
425 published visual inspections of their gut contents [40,41] (Purcell 1984a, Bardi & Marques 2007).
426 In all three specimens with fish prey we also found benthic and hard-bodied taxa (mysid, alpheid
427 shrimp, spider crab, copepod, benthic gastropod, and a sipunculid worm), as well as larvacean
428 prey sequences (Table S12). Their nematocysts are not able to subdue crustacean prey, and
429 their feeding reflex would not be triggered by a prey as small as a larvacean [42] (Purcell 1984b).
430 Therefore, we interpreted the presence of these taxa in the gut contents as secondary predation
431 in the gut contents of the fish prey (SM Figures Tables S23 and S67). In addition, we also detected
432 ctenophore prey in one specimen. This could be also a case of secondary predation, but we
433 suspect a ctenophore could be large enough to be prey of the man-o-war. If that is the case, this
434 would be the first record of *P. physalis* consuming gelatinous zooplankton, which would place the
435 man-o-war as a central species in the epipelagic ‘jelly-web’ [43] (Chi et al. 2020). Comparisons
436 with their surrounding prey field show these specimens were strongly selective for fish and
437 strongly exclusive of copepods (Fig. Fig 4).

438 *Apolemia* spp.

439 —These are among the longest siphonophores, with colonies attaining lengths as long
440 as 30m (Mackie et al. 1988) [9]. Their tentacles are different from other siphonophores since they
441 have no tentilla and carry birhopaloid nematocysts directly on the tentacles [27] (Damian-Serrano
442 et al. 2024b). *Apolemia* species are known to consume diverse prey including crustaceans,
443 molluscs, polychaetes, chaetognaths, fish, and gelatinous zooplankton [4,7] (Purcell 1981a, Choy

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444 ~~et al. 2017~~). While this may suggest these species are generalists, ~~Damian-Serrano et al. (2021a)~~
445 ~~Damian-Serrano et al. [26]~~ hypothesized that they may be gelatinous zooplankton specialists,
446 since they consume a larger proportion of this prey type than other siphonophores. In addition,
447 the nematocysts of *Apolemia* have similar traits to those in other gelatinous cnidarians ~~[27]~~
448 (~~Purcell & Mills 1988~~), and their apparent generality could be explained by the sheer number of
449 fine tentacles deployed for prey capture per colony, which would inevitably entangle almost
450 anything that swims by. ~~We found –All species of Apolemia analyzed here had consumed~~
451 ~~copepods, the A. rubriversa specimen had also consumed acopepod and~~ salp ~~prey sequences in~~
452 ~~Apolemia rubriversa, and the undescribed Apolemia species also had ctenophore, larvacean,~~
453 ~~mysid, and euphausiid prey sequences (Figs. Figs 2 and 4). The salp prey found in A. rubriversa~~
454 is congruent with its characterization as a gelatinous specialist ~~[26] in Damian-Serrano et al.~~
455 ~~(2021a), and may indicate a direct trophic pathway between siphonophores and consumers of~~
456 ~~microbial production~~. While the morphology-based predictions ~~derived from Damian-Serrano et~~
457 ~~al. (2021b)~~ indicate that *A. lanosa* is ~~likely~~ a gelatinous prey specialist ~~[27]~~, we only found copepod
458 prey in our sample. However, it is possible that the doliolid and hydromedusa reads we
459 conservatively labelled as potential cross-contamination could correspond to real prey. ~~We also~~
460 ~~analyzed samples from an undescribed Apolemia species, where we found a combination of~~
461 ~~gelatinous (ctenophore), soft-bodied (larvacean), and crustacean (mysid and euphausiid) prey,~~
462 ~~congruent with a generalist diet (Fig 2).~~ Considering the differences we found between species,
463 it seems possible that these coexisting species of midwater *Apolemia* are partitioning their trophic
464 niche by ~~varying differentiating the relative proportion of crustacean versus and~~ gelatinous prey
465 ~~they consume in their diets. Moreover, the salp prey found in A. rubriversa indicates a direct~~
466 ~~connection between phytoplankton consumers and siphonophores.~~

467 **Bargmannia spp.**

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468 — The three *Bargmannia* species considered here are frequently-observed in the
469 midwaters off Monterey Bay, and have relatively simple tentilla with large stenotele nematocysts
470 and an undifferentiated terminal filament [27] (Damian-Serrano et al. 2021b). ROVs have
471 recorded *Bargmannia elongata* consuming crustaceans and cephalopods. One *B. elongata*
472 specimen had euphausiid and ostracod prey, in agreement with the DAPC prediction for *B.*
473 *elongata* to feed mainly on large crustaceans, but also marginally on small crustaceans (Fig 5).
474 and during specimen collection we observed a mysid prey in a specimen *Bargmannia* *B.*
475 *amoena*. Our DNA metabarcoding identified this mysid as *Boreomysis* (METAXA assignment
476 score 54.68% for V7 on SILVA123.1), and found a copepod in another specimen. Nothing was
477 previously known, however, about the diet of *Bargmannia lata*. DNA metabarcoding confirmed
478 the identity of the mysid in *B. amoena* as *Boreomysis californica*, and found a copepod in another
479 specimen. One *B. elongata* specimen had euphausiid and ostracod prey, in agreement with the
480 DAPC prediction for *B. elongata* to feed mainly on large crustaceans, but also marginally on small
481 crustaceans. The two *B. lata* specimens we sequenced consumed a ctenophore and a copepod,
482 respectively.

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483 The diets of these three closely-related, coexisting species appear to be non-overlapping,
484 which could be a consequence of competitive trophic niche partitioning. The findings for *B. lata*
485 are not congruent with the morphology-based prediction to be a large-crustacean specialist (Fig.
486 Fig. 5). We suspect that the lack of taxon sampling among the pyrostephids in [26] Damian-
487 Serrano et al. (2021a) could have led to overfitting in the DAPC. Finding ctenophore prey also
488 supports the involvement of deep-sea siphonophores in the midwater ‘jelly web’.

489 **Other deep-sea physonects**

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490 — Undescribed physonect sp. L was predicted to be a fish specialist with a secondary
491 affinity for large crustacean prey (Fig. 5). However, we found this specimen consuming a

492 ctenophore. Other morphologically-similar deep-sea undescribed physonects ~~with close~~
493 ~~morphological affinity to our species~~ (GL and Zigzag) have been observed consuming fish and
494 squid prey [4] (Choy et al. 2017), thus it is possible that they are specialized in capturing and
495 digesting soft-bodied prey more generally. The rarely-observed *Resomia dunnii* was predicted to
496 be a generalist (consumer of all types of prey except gelatinous taxa), which is consistent with
497 the copepod prey we found in its gut contents.

498 *Forskalia* species are frequently found in both shallow and deep waters, and have been
499 observed to consume various crustaceans, molluscs, worms and fish [7] (Purcell 1981a).
500 However, mMorphology predicts *Forskalia* species to be large crustacean specialists [27]. We
501 found three midwater *Forskalia* specimens with copepod prey in the guts, one of them also had
502 consumed a sergestid shrimp. These results are fully congruent with those derived from visual
503 methods, and partly congruent with the morphological predictions. *Halistemma rubrum* tentilla
504 closely resemble those of *Forskalia*, and thus they are also predicted to be large-crustacean
505 specialists (Damian-Serrano et al. 2021b) [27]. This prediction is congruent with our identification
506 of a lophogastrid in the gut contents (Fig. Fig 4). On the other hand, *Lychnagalma utricularia* is
507 unique among the physonects for bearing a medusa-shaped floating vesicle at the end of their
508 large, coiled tentilla (Damian-Serrano et al. 2021b) [27]. They ~~Nonetheless, they~~ have been also
509 observed consuming large crustaceans through ROVs, such as consuming sergestid shrimp. We
510 found two specimens both with sergestid shrimp prey DNA, yet one of them was also digesting a
511 euphausiid (SM Figure Fig S1). This is consistent with their hypothesized large-crustacean
512 specialization (Fig. Fig 2 and, Fig. Fig 5). *Halistemma rubrum* tentilla closely resemble those of
513 *Forskalia*, and thus they are also predicted to be large crustacean specialists (Damian-Serrano
514 et al. 2021b). This prediction is congruent with our identification of a lophogastrid in the gut
515 contents (Fig. 4).

