The Organismal and Evolutionary Determinants of Siphonophore Predation

Alejandro Damian Serrano

Yale University Graduate School of Arts and Sciences, damianserrano.alejandro@gmail.com

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Abstract

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Alejandro Damian Serrano

2021

The open-ocean midwater is the largest habitat for animal life on Earth, and one of the most homogeneous environments in space and time. However, these waters harbor complex food webs involving predatory animals from manifold phyla, presenting unique adaptations to capturing the scarce prey resources. These conditions create a natural laboratory, with minimal abiotic disturbances, where interspecific interactions such as predation play a major role in shaping community dynamics and the evolution of form and function. Siphonophores play a central role in midwater food webs feeding across multiple trophic levels on different prey phyla (i.e. jellyfish, crustaceans, worms, fishes). Like other cnidarians, siphonophores capture prey using stinging capsules (nematocysts) in their tentacles, but unlike other cnidarians, their prey-capture nematocysts are often organized in complex prehensile batteries (tentilla) specialized for this function. Tentilla and their nematocysts come in a wide variety of shapes and sizes across species. This dissertation explores how tentacle and nematocyst morphology coevolved with prey-type specialization and selectivity in siphonophores, and how the role of siphonophore diversity in the midwater food web is determined by their evolved specializations and the prey encountered across their depth ranges in the water column. In order to collect siphonophore specimens, I used a combination of blue water SCUBA diving and Remotely Operated Vehicles (ROVs) during offshore expeditions. I imaged and measured several morphological characters of siphonophore tentilla and nematocysts using specimens deposited at the Yale Peabody Museum, and reconstructed the evolutionary history of these characters and diet on an expanded molecular phylogeny. Contrary to most theoretical expectations, I found that siphonophore predatory specialists can evolve into generalists, and that specialists can shift
their specific prey type. My results indicate that tentilla and nematocyst morphology evolved in correlation with evolutionary shifts in prey-type specialization. Dietary shifts are associated not only with the character states, but also with the mode of character evolution, and the patterns of correlations among characters. Moreover, I found evidence that distantly related small crustacean prey specialists, as well as fish prey specialists, have converged in the evolution of nematocyst shape and tentillum size. In addition, using multivariate discriminant analyses, I was able to generate dietary predictions for understudied species using tentilla morphology alone. Reviewing the literature on siphonophore feeding, I identified large gaps in knowledge and reported biases associated with visual methods. In order to bridge these gaps, I used ROV observation data to determine the diets and selectivities of deep-sea siphonophores, and DNA metabarcoding to detect prey types overlooked by visual methods. By comparing the vertical distributions of siphonophore and potential prey together with feeding observations, I found that most deep midwater siphonophore species are specialized and strongly selective for specific prey types. Using custom-built universal primers, I amplified and sequenced prey DNA from freshly-collected siphonophore gut contents. In addition, I collected and sequenced barcodes for several open-ocean species missing from public repositories to enhance the accuracy and resolution of my DNA-based gut content identifications. The metabarcoding results were largely congruent with previously published findings. In addition, they validated some of the dietary hypotheses generated from the morphology-based analyses, and revealed that small-crustacean specialists can occasionally prey on small soft-bodied animals. This dissertation advances our understanding on how open-ocean food web structure emerges from community composition and organismal traits. Incorporating explicit phylogenetic comparative methods into trophic ecology will enhance the value of descriptive systematic and oceanographic work, enabling its use to predict food web structure, nutrient flow, and ecosystem dynamics.
The Organismal and Evolutionary Determinants of Siphonophore Predation

A Dissertation
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of
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Doctor of Philosophy

by
Alejandro Damian Serrano

Dissertation Director: Casey W. Dunn

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Dedication

I dedicate this thesis to Dr. Jennifer Purcell, who’s pioneering efforts in siphonophore ecology and natural history inspired all of my work. I also dedicate this piece to Rebecca, my wife and love of my life, to my sister Andrea and to my ever-supporting parents, Gloria & José Ramón, who always encouraged me to go above and beyond in pursuit of my dreams.

Last but not least, to all my grandparents, living and beyond,

I hope this work makes you proud.
GENERAL INTRODUCTION

“Siphonophores do not convey the message of a favorite theme of unthinking romanticism that nature is but one gigantic whole, all its parts intimately connected and interacting in some higher, ineffable harmony. Nature revels in boundaries and distinctions; we inhabit a universe of structure. But since our universe of structure has evolved historically, it must present us with fuzzy boundaries, where one kind of thing grades into another.”

-Stephen J. Gould

The core question driving this dissertation lies at the intersection of macroevolution, organismal biology, and ecology: how do phylogenetic relatedness and trait evolution shape interspecific interactions? Interactions between species in an environment depend on many factors including community composition and functional traits (Wang & Brose 2018, Laigle et al. 2018). One of the greatest challenges in biological oceanography, and community ecology in general, is to reconstruct interactions and food web structure from community composition alone (Barton et al. 2013, Morales-Castilla et al. 2015). Many studies have attempted this through the analyses of relationships between simple traits (such as body size) and species interactions (Brose et al. 2019). However, many interspecific interactions depend on complex traits that are hard to record for a large number of species in the community (DeWitt & Langerhans 2003). In the past few decades, phylogenetic comparative biology has developed a myriad of tools to reconstruct ancestral states of traits and predict a species’ phenotypic makeup from its phylogenetic relationships (Garamszegi 2014). Attempts to bridge comparative biology and community ecology have been limited by several confounding factors, including the
functional pleiotropy of traits (DeLong 2017) and the strong influence of environmental heterogeneity driving the evolution and constraints around those traits (Menge & Sutherland 1987). If we wish to yield the potential of comparative biology to inform species traits and ultimately inform interspecific interactions, we first need to explore these connections in a system without such confounding effects.

The open ocean is the largest habitat for animal life on Earth, and one of the most uniform environments in space and time (Harbison 1992, Robison 2004). These waters harbor complex food webs involving predatory animals from manifold clades, presenting unique adaptations to capturing the scarce prey resources (Kunzig 2003). These conditions create a natural laboratory, with minimal abiotic disturbances, where interspecific interactions such as predation play a major role in shaping community dynamics and the evolution of form and function. Siphonophores are colonial gelatinous predators that play a central role in these food webs and have diversified to feed across multiple trophic levels on different prey taxa (Choy et al. 2017, Hetherington et al. 2021). Unlike most familiar predators, the siphonophore prey capture apparatus is physically and functionally decoupled from the rest of their bodies (Damian-Serrano et al. 2021). Siphonophores capture prey with stinging capsules (nematocysts) loaded in complex batteries on the side branches of their tentacles (tentilla), developed by specialized feeding bodies (gastrozooids). These tentilla are used exclusively for prey capture and present a broad diversity of shapes, sizes, and nematocyst compositions (Mapstone 2014). The spatial, developmental, and functional
independence of tentilla as structures for prey capture, the correlations between tentilla morphology and diet (Purcell 1984), as well as their morphological diversity, make siphonophores an ideal system for the study of trait evolution and its relationship to feeding interactions. My dissertation sets out to explore how tentilla and nematocyst morphology coevolved with prey-type specialization and selectivity in siphonophores, and how the role of siphonophore diversity in the midwater food web is determined by their evolved specializations and the prey encountered across their depth ranges in the water column.

This dissertation is composed of four chapters. Chapter 1 “The evolution of siphonophore tentilla for specialized capture in the open ocean” breaks with the expectation that specialists evolve from generalists becoming an evolutionary ‘dead end’. This work shows how the uniquely modular body plan of siphonophores releases their constraints to evolve out of their ancestral specializations by modifying their prey capture modules: the tentilla, revealing strong associations between the evolution of feeding specializations and tentilla morphology. This chapter is published as the cover of *PNAS* (Damian-Serrano et al. 2021). Chapter 2 “The evolutionary history of siphonophore tentilla: Novelties, convergence, and integration” dives further into the rich evolutionary history leading to the extant morphological diversity of siphonophore tentilla, with cases of structural novelties, adaptive convergence, and phenotypic integration. Also, this work generated testable, morphology-based, hypotheses on the diets of understudied siphonophore species, and compares the kinetic performance of tentilla discharge across various morphological forms. Chapter 3 “Prey selectivity
and dietary specialization in midwater siphonophores based on ROV observations” disentangles the effects of differential prey availability and intrinsic biological differences in determining the diets of midwater siphonophores. This chapter shows that midwater siphonophores are highly specialized and selective predators, that their specializations are driven by interspecific differences other than spatiotemporal distribution, and that neither depth nor niche overlap drive the differences in their trophic specializations. Finally, Chapter 4 “Characterizing the secret diets of siphonophores using DNA metabarcoding” applies state-of-the-art molecular technology to reveal the missing pieces in the diets of siphonophores that have been missed due to the limitations of visual methods such as microscopic gut inspections and ROV observations. This study also provided the first insights into the diets of some understudied deep-sea siphonophores, testing some of the dietary hypotheses generated in Chapter 2.

This thesis advances the field of evolutionary ecology by addressing fundamental questions about the evolution of specialization from a phylogenetic perspective in a unique system. In addition, it brings together tools across the fields of oceanography, food-web ecology, morphology, phylogenetics, and comparative biology. The results of this work advance our understanding of how food web structure emerges from community composition and organismal traits. Incorporating explicit phylogenetic comparative methods into trophic ecology enhances the value of descriptive systematic and oceanographic work, enabling its use to predict interspecific interactions, nutrient flow, and ecosystem dynamics.
References


CHAPTER 1

The Evolution of Siphonophore Tentilla for Specialized Prey Capture

Alejandro Damian-Serrano¹⁺, Steven H.D. Haddock², Casey W. Dunn¹

¹ Yale University, Department of Ecology and Evolutionary Biology, 165 Prospect St., New Haven, CT 06520, USA

² Monterey Bay Aquarium Research Institute, 7700 Sandholdt Rd., Moss Landing, CA 95039, USA

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Abstract

Predator specialization has often been considered an evolutionary ‘dead-end’ due to the constraints associated with the evolution of morphological and functional optimizations throughout the organism. However, in some predators, these changes are localized in separate structures dedicated to prey capture. One of the most extreme cases of this modularity can be observed in siphonophores, a clade of pelagic colonial cnidarians that use tentilla (tentacle side branches armed with nematocysts) exclusively for prey capture. Here we study how siphonophore specialists and generalists evolve, and what morphological changes are associated with these transitions. To answer these questions, we: (1) measured 29 morphological characters of tentacles from 45 siphonophore species, (2) mapped these data to a phylogenetic tree, and (3)
analyzed the evolutionary associations between morphological characters and prey type data from the literature. Instead of a dead-end, we found that siphonophore specialists can evolve into generalists, and that specialists on one prey type have directly evolved into specialists on other prey types. Our results show that siphonophore tentillum morphology has strong evolutionary associations with prey type, and suggest that shifts between prey types are linked to shifts in the morphology, mode of evolution, and evolutionary correlations of tentilla and their nematocysts. The evolutionary history of siphonophore specialization helps build a broader perspective on predatory niche diversification via morphological innovation and evolution. These findings contribute to understanding how specialization and morphological evolution have shaped present-day food webs.

**Keywords**

Siphonophores, nematocysts, predation, specialization, character evolution

**Introduction**

Most animal predators use specific structures to capture and subdue prey. Raptors have claws and beaks, snakes have fangs, wasps have stingers, and cnidarians have nematocyst-laden tentacles. The functional morphology of these structures is critical to their ability to successfully capture prey (1). Long-term adaptive evolution in response to the defense mechanisms of the prey (e.g., avoidance, escape, protective barriers) leads to modifications that can counter those defenses. The more specialized the diet of a predator is, the more
specialized its structures need to be to efficiently overcome the challenges posed by the prey. Characterizing the relationships between morphology and predatory specialization is necessary to understand how the phenotypic diversity of predators determines food-web structure. However, for many clades of predators, there is scarce knowledge on how these specializations evolved. The primary questions we set out to answer are: how do predator specialists and generalists evolve, and how does predatory specialization shape morphological evolution?

Siphonophores (Cnidaria: Hydrozoa) are a clade of gelatinous, colonial organisms that swim in the open ocean, feeding on a wide diversity of prey (often fish, crustaceans, and jellyfish). Siphonophore colonies have a modular body plan with different zooids specialized for different tasks. This modularity extends also within the feeding gastrozooids, which carry modular structures on the tentacle that are exclusively used for prey capture: the tentilla (Fig. 1.1). The tentilla have great morphological variation across species (2). Together with their well understood function, this makes them an ideal system to study the relationships between functional traits and prey specialization. Like a head of coral, a siphonophore is a colony bearing many feeding polyps (Fig. 1.1). Each feeding polyp has a single tentacle, which branches into a series of tentilla (side branches). Like other cnidarians, siphonophores capture prey with nematocysts, harpoon-like stinging capsules borne within specialized cells known as cnidocytes. Unlike the prey capture apparatus of most other cnidarians, siphonophore tentacles carry their cnidocytes in extremely complex and organized batteries (3) which are located in their tentilla. While nematocyst
batteries and clusters in other cnidarians are simple static scaffolds for cnidocytes, siphonophore tentilla have their own reaction mechanism, triggered upon encounter with prey. When it fires, a tentillum undergoes an extremely fast conformational change that wraps it around the prey, maximizing the surface area of contact for nematocysts to fire on the prey (4). In addition, some species have elaborate fluorescent or bioluminescent lures on their tentilla to attract prey with aggressive mimicry (5–7).

Siphonophores bear four major nematocyst types in their tentacles and tentilla (Fig. 1.1F). The largest type, heteronemes, have open-tip tubules characterized by bearing a distinctly wider spiny shaft at the proximal end of the everted tubule. These are typically found flanking the proximal end of the cnidoband. The most abundant type, haplonemes, have no distinct shaft, but similarly to heteronemes, their tubules have open tips and can be found in the cnidoband. Both heteronemes and haplonemes bear short spines along the tubule. Both can be toxic and penetrate the surface of some prey types. In the terminal filament, siphonophores bear two other types of nematocysts, characterized by their adhesive function, closed tip tubules, and lack of spines on the tubule. These are the desmonemes (a type of adhesive coiled-tubule spironeme), and rhopalonemes (a siphonophore-exclusive nematocyst type with wide tubules).

Many siphonophore species inhabit the deep pelagic ocean, which spans from ~200m to the abyssal seafloor (~4000m). This habitat has fairly homogeneous physical conditions and stable zooplankton abundances and
composition (8). With relatively predictable prey availability, ecological theory predicts that interspecific competition would inhibit the coexistence of closely-related species unless evolution towards specialization reduces the breadth of each species' niche (9–11). If this prediction holds true, we would expect the prey-capture apparatus morphologies of siphonophores to diversify with the evolution of specializations on a variety of prey types in different siphonophore lineages.

Specialization in resource acquisition and use has often been presented as an evolutionary ‘dead-end’ (12–16). The concept of a ‘dead-end’ can be problematic because it conflates very different macroevolutionary patterns. These patterns can pertain to the clade, such as higher extinction rates or lower diversification rates, or to the evolutionary lock-in of lineages to particular attributes. Here we exclusively focus on this last sense, in which feeding specialization can be considered a ‘dead-end’ if lineages that are feeding specialists do not give rise to feeding generalists or specialists on other prey. However, recent studies have found that ecological mechanisms such as interspecific competition can favor the evolution of generalists from specialists (17–19) and specialist resource switching (20, 21). In addition to studying relationships with morphology, we seek to identify what evolutionary transitions in trophic niche breadth are prevalent in these open-ocean tactile predators. To do so, we examine three alternative scenarios of siphonophore trophic specialization: (1) predatory specialists evolved from generalist ancestors; (2) predatory specialists evolved from specialist ancestors which targeted different
resources, switching their primary prey type; and (3) predatory generalists evolved from specialist ancestors. These scenarios are non-exclusive, and each could apply to different transitions along the siphonophore phylogeny.

In the past, the study of siphonophore tentilla and diets has been limited due to the inaccessibility of their oceanic habitat and the difficulties associated with the collection of fragile siphonophores. Thus, the morphological diversity of tentilla has only been characterized for a few taxa, and their evolutionary history remains largely unexplored. Contemporary underwater sampling technology provides an unprecedented opportunity to explore the trophic ecology (22) and functional morphology (23) of siphonophores. In addition, well-supported phylogenies based on molecular data are now available for these organisms (24). These advances allow for the examination of the evolutionary relationships between modern siphonophore form, function, and ecology. Our work builds upon previous pioneering studies that have explored the relationships between tentilla and diet, and have shown that siphonophores are a robust system for the study of predatory specialization via morphological diversification. Purcell (25, 26) showed clear relationships between diet, tentillum, and nematocyst characters in co-occurring epipelagic siphonophores for a small subset of extant epipelagic siphonophore species.

In this study, we present an extensive morphological characterization of tentilla and their nematocysts across a broad variety of shallow and deep-sea siphonophore species using modern imaging technologies, summarize the literature on siphonophore diets, expand the phylogenetic tree of siphonophores
by combining ribosomal gene sequences from a broad range of taxa with a transcriptome-based backbone tree, and explore the evolutionary histories and correlations between diet, tentillum, and nematocyst characters. Our results suggest that siphonophores can evolve new specializations and generalism by modifying the phenotypes and evolutionary correlations in their prey capture apparatus. These findings show how studying elusive non-bilaterian predators can challenge traditional views on the evolution of predatory specialization.

**Materials and Methods**

*Tentillum morphology* – The morphological work was carried out on siphonophore specimens fixed in 4% formalin from the Yale Peabody Museum Invertebrate Zoology (YPM-IZ) collection (accession numbers in Dryad repository). These specimens were collected intact across many years of fieldwork expeditions, using blue-water diving (41), remotely operated vehicles (ROVs), plankton net trawls, and human-operated submersibles. Tentacles were dissected from non-larval gastrozooids, sequentially dehydrated into 100% ethanol, cleared in methyl salicylate, and mounted onto slides with Canada Balsam or Permount mounting media. The slides were imaged as tiled z-stacks using differential interference contrast (DIC) on an automated stage at YPM-IZ (with the assistance of Daniel Drew and Eric Lazo-Wasem) and with laser point confocal microscopy using a 488 nm Argon laser that excited autofluorescence in the tissues. Thirty characters (defined in SI Appendix, Table S1.1) were measured using Fiji (42, 43). We did not measure the lengths of contractile
structures (terminal filaments, pedicles, gastrozooids, and tentacles) since they are too variable to quantify. We measured at least one specimen for 96 different species (raw data available in Dryad). Of these, we selected 38 focal species across clades based on specimen availability and phylogenetic representation. Three to five tentacle specimens from each one of these selected species were measured to capture intraspecific variation.

**Siphonophore phylogeny** – While the main goal of this work is not to elucidate a novel phylogeny for Siphonophora, we did expand on the most recent transcriptome-based phylogeny (24) to accommodate a larger taxon sampling. In order to do this, we ran a constrained analysis on an extensive 18S+16S dataset. The phylogenetic analysis included 55 siphonophore species and 6 outgroup cnidarian species (*Clytia hemisphaerica, Hydra circumcincta, Ectopleura dumortieri, Porpita porpita, Velella velella, Staurocladia wellingtoni*). The gene sequences we used in this study are available online (accession numbers in Dryad repository). Some of the sequences we used were accessioned in (27), and others we extracted from the transcriptomes in (24). Two new 16S sequences for *Frillagalma vityazi* (MK958598) and *Thermopalia* sp. (MK958599) sequenced by Lynne Christianson using the primers from (44) (read 3’ to 5’ F: TCGACTGTTTACCAAAAACATAGC, R: ACGGAATGAACATTTACATGTAAG) were included and accessioned to NCBI. Additional details on the phylogenetic inference methods can be found in the Supplementary Methods.

Unconstrained ML and Bayesian phylogenies were congruent (SI Appendix, Figs. S1.2 & S1.5). Given the broader sequence sampling of the
transcriptome phylogeny, we ran constrained inferences (using both ML and Bayesian approaches, which produced fully congruent topologies (SI Appendix, Fig. S1.4, Fig. S1.6)) after clamping the 5 nodes (SI Appendix, Fig. S1.3; blue circles in main-text Fig. 1.2) that were incongruent with the topology of the consensus tree in (24). This topology was then used to inform a Bayesian relaxed molecular clock time-tree in RevBayes, using a birth-death process (sampling probability calculated from the known number of described siphonophore species) to generate ultrametric branch lengths (SI Appendix, Figs. S1.7-S1.8). Scripts and tree files available in the Dryad repository.

**Feeding ecology** – We extracted categorical diet data for different siphonophore species from published sources, including seminal papers (4, 25, 32, 45–48), and ROV observation data (22, 49) with the assistance of Elizabeth Hetherington and C. Anela Choy (data available in Dryad repository). In order to detect coarse-level patterns in feeding habits, the data were merged into feeding guilds. For more details on how the diet data was curated and summarized into guilds, please see Supplementary Methods.

We also extracted copepod prey length data from (25). To calculate specific prey selectivities, we extracted quantitative diet and zooplankton composition data from (32), matched each diet assessment to each prey field quantification by site, calculated Ivlev’s electivity indices (50), and averaged those by species (data available in Dryad repository).

**Statistical analyses** – We used a series of phylogenetic comparative methods to test the evolutionary hypotheses presented in this study. We
reconstructed ancestral states using ML (R phytools::anc.ML (51)), and stochastic character mapping (R phytools::make.simmap) for categorical characters. When reconstructing the evolutionary history of feeding guilds, we fitted our SIMMAP model under the agnostic assumption that a generalist diet poses morphological challenges that are as distinct as each specialization is from each other. Thus, we do not impose any *a priori* constraints or weighting in the model for what state transition rates are permissible, letting the data determine the parameters. For more details on the data wrangling prior to these analyses, please see the Supplementary Methods. R scripts available in the Dryad repository.

In order to study the evolution of predatory specialization, we reconstructed components of the diet and prey selectivity on the phylogeny using ML (R phytools::anc.ML). To identify evolutionary associations of diet with tentillum and nematocyst characters, we compared the performance of a neutral evolution model to that of a diet-driven directional selection model. First, we collapsed the diet data into the five feeding guilds mentioned above (fish specialist, small crustacean specialist, large crustacean specialist, gelatinous specialist, generalist), based on which prey types they were observed consuming most frequently. Then, we reconstructed the feeding guild ancestral states using the ML function ace (package ape (52)), removing tips with no feeding data. The ML reconstruction was congruent with the consensus stochastic character mapping (SI Appendix, Fig. S1.15). Then, using the package *OUwie* (53), we fitted an OU model with multiple optima and rates of evolution (OUm) matched to
the reconstructed ancestral diet regimes, a single optimum OU model, and a BM null model, inspired by the analyses in (54). We then ranked the models in order of increasing parametric complexity (BM, OU, OUm), and compared the corrected Akaike Information Criterion (AICc) support scores (55) to the lowest (best) score, using a cutoff of 2 units to determine significantly better support. When the best fitting model was not significantly better than a less complex alternative, we selected the least complex model (SI Appendix, Fig. S1.9). In addition, we calculated and reported the model adequacy scores using the R package arbutus (56).

In order to study correlations between the rates of evolution between different characters, we fitted a set of evolutionary variance-covariance matrices (33) (R phytools::evol.vcv). For more details on the data wrangling preceding these analyses, please see Supplementary Methods. To test whether phenotypic integration changed across selective regimes determined by the reconstructed feeding guilds, we carried out character-pairwise variance-covariance analysis comparing alternative models (R phytools::evolvcv.lite), including those where correlations are the same across the whole tree and models where correlations differ between selective regimes (SI Appendix, Fig. S1.19). Number of taxa used in each pairwise comparison is reported in SI Appendix, Fig. S1.20. Finally, we compared regime-specific variance-covariance matrices to the general matrix and to their preceding regime matrix to identify the changes in character dependences unique to each regime (SI Appendix, Figs. S1.21-S1.22).
We carried out a linear discriminant analysis of principal components (DAPC) using the dapc function (R adegenet::dapc) (57). This function allowed us to incorporate more predictors than individuals. We generated discriminant functions for feeding guild, and for the presence of copepods, fish, and shrimp (large crustaceans) in the diet (SI Appendix, Figs. S1.10-S1.13). From these DAPCs we obtained the highest contributing morphological characters to the discrimination (characters in the top quartile of the weighted sum of the linear discriminant loadings controlling for the eigenvalue of each discriminant). In order to identify the sign of the relationship between the predictor characters and prey type presence in the diet, we then generated generalized logistic regression models (as a type of generalized linear model, or GLM using R stats::glm) and phylogenetic generalized linear models (R phylolm::phyloglm) with the top contributing characters (from the corresponding DAPC) as predictors (SI Appendix, Fig. S1.14). We also carried out these GLMs on the Ivlev’s selectivity indices for each prey type calculated from (32). In addition, we ran a series of comparative analyses to address hypotheses of diet-tentillum relationships posed in the literature. Additional details on the DAPC optimization are available in the Supplementary Methods.

Results

Novel phylogenetic relationships – In order to analyze the relationships between morphology and diet across the evolutionary history of siphonophores, we generated a siphonophore phylogeny that had broader taxonomic sampling
than was available in previously published analyses. We first inferred a new tree with the needed taxon sampling with publicly available ribosomal RNA genes (18S & 16S) and new data from one species. This tree is essentially an extended version of that published in (27), and the two are congruent. We then compared the new extended ribosomal RNA tree to a recently published siphonophore transcriptome phylogeny (24). The topology of the extended ribosomal RNA tree recapitulates the resolved nodes in (27) and most of the nodes in (24). Only five nodes in the unconstrained tree inference were incongruent with the transcriptome tree in (24), with four of them poorly supported (bootstrap values <84), and only one of them strongly supported (Frillagalma vityazi - Nanomia bijuga, 100 bootstrap support). We constrained the incongruent nodes to the (24) topology during estimation of the constrained 18S+16S tree inference (Fig. 1.2). Since the transcriptome-based placement of Nanomia bijuga is more consistent with the morphological data, that relationship was also constrained. Moreover, with the inclusion of sequences from Stephanomia amphytridis and multiple Erenna species, our tree reveals a novel sister relationship between the genus Erenna and Stephanomia.

We used the clade nomenclature defined in (27) and (24), including Codonophora to indicate the sister group to Cystonectae, Euphysonectae to indicate the sister group to Calycophorae, Clade A and B to indicate the two main lineages within Euphysonectae. In addition, we define two new clades within Codonophora (Fig. 1.2): Eucladophora as the clade containing Agalma elegans and all taxa that are more closely related to it than to Apolemia lanosa, and
Tendiculophora as the clade containing *Agalma elegans* and all taxa more closely related to it than to *Bargmannia elongata*. Eucladophora is characterized by bearing spatially differentiated tentilla with proximal heteronemes and a narrower terminal filament region. The etymology derives from the Greek *eu* + *kládos* + *phóros* for “true branch bearers”. Tendiculophora are characterized by bearing rhopalonemes and desmonemes in the terminal filament, having a pair of elastic strands, and developing proximally detachable cnidobands. The etymology of this clade is derived from the Latin *tendicula* for “snare or noose” and the Greek *phóros* for “carriers”.

*Evolutionary associations between diet and tentillum morphology* – We reconstructed the evolutionary history of feeding guilds using stochastic mapping on the new phylogeny (Fig. 1.2). Our reconstructions do not recover generalism as the ancestral siphonophore diet. None of the transitions in diet are consistent with scenario 1 (specialists evolving from generalists). Feeding guild specializations have shifted from an alternative ancestral state at least five times, consistent with instances supporting scenario 2 (specialists evolving to feed on a different resource). We also recover multiple independent origins of generalism from specialist ancestors (Fig. 1.3). Large crustacean specialists evolve into generalists twice independently, consistent with instances of scenario 3 (generalists evolving from specialists). This finding is particularly compelling given in that it is the opposite of known biases in ancestral state reconstruction. Nosil and Mooers (28) found that such methods tend to infer higher transition rates toward the more frequent state. In this case, that would lead to a bias for an
increased rate of transition from generalists (the rarer state across the tips) to specialists (the more common state across the tips). We observe the opposite, indicating strong evidence that these generalists are indeed a derived state.

To test whether measured morphological characters evolved in association with shifts in feeding ecology, we analyzed the evolutionary history of each character on the phylogeny, with the feeding guilds reconstructed on it as hypothetical selective regimes. We fit and compared alternative evolutionary models for each continuous character. The models compared were the Brownian Motion (BM) model of neutral divergent evolution (29), the Ornstein-Uhlenbeck (OU) model of stabilizing selection around a single fitted optimum state (30, 31), and an OU model with multiple optima (OUm) corresponding to each reconstructed selective regime (feeding guild). The model comparison shows that out of 30 characters, 10 show significantly stronger support for the diet-driven OUm (SI Appendix, Fig. S1.9). These characters include terminal filament nematocyst size and shape, involucrum length, elastic strand width, and heteroneme number. Most of these characters are found exclusively in Tendiculophora, thus this may reflect processes that could be unique to this clade. Five characters including cnidoband length, cnidoband shape, and haploneme length show maximal support for a diet-driven single-optimum OU model. The remaining 15 characters support BM (or OU with marginal AICc difference with BM).

In order to investigate the associations between the evolutionary history of morphological characters and specific prey types found in the diet, we used
phylogenetic logistic regressions. We found that several characters were significantly correlated with the gains and losses of specific prey types (Fig. 1.3, right). Shifts toward ostracod presence in diet correlated with reductions in pedicle width and total haploneme volume. Shifts to copepod presence in the diet were associated with reductions in haploneme width, cnidoband length and width, total haploneme and heteroneme volumes, and tentacle and pedicle widths. Consistently, transitions to decapod presence in the diet correlated with more coiled cnidobands (SI Appendix, Fig. S1.21). Evolutionary shifts in these characters may have allowed the inclusion of these prey types in the diet.

We tested some of the diet-morphology associations previously proposed in the literature (25, 26) for correlated evolution (Table 1.1). We found that most, such as heteroneme volume and copepod prey size, do show evidence for correlated evolution. The sole exception was the relationship between terminal filament nematocysts (rhopalonemes and desmonemes) and crustaceans in the diet. Analyses that do not take phylogeny into account do recover this correlation across the extant species studied, but it is not consistent with correlated evolution. The latter is likely a product of the larger species richness of crustacean-eating species with terminal filament nematocysts, rather than simultaneous evolutionary gains.

In addition to studying correlations with prey type presence/absence in the diet, we also tested for correlations between morphological characters and shifts in prey selectivity using phylogenetic linear models. Prey selectivity values were calculated from (32) by contrasting the gut content frequencies to the
corresponding environmental abundances of prey. We found that fish selectivity is associated with increased number of heteronemes per tentillum, increased roundness of nematocysts (desmonemes and haplonemes), larger heteronemes, reduced heteroneme/cnidoband length ratios, smaller rhopalonemes, lower haploneme surface area to volume ratio (SA/V), and larger the cnidoband, elastic strand, pedicle and tentacle widths. Decapod-selective diets were associated with increasing cnidoband size and coiledness, haploneme row number, elastic strand width, and heteroneme number. Copepod-selective diets evolved in association with smaller heteroneme and total nematocyst volumes, smaller cnidobands, rounder rhopalonemes, elongated heteronemes, narrower haplonemes with higher SA/V ratios, and smaller heteronemes, tentacles, pedicles, and elastic strands. Selectivity for ostracods was associated with reductions in size and number of heteroneme nematocysts, cnidoband size, number of haploneme rows, heteroneme number, and cnidoband coiledness. Heteroneme length and elongation also correlated negatively with chaetognath selectivity (S21). These results indicate that not only diet but also differential feeding selectivity has evolved in correlation with changes in the prey capture apparatus of siphonophores. For each prey type studied, tentillum morphology is a much better predictor of prey selectivity than of prey presence in the diet, despite prey selectivity data being available for a smaller subset of species. Interestingly, many of the morphological predictors had opposite slope signs when predicting prey selectivity versus predicting prey presence in the diet (Table 1.2).
Evolution of relationships between characters with diet — Phenotypic integration results in correlation patterns between morphological characters and their rates of evolution. To study these patterns, we fit a set of evolutionary variance-covariance matrices (33). The quantitative characters we measured from tentilla and their nematocysts are highly correlated. The variance-covariance matrices (SI Appendix, Fig. S1.21-S1.23) clearly reveal the diagonal blocks that constitute the evolutionary modules, such as the heteroneme block, the terminal filament nematocyst block, and the cnidoband-pedicle-tentacle block. These results were not sensitive to the transformation of inapplicable states and taxon sampling. These results indicate that siphonophore tentilla and nematocysts are phenotypically integrated and co-evolve within discrete evolutionary modules.

In order to test whether rate covariance matrices changed with evolutionary shifts in feeding guild regimes, we compared the rate covariance terms between characters across the subtrees occupied by the different feeding guild regimes (SI Appendix, Fig. S1.21). We found that half (48%) of the character pairs presented significantly distinct correlation coefficients across different regimes (SI Appendix, Fig. S1.19), indicating that the mode of phenotypic integration also shifts with trophic niche. When contrasting the regime-specific rate correlation matrices to the whole-tree matrix and to the preceding ancestral regime matrix, we were able to identify the character
dependencies that are unique to each predatory niche (SI Appendix, Figs. S1.22-S1.23).

We were able to identify specific character correlations that shifted with the evolution of new diets. Under the majority of stochastic character mapping outcomes, large crustacean specialists are the ancestral feeding regime, and all other feeding regimes evolve from this ancestral specialization. Compared to the rate correlation matrix estimated over the whole tree, large crustacean specialists present strong negative correlations between haploneme elongation and heteroneme size, and between rhopaloneme elongation and tentillum size, as well as with involucrum length. Within generalist clades (Forskalia and the Agalma-Athorybia clade), terminal filament nematocyst (desmoneme and rhopaloneme) sizes became negatively correlated with the sizes of most characters, meaning that as some tentilla became larger, their individual terminal nematocysts became smaller, observed to the extreme in Agalma. In addition, heteroneme and rhopaloneme elongation became positively correlated with cnidoband size. When large crustacean specialists switched to small crustacean prey in Cordagalma and calycophorans, haploneme size became inversely correlated with heteroneme elongation, which in turn developed a strong positive relationship with tentillum size. The extremes of this gradient can be seen in Cordagalma and Hippopodius, genera subspecialized in copepods and ostracods respectively. With the evolution of fish prey specialization in cystonects and within Clade B (Fig. 1.2), haploneme elongation became negatively correlated with heteroneme elongation (signal driven by Clade B, since cystonects lack
tentacular heteronemes), and the surface area to volume ratio of haploneme nematocysts switched from a strong negative relationship with cnidoband size (found in every other regime) to a positive correlation. This is consistent with Clade B haplonemes becoming rounder, more similar to cystonect haplonemes specialized in fish prey penetration and envenomation. Gelatinous specialization, albeit appearing only once in our tree, also carries a unique signature in character rate correlation shifts, with an increase in the strength of the correlation between heteroneme shape and shaft width, consistent with the appearance of birrhopaloid nematocysts with swollen shafts. These are likely effective at anchoring gelatinous tissue in a similar way to the nematocysts of the Narcomedusae (26).