516 ***Nanomia* spp.**

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517 —These are among the most common siphonophores in both Atlantic and Pacific waters,
518 both in epipelagic and midwater environments. We have observed that epipelagic *Nanomia* tend
519 to have smaller tentilla than their mesopelagic counterparts, which may ~~account for their~~
520 ~~specialization explain their tendency to capture~~ ~~on~~ smaller crustaceans (such as copepods [7])
521 instead of larger crustaceans (such as krill). Midwater ROV observations of deep-dwelling
522 *Nanomia* have predominantly reported interactions with krill prey, as well as with the occasional
523 chaetognath or sergestid shrimp [4] (Choy et al. 2017). We identified one specimen of
524 mesopelagic *Nanomia* with krill and stomatopod DNA in its gut contents, ~~in agreement congruent~~
525 with its hypothesized large-crustacean specialist characterization (Fig.–Fig. 5). However,
526 ~~e~~Epipelagic *Nanomia* ~~might not be~~ ~~as~~ ~~seems to be less~~ specialized on large crustacean prey,
527 since the literature reports a combination of copepod, decapod, mysid, and chaetognath prey
528 (Purcell, 1981a [7]). In the North Pacific Ocean, our metabarcoding identified copepod prey in an
529 epipelagic *Nanomia* off California, a hyperiid amphipod prey in an epipelagic *Nanomia* off Hawaii
530 (SM–Figure Fig. S1). The hyperiid amphipod could have been a commensal or parasite on the
531 *Nanomia* instead of prey, though this is unlikely since only the gastrozooids were dissected while
532 amphipods tend to colonize the nectophores or bracts. In the North Atlantic Ocean, we sampled
533 14 specimens of epipelagic *Nanomia*, seven of which contained copepod prey (Fig.–Fig. 2). Upon
534 visual inspection of the sampled gastrozooids we could identify *Temora*, *Centropages*, and
535 *Acartia* copepods, the most abundant genera in the plankton sample, whose identity was also
536 ~~validated supported~~ by the metabarcoding results (genus and species-level assignment scores:
537 *Centropages* sp. 54.99% for barcode V5-V7L, *Acartia tonsa* 91.5% for barcode V5-V7S, and
538 *Temora discaudata* 91.81% for barcode V7p+V8, using the SILVA123.1 database). The
539 corresponding environmental plankton samples showed that these waters were dominated by
540 cladocerans, and thus these *Nanomia* were positively selecting for copepod prey (LI values
541 between 0.69 and 0.72) and selecting against cladoceran prey, ~~which was~~ ~~(not detected in the~~

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542 guts ([Fig 4 and Fig S1](#)). The exclusion of the overabundant cladocerans from the diet of Atlantic
543 *Nanomia* suggests that their specialization, if any, could be copepod-specific.

544 **Calycophorans**

545 These siphonophores are characterized by their lack of a pneumatophore (gas-filled apical
546 vesicle) and their structurally-homogeneous tentilla-[\[27\]](#)([Damian Serrano et al. 2021b](#)). However,
547 these tentilla present a great variation in nematocyst number and size, which may translate into
548 dietary differences[\[26\]](#)([Damian Serrano et al. 2021a](#)). We provided the first insights into the diets
549 of two highly abundant deep-sea calycophorans, *Lensia conoidea* and *Chuniphyes multidentata*,
550 which morphology predicted as small-crustacean specialists. Both sequenced specimens
551 contained copepod DNA ([Fig S1](#)), supporting these predictions ([Fig. Fig 5](#)). While ~~g~~elatinous
552 prey has been reported for *Desmophyes annectens* from ROV observations [\[4\]](#), ~~however~~ we
553 found only copepod prey sequences. We ~~found did find~~ gelatinous prey, however, in *Diphyes-D.*
554 *dispar* (salp prey), *Muggiae atlantica* (larvacean), and *Sphaeronectes christiansonae* (nausithoid
555 medusa). The latter constitutes the first record of *S. christiansonae* feeding. While these medusae
556 can be very small, the minute size of this siphonophore may render this interaction dubious. The
557 far more common epipelagic *Sphaeronectes* species, *Sphaeronectes-koellikeri*, appears to be a
558 copepod specialist according to visual gut content analysis, since they appear to feed exclusively
559 on copepods [7]-[\(Purcell 1981a\)](#). We sequenced the gut contents of two specimens of this
560 species, one of them indeed was consuming a copepod, yet the other was consuming a crab
561 larva. The latter constitutes a novel prey type for this species, yet still within the expected range
562 of a small-crustacean specialist. Another ~~validated expectation dietary hypothesis occurred was~~
563 supported with for Sulculeolaria-S. chuni, ~~a Their~~ visually-assessed diet appears comprised
564 exclusively of copepods ~~s specialist[7] in Purcell (1981a), for which~~ and we detected copepod prey
565 in an ~~Atlantic~~ specimen from the Atlantic ocean. On the other hand, while Muggiae atlantica has
566 also been observed feeding exclusively on copepods [7], our specimen had only larvacean

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567 sequences that could correspond to prey. While the read abundance for this OTU was low, the
568 small size of this prey type is reasonable given the small tentilla and gastrozooids of *M. atlantica*
569 [27,42].

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570 The calyptophoran *Vogtia* is the closest relative to *Hippopodius*, the only siphonophore
571 known to be an ostracod specialist [7] (Purcell 1981a). Like many other hard-to-access
572 mesopelagic taxa, the diet of *Vogtia* has remained unknown, though tentillum morphology
573 predicted them to be generalists [27] (Damian Serrano et al. 2021b). Pugh (1986) An
574 oceanographic study [44] found spatial correlations between ostracods and *Vogtia* species, and
575 even mentions a *Vogtia* sp. specimen which had the exoskeleton of an ostracod in its gut contents.
576 Our DNA metabarcoding on *Vogtia serrata* has revealed one specimen feeding on an ostracod
577 (with high selectivity), and a specimen feeding on a sergestid shrimp and a bivalve (with high
578 selectivity, LI = 0.5). These results are consistent with the generalist morphological prediction,
579 and congruent with the single visual finding of an ostracod in a congener-[44]from Pugh (1986).
580 The presence in the gut contents of one of our specimens of an ostracod and a bivalve (likely a
581 pediveliger larva), which has a very similar shape to an ostracod (with two hard valves), indicates
582 phylogenetic conservatism of prey traits within Hippopodiidae.

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583 Comparisons with visual methods

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584 We report the first insights into the diets of nine siphonophore species and reveal 29 novel
585 predator-prey interactions (Fig. Fig 2 and, Fig. Fig 5). When comparing our metabarcoding
586 findings with the published visual observations from gut content inspections and submersible
587 dives, we found five interactions congruent with ROV observations, and eight interactions (six of
588 them involving copepods) congruent with visual gut content inspections of SCUBA-collected
589 colonies (Fig. Fig 5).