**Discussion**

Several studies (12–16) have suggested that resource specialization can be an irreversible state due to the constraints posed by extreme phenotypic specialization. Our results show that this is not the case for siphonophores, where the prey type on which they specialize has shifted at least 5 times. We find no support for any transitions from generalist to specialist (scenario 1, as described in the Introduction). We do find support for at least 3 instances of specialists switching from one prey type to another prey type, (scenario 2) and two switches from specialist to generalist (scenario 3). This is consistent with the findings of recent studies on phytophagous insects (19), where the rate of evolution from generalists to specialists is comparable to the reverse, thus specialization does not limit further evolution. Our results are also consistent with
analyses of lepidopterans (21), where specialized resource switching is the primary transition type while niche breadth remains fairly constant. These results show how an ancestor specialized for feeding on a particular prey can still give rise to multiple lineages and to novel feeding guilds, including generalists. For example, we find that, Eucladophora, a large clade of siphonophores that contains the majority of extant species and diverse feeding strategies, arose from a most recent common ancestor that was a specialist on large crustacean prey. Though ‘evolutionary dead-end’ is a problematic term that could apply to several evolutionary patterns, this result is inconsistent with multiple specific uses of this term.

The evolutionary history of tentilla shows that siphonophores are an example of trophic niche diversification via morphological innovation and evolution, which allowed transitions between specialized trophic niches. In more familiar predators, the prey capture apparatus (such as claws and jaws) is well integrated in the body, leading to trade-offs and whole-body adaptations for feeding specialization. The extreme modularity of the siphonophore prey capture apparatus could release them from constraints typically imposed by adaptation to ecological specialization. This evolutionary mechanism is particularly important in a deep open-ocean ecosystem, which is a relatively homogeneous physical environment, where the primary niche heterogeneity available is the potential interactions between organisms (8).

While selection acting on character states is a widely studied phenomenon, recent studies have shown that selection can also act upon the
patterns of character correlations and phenotypic dependencies (33–39). This evolution of character relationships can allow lineages to explore new regions of the morphospace and facilitate the appearance of ecological novelties. Our results show that the patterns of phenotypic integration in siphonophore tentilla vary among clades, and appear to display different relationships across shifting feeding specializations. Similar to what has been found in the feeding morphologies of fish (33, 40), siphonophore tentilla may have accommodated new diets by altering the correlations between characters. For example, changes in the size and shape relationships between nematocyst types gave rise to the nematocyst complements specialized in ensnaring prey with different combinations of defensive traits.

Our results unambiguously show that tentillum morphology evolved with diet and strongly support deviations from the generalist-to-specialist evolution scenario. However, the conclusions we can draw from these analyses are limited in several ways. The biggest challenge at present is the sparse dietary data available in the literature. Additional dietary data could reveal transitions from generalists to specialists we were unable to detect for two reasons: some of the taxa in our dataset have a very limited number of feeding observations, which could lead to apparent specialization; and some of the taxa not included in our dataset could be undiscovered generalists. When interpreting these results, it is important to remember that diet is also dependent on environmental prey availability, which was not available in most of the sources we used (except Purcell 1981 & Purcell 1984). We integrated published dietary data collected
using different methods bearing different inherent biases. Gut content inspections (used in the majority of our literature sources) are very effective at detecting small hard-bodied prey, but can fail to detect rapidly-digested soft-bodied prey. ROV observations can be biased towards large (often gelatinous) prey, and can easily oversee smaller prey items. In addition, selectivity differences across siphonophore species could be also driven by other phenotypes not accounted for in this study. Finally, further observations on behavior, digestion biochemistry, and toxin composition are necessary to assess their relative importance in determining diet.

We hypothesize that siphonophores are able to evolve from specialization for one prey type to other states due to their prey-capture apparatus being extremely independent from the rest of the bodies in terms of location and function. We also hypothesize that the homogeneous midwater environment they live in favors the evolution of extreme morphological adaptations for prey capture. Testing these hypotheses will be interesting in its own right, and also give a better sense of how generalizable our results are beyond siphonophores. It is important to note that our hypotheses only apply to organisms with access to a broad-enough diversity of resources (such as prey, or hosts) on which to specialize, and only when said resources pose distinct challenges that require anatomical modifications. Otherwise, there would not be enough variation to detect these patterns in the first place.
Conclusions

Most studies on the evolution of predation have focused on vertebrate systems with an integrated feeding apparatus serving multiple functions. This has led to a narrow understanding of the evolutionary outcomes of specialization, where extreme morphological evolution constrains further shifts in their ecology. Siphonophores differ in many ways from commonly-known predators, using modular weapons for prey capture (the tentilla) that are fully decoupled from other structures and body functions. Our analysis of the evolutionary history of dietary specialization and morphological change in these elusive animals has revealed notable deviations from traditional expectations. While much of the feeding ecology literature focuses on how predatory generalists evolve into predatory specialists, in siphonophores we find predatory specialists can evolve into generalists, and that specialists on one prey type have directly evolved into specialists on other prey types. We find that the character states, evolutionary optima, and evolutionary correlations of many morphological characters have evolved following these ecological shifts. We find that the relationships between form and ecology hold across a large set of siphonophore taxa and characters. These findings are central to understanding the evolutionary mechanisms driving the emergence of food web complexity.

Acknowledgments

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Award to C.W.D., and NSF-OCE 1829835 to C.W.D., OCE-1829805 to S.H.D.H., and OCE-1829812 to C. Anela Choy). A.D.S. was supported by a Fulbright Spain Graduate Studies Scholarship. We wish to thank the crew and scientists of the R/V Western Flyer, who participated in the collection of many of the specimens used in this study. We also want to thank Lynne Christianson and Shannon Johnson from the Monterey Bay Aquarium Research Institute for their assistance in the field as well as for sequencing some of the species included in this phylogeny. In addition, we wish to thank Lourdes Rojas, Daniel Drew, and Eric Lazo-Wasem for their assistance in imaging the fixed specimens and managing the collections. We thank Dennis Pilarczyk for organizing the prey selectivity data, Michael Landis for helping design the Bayesian analyses, and Joaquin Nunez for reviewing this manuscript. Furthermore, we thank Elizabeth D. Hetherington and C. Anela Choy for collating the data on siphonophore feeding and for reviewing the manuscript. Finally, we thank Philip Pugh, who confirmed many of our specimen identifications and taught us valuable knowledge about siphonophores.

References


41. S. H. Haddock, J. N. Heine, *Scientific blue-water diving* (California Sea Grant College Program, 2005).


Figures and Tables

Table 1.1. Tests of correlated evolution between siphonophore morphological characters and aspects of the diet found correlated in the literature. We report the direction and significance of the evolutionary association, the number of taxa used for the analysis, and the literature source where the morphology-diet association was first reported.

<table>
<thead>
<tr>
<th>Character</th>
<th>Aspect of diet</th>
<th>Test of evolutionary association</th>
<th>Relationship sign</th>
<th>P-value</th>
<th>Number of taxa</th>
<th>Association first report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiated cnidobands</td>
<td>Hard bodied prey</td>
<td>Pagel’s test</td>
<td>+</td>
<td>0.017</td>
<td>19</td>
<td>(25)</td>
</tr>
<tr>
<td>Heteroneme volume</td>
<td>Copepod prey size</td>
<td>pGLS</td>
<td>+</td>
<td>0.002</td>
<td>8</td>
<td>(25)</td>
</tr>
<tr>
<td>Terminal filament nematocysts</td>
<td>Crustacean diet</td>
<td>Pagel’s test</td>
<td>Non-Significant</td>
<td>0.200</td>
<td>19</td>
<td>(26)</td>
</tr>
<tr>
<td>Number of nematocyst types</td>
<td>Soft-bodied prey</td>
<td>Phylogenetic logistic regression</td>
<td>-</td>
<td>0.040</td>
<td>22</td>
<td>(26)</td>
</tr>
</tbody>
</table>

Table 1.2. Discriminant analysis of principal components for the presence of specific prey types using the morphological data. Top quartile variable (character) contributions to the linear discriminants are ordered from highest to lowest. Logistic regressions and GLMs were fitted to predict prey type presence and selectivity respectively. The sign of the slope of each predictor is reported, marked with an asterisk if significant (p-value < 0.05), and highlighted grey if it differs between prey presence in diet and prey selectivity. Pseudo-$R^2$ (%) approximates the percent variance explained by the model.
<table>
<thead>
<tr>
<th>Prey type</th>
<th>DAPC</th>
<th>GLM for prey type presence (22 taxa)</th>
<th>Best fitting GLM for prey type selectivity. Data from (32) (7 taxa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Top quartile variable contributions</td>
<td>Sign        R²</td>
</tr>
<tr>
<td>Discrimination (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copepods</td>
<td>95.4</td>
<td>Total nematocyst volume</td>
<td>-           67.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tentacle width</td>
<td>-           +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Haploneme elongation</td>
<td>-           +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Haploneme surface area/volume ratio</td>
<td>+           -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Haploneme row number</td>
<td>+           +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cnidoband length</td>
<td>-           +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cnidoband width</td>
<td>-           -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cnidoband free length</td>
<td>+           +</td>
</tr>
<tr>
<td>Fish</td>
<td>68.1</td>
<td>Total haploneme volume</td>
<td>-           45.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heteroneme volume</td>
<td>+           -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total nematocyst volume</td>
<td>-           +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total heteroneme volume</td>
<td>-           -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cnidoband length</td>
<td>-           -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cnidoband free length</td>
<td>+           +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Involucrum length</td>
<td>-           -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pedicle width</td>
<td>+           +</td>
</tr>
<tr>
<td>Large crustaceans</td>
<td>81.8</td>
<td>Involucrum length</td>
<td>+*          73.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total heteroneme volume</td>
<td>-           -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elastic strand width</td>
<td>-           +*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rhopaloneme length</td>
<td>+           +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heteroneme volume</td>
<td>+           -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Haploneme elongation</td>
<td>-           +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Desmoneme length</td>
<td>-           -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tentacle width</td>
<td>+           +</td>
</tr>
</tbody>
</table>
Figure 1.1. Siphonophore anatomy. A - *Nanomia* sp. siphonophore colony (photo by Catriona Munro). B, C - Illustration of a *Nanomia* colony, gastrozooid, and tentacle closeup (by Freya Goetz). D - *Nanomia* sp. Tentillum illustration and main parts. E - Differential interference contrast micrograph of the tentillum illustrated in D. F - Nematocyst types (illustration reproduced with permission from Mapstone 2014), hypothesized homologies, and locations in the tentillum. Undischarged to the left, discharged to the right.
Figure 1.2. Bayesian time-tree inferred from 18S + 16S concatenated sequences and constrained to be congruent with a published transcriptome phylogeny. Branch lengths estimated using a relaxed molecular clock. Species names in red indicate replicated representation in the morphology data. All data were publicly available, apart from new sequences produced for *Thermopalia taraxaca* and *Frillaglma vityazi* (bold). Nodes labeled with Bayesian posteriors (BP). Green circles indicate BP = 1. Blue circles indicate nodes constrained to be congruent with Munro *et al.* (2018). Tips with black squares indicate the species with transcriptomes used in Munro *et al.* (2018). Tips with purple squares indicate genus-level correspondence to taxa included in Munro *et al.* (2018). The main clades are labeled: with black bars for described taxonomic units, and grey bars for operational phylogenetic designations.
Figure 1.3. Left - Subset phylogeny showing the mapped feeding guild regimes that were used to inform the *OUwie* analyses. Right - Grid showing the prey items consumed from which the feeding guild categories were derived. Diet data were obtained from the literature review, available in the Dryad repository.
Supplementary Information

Supplementary Methods

Phylogenetic inference — We aligned the sequences using MAFFT (1) (alignments available in Dryad). We inferred a Maximum Likelihood (ML) phylogeny (S2) from 16S and 18S ribosomal rRNA genes using IQTree (2) with 1000 bootstrap replicates (iqtree -s alignment.fa -nt AUTO -bb 1000). We used ModelFinder (3) implemented in IQTree v1.5.5. to assess the relative model fit. ModelFinder selected GTR+R4 for having the lowest Bayesian Information Criterion score. Additionally, we inferred a Bayesian tree with each gene as an independent partition in RevBayes (4) (S5), which was topologically congruent with the unconstrained ML tree. The alpha priors were selected to minimize prior load in site variation.

Diet data curation — We removed the gelatinous prey observations for Praya dubia eating a ctenophore and a hydromedusa, and for Nanomia sp. eating Aegina since we believe these are rare events that have a much larger probability of being detected by ROV methods than their usual prey, and it is not clear whether the medusae were attempting to prey upon the siphonophores. Personal observations on feeding (from SHDH, Anela Choy, and Philip Pugh) were also included for Resomia ornicephala, Lychnagalma utricularia, Bargmannia amoena, Erenna richardi, Erenna laciniata, Erenna sirena, and Apolemia rubriversa. The feeding guilds declared in this study are: small-crustacean specialist (feeding mainly on copepods and ostracods), large
crustacean specialist (feeding on large decapods, mysids, or krill), fish specialist (feeding mainly on actinopterygian larvae, juveniles, or adults), gelatinous specialist (feeding mainly on other siphonophores, medusae, ctenophores, salps, and/or doliolids), and generalist (feeding on a combination of the aforementioned taxa, without favoring any one prey group). These were selected to minimize the number of categories while keeping the most different types of prey separate. The gut content observations on *Forskalia* sp. were synonymized to an arbitrary *Forskalia* species on the tree (*F. tholoides*) for comparative analyses.

*Data wrangling for comparative analyses* — For comparative analyses, we removed species present in the tree but not represented in the morphology data, and *vice versa*. Although we measured specimens labeled as *Nanomia bijuga* and *Nanomia cara*, we are not confident in some of the species-level identifications, and some specimens were missing diagnostic zooids. Thus, we decided to collapse these into a single taxonomic concept (*Nanomia* sp.). All *Nanomia* sp. observations were matched to the phylogenetic position of *Nanomia bijuga* in the tree. We carried out all phylogenetic comparative statistical analyses in the programming environment R (5), using the Bayesian ultrametric species tree (S8), and incorporating intraspecific variation estimated from the specimen data as standard error whenever the analysis tool allowed it. R scripts and summarized species-collapsed data available in the Dryad repository. For each character (or character pair) analyzed, we removed species with missing data and reported the number of taxa included. We tested each
character for normality using the Shapiro-Wilk test (6), and log-transformed those that were non-normal.

Data wrangling for the variance-covariance analyses — When fitting all variance-covariance terms simultaneously (S16-18), we selected the largest set of characters that would allow the analysis to run without computational singularities. This excluded many of the morphometric characters which are linearly dependent on other characters. Since the functions do not tolerate missing data, we ran the analyses in two ways: One including all taxa but transforming absent states to zeroes, and another removing the taxa with absent states. These analyses could only be carried out on the subset of taxa for which diet data is available, and only among character pairs that are not computationally singular for that taxonomic subset. Gelatinous specialist correlations could only be estimated for a small subset of characters present in Apolemia (S21F, S22E, S23D) and should be interpreted with care.

Comparative tools used to test character associations — To test for correlated evolution among binary characters, we used Pagel’s test (7). To characterize and evaluate the relationship between continuous characters, we used phylogenetic generalized least squares regressions (PGLS) (8). To compare the evolution of continuous characters with categorical aspects of the diet, we carried out a phylogenetic logistic regression (R nlme::gls using the ‘corBrownian’ function for the argument ‘correlation’).
**DAPC optimization** — Some taxa have inapplicable states for certain absent characters (such as the length of a nematocyst subtype that is not present in a species), which are problematic for DAPC analyses. We tackled this by transforming the absent states to zeroes. This approach allows us to incorporate all the data, but creates an attraction bias between small character states (*e.g.* small tentilla) and absent states (*e.g.* no tentilla). Absent characters are likely to be very biologically relevant to prey capture and we believe they should be accounted for in a predictive approach. We limited the number of linear discriminant functions retained to the number of groupings in each case. We selected the number of principal components retained using the a-score optimization function (*R adegenet::optim.a.score*) (9) with 100 iterations, which yielded more stable results than the cross validation function (*R adegenet::xval*). This optimization aims to find the compromise value with highest discrimination power with the least overfitting. The discriminant analysis for feeding guild (7 principal components, 4 discriminants) produced 100% discrimination, and the highest loading contributions were found for the characters (ordered from highest to lowest): Involucrum length, heteroneme volume, heteroneme number, total heteroneme volume, tentacle width, heteroneme length, total nematocyst volume, and heteroneme width (S10).


Table S1.1: Character definitions.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haptoneme</td>
<td>Nematocty with no shaft</td>
</tr>
<tr>
<td>Heteroneme</td>
<td>Nematocty with a distinct shaft</td>
</tr>
<tr>
<td>Desmoneme</td>
<td>Small oval-shaped adhesive nematocty with thick ovoid tubule</td>
</tr>
<tr>
<td>Rhopaloneme</td>
<td>Small rod-like nematocty found on the terminal filament</td>
</tr>
<tr>
<td>Terminal filament</td>
<td>Distal extension of the tentillum beyond the cridiband</td>
</tr>
<tr>
<td>Cridiband</td>
<td>Distinct packing of nematoctys on the dorsal side of the tentillum</td>
</tr>
<tr>
<td>Tentacle</td>
<td>Tubular projection from the gastrostomial basegaster</td>
</tr>
<tr>
<td>Tentillum</td>
<td>Evenly spaced dorsal evagination of the tentillum carrying ordered and functional nematoctys</td>
</tr>
<tr>
<td>Inviolulum</td>
<td>Extension of the pedicle covering part of the cridiband</td>
</tr>
<tr>
<td>Pedicle</td>
<td>Proximal region of the tentillum between the cridiband and the tentillum</td>
</tr>
<tr>
<td>Elastic strand</td>
<td>Mesoglea derived collagenous double strand underlying the cridiband of some isoponopores</td>
</tr>
</tbody>
</table>

Table S1.2: Definitions of the continuous morphological and kinematic characters measured.

<table>
<thead>
<tr>
<th>Character</th>
<th>Definition</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cridiband length</td>
<td>Distance from the base to tip of the cridiband in natural position</td>
<td>micrometers</td>
</tr>
<tr>
<td>Cridiband free length</td>
<td>Distance from the base to the tip of the cridiband when stretched straight</td>
<td>micrometers</td>
</tr>
<tr>
<td>Cridiband width</td>
<td>Diameter of the cridiband at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Inviolulum length</td>
<td>Length of the inviolulum from the base of the cridiband to its most distal extent</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneme length</td>
<td>Length of the heteronemess</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneme width</td>
<td>Diameter of the heteronemess at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneme shaft length</td>
<td>Length of the heteroneme shaft</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneme shaft width</td>
<td>Width of the heteroneme shaft</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneme number</td>
<td>Number of heteronemess in each tentillum (# in each row²)</td>
<td></td>
</tr>
<tr>
<td>Haptoneme length</td>
<td>Length of the haptonemess</td>
<td>micrometers</td>
</tr>
<tr>
<td>Haptoneme width</td>
<td>Diameter of the haptonemess at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Rhopaloneme length</td>
<td>Length of the rhopalones</td>
<td>micrometers</td>
</tr>
<tr>
<td>Rhopaloneme width</td>
<td>Diameter of the rhopalones at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Desmoneme length</td>
<td>Length of the desmonemess</td>
<td>micrometers</td>
</tr>
<tr>
<td>Desmoneme width</td>
<td>Diameter of the cridiband at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Inviolulum length</td>
<td>Length of the inviolulum from the base of the cridiband to its most distal extent</td>
<td>micrometers</td>
</tr>
<tr>
<td>Elastic strand width</td>
<td>Diameter of the descending elastic strand at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Pedicle width</td>
<td>Diameter of the pedicle</td>
<td>micrometers</td>
</tr>
<tr>
<td>Tentacle width</td>
<td>Diameter of the tentacle</td>
<td>micrometers</td>
</tr>
<tr>
<td>Haptoneme row number</td>
<td>Number of haptoneme rows running parallel to the length of the cridiband</td>
<td>micrometers</td>
</tr>
<tr>
<td>Cridiband coiledness</td>
<td>Cridiband free length / Cridiband length</td>
<td>adimensional</td>
</tr>
<tr>
<td>Heteroneme elongation</td>
<td>Heteroneme Length/Width</td>
<td>adimensional</td>
</tr>
<tr>
<td>Haptoneme elongation</td>
<td>Haptoneme Length/Width</td>
<td>adimensional</td>
</tr>
<tr>
<td>Desmoneme elongation</td>
<td>Desmoneme Length/Width</td>
<td>adimensional</td>
</tr>
<tr>
<td>Rhopaloneme elongation</td>
<td>Rhopalone Length/Width</td>
<td>adimensional</td>
</tr>
<tr>
<td>Heteronene shaft extension</td>
<td>Heteroneme shaft length / Heteroneme capsule length</td>
<td>adimensional</td>
</tr>
<tr>
<td>Nematocty surface area</td>
<td>$4\pi r^2\left(1.69 h + (\text{Width}^2 + \text{Width}^2 + \text{Width}^2) / (1.89)\right)$</td>
<td>micrometers squared</td>
</tr>
<tr>
<td>Nematocty volume</td>
<td>Ellipsoid formula: $\left(4\pi / 3\right) r^3 \left(1 - (3/2)(r/\text{Width})^2\right)$</td>
<td>micrometers cubed</td>
</tr>
<tr>
<td>Nematocyst SA/V ratio</td>
<td>Nematocyst surface area / Nematocyst volume</td>
<td>$1 / \text{micrometers}$</td>
</tr>
<tr>
<td>Total haptoneme volume</td>
<td>Haptoneme volume * Haptoneme row number / (Cridiband free length / Haptoneme width)</td>
<td>micrometers cubed</td>
</tr>
<tr>
<td>Total heteroneme volume</td>
<td>Heteroneme volume * Heteroneme number</td>
<td>micrometers cubed</td>
</tr>
<tr>
<td>Total nematocyst volume</td>
<td>Total haptoneme volume + Total heteroneme volume</td>
<td>micrometers cubed</td>
</tr>
</tbody>
</table>
Figure S1.2: Maximum likelihood IQTree inference, unconstrained. Node labels are bootstrap support values.

Figure S1.3: Topology used to constrain analyses (minimal topological statements based on the incongruences between the unconstrained tree and Munro et al. (2018)).
Figure S1.4: Constrained IQTree ML inference. Node labels are bootstrap support values.

Figure S1.5: Unconstrained Bayesian topology inference in RevBayes (node labels are Bayesian posteriors).
Figure S1.6: Clade constrained Bayesian inference in RevBayes (node labels are Bayesian posteriors).
Figure S1.7: Unconstrained ultrametric Bayesian time tree branch length and topology inference in RevBayes (node labels are Bayesian posteriors). Arbitrary rooting.
Figure S1.8: Ultrametric Bayesian time tree branch length inference in RevBayes (node labels are bayesian posteriors). Topology clamped to the Bayesian constrained topology inference in Fig. SM3. Tree rooted using outgroup constraint.
Table S1.9: Model support (delta AICc) for each morphological character analyzed on the feeding guild reconstruction regime tree. OU1 = Single-optimum Ornstein-Uhlenbeck. OUm = Multi-optima Ornstein-Uhlenbeck. Model adequacy scores calculated for the best supported model only. Msig = mean of squared contrasts. Cvar = coefficient of variation of the absolute value of the contrasts. Svar = Slope of a linear model fitted to the absolute value of the contrasts against their expected variances. Sasr = slope of the contrasts against the ancestral state inferred at each corresponding node. Shgt = slope of the contrasts against node depth. Dcfd = Kolmogorov-Smirnov D-statistic comparing contrasts to a normal distribution with SD equal to the root of the mean of squared contrasts.

<table>
<thead>
<tr>
<th>Character</th>
<th>N</th>
<th>dAICc BM</th>
<th>dAICc OU1</th>
<th>dAICc OUm</th>
<th>Msig</th>
<th>Cvar</th>
<th>Svar</th>
<th>Sasr</th>
<th>Shgt</th>
<th>Dcfd</th>
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</thead>
<tbody>
<tr>
<td>Haplomene elongation</td>
<td>21</td>
<td>0.953</td>
<td>713.671</td>
<td>0.801</td>
<td>0.038</td>
<td>0.156</td>
<td>0.362</td>
<td>0.096</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heteromene shaft width μm</td>
<td>19</td>
<td>1.051</td>
<td>632.503</td>
<td>0.767</td>
<td>0.801</td>
<td>0.128</td>
<td>0.062</td>
<td>0.4</td>
<td>0.813</td>
<td></td>
</tr>
<tr>
<td>Cnidoband width μm</td>
<td>21</td>
<td>1.595</td>
<td>761.241</td>
<td>0.781</td>
<td>0.723</td>
<td>0.072</td>
<td>0.09</td>
<td>0.31</td>
<td>0.228</td>
<td></td>
</tr>
<tr>
<td>Heteromene shaft free length μm</td>
<td>19</td>
<td>1.649</td>
<td>628.334</td>
<td>0.791</td>
<td>0.402</td>
<td>0.941</td>
<td>0.068</td>
<td>0.575</td>
<td>0.464</td>
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</tr>
<tr>
<td>Heteromene volume μm3</td>
<td>19</td>
<td>2.105</td>
<td>629.217</td>
<td>0.779</td>
<td>0.004</td>
<td>0.39</td>
<td>0.338</td>
<td>0.637</td>
<td>0.392</td>
<td></td>
</tr>
<tr>
<td>Haplomene width μm</td>
<td>21</td>
<td>2.452</td>
<td>766.546</td>
<td>0.779</td>
<td>0.599</td>
<td>0.316</td>
<td>0.791</td>
<td>0.995</td>
<td>0.286</td>
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<tr>
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<td>764.406</td>
<td>0.815</td>
<td>0.791</td>
<td>0.368</td>
<td>0.26</td>
<td>0.963</td>
<td>0.286</td>
<td></td>
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<tr>
<td>Heteromene width μm</td>
<td>19</td>
<td>2.516</td>
<td>634.229</td>
<td>0.805</td>
<td>0.009</td>
<td>0.292</td>
<td>0.208</td>
<td>0.709</td>
<td>0.38</td>
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<tr>
<td>Tentacle width μm</td>
<td>22</td>
<td>2.702</td>
<td>383.12</td>
<td>0.835</td>
<td>0.496</td>
<td>0.344</td>
<td>0.867</td>
<td>0.086</td>
<td>0.444</td>
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</tr>
<tr>
<td>Heteromene to CB</td>
<td>19</td>
<td>0.127 NA</td>
<td>8.111</td>
<td>0.336</td>
<td>0.004</td>
<td>0.068</td>
<td>0.026</td>
<td>0.434</td>
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<td></td>
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<tr>
<td>Haplomene surface area/volume</td>
<td>21</td>
<td>2.280</td>
<td>757.267</td>
<td>0.747</td>
<td>0.563</td>
<td>0.392</td>
<td>0.583</td>
<td>0.927</td>
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</tr>
<tr>
<td>Heteromene elongation</td>
<td>19</td>
<td>0.217</td>
<td>618.621</td>
<td>0.819</td>
<td>0.601</td>
<td>0.012</td>
<td>0.707</td>
<td>0.062</td>
<td>0.715</td>
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<tr>
<td>Total nematocyst volume</td>
<td>22</td>
<td>0.57</td>
<td>378.672</td>
<td>0.809</td>
<td>0.501</td>
<td>0.006</td>
<td>0.088</td>
<td>0.266</td>
<td>0.501</td>
<td></td>
</tr>
<tr>
<td>Heteromene free length μm</td>
<td>19</td>
<td>0.746</td>
<td>627.372</td>
<td>0.811</td>
<td>0.865</td>
<td>0.593</td>
<td>0.156</td>
<td>0.368</td>
<td>0.679</td>
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<tr>
<td>Total hapolomene volume</td>
<td>21</td>
<td>1.281</td>
<td>730.592</td>
<td>0.829</td>
<td>0.452</td>
<td>0.038</td>
<td>0.134</td>
<td>0.096</td>
<td>0.819</td>
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</tr>
<tr>
<td>Cnidoband length μm</td>
<td>21</td>
<td>1.439</td>
<td>763.478</td>
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<td>0.328</td>
<td>0.004</td>
<td>0.11</td>
<td>0.098</td>
<td>0.803</td>
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</tr>
<tr>
<td>Cnidoband free length μm</td>
<td>21</td>
<td>2.219</td>
<td>760.518</td>
<td>0.843</td>
<td>0.35</td>
<td>0.012</td>
<td>0.066</td>
<td>0.05</td>
<td>0.911</td>
<td></td>
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<tr>
<td>Cnidoband colledness</td>
<td>21</td>
<td>2.669</td>
<td>765.921</td>
<td>0.807</td>
<td>0.002</td>
<td>0.008</td>
<td>0.076</td>
<td>0.781</td>
<td></td>
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<tr>
<td>Haplomeone row number</td>
<td>21</td>
<td>4.177</td>
<td>729.95</td>
<td>0.825</td>
<td>0.004</td>
<td>0.002</td>
<td>0.006</td>
<td>0.006</td>
<td>0.346</td>
<td></td>
</tr>
<tr>
<td>Haplomene free length μm</td>
<td>21</td>
<td>5.497</td>
<td>778.011</td>
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<td>0.388</td>
<td>0.032</td>
<td>0.002</td>
<td>0.052</td>
<td>0.306</td>
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<tr>
<td>Heteromene length extension</td>
<td>19</td>
<td>6.17</td>
<td>611.533</td>
<td>0.775</td>
<td>0.0068</td>
<td>0.665</td>
<td>0.124</td>
<td>0.164</td>
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</tr>
</tbody>
</table>

- **Brownian Motion Supported**
- **Single Optimum OU Supported**
- **Multiple Optima OU Supported**
**Figure S1.10:** DAPC for Feeding guilds. Six PCs retained after a-score optimization (100 iterations). Four LDA functions used. Discriminant power on training set: 100%. Prediction posterior distribution heat map in main text Figure 6. Variable contribution (top quartile) calculated by the sum of the LDA variable loadings weighted by the eigenvalue of each LDA.
Figure S1.11: DAPC for copepod presence in the diet. Eight PCs retained after a-score optimization (100 iterations). One LDA functions used. Discriminant power on training set: 95.4%. Grayscale heat map shows the posterior probability distribution of the predictions. Variable contribution (top quartile) calculated by the sum of the LDA variable loadings weighted by the eigenvalue of each LDA.
Figure S1.12: DAPC for fish presence in the diet. 3 PCs retained after a-score optimization (100 iterations). 1 LDA function used. Discriminant power on training set: 68.1%. Grayscale heat map shows the posterior probability distribution of the predictions. Variable contribution (top quartile) calculated by the sum of the LDA variable loadings weighted by the eigenvalue of each LDA.
Figure S1.13: DAPC for large crustacean presence in the diet. Four PCs retained after a-score optimization (100 iterations). One LDA function used. Discriminant power on training set: 81.8%. Grayscale heat map shows the posterior probability distribution of the predictions. Variable contribution (top quartile) calculated by the sum of the LDA variable loadings weighted by the eigenvalue of each LDA.
Table S1.14: Logistic regressions between continuous morphological characters and prey type presences. Ntaxa = number of taxa used in the analyses after removing taxa with missing diet data and inapplicable character states. phyloGLM = Phylogenetic generalized logistic regression model. GLM = Generalized logistic regression model. P = p-value. b = slope. Only cases with significant GLM fits were retained. Cells colored blue indicate phyloGLM p-value < 0.05. Cells colored green indicate GLM p-value < 0.05.