590 The published records on the diets of siphonophores appear to differ in prey-type
591 composition between epi- and deep-pelagic habitats ([Hetherington et al. In review](#)). However,
592 [Hetherington et al. \(in review\) hypothesized that](#) the different methodological limitations inherent
593 to each visual method (small prey underestimated by submersibles, soft-bodied prey
594 underestimated by gut content inspections) [may be hypothesized to](#) be responsible for such
595 differences [\[8\]](#). Our approach has detected prey types, such as larvaceans, ctenophores,
596 bivalves, and ostracods previously missed by visual methods. The gelatinous animals (i.e.
597 ctenophores, medusae, salps) identified by submersibles as prey of deep-pelagic siphonophores
598 were found present in the gut contents of several deep species (*Apolemia* sp., *B. lata*, undescribed
599 physonect L, and *S. christiansonae*), supporting the validity of these observations. However, the
600 gelatinous prey recorded by submersibles in prayids such as *Praya dubia* and *D. annectens*
601 [\[4\]\(Chey et al., 2017, Fig. 5\)](#) were not recovered in our [prayid-*D. annectens*](#) samples ([Fig 5](#)),
602 suggesting that either our sample sizes were not large enough, or that ROVs had observed
603 accidental entanglement of jellies on their tentacle nets which did not end in ingestion. In addition,
604 we found several small crustaceans in the gut contents of epipelagic species (*Forskalia* sp.,
605 *Nanomia* sp., *S. koellikeri*, *S. chuni*, and *D. dispar*) in agreement with visual gut contents
606 observations in shallow habitats. On the other hand, we also found gelatinous and soft-bodied
607 invertebrate prey in shallow-dwelling species (*P. physalis*, *D. dispar*, and *M. atlantica*); as well as
608 small-bodied animals among the prey [of](#) deep-pelagic [species-siphonophores](#) (*Apolemia* spp.,
609 *Bargmannia* spp., *R. dunnii*, *V. serrata*, *D. annectens*, *C. multidentata*, and *L. conoidea*) ([Fig-Fig](#)
610 2). Copepods and ctenophores were the most frequent prey among bathypelagic siphonophores,
611 while other crustaceans (such as ostracods, decapods, and krill) appeared as prey more
612 frequently among the mesopelagic taxa. While these findings are consistent with the hypothesis
613 that small prey is underestimated in submersible observations and rapidly-digested, soft-bodied
614 prey is underestimated by gut content inspections, our sample sizes are insufficient to determine
615 whether the relative contribution of these prey differs between habitats.

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616 DNA metabarcoding was able to detect prey both small and large, gelatinous and hard-
617 bodied, for both deep and shallow-dwelling [siphonophore](#) species. These results show that the
618 trophic roles of siphonophores in epi- and deep-pelagic food webs could be more similar than
619 previously-published records may indicate, due to the biases brought by the different diet-
620 assessment methods applied in each habitat. Vertical migration is an important driver of pelagic
621 food web structure [\[45,46\]](#), ([Sutton 2013, Kelly et al. 2019](#)). We found copepods, decapods, and
622 euphausiids in the gut contents of both meso- and epipelagic siphonophores. These prey taxa
623 are well-known vertical migrators [\[47-49 \(Longhurst 1976, Hopkins et al. 1994, Cohen & Forward
624 2009\)\]](#), suggesting that there might be some vertical trophic connectivity between these habitats
625 as prey migrates between them. In addition, a few siphonophore species (including *V. serrata* and
626 *L. conoidea* in this study) are also known diel vertical migrators-[\[50\]\(Pugh 1984\)](#), but their patterns
627 of feeding with depth remain unknown. Finally, our selectivity estimates (for four epipelagic and
628 two mesopelagic species) indicate that siphonophores may play a similar role as selective,
629 [specialized](#) predators across all depths in the water column.

630 Comparisons with prey field

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631 We examined 8 prey-field samples that corresponded to the colocalized ambient prey of
632 15 out of 47 specimens (some trawls correspond to more than one sampled specimen). The
633 epipelagic plankton samples from Bermuda (colocalized with the *P. physalis* specimens) were
634 dominated by copepods, followed by decapod larvae and chaetognaths. While fish larvae were
635 scarce in these samples, they were still far more abundant than in any other sampled location.
636 The Atlantic epipelagic plankton samples (colocalized with the *S. chuni* and Atlantic shallow
637 *Nanomia* specimens) were dominated by cladocerans, followed by copepods, larvaceans and
638 salps. The Pacific epipelagic plankton sample from California (colocalized with the *D. dispar*
639 specimens) was also dominated by copepods, followed by cladocerans and larvaceans. The

640 quantified midwater tucker trawl from California (colocalized with *V. serrata* specimen D1137-D8
641 and *Forskalia* sp. ~~s~~Specimen D1137-D9) was also dominated by copepods (albeit larger species),
642 followed by euphausiids (both adult and larval), chaetognaths, and ostracods.

643 We found both positive and negative selectivity when comparing identified siphonophore
644 prey to quantified co-localized prey fields. We found strong negative (<-0.5) selectivity for
645 copepods in *P. physalis* specimens and in one specimen of *V. serrata*. However, in 11
646 siphonophore specimens from 4 species (out of the 6 species that were quantitatively assessed),
647 we found strong positive selectivity (>0.5) for a specific prey type (~~SM-Figure-Fig S1~~). These
648 cases include: selectivity for fish in *P. physalis*; selectivity for copepods in *S. chuni*, and Atlantic
649 *Nanomia* sp., selectivity for ostracods in *V. serrata*, and selectivity for salps in *D. dispar* (~~Fig-Fig~~
650 4). These selectivity values suggest a strong influence of predator-specific differences in prey
651 capture capabilities for different prey types. However, more replication is necessary in order to
652 test for prey-type specialization.

653 Epipelagic siphonophores are known to be highly selective and specialized carnivores
654 [~~7,26,51,52~~] (~~Purcell 1981a, Purcell & Mills 1988, Mills 1995, Damian-Serrano et al. 2021a~~). ROV
655 observations have revealed that some deep-sea siphonophores are also highly specialized [4]
656 (~~Choy et al. 2017~~). However, the lack of paired diet and planktonic community samples has limited
657 an assessment of their feeding selectivity. For both the shallow- and deep-dwelling siphonophore
658 species assessed here, we found their prey belonged to the less-abundant components of the
659 co-localized planktonic community, demonstrating high prey-type selectivity. However, the
660 selectivity index values presented in this study should be interpreted with care, since the prey
661 field data is quantitative (abundance-based) but the gut content values are only binary at the
662 specimen level, and frequency-based at the predator species level. Overall, crustaceans
663 (especially copepods) were identified as the most frequent prey type among siphonophore diets.
664 Copepods are typically the most abundant prey type in planktonic communities, thus being able

665 to feed on them is likely an advantageous strategy for any planktivorous predator [53] (Turner
666 2004). Fish prey were detected only in the Portuguese man-o-war samples, in agreement with
667 published observations of man-o-war feeding [40,41].

668 Our findings are congruent with the idea that siphonophores span multiple trophic
669 positions, consuming prey across low (salps, larvaceans, copepods, ostracods) and high (fish,
670 ctenophores, medusae) trophic levels. We found larvaceans and salps as prey of shallow- and
671 deep-dwelling siphonophores. These thaliaceans-urochordates have an important role in the
672 biological carbon pump, sequestering carbon from phytoplanktonic producers into the deep sea
673 by means of fecal matter production and carcass depositions [54,55] (Robison et al. 2005, Luo et
674 al. 2020). The role of predation on gelatinous herbivores is often underestimated in oceanic food-
675 web models, or primarily attributed to vertebrate predators [56] (Henschke et al. 2016). Our results
676 show that some siphonophores like *Apolemia* sp., *A. lanosa*, *M. atlantica*, and *D. dispar* may play
677 an important mid-trophic role incorporating this gelatinous-soft-bodied herbivore productivity into
678 the food web, and providing an alternative avenue to transfer carbon into the deep sea.

679 Comparisons with morphology predictions

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680 Comparing our metabarcoding findings with the morphology-based predictions [27] from
681 Damian-Serrano et al. (2021b), we found support for 10 of the 16 predicted interactions between
682 siphonophores and prey. Among the physonects, our results supported the predictions of *B.*
683 *elongata* eating krill and ostracods, *R. dunnii* eating copepods, *Forskalia* sp. eating decapods, and
684 *H. rubrum* eating lophogastrids. Among the calycophorans, we found support for the predictions
685 of *V. serrata* eating decapods, ostracods, and molluscs; also *C. multidentata* and *L. conoidea*
686 eating copepods. Among the siphonophore species studied there were 70 predicted interactions
687 that were not found among the metabarcoding results (Fig–Fig 5). Out of the 10 taxa with both
688 morphology-based predictions and metabarcoding results, six had all prey congruent with the

689 predictions, three had all prey incongruent with the predictions, and *Forskalia* sp. presented both
690 cases.

691 Food-web structure is determined largely by community composition and its patterns in
692 time and space, as the organismal assemblages determine what predators are present and what
693 prey is available to them (Getelli & Graves 1996, Giannelli et al. 2005, Cehn [57-59] n et al. 2012).
694 However, organismal traits constrain which predators can eat which prey [60,61] (Lajgle et al.
695 2018, Maureaud et al. 2020). The most commonly-studied trait to predict oceanic food web
696 structure has been size (Ward et al. 2012, Zhang et al. 2014) [62,63]. This is due to the importance
697 of gape size in most predators (i.e. fish, squids, crustaceans etc.) with singular and rigid buccal
698 openings (Scharf et al. 2000; Cesta [64,65] 2010). Siphonophores differ from most predators by
699 having many gastrozooid mouths along their length, all capable of stretching out significantly
700 (Pages & Madin 2010) to ingest prey [66], sometimes utilizing multiple zooids to wrap around
701 large prey (Hardy 1956) [67]. While prey size is still an important constraint for siphonophore-prey
702 interactions [42] (Purcell 1984b), siphonophore size is far less relevant. Moreover, some studies
703 have found that phylogenetically-conserved predator traits other than size may also be important
704 predictors of food web structure [68,69]. (Gilljam et al. 2011, Jacob et al., 2011). Damian-Serrano
705 et al. (2021a) found that diet is a strong predictor of both extant and ancestral siphonophore
706 tentilla morphology, as well as of its evolutionary dynamics [26]. Damian-Serrano et al. (2021b)
707 used these relationships in reverse to These relationships were utilized predict the diets of
708 understudied siphonophore species based on the morphology of their tentilla and nematocysts
709 [27]. We Here, we were able to test these predictions for ten species and found that most of the
710 prey items found were congruent with these predictions, indicating that tentilla morphology is a
711 strong predictor of siphonophore diets. This finding suggests that at least some components of
712 the open-ocean food web are may be structured by variation in complex morphological traits
713 exclusive to specific predator groups.

714 Siphonophores are hypothesized to easily evolve between feeding specializations and
715 into a generalist diet due to their modular body plan and their functionally-specialized tentilla [26]
716 (Damian-Serrano et al. 2021a). Our results show that closely-related species, such as those
717 within the genera *Bargmannia*, *Apolequia*, and *Nanomia*, ~~may have evolved distinct feeding~~
718 ~~specializations appear to feed on different prey. We hypothesize that these species could be~~
719 ~~further subspecializing to avoid competition or adapt to different prey fields at different depth~~
720 ~~habitats. These results hypothesis are is congruent with the conclusions from Damian-Serrano~~
721 ~~et al. (2021a), further indicating conclusions in Damian-Serrano et al. [26] that siphonophore~~
722 ~~dietary evolution can drive rapid morphological shifts even within the same genus.~~ Moreover,
723 we find that *Apolequia* sp., as well as *V. serrata*, could be generalists feeding on a variety of
724 crustacean and soft-bodied prey. ~~These results suggest that If a more extensive and quantitative~~
725 ~~sampling of these taxa was to validate this trophic reclassification, that would suggest that a~~
726 ~~generalist diet may have evolved not just three (as proposed in Damian-Serrano et al. [26]), but~~
727 ~~up to five times independently, thus further reinforcing their conclusions from Damian-Serrano et~~
728 ~~al. (2021a) on the evolution of feeding guilds.~~

729 Methodological considerations

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730 While DNA-based tools can detect prey unrecognized by visual methods, they are not free
731 of shortcomings. Since all life stages of an animal have the same genetic signature,
732 metabarcoding tools are unable to distinguish between larval, juvenile, or adult prey. These
733 ontogenetic stages can have vastly different ecological implications and pose different challenges
734 during prey capture. In addition, the application of metabarcoding to predator diets is usually not
735 quantitative, since too many sources of variation may lead to differences in read abundance. For
736 example, different animal clades have different sizes, cell densities (due to variable acellular
737 mesoglea content), digestion rates, number of copies of the target gene, or primer affinities during

738 the PCR ([Deagle & Tollit 2007](#), [Treedsson et al. 2009](#), [Valentini et al. 2010](#))[\[70-72\]](#)[\[99\]](#). Due to the
739 difficulties inherent to locating and sampling the species examined in this study, frequency-based
740 quantitative comparisons were not possible for most species either. In addition, the sample size
741 limitations of this study may have biased the results towards higher apparent specialization, and
742 may have missed some important components of the diets of some target species. This caveat is
743 also common in submersible observation data and limits the reliability of comparisons across
744 these methods.

745 Siphonophores differ from other consumers in several ways which impose further
746 limitations to the value of gut content metabarcoding. The most important aspect is their feeding
747 mode and feeding rate, especially as deep-sea ambush predators, which typically consume one
748 prey at a time and do not get a chance to capture another until far after the former has been
749 digested-[\[9\]](#)[\(Mackie et al. 1988\)](#). Therefore, most siphonophores are found with empty guts or
750 digesting one or few prey items at a time. [Thus](#)[Thus](#), the sample size required for frequency-
751 based analyses is much higher than for other consumers which feed more frequently. [Our prey](#)
752 [frequency results \(Fig. 2\) are consistent with this idea.](#) Moreover, except for a couple species
753 such as *Rhizophysa eysenhardtii* and *Rosacea cymbiformis* which are diurnal feeders-[\[17\]](#)[\(Purcell](#)
754 [1981a\)](#), most species also feed during the night. In the open ocean, diel vertical migration
755 drastically changes the prey field composition for siphonophores at night [\[45\]](#)[\(Sutton 2013\)](#). Given
756 the fieldwork limitations in this study, we were only able to collect siphonophore gut contents
757 during the day, thus likely biasing their diet towards their diurnal prey captures. [Moreover,](#)
758 [metabarcoding can only ascertain the taxon of the prey and not the life stage, thus being unable](#)
759 [to distinguish between small larval and large adult prey.](#) Finally, secondary predation (the prey of
760 the prey) cannot be empirically distinguished from direct predation, and thus we must rely on
761 natural-history based assumptions.