<table>
<thead>
<tr>
<th>Character</th>
<th>Prey type</th>
<th>Ntaxa</th>
<th>phyloGLM AIC</th>
<th>phyloGLM P</th>
<th>GLM AIC</th>
<th>GLM P</th>
<th>GLM b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cnidoband coiledness</td>
<td>Decapod diet</td>
<td>21</td>
<td>23.701</td>
<td>0.029</td>
<td>2.327</td>
<td>21.762</td>
<td>0.016</td>
</tr>
<tr>
<td>Haploneme surface area:volume</td>
<td>Copepod diet</td>
<td>21</td>
<td>19.143</td>
<td>0.017</td>
<td>3.246</td>
<td>17.355</td>
<td>0.017</td>
</tr>
<tr>
<td>Haploneme width µm</td>
<td>Copepod diet</td>
<td>21</td>
<td>18.844</td>
<td>0.017</td>
<td>-3.098</td>
<td>16.997</td>
<td>0.019</td>
</tr>
<tr>
<td>Pedicle width µm</td>
<td>Copepod diet</td>
<td>21</td>
<td>22.182</td>
<td>0.032</td>
<td>1.16</td>
<td>23.723</td>
<td>0.024</td>
</tr>
<tr>
<td>Tentacle width µm</td>
<td>Copepod diet</td>
<td>22</td>
<td>22.038</td>
<td>0.026</td>
<td>-1.543</td>
<td>23.634</td>
<td>0.025</td>
</tr>
<tr>
<td>Cnidoband length µm</td>
<td>Copepod diet</td>
<td>21</td>
<td>23.431</td>
<td>0.042</td>
<td>-0.864</td>
<td>24.178</td>
<td>0.025</td>
</tr>
<tr>
<td>Cnidoband width µm</td>
<td>Copepod diet</td>
<td>21</td>
<td>22.887</td>
<td>0.036</td>
<td>1.545</td>
<td>23.658</td>
<td>0.027</td>
</tr>
<tr>
<td>Heteroneme number</td>
<td>Copepod diet</td>
<td>17</td>
<td>20.52</td>
<td>0.059</td>
<td>-0.718</td>
<td>19.615</td>
<td>0.03</td>
</tr>
<tr>
<td>Total haploneme volume</td>
<td>Copepod diet</td>
<td>21</td>
<td>23.507</td>
<td>0.03</td>
<td>-0.581</td>
<td>25.232</td>
<td>0.031</td>
</tr>
<tr>
<td>Total heteroneme volume</td>
<td>Copepod diet</td>
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<td>17.156</td>
<td>0.032</td>
<td>-0.533</td>
<td>16.369</td>
<td>0.031</td>
</tr>
<tr>
<td>Pedicle width µm</td>
<td>Ostracod diet</td>
<td>21</td>
<td>17.523</td>
<td>0.041</td>
<td>-1.43</td>
<td>15.165</td>
<td>0.035</td>
</tr>
<tr>
<td>Heteroneme shaft free length µm</td>
<td>Copepod diet</td>
<td>19</td>
<td>23.955</td>
<td>0.076</td>
<td>-1.53</td>
<td>23.378</td>
<td>0.04</td>
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<tr>
<td>Haploneme width µm</td>
<td>Fish diet</td>
<td>21</td>
<td>28.118</td>
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<td>1.268</td>
<td>27.551</td>
<td>0.043</td>
</tr>
<tr>
<td>Tentacle width µm</td>
<td>Fish diet</td>
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<td>28.927</td>
<td>0.058</td>
<td>0.804</td>
<td>28.771</td>
<td>0.044</td>
</tr>
<tr>
<td>Haploneme surface area:volume</td>
<td>Fish diet</td>
<td>21</td>
<td>28.258</td>
<td>0.098</td>
<td>-1.329</td>
<td>27.596</td>
<td>0.044</td>
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<tr>
<td>Total haploneme volume</td>
<td>Ostracod diet</td>
<td>21</td>
<td>20.028</td>
<td>0.043</td>
<td>-0.619</td>
<td>17.733</td>
<td>0.046</td>
</tr>
<tr>
<td>Heteroneme volume µm3</td>
<td>Copepod diet</td>
<td>19</td>
<td>24.282</td>
<td>0.091</td>
<td>-0.521</td>
<td>24.297</td>
<td>0.046</td>
</tr>
<tr>
<td>Pedicle width µm</td>
<td>Fish diet</td>
<td>21</td>
<td>28.21</td>
<td>0.074</td>
<td>0.815</td>
<td>27.839</td>
<td>0.049</td>
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</tbody>
</table>
Figure S1.15: Stochastic character mapping of feeding guilds.
Figure S1.16: Rate covariance matrix for the whole tree using all taxa (45 species), transforming inapplicable states to zeroes. Covariances scaled to correlations. All characters estimated simultaneously under Brownian Motion.
Figure S1.17: Rate covariance matrix for the whole tree using only taxa without inapplicable states (24 species). Covariances scaled to correlations. All characters estimated simultaneously under Brownian Motion.
Figure S1.18: Rate covariance matrix for the whole tree using only taxa with diet data (22 species), transforming inapplicable states to zeroes. Covariances scaled to correlations. All characters estimated simultaneously under Brownian Motion.
Figure S1.19: Best models (lowest AIC) supported in a pairwise character rate covariance analysis comparing correlated Brownian Motion models across the five selective regimes. Selective regimes were mapped onto the tree using an ancestral state reconstruction of the feeding guilds. Blank cells represent computationally singular contrasts.
Figure S1.20: Number of taxa used for each pairwise contrast in the variance-covariance analyses, given the number of taxa without inapplicable states.
Figure S1.21: Pairwise estimated rate covariance matrices across the five selective regimes, using only taxa with diet data. Covariances scaled to correlations. Selective regimes were mapped onto the tree (22 species with diet data) using a stochastic mapping of the feeding guilds. Tree is pruned to taxa with no inapplicable states for a given character pair. Not all regimes are represented in all contrasts. Question marks represent computationally singular contrasts.
Figure S1.22: Scaled differences between the regime-specific covariance matrices in Fig. S1.21 and the whole tree covariance matrix.
Figure S1.23: Scaled differences between the regime-specific covariance matrices in S1.21 and the covariance matrices in their preceding regime, the large-crustacean specialist regime (Fig. S1.21C).
CHAPTER 2

The Evolutionary History of Siphonophore Tentilla: Novelties, Convergence, and Integration

Alejandro Damian-Serrano¹⁺, Steven H.D. Haddock², Casey W. Dunn¹

¹ Yale University, Department of Ecology and Evolutionary Biology, 165 Prospect St., New Haven, CT 06520, USA.

² Monterey Bay Aquarium Research Institute, 7700 Sandholdt Rd., Moss Landing, CA 95039, USA.

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Abstract

Siphonophores are free-living predatory colonial hydrozoan cnidarians found in every region of the ocean. Siphonophore tentilla (tentacle side branches) are unique biological structures for prey capture, composed of a complex arrangement of cnidocytes (stinging cells) bearing different types of nematocysts (stinging capsules) and auxiliary structures. Tentilla present an extensive morphological and functional diversity across species. While associations between tentillum form and diet have been reported, the evolutionary history giving rise to this morphological diversity is largely unexplored. Here we examine the evolutionary gains and losses of novel tentillum substructures and nematocyst types on the most recent siphonophore
phylogeny. Tentilla have a precisely coordinated high-speed strike mechanism of synchronous unwinding and nematocyst discharge. Here we characterize the kinematic diversity of this prey capture reaction using high-speed video and find relationships with morphological characters. Since tentillum discharge occurs in synchrony across a broad morphological diversity, we evaluate how phenotypic integration is maintaining character correlations across evolutionary time. We found that the tentillum morphospace has low dimensionality, identified instances of heterochrony and morphological convergence, and generated hypotheses on the diets of understudied siphonophore species. Our findings indicate that siphonophore tentilla are phenotypically integrated structures with a complex evolutionary history leading to a phylogenetically structured diversity of forms which are predictive of kinematic performance and feeding habits.

**Keywords**

Siphonophore, tentilla, nematocysts, character evolution

**Introduction**

Siphonophores have fascinated zoologists for centuries for their extremely subspecialized colonial organization and integration. Today we have a comprehensive taxonomic coverage on the morphological diversity of this group due to the extensive work of siphonophore taxonomists in the past few decades (Pugh, 1983, 2001; Pugh & Harbison, 1986; Pugh & Youngbluth, 1988; Hissmann, 2005; Haddock *et al.* 2005; Dunn *et al.* 2005; Bardi & Marques, 2007; Pugh & Haddock, 2010; Pugh & Baxter, 2014), which has been elegantly synthesized in detailed synopses (Totton & Bargmann, 1965, Mapstone 2014). In
addition, recent advances in phylogenetic analyses of siphonophores (Munro et al. 2018; Damian-Serrano et al. 2021) have provided a macroevolutionary context to interpret this diversity. With these assets in hand, we can now begin to study siphonophores from a comparative perspective across taxa, focusing on the diversity and evolutionary history of specific structures. Here we focus on one such structure: the tentillum. Like many cnidarians, siphonophores bear tentacle side branches (tentilla) with nematocysts (Fig 2.1C-E). But unlike other cnidarians, most siphonophore tentilla are dynamic structures that react to prey encounters by rapidly unfolding the nematocyst battery to slap around the prey (Fig 2.1F). The acrorhagi in some anthozoans can be autonomously reactive (Williams 1991), but nowhere close to the complexity, speed and coordination of tentillum discharge. This maximizes the surface area of contact between the nematocysts and the prey they fire upon.

Siphonophore tentilla are defined as lateral, monostichous (branching on one side only) evaginations of the tentacle (including its gastrovascular lumen), armed with epidermal nematocysts (Totton and Bargmann 1965). The most complex ones are typically composed of (1) a flexible pedicle that provides the connection to the tentacle, (2) an epidermis-derived cnidoband that contains the penetrant and entangling haploneme and heteroneme nematocysts, (3) a rigid mesoglea-derived, collagen-based strand (called ‘elastic strand’ though not very elastic) that runs ascending parallel and attached to the cnidoband with a descending portion detached from the cnidoband but firmly attached to the pedicle and the distal end of the cnidoband, (4) a terminal filament loaded with
adhesive desmoneme and rhopaloneme nematocysts, and (5) an epithelial expansion named ‘involucrum’ that arises from the pedicle and in some cases can completely cover the cnidoband (Fig 2.1D, Fig 2.2). A gastrodermis-derived axial tube is occasionally present in the cnidoband, but is often greatly reduced in the terminal filament (Totton & Bargmann 1965; Mackie et al. 1987; Mapstone 2014). The complexity of these structures varies greatly across siphonophores, yet the evolutionary history of this complexity remains unexplored. Tentillum discharge is typically elicited by adhesion of prey onto the terminal filament. During tentillum discharge, the distal end of the cnidoband shoots out, sometimes directed forward by the involucrum. The proximal end of the cnidoband detaches from the pedicle and slings forward. Nematocysts discharge as they come in contact with the surface of the prey, the proximal heteronemes being the last ones to make contact. The structural integrity of the line connecting the tentacle to the prey for reeling is maintained by the elastic strand attachment to the cnidoband and pedicle (Fig 1F). In addition, siphonophore tentilla present a remarkable diversity of morphologies (Fig 2.2), sizes, and nematocyst complements (Fig 2.3). In Figure 2 we showcase a few of these different morphologies. Our overarching aim is to organize all this phenotypic diversity in a phylogenetic context, and identify the evolutionary processes that generated it.

Nematocysts are unique biological weapons for defense and prey capture exclusive to the phylum Cnidaria. Mariscal (1974) reported that hydrozoans have the largest diversity of nematocyst types among cnidarians. Among them,
siphonophores present the greatest variety of types (Mapstone, 2014), and vary widely across taxa in which and how many types they carry on their tentacles (Fig 2.3). Werner (1965) noted that there are nine types of nematocyst found in siphonophores, of which four, anacrophore rhopalonemes, acrophore rhopalonemes, homotrichous anisorhizas, and birhopaloids, are unique to them. Heteroneme and haploneme nematocysts serve penetrant and entangling functions, while rhopalonemes and desmonemes work by adhering to the surface of the prey. While recent descriptive studies have expanded and confirmed our understanding of this diversity, the evolutionary history of nematocyst type gain and loss in siphonophores remains unexplored. Thus, here we reconstruct the evolution of shifts, gains, and losses of nematocyst types, subtypes, and other major categorical traits that led to the extant diversity we see in siphonophore tentilla.

Distantly related organisms that evolved to feed on similar resources often evolve similar adaptations (Winemiller et al. 2015). In Damian-Serrano et al. (2021), we found strong associations between piscivory and haploneme shape (elongation) across distantly related siphonophore lineages. These associations could have been produced by convergent changes in the adaptive optima of these characters. Here we set out to test this hypothesis using comparative model fitting methods. Analyzing the diversity of morphological states from a phylogenetic perspective allows us to identify the specific evolutionary processes that gave rise to it. Here we fit and compare a variety of macroevolutionary models to morphological measurement data from
siphonophore tentilla to identify instances of neutral divergence, stabilizing selection, changes in the speed of evolution, and convergent evolution.

In Damian-Serrano et al. (2021) we fit discriminant analyses to identify characters that are predictive of feeding guild. These discriminant analyses can be used to generate hypotheses on the diets of ecologically understudied siphonophore species for which we have morphology data. Here we present a Bayesian prediction for the feeding guild of 45 species using the discriminant functions and morphological dataset in Damian-Serrano et al. (2021). As mentioned above, tentilla are far from being passive structures and are in fact violently reactive weapons for prey capture (Mackie et al. 1987; Damian-Serrano et al. 2021; Damian-Serrano 2021). While we now have detailed characterizations of tentillum morphologies across many species, the diversity of dynamic performances and their relationships to the undischarged morphologies have not been examined to date. To address this gap, we set out to record high-speed video of the in vivo discharge dynamics of several siphonophore species at sea (Damian-Serrano 2021), and compare the kinematic attributes to their morphological characters.

In Damian-Serrano et al. (2021), we collected a morphological dataset on siphonophore tentilla and nematocysts using microscopy techniques, and expanded the taxon sampling of the phylogeny to disentangle the evolutionary history. The analyses we carried out led to generalizable insights into the evolution of predatory specialization. The primary findings of that work were that generalists evolved from crustacean-specialist ancestors, and that feeding
specializations were associated with distinct modes of evolution and character integration patterns. The work we present here is complementary to Damian-Serrano et al. (2021), showcasing a far more detailed account of the evolutionary history of tentillum morphology. In this study, we set out to examine seven core questions: (1) what is the evolutionary history of morphological novelties in siphonophore tentilla, (2) what models of evolution best describe the evolutionary history of tentillum and nematocyst characters, (3) are siphonophore tentilla phenotypically integrated, (4) does siphonophore feeding guild explain tentillum morphospace differentiation and disparity, (5) are any of the similarities between the tentilla of siphonophores in the same feeding guild convergent, (6) what prey should we expect understudied siphonophore species to feed upon based on their tentillum morphology, and (7) are there any differences in tentillum discharge performance predicted from tentillum morphology.

Methods

All character data and the phylogeny analyzed here were published in Damian-Serrano et al. (2021) and are available in the associated Dryad repository (Damian-Serrano et al. 2020). Details on the specimen collection, microscopy, and measurements can be found in the aforementioned publication.

To facilitate access, we re-included here the character definitions (S2.15) in the Supporting Information. We also made all the microscopy images available through the Yale Peabody Museum collections website (https://collections.peabody.yale.edu/). These images are flat projections of the z-
stacks, which will be available upon request from the Invertebrate Zoology collection. In this dataset, multiple specimens of each species were measured when possible. For each specimen there was a single measurement taken of each character, giving a greater focus to capturing species and intraspecific specimen diversity than to capturing intra-individual variation. These measurements should not be used for diagnostic nor taxonomic purposes, since they do not capture the full span of intra-individual nor intra-specific variation. Since the goal of these morphological measurements was comparative and not diagnostic, it is not as relevant whether a specimen is representative of the taxon. Moreover, desmoneme, rhopaloneme, and heteroneme sizes are extremely uniform in siphonophore tentilla. For example, in the description of *Sphaeronectes haddocki* (Pugh et al. 2009), they describe the mastigophore size range is 65.4x10.4 - 63.6x9.1 µm; or in Purcell (1984), *Agalma okenii* stenoteles are shown to range between 112.5x20 - 135x24 µm. The error margins on our mean values match the ranges measured in other published studies where multiple nematocysts were measured per specimen. Our evolutionary models and phylogenetic signal calculations incorporate these margins as standard errors. When a homologous nematocyst type had subspecialized into two forms or size classes (such as the isorhizas of cystonects, or the central v.s. edge cnidoband anisorhizas), only one class was consistently measured. We took the largest in the case of cystonect isorhizas, and the central ones in the case of cnidoband anisorhizas, since either class is homologous to the single class in other taxa. Due to the small intra-specific sample sizes, the normality of the
measurement distributions within species could not be ascertained. We log-
transformed all the continuous characters that did not pass Shapiro-Wilks
normality tests across species, and used the ultrametric constrained Bayesian
time tree in all comparative analyses. In the species measured for comparative
analyses, between 3 and 11 specimens were typically measured with the
exception of Agalma clausi, Chuniphyes moserae, Forskalia formosa, F.
tholoides, Kephyes ovata, Physonect sp., and Physophora gilmeri with one
specimen each, and Erenna sirena with two specimens. The number of
specimens included per species was limited by specimen availability, since
finding and collecting certain siphonophore species can be extremely
challenging.

Inapplicable characters were recorded as NA states, and species with
states that could not be measured due to technical limitations were removed
before the analyses. We used the feeding guild categories detailed in (Damian-
Serrano et al. 2021) with one modification: including all Forskalia spp. as
generalists instead of as a single Forskalia species on the tree after a
reinterpretation of the data in (Purcell, 1981). In order to characterize the
evolutionary history of tentillum morphology, we fitted different models
generating the observed data distribution given the phylogeny for each
continuous character using the function fitContinuous in the R package geiger
(Harmon et al. 2007). These models include a non-phylogenetic white-noise
model (WN), a neutral divergence Brownian Motion model (BM), an early-burst
decreasing rate model (EB), and an Ornstein-Uhlenbeck (OU) model with
stabilizing selection around a fitted optimum trait value. In the same way as Damian-Serrano et al. (2021) we then ordered the models by increasing parametric complexity (WN, BM, EB, OU), and compared their corrected Akaike Information Criterion (AICc) scores (Sugiura, 1978). We used the lowest (best) score using a delta cutoff of 2 units to determine significance relative to the next simplest model (S2.10). We calculated model adequacy scores using the R package arbutus (Pennell et al. 2015) (S2.11), and calculated phylogenetic signals in each of the measured characters using Blomberg’s K (Blomberg et al. 2003) (S2.10). To reconstruct the ancestral character states of nematocyst types and other categorical traits, we used stochastic character mapping (SIMMAP) using the package phytools (Revell, 2012).

In order to examine the phenotypic integration in the tentillum, we explored the correlational structure among continuous characters and among their evolutionary histories using principal component analysis (PCA) and phylogenetic PCA (Revell, 2012). Since the character dataset contains gaps due to missing data and inapplicable character states, we carried out these analyses on a subset of species and characters that allowed for the most complete dataset. This was done by removing the terminal filament characters (which are only shared by a small subset of species), and then removing species which had inapplicable states for the remaining characters (apolemiids and cystonects). In addition, we obtained the correlations between the phylogenetic independent contrasts (Felsenstein, 1985) using the package rphylip (Revell and Chamberlain, 2014) accounting for intraspecific variation. Using these contrasts, we identified
multivariate correlational modules among characters. To test and quantify phenotypic integration between these multivariate modules, we used the phylogenetic phenotypic integration test in the package *geomorph* (Adams *et al.* 2016).

When comparing the morphospaces of species in different feeding guilds, we carried out a PCA on the complete character dataset while transforming inapplicable states of absent characters to zeros (i.e. cnidoband length = 0 when no cnidoband is present) to account for similarity based on character presence/absence. Using these principal components, we examined the occupation of the morphospace across species in different feeding guilds using a phylogenetic MANOVA with the package *geiger* (Harmon *et al.* 2007) to assess the variation explained, and a morphological disparity test with the package *geomorph* (Adams *et al.* 2016) to assess differences in the extent occupied by each guild.

In order to detect and evaluate instances of convergent evolution, we used the package *SURFACE* (Ingram and Mahler, 2013). This tool identifies OU regimes and their optima given a tree and character data, and then evaluates where the same regime has appeared independently in different lineages. We applied these analyses to the haploneme nematocyst length and width characters as well as to the most complete dataset without inapplicable character states with 43 species and 186 specimens.

In order to generate hypotheses on the diets of siphonophores using tentillum morphology, we used the discriminant analyses of principal
components (DAPC) (Jombart et al. 2010) trained in (Damian-Serrano et al. 2021). We predict the feeding guilds of species in the dataset for which there are no published feeding observations using their morphological data as inputs, and presenting the predictive output in the form of posterior probabilities for each guild category.

To observe the discharge behavior of different tentilla, we recorded high speed footage (1000-3000 fps) of tentillum and nematocyst discharge by live siphonophore specimens (26 species) using a Phantom Miro 320S camera mounted on a stereoscopic microscope. We mechanically elicited tentillum and nematocyst discharge using a fine metallic pin. We used the Phantom PCC software to analyze the footage. For the 10 species recorded, we measured total cnidoband discharge time (ms), heteroneme filament length (µm), and discharge speeds (mm/s) for cnidoband, heteronemes, haplonemes, and heteroneme shafts when possible (all data and code is available in the Github repository https://github.com/dunnlab/tentilla_organismal/).

Results

Evolutionary history of tentillum morphology – The phylogeny of Damian-Serrano et al. (2021) had revealed for the first time that the genus *Erenna* is the sister to *Stephanomia amphytridis*. *Erenna* and *Stephanomia* bear the largest tentilla among all siphonophores, thus their monophyly indicates that there was a single evolutionary transition to giant tentilla. Siphonophore tentilla range in size from ~30 µm in some *Cordagalma* specimens to 2-4 cm in *Erenna* species,
and up to 8 cm in *Stephanomia amphytridis* (Pugh and Baxter 2014). Most siphonophore tentilla measure between 175 and 1007 µm (1st and 3rd quartiles), with a median of 373 µm. The extreme gain of tentillum size in this newly recognized clade may have important implications for access to large prey size classes such as adult deep-sea fishes.

The buttons on *Physalia* tentacles (see one of our imaged specimens https://collections.peabody.yale.edu/search/Record/YPM-IZ-106663) were not traditionally regarded as tentilla, but Bardi and Marques (2007), Munro et al. (2018), and our own observations confirm that the buttons contain evaginations of the gastrovascular lumen, thus satisfying all the criteria for the definition given in the Introduction. In this light, and given that most Cystonectae bear conspicuous tentilla, we conclude, in agreement with Munro et al. (2018), that tentilla were present in the most recent common ancestor of all siphonophores, and secondarily lost twice, once in *Apolemia* and again in *Bathyphysa conifera*. In order to gain a broad perspective on the evolutionary history of tentilla, we reconstructed the phylogenetic positions of the main categorical character shifts (such as gains and losses of nematocyst types) using stochastic character mapping (S2.1-9) and manual reconstructions. This phylogenetic roadmap of evolutionary novelties is summarized in Fig 2.4.

The phylogenetic position of siphonophores within Hydroidolina has been inconsistent across different studies. In Cartwright et al. (2008), they are reported as sister to Aplanulata, in Cartwright & Nawrocki (2010) they appear to be sister to Leptothecata, while in Kayal et al. (2015) they appear as sister to all
other Hydroidolina. However, in the first two cases the node support for these relationships is weak, and in the last case the results are based on mitochondrial genes only. In Bentlage and Collins (2020), siphonophores appear as sister to the clade composed of Filifera III and Filifera IV, with strong node support. In any case, their affinities are congruent with the assumption that haploneme nematocysts are ancestrally present in siphonophore tentacles since they are present in the tentacles of many other hydrozoans (Mariscal 1974). Haplonemes are toxin-bearing open-ended nematocysts characterized by the lack of a shaft preceding the tubule. Two subtypes are found in siphonophores: the isorhizas of homogeneous tubule width, and the anisorhizas with a slight enlargement of the tubule near the base. In Cystonectae, haplonemes diverged into spherical isorhizas of two size classes. There is one size of haplonemes in Codonophora, which consist of elongated anisorhizas. Haplonemes were likely lost in the tentacles of Apolemia but retained as spherical isorhizas in other Apolemia tissues (Siebert et al. 2013). While heteronemes exist in other tissues of cystonects, they appear in the tentacles of codonophorans exclusively, — as birhopaloids in Apolemia, stenoteles in eucladophoran physonects (except Agalma & Athorybia spp.), and microbasic mastigophores in calycophorans and in the Agalma-Athorybia clade. The four nematocyst types unique to siphonophores appear in two events in the phylogeny (Fig 2.4): birhopaloids arose in the lineage leading to Apolemia (Fig 2.4, branch 11), while rhopalonemes (acrophore and anacrophore) and elongated homotrichous anisorhizas arose in the lineage leading to Tendiculophora (Fig 2.4, branch 3).
Nematocyst type gain and loss is also associated with prey capture functions. For example, the loss of desmonemes and rhopalonemes in piscivorous *Erenna*, retaining solely the penetrant (and venom injecting) anisorhizas and stenoteles (two size classes) is reminiscent of the two size classes of penetrant isorhizas in the fish-specialist cystonects. Moreover, with the gain of anisorhizas, desmonemes, and rhopalonemes, the Tendiculophora gained versatility in entangling and adhesive functions of the cnidoband and terminal filament, which may have allowed their feeding niches to diversify. Part of the effectiveness of calycophoran cnidobands at entangling crustaceans may be attributed to the subspecialization of their heteronemes. These shifted from the ancestral stenotele to the microbasic mastigophore (or eurytele in some species) with a long, barbed shaft armed with many long spines. This heteroneme subtype could be better at interlocking with and adhering to the setae of crustacean legs and antennae. In those species that have a functional terminal filament, the desmonemes and rhopalonemes play a fundamental role in the first stages of adhesion of the prey. In many species, the tugs of the struggling prey on the terminal filament trigger the cnidoband discharge (Mackie et al. 1987 and pers. obs.). The adhesive terminal filament has been lost several times in the Euphysonectae (*Frillagalma, Lychnagalma, Physophora, Erenna*, and some species of *Cordagalma*). In these species, we hypothesize that a different trigger mechanism is at play, possibly involving the prey actively biting or grasping the tentillum or lure.
The clades defined in Damian-Serrano et al. (2021) are characterized by unique evolutionary innovations in their tentilla. The clade Eucladophora (containing Pyrostephidae, Euphysonectae, and Calycophorae) encompasses all of the extant siphonophore species (178 of 186) except Cystonects and Apolemia. Innovations that arose along the lineage leading to this group (Fig 2.4, branch 2) include spatially segregated heteroneme and haploneme nematocysts, terminal filaments, and elastic strands. Pyrostephids (Fig 2.4, branch 7) evolved a unique bifurcation of the axial gastrovascular canal of the tentillum known as the “saccus” (Totton and Bargmann 1965). The lineage leading to the clade Tendiculophora (clade containing Euphysonectae and Calycophorae, see Fig 2.4, branch 3) subsequently acquired further novelties such as the desmonemes and rhopalonemes (acrophore subtype present in euphysonects, anacrophore subtype present in calycophorans) on the terminal filament, which bears no other nematocyst type. These are arranged in sets of 2 parallel rhopalonemes for each single desmoneme (Skaer 1988, 1991). The involucrum is an expansion of the epidermal layer that can cover part or all of the cnidoband (Fig 2.2). This structure, together with differentiated larval tentilla, appeared in the branch leading to Clade A physonects (Fig 2.4, branch 6).

Among Clade A euphysonects, several interesting novelties have arisen. The clade composed of Forskalia and Cordagalma (Fig 2.4, branch 10) lost their involucrum, while Halistemma rubrum had it greatly reduced to a vestigial form. Other Halistemma species have retained their ancestral involucrum (Mapstone 2004; Pugh and Baxter 2014). Frillagalma lost its terminal filament, and gained
an encapsulated cnidoband (cnidosac) followed by their characteristic serial, fluid-filled, vesicles which may act as a lure for prey. The branch leading to the clade comprising *Lychnagalma* and *Physophora* (Fig 2.4, branch 8) similarly encapsulated their cnidoband — losing their terminal filament and shifting the coiled cnidoband shape to a much more convoluted morphology. *Lychnagalma* subsequently gained its characteristic floating medusa-shaped vesicle, while *Physophora* completely inverted the orientation of its cnidoband, placing its heteronemes near the distal end. The clade composed of *Agalma* and *Athorybia* (Fig 2.4, branch 9) modified their terminal filament into two thick terminal filaments with minute rhopaloneme nematocysts separated by a central, fluid-filled ampulla.

Calycophorans evolved novelties such as larger desmonemes at the distal end of the cnidoband, pleated pedicles with a “hood” (here considered homologous to the involucrum) at the proximal end of the tentillum, anacrophore rhopalonemes, and microbasic mastigophore-type heteronemes (Fig 2.4, branch 5). While calycophorans have diversified into most of the extant described siphonophore species (108 of 186), their tentilla have not undergone any major categorical gains or losses since their most recent common ancestor. Nonetheless, they have evolved a wide variation in nematocyst and cnidoband sizes. Ancestrally (and retained in most prayomorphs and hippopodiids), the calycophoran tentillum is recurved where the proximal and distal ends of the cnidoband are close together. Diphyomorph tentilla are slightly different in shape, with straighter cnidobands.
Evolution of tentillum and nematocyst characters – Most (74%) characters present a significant phylogenetic signal, yet only total nematocyst volume, haploneme length, and heteroneme-to-cnidoband length ratio had a phylogenetic signal with K larger than 1. Total nematocyst volume and cnidoband-to-heteroneme length ratio showed strongly conserved phylogenetic signals. The majority (67%) of log-transformed characters were best fitted by BM models, indicating a history of neutral constant divergence. We did not find any relationship between phylogenetic signal and specific model support, where characters with high and low phylogenetic signal were broadly distributed among the best fitted for each model. One-third of the characters measured in Damian-Serrano et al. (2021) did not recover significant support for any of the phylogenetic models tested, indicating they are either not phylogenetically conserved, or they evolved under a complex evolutionary process not represented among the models tested (S2.10). Haploneme nematocyst length was the only character with support for an EB model of decreasing rate of evolution with time. No character had support for a single-optimum OU model (when not informed by feeding guild regime priors). The model adequacy tests (S2.11) indicate that many characters may have a relationship between the states and the rates of evolution (Sasr) not captured in the basic models compared here, accompanied by a signal of unaccounted rate heterogeneity (Cvar). No characters show significant deviations in the overall rate of evolution estimated (Msig). Some characters show a perfect fit (no significant deviations across all metrics) under BM evolution, such as heteroneme elongation, length,
width & volume, haploneme width & SA/V, tentacle width and pedicle width. Haploneme row number and rhopaloneme elongation have significant deviations across four metrics, indicating that BM (best model) is a poor fit. These characters likely evolved under complex models which would require many more data points than we have available to fit with accuracy.

**Phenotypic integration of the tentillum** – Phenotypically integrated structures maintain evolutionary correlations between their constituent characters. Of the phylogenetic correlations among tentillum and nematocyst characters examined here (Fig 2.5a, lower triangle), 81.3% were positive and 18.7% were negative, while of the ordinary correlations (Fig 2.5a, upper triangle) 74.6% were positive and 25.4% were negative. Half (49.9%) of phylogenetic correlations were >0.5, while only 3.6% are < -0.5. Similarly, among the correlations across extant species, 49.1% were >0.5 and only 1.5% were < -0.5. In addition, we found that 13.9% of character pairs had opposing phylogenetic and ordinary correlation coefficients (Fig 2.5B). Just 4% of character pairs have negative phylogenetic and positive ordinary correlations (such as rhopaloneme elongation ~ heteroneme-to-cnidoband length ratio and haploneme elongation, or haploneme elongation ~ heteroneme number), and only 9.9% of character pairs had positive phylogenetic correlation yet negative ordinary correlation (such as heteroneme elongation ~ cnidoband convolution and involucrum length, or rhopaloneme elongation with cnidoband length). These disparities could be explained by Simpson’s paradox (Blyth 1972): the reversal of the sign of a relationship when a third variable (or a phylogenetic topology, as suggested
by Uyeda et al. (2018)) is considered. However, no character pair had correlation coefficient differences larger than 0.64 between ordinary and phylogenetic correlations (heteroneme shaft extension ~ rhopaloneme elongation has a Pearson’s correlation of 0.10 and a phylogenetic correlation of -0.54). Rhopaloneme elongation shows the most incongruence between phylogenetic and ordinary correlations with other characters. We identified four hypothetical modules among the tentillum characters: (1) The tentillum scaffold module including cnidoband length & width, nematocyst row number, pedicle & elastic strand width, tentacle width; (2) the heteroneme module including heteroneme length & width, shafts length & width; (3) the haploneme module including length and width; and (4) the terminal filament module including desmoneme and rhopaloneme length and width. The phenotypic integration test showed significant integration signal between all modules, tentillum and haploneme modules sharing the greatest regression coefficient (S2.12).

In the non-phylogenetic PCA morphospace using only characters derived from simple measurements (Fig 2.7), PC1 (aligned with tentillum and tentacle size) explained 69.3% of the variation in the tentillum morphospace, whereas PC2 (aligned with heteroneme length, heteroneme number, and haploneme arrangement) explained 13.5%. In a phylogenetic PCA, 63% of the evolutionary variation in the morphospace is explained by PC1 (aligned with shifts in tentillum size), while 18% is explained by PC2 (aligned with shifts in heteroneme number and involucrum length).
Evolution of nematocyst shape – The greatest evolutionary change in haploneme nematocyst shape occurred in a single shift towards elongation in the branch leading to Tendiculophora, which contains the majority of described siphonophore species, i.e. all siphonophores other than Cystonects, Apolemia, and Pyrostephidae. There is one secondary return to more oval, less elongated haplonemes in Erenna, but it does not reach the sphericity present in Cystonectae or Pyrostephidae (Fig 2.6). Heteroneme evolution presents a less discrete evolutionary history. Tendiculophora evolved more elongate heteronemes before diversifying, but the difference between theirs and other siphonophores is much smaller than the variation in elongation within Tendiculophora, bearing no phylogenetic signal within this clade. In this clade, the evolution of heteroneme elongation has diverged in both directions, and there is no correlation with haploneme elongation (Fig 2.6), which has remained fairly constant (elongation between 1.5 and 2.5).

Haploneme and heteroneme elongation share 21% of their variance across extant values, and 53% of the variance in their shifts along the branches of the phylogeny. However, much of this correlation is due to the sharp contrast between Pyrostephidae and their sister group Tendiculophora. We searched for regime shifts in the evolution of haploneme nematocyst length and width using SURFACE (Ingram and Mahler 2013). SURFACE identified eight distinct OU regimes in the evolutionary history of haploneme length and width (Fig 2.7A). The different regimes are located in (1) cystonects, (2) most of Tendiculophora,
(3) most diphyomorphs, (4) *Cordagalma ordinatum*, (5) *Stephanomia amphytridis*, (6) pyrostephids, (7) *Diphyes dispar* + *Abylopsis tetragona*, and (8) *Erenna* spp.

*Morphospace occupation* – In order to examine the occupation structure of the morphospace across all siphonophore species in the dataset, we cast a PCA on the data after transforming inapplicable states (due to absence of character) to zeroes. This allows us to accommodate species with many missing characters (such as cystonects or apolemiids), and to account for common absences as morphological similarities. In this ordination, PC1 (aligned with cnidoband size) explains 47.45% of variation and PC2 (aligned with heteroneme volume and involucrum length) explains 16.73% of variation. When superimposing feeding guilds onto the morphospace (Fig 2.8), we find that the morphospaces of each feeding guild are only slightly overlapping in PC1 and PC2. A phylogenetic MANOVA showed that feeding guilds explain 27.63% of variance across extant species (p value < 0.000001), and 20.97% of the variance when accounting for phylogeny, an outcome significantly distinct from the expectation under neutral evolution (p-value = 0.0196). In addition, a morphological disparity analysis accounting for phylogenetic structure shows that the morphospace of fish specialists is significantly broader than that of generalists and other specialists, and the gelatinous morphospace is significantly smaller than that of all other feeding guilds. This is mainly due to the large morphological disparities between cystonects and piscivorous euphysonects, and to the narrow taxonomic diversity of gelatinous specialists.
(Apolemia spp.). There are no significant differences among the morphospace disparities of the other feeding guilds.

Convergent evolution – Convergence is a widespread evolutionary phenomenon where distantly related clades independently evolve similar phenotypes. When the dimensionality of the state space is small as it is in tentilla morphology, convergence is more likely given the same amount of evolutionary change. Using the package SURFACE (Ingram and Mahler 2013), we identified convergence in haploneme nematocyst dimensions and in morphospace position. In Damian-Serrano et al. (2021), we identified haploneme nematocyst shape as one of the traits associated with the convergent evolution of piscivory. Here we find that indeed wider haploneme nematocysts have convergently evolved in the piscivorous cystonects and Erenna spp. (Fig 2.7A). Independent shifts in width are responsible for this convergent loss of elongation. When integrating many traits into a couple principal components, we find two distinct convergences between euphysonects and calycophorans with a reduced prey capture apparatus. Those convergences are between Frillagalma vityazi and calycophorans, and between the extremely small haplonemes in the euphysonect Cordagalma ordinatum and copepod specialist calycophorans such as Sphaeronectes koellikeri (Fig 2.7B).