762 **Conclusions**

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This study uses DNA metabarcoding technology to investigate the diets of a diverse range of siphonophores. We identified 55 unique prey items in the gut contents of 24 siphonophore species, the majority of which were crustaceans (most of which were copepods), in addition to fishes, molluscs, and gelatinous *species taxa* (Figs. Figs 2 and -4). Our results expand the existing knowledge on siphonophore diets, detecting prey types previously missed by visual methods, and providing insights into the diets of several understudied siphonophore species. We show that whole gastrozooids can be utilized for DNA metabarcoding of diets without need for further dissection or the use of predator-blocking primers. We identified representatives from diverse animals (Fig.-Fig 3, SM-Figures-Tables S56-S112), which demonstrates the phylogenetic range of taxa that can be amplified with our primer pairs. By comparing the taxonomic composition of the gut contents to that of the environmental planktonic community, we find support for the idea that ~~most of the examined both shallow and deep-dwelling~~ siphonophore species ~~are specialized~~selectively prey on distinct components of zooplankton and microneuston communities (Fig.-Fig 4). Many of the prey types found in both shallow and deep-dwelling species match published records based on visual methods, but some prey types appear underrepresented by those methods. Moreover, we find that many of the tentillum morphology-based dietary predictions for these species were supported by the metabarcoding results (Fig.-Fig 5).

Overall, we provide novel insights into the ecology and natural history of several siphonophore species, revealing that siphonophores across all depths are ~~specialized and~~ selective predators which have diversified their feeding habits to consume fish, crustaceans, gelatinous predators, gelatinous filter-feeders, meroplanktonic larvae, and other pelagic invertebrates. Our results reveal a significant involvement of deep- and shallow-dwelling siphonophores in the open-ocean ‘jelly web’, highlight suspected biases from visual methods, and support the hypothesized value of tentilla morphology to predict their diets. This study also

787 demonstrates the suitability and effectiveness of DNA metabarcoding to identify the prey
788 consumed by gelatinous predators.

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1176 Figures

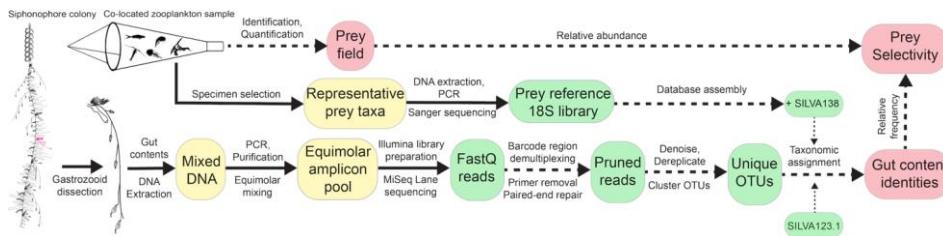


Figure 1. Gut content metabarcoding workflow used in this study. Siphonophore colony

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1179 illustrated by Freya Goetz. Silhouettes in the plankton net downloaded from phylopic.org. Solid
 1180 arrows indicate physical material transfer and processing, dashed lines indicate information
 1181 transfers and processing. Yellow islands indicate elements processed in the laboratory bench,
 1182 green islands represent bioinformatic datasets processed in the high-performance computing
 1183 cluster, and red islands represent curated data products.

Species	Vertical habitat	N sampled	N with prey	Literature-based guild	Tentile-based prediction of guild	Metabarcoding prey	Photo
<i>Physalia physalis</i>	Pelagic	5	3	Fish		Fish, ctenophore	A
Undescribed physocnemoid	Mesopelagic	1	0				B
Undescribed physocnemoid L	Bathypelagic	2	1		Fish	Ctenophore	
<i>Erenna sinena</i>	Bathypelagic	2	0	Fish			
<i>Erenna cornuta</i>	Bathypelagic	1	0				
<i>Stephanonema amphipodis</i>	Bathypelagic	3	0				
<i>Apoenia rubrivena</i>	Mesopelagic	7	2	Generalist		Copepod, mysid	
<i>Apoenia</i> sp.	Mesopelagic	3	3	Generalist		Copepod, mysid, euphausiid, ctenophore, tunicate	
<i>Apoenia lansae</i>	Bathypelagic	3	1		Generalist	Copepod	
<i>Nanomia</i> sp. shallow Pacific	Epipelagic	12	2	Large crustacean		Copepod	
<i>Nanomia</i> sp. shallow Atlantic	Epipelagic	14	9			Copepod, amphipod	
<i>Halestoma rubrum</i>	Epipelagic	1	1		Large crustacean	Lophognathid	
<i>Nanomia</i> sp. deep	Mesopelagic	3	1	Large crustacean		Euphausiid, stomatopod	
<i>Lycophamia uliculae</i>	Mesopelagic	4	2	Large crustacean		Euphausiid, euphausiid	
<i>Resonia omicroncephala</i>	Mesopelagic	1	0	Large crustacean		Euphausiid, euphausiid	
<i>Crassoeus lethetica</i>	Mesopelagic	2	0	Large crustacean			
<i>Ulyssesia fluoranthraea</i>	Mesopelagic	1	0	Large crustacean			
<i>Praya dubia</i>	Mesopelagic	2	0	Large crustacean			
<i>Dennoubus punctatus</i>	Mesopelagic	1	1			Copepod	
<i>Bargmannia elongata</i>	Mesopelagic	2	1		Large crustacean	Ostracod, euphausiid	
<i>Bargmannia amanca</i>	Bathypelagic	3	2	Large crustacean		Copepod, mysid	
<i>Bargmannia late</i>	Bathypelagic	3	2		Large crustacean	Copepod, ctenophore	
<i>Murina claudiae</i>	Bathypelagic	2	0		Large crustacean		
<i>Forskalia</i> sp.	Epipelagic	3	3	Generalist		Copepod, decapod	
<i>Agalma okenii</i>	Epipelagic	3	0	Generalist			
<i>Frigalpina vityazi</i>	Mesopelagic	3	0		Generalist		
<i>Vogtia serrata</i>	Mesopelagic	3	2		Generalist	Ostracod, decapod, bivalve	
<i>Resonia dunni</i>	Bathypelagic	3	1		Generalist	Copepod	
<i>Sulcodeliaris chuni</i>	Epipelagic	1	1	Small crustacean			
<i>Mugiloides atlanticus</i>	Epipelagic	5	1	Small crustacean			
<i>Diphyes dipter</i>	Epipelagic	15	3	Small crustacean			
<i>Contaglione cordatum</i>	Epipelagic	1	0	Small crustacean			
<i>Sphaeromedusoides kofoidi</i>	Epipelagic	10	2	Small crustacean			
<i>Sphaeromedusoides christiansenae</i>	Mesopelagic	1	1				
<i>Lemisia conoides</i>	Mesopelagic	1	1	Small crustacean			
<i>Chuniphyes multidentata</i>	Mesopelagic	5	1	Small crustacean			
<i>Gymnophore leptoizaus</i>	Mesopelagic	3	0	Small crustacean			
<i>Kophyres ovata</i>	Mesopelagic	2	0	Small crustacean			
<i>Chuniphyes moerense</i>	Bathypelagic	1	0	Small crustacean			

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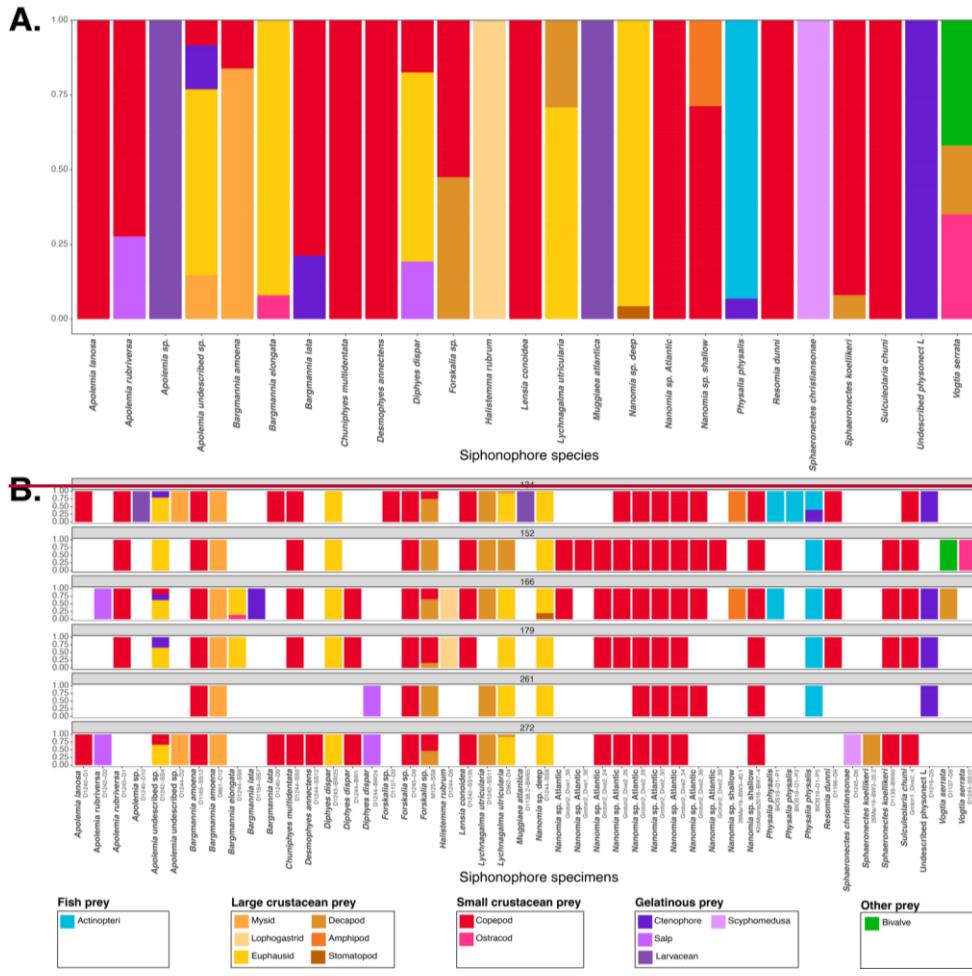
Species	Vertical habitat	N sampled	N with prey	Literature-based guild	Tentilla-based prediction of guild	Metabarcoding prey	Photo
<i>Physalia physalis</i>	Mesopelagic	5	3	Fish		Fish, ctenophore	A
Undescribed physocorect L	Mesopelagic	1	0				
<i>Erenna sinuosa</i>	Bathypelagic	2	1	Fish	Fish	Ctenophore	B
<i>Erenna cornuta</i>	Bathypelagic	1	0				
<i>Stephanomia amphitrichis</i>	Bathypelagic	3	0				
<i>Apoenia rubinversa</i>	Mesopelagic	7	2	Gelatinous		Copepod, salp	C
<i>Apoenia sp.</i>	Mesopelagic	3	3	Gelatinous	Gelatinous	Copepod, mysid, euphausiid, ctenophore, tunicate	D
<i>Apoenia lamosa</i>	Bathypelagic	3	1			Copepod	E
<i>Nanomia sp. shallow Pacific</i>	Epipelagic	12	2	Large crustacean		Copepod, amphipod	F
<i>Nanomia sp. shallow Atlantic</i>	Epipelagic	14	9			Copepod, amphipod	G
<i>Halistemma rubrum</i>	Epipelagic	1	1			Lophognathid	H
<i>Nanomia sp. deep</i>	Mesopelagic	3	1	Large crustacean	Large crustacean	Euphausiid, stomatopod	I
<i>Lychamagia utricularia</i>	Mesopelagic	4	2	Large crustacean	Large crustacean	Decapod, euphausiid	J
<i>Resomia ornicephala</i>	Mesopelagic	1	0				
<i>Crassco latifrons</i>	Mesopelagic	2	0				
<i>Lilypais fluoracanthia</i>	Mesopelagic	1	0				
<i>Praya dubia</i>	Mesopelagic	2	0	Large crustacean	Large crustacean		
<i>Desmophyes annectens</i>	Mesopelagic	1	1			Copepod	
<i>Bargmannia elongata</i>	Mesopelagic	2	1			Ostracod, euphausiid	
<i>Bargmannia amboina</i>	Bathypelagic	3	2	Large crustacean	Large crustacean	Copepod, mysid	
<i>Bargmannia lata</i>	Bathypelagic	3	2			Copepod, ctenophore	
<i>Murru claudianus</i>	Bathypelagic	2	0				
<i>Forskalia sp.</i>	Epipelagic	3	3		Generalist		
<i>Agalma okunii</i>	Epipelagic	3	0		Generalist		
<i>Friggalima vitazi</i>	Mesopelagic	3	0		Generalist		
<i>Vogtia serata</i>	Mesopelagic	3	2		Generalist		
<i>Resomia durni</i>	Bathypelagic	3	1			Ostracod, decapod, bivalve	
<i>Sulcicladaria chuni</i>	Epipelagic	1	1	Small crustacean		Copepod	
<i>Muggiae atlantica</i>	Epipelagic	5	1	Small crustacean		Larvacean	
<i>Diphyes dispar</i>	Epipelagic	15	3	Small crustacean		Copepod, euphausiid, salp	
<i>Cordigalma ordinatum</i>	Epipelagic	1	0	Small crustacean			
<i>Sphaeromedes koellikeri</i>	Epipelagic	10	2	Small crustacean		Copepod, decapod	
<i>Sphaeromedes christiansonneae</i>	Mesopelagic	1	1			Siphonophores	
<i>Lensis conoidea</i>	Mesopelagic	1	1			Copepod	
<i>Chuniphysa multidentata</i>	Mesopelagic	5	1	Small crustacean		Copepod	
<i>Gymnophra laeviszakula</i>	Mesopelagic	3	0	Small crustacean		Copepod	
<i>Kephys ovata</i>	Mesopelagic	2	0	Small crustacean			
<i>Chuniphys moserae</i>	Bathypelagic	1	0	Small crustacean			