Functional morphology of tentillum and nematocyst discharge – Tentillum and nematocyst discharge high speed videos and measurements are available in the Supplementary Information. While the sample sizes of these measurements were insufficient to draw reliable statistical results at a
phylogenetic level, we did observe patterns that may be relevant to their functional morphology. For example, cnidoband length is strongly correlated with discharge speed (p value = 0.0002). This explains much of the considerable difference between euphysonect and calycophoran tentilla discharge speeds (average discharge speeds: 225.0 mm/s and 41.8 mm/s respectively; t-test p value = 0.011), since the euphysonects have larger tentilla than the calycophorans among the species recorded. In addition, we observed that calycophoran haploneme tubules fire faster than those of euphysonects (t-test p value = 0.001). Haploneme nematocysts discharge 2.8x faster than heteroneme nematocysts (t-test p value = 0.0012). Finally, while all nematocyst evert a twisted filament in a subtle solenoid motion, we observed that the stenotele filament of the Euphysonectae discharges in a distinctively coiled solenoid fashion that “drills” itself like a corkscrew through the medium it penetrates as it everts. This is particularly conspicuous in the stenoteles of *Frillagalma vityazi* (Damian-Serrano 2021), and is very different from how typical nematocysts, such as *Hydra* stenoteles, evert (Holstein and Tardent 1984, Nüchter et al. 2006).

*Generating dietary hypotheses using tentillum morphology* – For many siphonophore species, no feeding observations have yet been published. To help bridge this gap of knowledge, we generated hypotheses about the diets of these understudied siphonophores based on their known tentacle morphology using one of the linear discriminant analyses of principal components (DAPC) fitted in Damian-Serrano et al. (2021). This provides concrete predictions to be tested in future work and helps extrapolate our findings to many poorly known
species that are extremely difficult to collect and observe. The discriminant analysis for feeding guild (7 principal components, 4 discriminants) produced 100% discrimination, and the highest loading contributions were found for the characters (ordered from highest to lowest): Involucrum length, heteroneme volume, heteroneme number, total heteroneme volume, tentacle width, heteroneme length, total nematocyst volume, and heteroneme width. We used the predictions from this discriminant function to generate hypotheses about the feeding guild of 45 species in the morphological dataset (Fig 2.10). This extrapolation predicts that two other Apolemia species are gelatinous prey specialists like Apolemia rubriversa, and predicts that Erenna laciniata is a fish specialist like Erenna richardi. When predicting soft- and hard-bodied prey specialization, the DAPC achieved 90.9% discrimination success, only marginally confounding hard-bodied specialists with generalists (S2.13). The main characters driving this discrimination are involucrum length, heteroneme number, heteroneme volume, tentacle width, total nematocyst volume, total haploneme volume, elastic strand width, and heteroneme length.

Discussion

On the evolution of tentillum morphology – The evolutionary history of siphonophore tentilla shows three major transition points which have structured the morphological diversity we see today. First, the earliest split between codonophorans and cystonects divides lineages with penetrating isorhizas (cystonects) from those which utilize heteronemes (codonophorans) for prey capture. Second, the split between apolemiids and eucladophorans divided the
simple-tentacled *Apolemia* from the lineage that evolved composite tentilla with heteronemes and haplonemes. Finally, the branch leading to tendiculophorans fostered innovations such as the elastic strands and the terminal filament nematocysts which produced the most complex tentillum structures and greatest morphological diversity we observe among siphonophores.

Siphonophore tentilla are extraordinarily complex and highly diverse. Our analyses show, however, that the siphonophore tentillum morphospace actually has a fairly low extant dimensionality due to having an evolutionary history with many synchronous, correlated changes. This can be due to many causes including structural constraints, developmental constraints, or selection that reduces the viable state space. Though siphonophore development has not been extensively studied, what is known suggests that developmental constraints alone could not explain the highly correlated evolutionary changes we observe. The nematocysts that arm the tentillum are developed in a completely separate region of the gastrozooid (Carré, 1972) and then migrate and assemble within the tentillum later on (Skaer, 1988). This lack of proximity and physical independence of development between traits makes developmental constraints unlikely. Surprisingly, many of the strong correlations we find are between nematocyst and structural tentillum characters. Therefore, we hypothesize the genetic correlations and phenotypic integration between tentillum and nematocyst characters are maintained through natural selection on separate regulatory networks, out of the necessity to work together and meet the spatial, mechanical, and functional constraints of their prey capture behavior.
In order to adequately test these hypotheses, future work would need to study the genetic mechanisms underlying the development of tentilla from a comparative, evolutionary approach. Fortunately, the unique biology of siphonophore tentacles displays the full developmental sequence of tentilla along each tentacle, making siphonophores an ideal system for the comparative study of development.

In Damian-Serrano et al. (2021) we examined the covariance terms in the multivariate rate matrix for the evolution of tentillum and nematocyst characters. Building on this work, here we examine the correlations among the trait values while accounting for phylogenetic structure. The results for both analyses indicate that tentilla are not only phenotypically integrated (with widespread evolutionary correlations across structures) but also show patterns of evolutionary modularity, where different sets of characters appear to evolve in stronger correlations among each other than with other characters (Wagner, 1996). This may be indicative of the underlying genetic and developmental dependencies among closely-related nematocyst types and other homologous structures. In addition, these evolutionary modules point to hypothetical functional modules. For example, the coiling degree of the cnidoband and the extent of the involucrum have correlated rates of evolution, while the involucrum may help direct the whiplash of the uncoiling cnidoband distally (towards the prey). The evolutionary innovation of the Tendiculophora tentilla with shooting cnidobands and modular regions may have facilitated further dietary diversification. A specific instance of this may have been the access to the
abundant small crustacean prey such as copepods. The rapid darting escape response of copepods may preclude their capture in siphonophores without shooting cnidobands. Dietary diversification may be related to the far greater number of species in Tendicilophora than its relatives Cystonectae, Apolemiidae, and Pyrostephidae.

**Heterochrony and convergence in the evolution of tentilla with diet** - In addition to identifying shifts in prey type, Damian-Serrano et al. (2021) revealed the specific morphological changes in the prey capture apparatus associated with these changes. Copepod-specialized diets have evolved independently in *Cordagalma* and some calycophorans. These evolutionary transitions happened together with transitions to smaller tentilla with fewer and smaller cnidoband nematocysts. We found that these morphological transitions evolved convergently in these taxa. Tentilla are expensive single-use structures (Mackie *et al.* 1987), therefore we would expect that specialization in small prey would beget reductions in the size of the prey capture apparatus to the minimum required for the ecological performance. Such a reduction in size would require extremely fast rates of trait evolution in an ordinary scenario. However, *Cordagalma*’s tentilla strongly resemble the larval tentilla (only found in the first-budded feeding body of the colony) of their sister genus *Forskalia*. This indicates that the evolution of *Cordagalma* tentilla could be a case of paedomorphic heterochrony associated with predatory specialization on smaller prey. This developmental shift may have provided a shortcut for the evolution of a smaller prey capture apparatus.
Our work identifies yet another novel example of convergent evolution. The region of the tentillum morphospace occupied by calycophorans was independently (and more recently) occupied by the physonect Frillagalma vityazi. Like calycophorans, Frillagalma tentilla have small C-shaped cnidobands with a few rows of anisorhizas. Unlike calycophorans, they lack paired elongate microbasic mastigophores. Instead, they bear exactly three oval stenoteles, and their cnidobands are followed by a branched vesicle, unique to this genus. Their tentillum morphology is very different from that of other related physonects, which tend to have long, coiled, cnidobands with many paired oval stenoteles. Our SURFACE analysis clearly indicates a regime convergence in the cnidoband morphospace between Frillagalma and calycophorans (Fig 2.9B). Most studies on calycophoran diets have reported their prey to consist primarily of small crustaceans, such as copepods or ostracods (Purcell, 1981, 1984). The diet of Frillagalma vityazi is unknown, but this morphological convergence suggests that they evolved to capture similar kinds of prey. The DAPCs in Damian-Serrano et al. (2021) predict that Frillagalma has a generalist niche with both soft and hard-bodied prey, including copepods.

**Evolution of nematocyst shape** – A remarkable feature of siphonophore haplonemes is that they are outliers to all other Medusozoa in their surface area to volume relationships, deviating significantly from sphericity (Thomason, 1988). This suggests a different mechanism for their discharge that could be more reliant on capsule tension than on osmotic potentials (Carré & Carré, 1980), and strong selection for efficient nematocyst packing in the cnidoband (Thomason,
1988; Skaer, 1988). Our results show that Codonophora underwent a shift towards elongation and Cystonectae towards sphericity, assuming the common ancestor had an intermediate state. Since we know that the haplonemes of other hydrozoan outgroups are generally spheroidal, it is more parsimonious to assume that cystonects are simply retaining this ancestral state. We observe a return to more rounded (ancestral) haplonemes in *Erenna*, concurrent with a secondary gain of a piscivorous trophic niche, like that exhibited by cystonects. Our SURFACE analysis shows that this transition to roundness is convergent with the regime occupied by cystonects (Fig 2.9A). Purcell (1984) showed that haplonemes have a penetrating function as isorhizas in cystonects and an adhesive function as anisorhizas in Tendiculophora. It is no coincidence that the two clades that have converged to feed primarily on fish have also converged morphologically toward more compact haplonemes. Isorhizas in cystonects are known to penetrate the skin of fish during prey capture, and to deliver the toxins that aid in paralysis and digestion (Hessinger, 1988). *Erenna*’s anisorhizas are also able to penetrate human skin and deliver a painful sting (Pugh, 2001 and pers. obs.), a common feature of piscivorous cnidarians like the Portuguese man-o-war or box jellies.

The implications of these results for the evolution of nematocyst function are that an innovation in the discharge mechanism of haplonemes may have occurred during the main shift to elongation. Elongate nematocysts can be tightly packed into cnidobands. We hypothesize this may be a Tendiculophora lineage-specific adaptation to packing more nematocysts into a limited tentillum
space, as suggested by Skaer (1988). Thomason (1988) hypothesized that smaller, more spherical nematocysts, with a lower surface area to volume ratio, are more efficient in osmotic-driven discharge and thus have more power for skin penetration. The elongated haplonemes of crustacean-eating Tendiculophora have never been observed penetrating their crustacean prey (Purcell, 1984), and are hypothesized to entangle the prey through adhesion of the abundant spines to the exoskeletal surfaces and appendages. Entangling requires less acceleration and power during discharge than penetration, as it does not rely on point pressure. In fish-eating cystonects and *Erenna* species, the haplonemes are much less elongated and very effective at penetration, in congruence with the osmotic discharge hypothesis. Tendiculophora, composed of the clades Euphysonectae and Calycophorae, includes the majority of siphonophore species. Within these clades are the most abundant siphonophore species, and a greater morphological and ecological diversity is found. We hypothesize that this packing-efficient haploneme morphology may have also been a key innovation leading to the diversification of this clade. However, other characters that shifted concurrently in the lineage leading to this clade could have been equally responsible for their extant diversity.

All cnidarians are characterized by bearing nematocysts used primarily for defense and prey capture. The patterns we revealed in siphonophores may reflect more general patterns in the evolution of nematocysts across cnidarians. Siphonophore tentilla are unique in many ways, but also bear similarities to other structures found in other cnidarians. For example, many anemones bear
specialized, nematocyst-laden filaments named acontia, which they use for
defense and territorial competition (Shick 2012). These filaments also carry
tightly packed, extremely elongated nematocysts (mastigophores and isorhizas).
This extreme elongation may have also arisen as an adaptation to pack a higher
number of nematocysts in a small space. While siphonophore nematocyst
elongation may be an outlier among Medusozoa, similar morphologies can be
commonly found across Actiniaria and Hexacorallia. These morphological shifts
may also involve changes to the discharge mechanisms and nematocyst
function. Answering this question requires further research on the discharge
mechanics of nematocysts beyond model organisms like _Hydra_. As shown in
Figure 3, siphonophores bear a large variety of nematocyst types and subtypes.
Different heteroneme subtypes vary widely in shaft and filament complexity,
-ranging from the simplest mastigophores to 3-spined stenoteles or double-
bulged birhopaloids. However, the functional differences between these
subtypes is still poorly known. Further research is necessary to fully
comprehend the evolutionary and ecological implications of these transitions in
nematocyst subtype.

_Generating hypotheses on siphonophore feeding ecology –_ One
motivation for our research is to understand the links between prey-capture
tools and diets so we can generate hypotheses about the diets of predators
based on morphological characteristics. Indeed, our discriminant analyses were
able to distinguish between different siphonophore diets based on
morphological characters alone. The models produced by these analyses
generated testable predictions about the diets of many species for which we only have morphological data of their tentacles. For example, the unique tentilla morphology of *Frillagalma* is predicted to render a generalist diet, or one of the undescribed deep-sea physonect species examined is predicted to be a fish specialist, which if true would show a third instance of independently evolved piscivory. While the limited dataset used here is informative for generating tentative hypotheses, the empirical dietary data are still scarce and insufficient to cast robust predictions. This reveals the need to extensively characterize siphonophore diets and feeding habits. In future work, we will test these ecological hypotheses and validate these models by directly characterizing the diets and feeding habits of some of those siphonophore species. Predicting diet using morphology is a powerful tool to reconstruct food web topologies from community composition alone. In many of the ecological models found in the literature, interactions among the oceanic zooplankton have been treated as a black box (Mitra, 2009). The ability to predict such interactions, including those of siphonophores and their prey, will enhance the taxonomic resolution of nutrient-flow models constructed from plankton community composition data.

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Figure 2.1. Siphonophore anatomy. A - *Nanomia* sp. siphonophore colony (photo by Catriona Munro). B, C - Illustration of a *Nanomia* colony, gastrozooid, and tentacle closeup (by Freya Goetz). D - *Nanomia* sp. Tentillum illustration and main parts. E - Differential interference contrast micrograph of the tentillum illustrated in D (Specimen: YPM IZ 106704). Figure reproduced from Damian-Serrano et al. 2021 with permission. F. Action strip showing the behavior of tentilla during prey capture, illustrated by Riley Thompson.
Figure 2.2. Tentillum diversity. The illustrations delineate the pedicle, involucrum, cnidoband, elastic strands, and terminal structures. Heteroneme nematocysts (stenoteles in C,E,F,G and mastigophores in H,I) are only depicted for some species. A - *Erenna laciniata* bears giant tentilla with a flicking bioluminescent lure, 10x. B - *Lychnagalma utricularia* has a large convoluted cnidoband and unique buoyant medusa-shaped vesicle, 10x. C - *Agalma elegans* has dual terminal filaments and ampulla, 10x. D - *Resomia ornicephala* presents a zig-zag cnidoband and flap-shaped fluorescent involucrum, 10x. E - *Frillagalma vityazi* has a minute encapsulated cnidoband with just three stenoteles, 20x. F - *Bargmannia amoena* presents a simple tentillum with massive round stenoteles, 10x. G - *Cordagalma* sp. has a greatly reduced tentillum with long terminal cnidocils (nematocyst-triggering sensory cilia), reproduced from Carré 1968. H - *Lilyopsis fluoracantha* tentilla bear a pleated cnidoband flanked by long mastigophores, 20x. I - *Abylopsis tetragona* exemplifies a typical calycophorans tentillum with desmonemes clustered at the distal end of the cnidoband, 20x.
Figure 2.3. Phylogenetic distribution of nematocyst types, subtypes, functions, and locations in the tentacle across the major siphonophore clades. Illustrations reproduced with permission from Mapstone (2014). Undischarged capsules to the left, discharged to the right. Agalmatidae* here refers only to the genera Agalma, Athorybia, Halistemma, and Nanomia.
Figure 2.4. Siphonophore cladogram with the main categorical character gains (green) and losses (red) mapped. Some branch lengths were modified from the Bayesian chronogram to improve readability. The main visually distinguishable tentillum types are sketched next to the species that bear them, showing the location and arrangement of the main characters. In large, complex-shaped euphysonectententilla, haplonemes were omitted for simplification. The hypothesized phylogenetic placement of the rhizophysid *Bathyphysa conifera*, for which no molecular data are yet available, was added manually (dashed line). Some branches have been numbered 1-11 to facilitate their reference in the text.
Figure 2.5. A. Correlogram showing strength of ordinary (upper triangle) and phylogenetic (lower triangle) correlations between characters. Both size and color of the circles indicate the strength of the correlation ($R^2$). B. Scatterplot of phylogenetic correlation against ordinary correlation showing a strong linear relationship ($R^2 = 0.92$, 95% confidence between 0.90 and 0.93). Light red and blue boxes indicate congruent negative and positive correlations respectively. Darker red and blue boxes indicate strong ($<-0.5$ or $>0.5$) negative and positive correlation coefficients respectively.
Figure 2.6. Phylomorphospace showing haploneme and heteroneme elongation (log scaled). Orange area delimits rod-shaped haplonemes, the blue area covers oval and round-shaped haplonemes. Smaller dots and lines represent phylogenetic relationships and ancestral states of internal nodes under BM. Species nodes in red lack either haplonemes or heteronemes, and their values are projected onto the axis of the nematocyst type they bear. Cystonects have no tentacle heteronemes and are projected onto the haploneme axis. Apolemiids have no tentacle haplonemes and are projected onto the heteroneme axis. Silhouettes on the right side represent haploneme shapes along the y axis.
Figure 2.7. SURFACE plots showing convergent evolutionary regimes modelled under OU for (A) haploneme nematocyst length & width, and (B) for PC1 & PC2 of all continuous characters with the exception of terminal filament nematocysts, and removing taxa with inapplicable character states. Node numbers on the tree label different regimes, regimes of the same color are identified as convergent. Small circles on the scatterplots indicate species values, large circles indicate the average position of the OU optima ($\theta$) for a given combination of convergent regimes.
Figure 2.8. PCA of the simple-measurement continuous characters principal components, excluding ratios and composite characters. A. Variance explained by each variable in the PC1-PC2 plane. Axis labels include the phylogenetic signal (K) for each component and p-value. B. Phylogenetic relationships between the species points and reconstructed ancestors distributed in that same space.
Figure 2.9. Phylomorphospace showing PC1 and PC2 from a PCA of continuous morphological characters with inapplicable states transformed to zeroes, overlapped with polygons conservatively defining the space occupied by each feeding guild. Lines between species coordinates show the phylogenetic relationships between them. Grey points indicate species with no feeding guild information.
Figure 2.10. Hypothetical feeding guilds for siphonophore species predicted by a 6 PCA DAPC. Cell darkness indicates the posterior probability of belonging to each guild. The training dataset was transformed so inapplicable states are computed as zeroes. Species are sorted and colored according to their predicted feeding guild.
Figure S2.1: Stochastic character mapping of tentilla presence/absence.
Figure S2.2: Stochastic character mapping of cnidoband proximal heteronemes presence/absence.
Figure S2.3: Stochastic character mapping of terminal filament nematocysts (desmonemes & rhopalonemes) presence/absence.
Figure S2.4: Stochastic character mapping of actively-discharging cnidobands presence/absence.
Figure S2.5: Stochastic character mapping of elastic strands presence/absence.
Figure S2.6: Stochastic character mapping of cnidoband distal desmonemes presence/absence.
Figure S2.7: Stochastic character mapping of coiled cnidoband phenotype presence/absence.
Figure S2.8: Stochastic character mapping of heteroneme subtype.
Figure S2.9: Stochastic character mapping of haploneme subtype.
Table S2.10: Model support (delta AICc), phylogenetic signal (Blomberg’s K), and phylogenetic signal permutation test p-value for each continuous character. Ntaxa = Number of taxa used in the analyses after removing those where the character state is inapplicable or the data is missing.