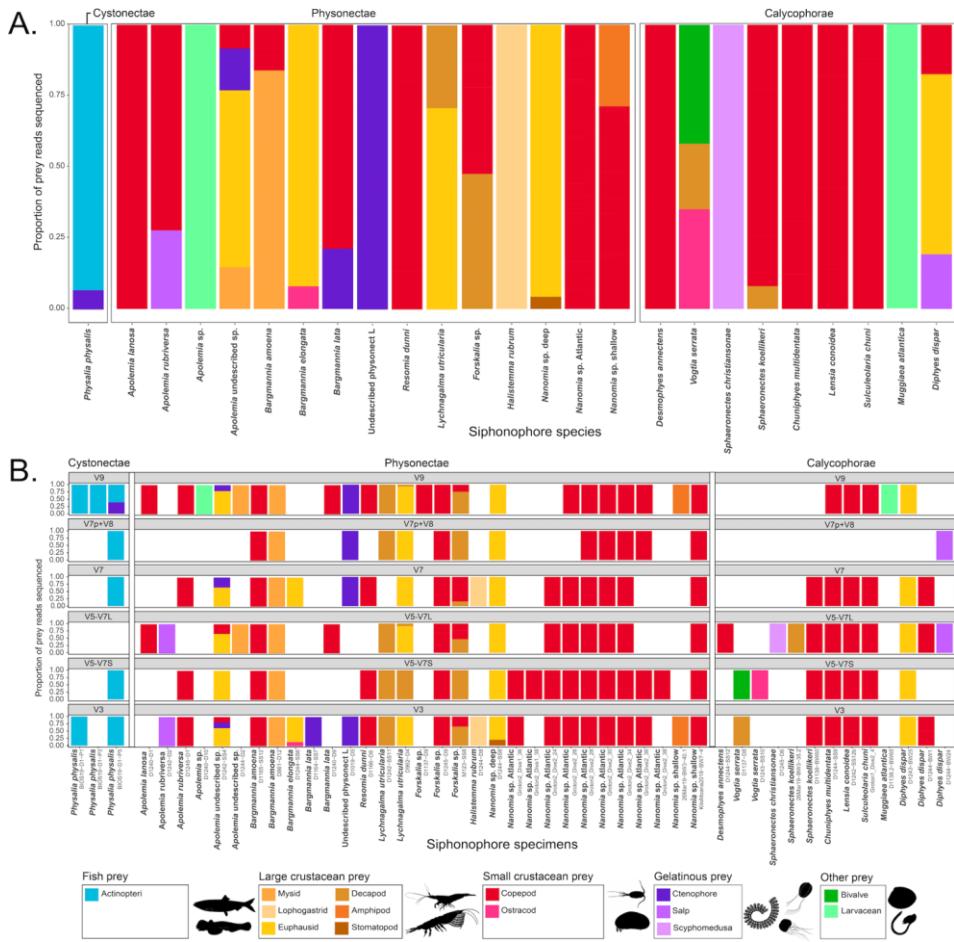
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Figure 2. Summary table of the siphonophore species sampled for this study indicating**Formatted:** Font: Bold

their vertical habitat, the number of specimens sampled, the number of specimens with recognizable prey sequences, and hypothesized feeding guild. Guilds are based on published feeding records used in Damian-Serrano et al.- [26] (2021a), predicted feeding guild from the DAPC analysis in Damian-Serrano et al. [27] (2021b) based on tentilla morphology, and prey found in this study. Photo credits: (A) [Casey Dunn-W.D.](#), (B, D) [Schmidt Ocean Stephan Siebert, CC BY licensed and reprinted from Munro et al. \[73\]](#), (C) [MBARI](#), (D) [reprinted from https://www.theredshrimp.com/ with permission from Reyn Yoshioka, original copyright \(2018\)](#), (E) [Steven Haddock, MBARI](#), (F) [reprinted from https://biolum.eemb.ucsb.edu/organism/pictures/bargmannia.html with permission from Steven Haddock, original copyright \(1997\)](#) Pugh et al. 2020, (G-H) [MBARI](#), (G, I) [Russell Hepcroft Alejandro Damian-Serrano](#), (H) [NOAA, CC BY licensed, reprinted from https://www.flickr.com/photos/noaaphotolib/19988388271](#), (J) [reprinted from https://www.flickr.com/photos/noaaphotolib/19988388271](#)

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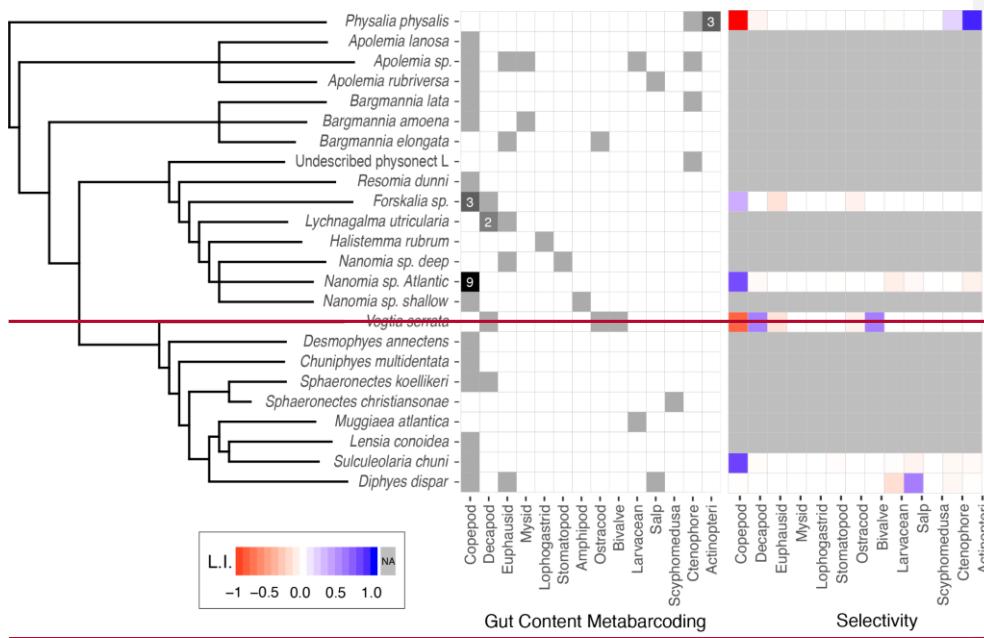


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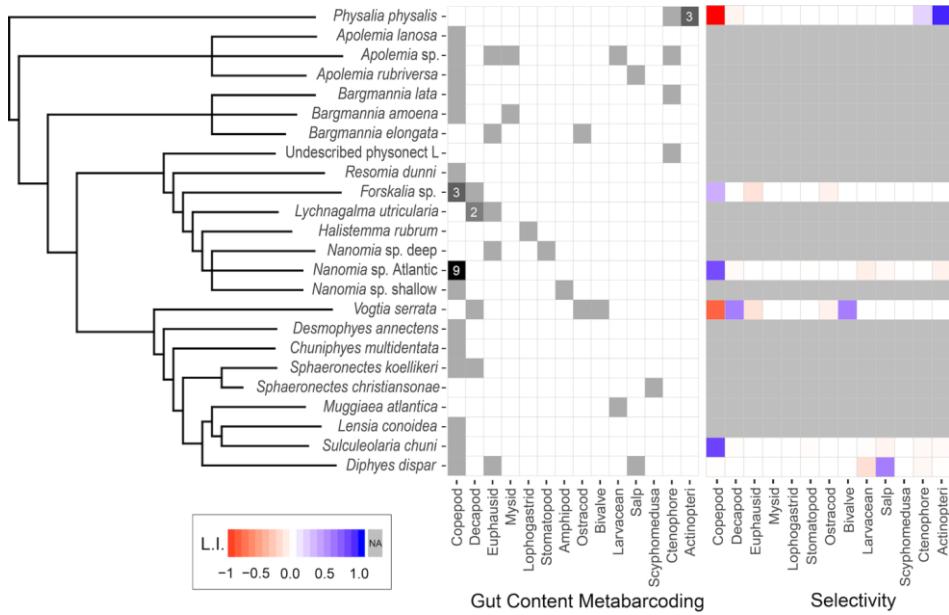
1203 **Figure 3. Relative log-abundances of prey reads colored by taxon.** (A) For each
 1204 **siphonophore species**, and (B) **Relative read log abundance of prey colored by prey taxon** for
 1205 each **siphonophore specimen** and barcode.

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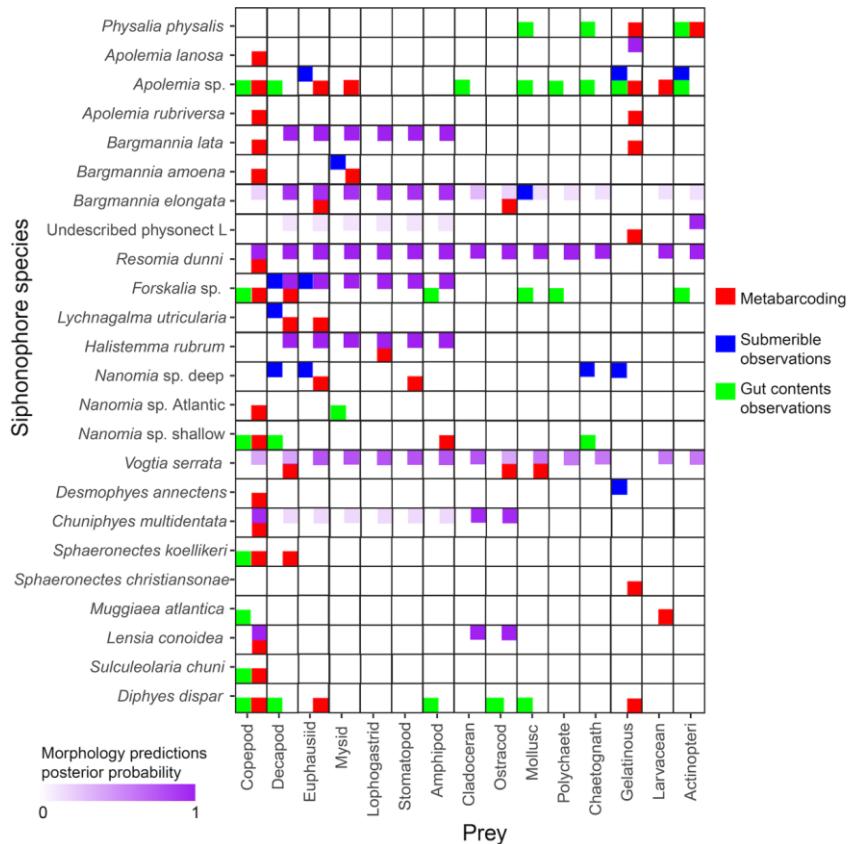
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1208 **Figure 4. Species-wise grid with the frequency of the major prey types identified from the**
 1209 **metabarcoding data (left) and the average prey-type selectivity, estimated in comparison with**
 1210 **the local planktonic community composition (right).** Gut content cells in white indicate absence,
 1211 and cells in grey indicate presence in one specimen, or more than one specimen if labeled with a
 1212 number. Selectivity colors mapped to Strauss' L.I. values. The siphonophore cladogram (left) is a
 1213 simplified version of the phylogenetic tree published in Damian-Serrano et al. [26] (2021a).

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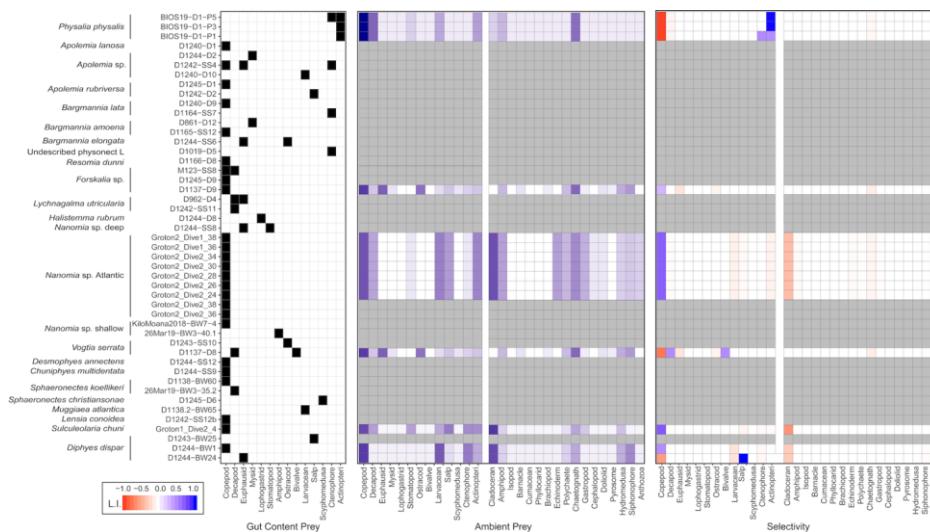
1214 **Figure 5. Feeding interactions between siphonophores species and their prey from**
 1215 **different data sources. Including prey identified by our metabarcoding results (red),**
 1216 **Submerible observations (blue), and Gut contents observations (green).**

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1217 observations published submersible observations (blue), observations published visual gut
 1218 content analyses (green), and prey types predicted by the morphology-based DAPC model in
 1219 Damian-Serrano et al.-[27](2024b). Gelatinous prey refers to ctenophores, medusae, and salps.
 1220 Larvaceans were excluded as their own category since they are not gelatinous when swimming
 1221 freely outside their mucous ‘houses’, which would be the only times they would be able to
 1222 trigger a prey-capture response in siphonophore tentacles.

1223 Supporting information

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1224 **Fig S1. Species-wise grid with the frequency of the major prey types identified from the**
1225 metabarcoding data and the average prey-type selectivity. Gut content cells in white
 1226 indicate absence, and cells in grey indicate presence in one specimen, or more than one
 1227 specimen if labeled with a number. Selectivity colors mapped to Strauss' L.I. values.

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1228 **Table S1. Read abundances assigned to each DNA source interpretation category for**
1229 each siphonophore species.

1230 **Table S2. Read abundances assigned to each DNA source interpretation category for**
1231 each siphonophore species by barcode.

1232 **Table S3. Read abundances assigned to each DNA source interpretation category for**
1233 each siphonophore specimen.

1235 [Table S4. Read abundances assigned to each DNA source interpretation category for](#)
1236 [each siphonophore specimen by barcode.](#)

1237 [Table S5. Read abundances assigned to each OTU broad taxon for each siphonophore](#)
1238 [species.](#)

1239 [Table S6. Read abundances assigned to each OTU broad taxon for each siphonophore](#)
1240 [species by barcode.](#)

1241 [Table S7. Read abundances assigned to each OTU broad taxon for each siphonophore](#)
1242 [specimen.](#)

1243 [Table S8. Read abundances assigned to each OTU broad taxon for each siphonophore](#)
1244 [specimen by barcode.](#)

1245 [Table S9. Read abundances assigned to each prey OTU broad taxon for each](#)
1246 [siphonophore species.](#)

1247 [Table S10. Read abundances assigned to each prey OTU broad taxon for each](#)
1248 [siphonophore specimen.](#)

1249 [Table S11. Read abundances assigned to each prey OTU broad taxon for each](#)
1250 [siphonophore species by barcode.](#)

1251 [Table S12. Read abundances assigned to each prey OTU broad taxon for each](#)
1252 [siphonophore specimen by barcode.](#)

1253 [Table S13. Number of unique sequences assigned to each barcode in each DNA source](#)
1254 [interpretation category.](#)

1255 [Table S14. Number of unique sequences assigned to each barcode in each OTU broad](#)
1256 [taxon.](#)

1257 [Table S15. Specimen collection metadata and Yale Peabody Museum catalog numbers](#)
1258 [for voucher specimens.](#)

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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/> 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 to enhance the reproducibility of your results. [Protocols.io](https://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>. Additionally, PLOS ONE offers an option for publishing peer-reviewed Lab Protocol articles, which describe protocols hosted on protocols.io. Read more information on sharing protocols at https://plos.org/protocols?utm_medium=editorial-email&utm_source=authorletters&utm_campaign=protocols.

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
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"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.)"

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

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

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>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-posed 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 migrants 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>

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>

>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 gastrozoid 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).**



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