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<th>Non-phylogenetic dAIC</th>
<th>BM dAIC</th>
<th>EB dAIC</th>
<th>OU dAIC</th>
<th>K</th>
<th>p-value</th>
<th>Ntaxa</th>
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<td>0.082</td>
<td>0.827</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Desmoneme width μm</td>
<td>3.96</td>
<td>2.46</td>
<td>2.121</td>
<td>0.553</td>
<td>0.004</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Cnidoband length μm</td>
<td>4.094</td>
<td>1.911</td>
<td>2.315</td>
<td>0.321</td>
<td>0.015</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Heteroneme number</td>
<td>4.262</td>
<td>2.362</td>
<td>2.219</td>
<td>0.866</td>
<td>0.001</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Heteroneme shaft free length μm</td>
<td>4.553</td>
<td>2.324</td>
<td>2.321</td>
<td>0.331</td>
<td>0.126</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Rhopaloneme length μm</td>
<td>5.599</td>
<td>2.46</td>
<td>2.457</td>
<td>0.589</td>
<td>0.001</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Heteroneme/Cnidoband length</td>
<td>5.671</td>
<td>1.862</td>
<td>2.342</td>
<td>1.068</td>
<td>0.001</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Pedicle width μm</td>
<td>6.566</td>
<td>2.253</td>
<td>2.315</td>
<td>0.541</td>
<td>0.001</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Haploneme width μm</td>
<td>7.495</td>
<td>2.218</td>
<td>2.304</td>
<td>0.553</td>
<td>0.001</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Heteroneme width μm</td>
<td>7.53</td>
<td>2.324</td>
<td>1.847</td>
<td>0.502</td>
<td>0.001</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Heteroneme elongation</td>
<td>14.169</td>
<td>0.819</td>
<td>2.23</td>
<td>0.508</td>
<td>0.001</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Haploneme row number</td>
<td>19.566</td>
<td>2.114</td>
<td>2.315</td>
<td>0.442</td>
<td>0.001</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Total nematoctyl volume μm3</td>
<td>21.007</td>
<td>2.213</td>
<td>2.292</td>
<td>1.3</td>
<td>0.001</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Cnidoband width μm</td>
<td>5.69</td>
<td>0.307</td>
<td>2.623</td>
<td>0.374</td>
<td>0.001</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Haploneme free length μm</td>
<td>12.337</td>
<td>7.125</td>
<td>9.439</td>
<td>1.079</td>
<td>0.001</td>
<td>0.43</td>
<td></td>
</tr>
</tbody>
</table>
Table S2.11: P-values of the model adequacy score tests for the best model supported for each morphological character. Cvar = coefficient of variation of the absolute value of the contrasts. Svar = Slope of a linear model fitted to the absolute value of the contrasts against their expected variances. Sasr = slope of the contrasts against the ancestral state inferred at each corresponding node. Shgt = slope of the contrasts against node depth. Dcfd = Kolmolgorov-Smirnov D-statistic comparing contrasts to a normal distribution with SD equal to the root of the mean of squared contrasts. P-values < 0.05 were highlighted in grey, indicating significant deviations between the model and the observed data.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Best model</th>
<th>Msig</th>
<th>Cvar</th>
<th>Svar</th>
<th>Sasr</th>
<th>Shgt</th>
<th>Dcfd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmoneme length μm</td>
<td>WN</td>
<td>0.889</td>
<td>0.224</td>
<td>0.084</td>
<td>0.32</td>
<td>0.146</td>
<td>0</td>
</tr>
<tr>
<td>Heteroneme shaft extension</td>
<td>WN</td>
<td>0.861</td>
<td>0</td>
<td>0.577</td>
<td>0</td>
<td>0.533</td>
<td>0.042</td>
</tr>
<tr>
<td>Total heteroneme volume</td>
<td>WN</td>
<td>0.895</td>
<td>0.577</td>
<td>0.006</td>
<td>0.026</td>
<td>0.078</td>
<td>0.603</td>
</tr>
<tr>
<td>Rhopaloneme width μm</td>
<td>WN</td>
<td>0.823</td>
<td>0.42</td>
<td>0.182</td>
<td>0.014</td>
<td>0.531</td>
<td>0.006</td>
</tr>
<tr>
<td>Haploneme free length μm</td>
<td>EB</td>
<td>0.841</td>
<td>0.052</td>
<td>0.036</td>
<td>0.168</td>
<td>0.226</td>
<td>0.843</td>
</tr>
<tr>
<td>Heteroneme volume μm3</td>
<td>BM</td>
<td>0.855</td>
<td>0.731</td>
<td>0.228</td>
<td>0.897</td>
<td>0.775</td>
<td>0.104</td>
</tr>
<tr>
<td>Involucrum length μm</td>
<td>BM</td>
<td>0.839</td>
<td>0.01</td>
<td>0.018</td>
<td>0.116</td>
<td>0.09</td>
<td>0.987</td>
</tr>
<tr>
<td>Tentacle width μm</td>
<td>BM</td>
<td>0.817</td>
<td>0.841</td>
<td>0.402</td>
<td>0.386</td>
<td>0.785</td>
<td>0.48</td>
</tr>
<tr>
<td>Cnidoband coiledness</td>
<td>BM</td>
<td>0.873</td>
<td>0</td>
<td>0.028</td>
<td>0.016</td>
<td>0.144</td>
<td>0.41</td>
</tr>
<tr>
<td>Total haploneme volume</td>
<td>BM</td>
<td>0.807</td>
<td>0.228</td>
<td>0.004</td>
<td>0.006</td>
<td>0.024</td>
<td>0.398</td>
</tr>
<tr>
<td>Cnidoband free length μm</td>
<td>BM</td>
<td>0.825</td>
<td>0.076</td>
<td>0.002</td>
<td>0</td>
<td>0.006</td>
<td>0.681</td>
</tr>
<tr>
<td>Haploneme free length μm</td>
<td>BM</td>
<td>0.859</td>
<td>0.392</td>
<td>0.386</td>
<td>0.056</td>
<td>0.591</td>
<td>0.284</td>
</tr>
<tr>
<td>Rhopaloneme elongation</td>
<td>BM</td>
<td>0.873</td>
<td>0.022</td>
<td>0.006</td>
<td>0.004</td>
<td>0.048</td>
<td>0.104</td>
</tr>
<tr>
<td>Desmoneme width μm</td>
<td>BM</td>
<td>0.813</td>
<td>0.877</td>
<td>0.531</td>
<td>0.014</td>
<td>0.941</td>
<td>0.014</td>
</tr>
<tr>
<td>Cnidoband length μm</td>
<td>BM</td>
<td>0.829</td>
<td>0.096</td>
<td>0</td>
<td>0</td>
<td>0.004</td>
<td>0.901</td>
</tr>
<tr>
<td>Heteroneme number</td>
<td>BM</td>
<td>0.823</td>
<td>0.312</td>
<td>0</td>
<td>0.004</td>
<td>0.02</td>
<td>0.869</td>
</tr>
<tr>
<td>Heteroneme shaft free length μm</td>
<td>BM</td>
<td>0.877</td>
<td>0.468</td>
<td>0.565</td>
<td>0.034</td>
<td>0.841</td>
<td>0.851</td>
</tr>
<tr>
<td>Rhopaloneme length μm</td>
<td>BM</td>
<td>0.829</td>
<td>0.525</td>
<td>0.547</td>
<td>0.01</td>
<td>0.917</td>
<td>0.08</td>
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<tr>
<td>Heteroneme/cnidoband length</td>
<td>BM</td>
<td>0.839</td>
<td>0.01</td>
<td>0</td>
<td>0.004</td>
<td>0.008</td>
<td>0.715</td>
</tr>
<tr>
<td>Cnidoband width μm</td>
<td>BM</td>
<td>0.907</td>
<td>0.977</td>
<td>0</td>
<td>0.002</td>
<td>0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>Pedicle width μm</td>
<td>BM</td>
<td>0.817</td>
<td>0.931</td>
<td>0.476</td>
<td>0.088</td>
<td>0.969</td>
<td>0.813</td>
</tr>
<tr>
<td>Haploneme width μm</td>
<td>BM</td>
<td>0.881</td>
<td>0.805</td>
<td>0.42</td>
<td>0.294</td>
<td>0.511</td>
<td>0.15</td>
</tr>
<tr>
<td>Heteroneme width μm</td>
<td>BM</td>
<td>0.849</td>
<td>0.142</td>
<td>0.156</td>
<td>0.356</td>
<td>0.819</td>
<td>0.278</td>
</tr>
<tr>
<td>Heteroneme elongation</td>
<td>BM</td>
<td>0.933</td>
<td>0.094</td>
<td>0.07</td>
<td>0.581</td>
<td>0.791</td>
<td>0.777</td>
</tr>
<tr>
<td>Haploneme row number</td>
<td>BM</td>
<td>0.863</td>
<td>0</td>
<td>0.002</td>
<td>0.004</td>
<td>0.008</td>
<td>0.012</td>
</tr>
<tr>
<td>Total nematocyst volume</td>
<td>BM</td>
<td>0.809</td>
<td>0.521</td>
<td>0.024</td>
<td>0.016</td>
<td>0.198</td>
<td>0.837</td>
</tr>
<tr>
<td>Haploneme surface: volume</td>
<td>BM</td>
<td>0.831</td>
<td>0.945</td>
<td>0.130</td>
<td>0.503</td>
<td>0.426</td>
<td>0.170</td>
</tr>
</tbody>
</table>
Figure S2.12: Heatmap showing the phenotypic integration between character modules accounting for phylogeny. Text in cells shows p-values. Color indicates the partial least squares (PLS) multivariate correlation coefficients.
Figure S2.13: DAPC for soft-bodied vs. hard bodied prey specialization. Six PCs retained after a-score optimization (100 iterations). Two LDA functions used. Discriminant power on training set: 90.9%. Grayscale heat map shows the posterior probability distribution of the predictions. Variable contribution (top quartile) calculated by the sum of the LDA variable loadings weighted by the eigenvalue of each LDA.
Figure S2.14: Heatmap summarizing the morphological diversity measured in Damian-Serrano et al. 2020 for 96 species of siphonophores clustered by similarity (raw data published in Damian-Serrano (2020)). Missing values from absent characters presented as dark grey cells, missing values produced from technical difficulties presented as white cells. Values scaled by character.
Table S2.15: Character definitions used in Damian-Serrano et al. (2021).

### S1.1) Definitions of homologous structures used throughout this work.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haploneure</td>
<td>Nematocyst with no shaft</td>
</tr>
<tr>
<td>Heteroneure</td>
<td>Nematocyst with a distinct shaft</td>
</tr>
<tr>
<td>Desmonemeure</td>
<td>Small oval-shaped adhesive nematocyst with thick coiled tubule</td>
</tr>
<tr>
<td>Rhopaloneure</td>
<td>Small rosette-like nematocyst found on the terminal filament</td>
</tr>
<tr>
<td>Terminal filament</td>
<td>Distal extension of the tentillum beyond the cristaribd</td>
</tr>
<tr>
<td>Cristaribd</td>
<td>Distinct pouch of nematocyst on the dorsal side of the tentillum</td>
</tr>
<tr>
<td>Tentacle</td>
<td>Tubular projection from the gastrostomial bodywater</td>
</tr>
<tr>
<td>Tentillum</td>
<td>Evenly spaced dorsal evagination of the tentacle carrying ordered and functional nematocysts</td>
</tr>
<tr>
<td>Involution</td>
<td>Extension of the pedicle covering part of the cristaribd</td>
</tr>
<tr>
<td>Pedicle</td>
<td>Proximal region of the tentillum between the cristaibd and the tentacle</td>
</tr>
<tr>
<td>Elastic strand</td>
<td>Mesoglea derived collagenous double strand underlying the cristaibd of some siphonophores</td>
</tr>
</tbody>
</table>

### S1.2) Definitions of the continuous morphological and kinematic characters measured.

<table>
<thead>
<tr>
<th>Character</th>
<th>Definition</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cristallaria length</td>
<td>Distance from the base to the tip of the cristallaria in natural position</td>
<td>micrometers</td>
</tr>
<tr>
<td>Cristallaria free length</td>
<td>Distance from the base to the tip of the cristallaria when stretched straight</td>
<td>micrometers</td>
</tr>
<tr>
<td>Cristallaria width</td>
<td>Diameter of the cristallaria on the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Involution length</td>
<td>Length of the involution from the base of the cristallaria to its most distal extent</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneure length</td>
<td>Length of the heteroneure</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneure width</td>
<td>Diameter of the heteroneure at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneure shaft length</td>
<td>Length of the heteroneure shaft</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneure shaft width</td>
<td>Width of the heteroneure shaft</td>
<td>micrometers</td>
</tr>
<tr>
<td>Heteroneure number</td>
<td>Number of heteroneures in each tentillum (# in each row*2)</td>
<td>micrometers</td>
</tr>
<tr>
<td>Haplooneure length</td>
<td>Length of the haplooneure</td>
<td>micrometers</td>
</tr>
<tr>
<td>Haplooneure width</td>
<td>Diameter of the haplooneure at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Rhopaloneure length</td>
<td>Length of the rhopaloneure</td>
<td>micrometers</td>
</tr>
<tr>
<td>Rhopaloneure width</td>
<td>Diameter of the rhopaloneure at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Desmonemeure length</td>
<td>Length of the desmonemeure</td>
<td>micrometers</td>
</tr>
<tr>
<td>Desmonemeure width</td>
<td>Diameter of the cristaibd at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Involution length</td>
<td>Length of the involution from the base of the cristaibd to its most distal extent</td>
<td>micrometers</td>
</tr>
<tr>
<td>Elastic strand width</td>
<td>Diameter of the descending elastic strand at the widest point</td>
<td>micrometers</td>
</tr>
<tr>
<td>Pedicle width</td>
<td>Diameter of the pedicle</td>
<td>micrometers</td>
</tr>
<tr>
<td>Tentacle width</td>
<td>Diameter of the tentacle</td>
<td>micrometers</td>
</tr>
<tr>
<td>Haplooneure row number</td>
<td>Number of haplooneures running parallel to the length of the cristaibd</td>
<td>micrometers</td>
</tr>
<tr>
<td>Cristallaria ciliatedness</td>
<td>Cristallaria free length / Cristallaria length</td>
<td>adimensional</td>
</tr>
<tr>
<td>Heteroneure elongation</td>
<td>Heteroneure Length / Width</td>
<td>adimensional</td>
</tr>
<tr>
<td>Haplooneure elongation</td>
<td>Haplooneure Length / Width</td>
<td>adimensional</td>
</tr>
<tr>
<td>Desmonemeure elongation</td>
<td>Desmonemeure Length / Width</td>
<td>adimensional</td>
</tr>
<tr>
<td>Rhopaloneure elongation</td>
<td>Rhopaloneure Length / Width</td>
<td>adimensional</td>
</tr>
<tr>
<td>Heteroneure shaft extension</td>
<td>Heteroneure shaft length / Heteroneure capsule length</td>
<td>adimensional</td>
</tr>
<tr>
<td>Nematocyst volume</td>
<td>Ellipsoid formula : ( (4/3)\pi \times \text{length} \times \text{width} \times \text{depth} )</td>
<td>micrometers squared</td>
</tr>
<tr>
<td>Nematocyst SAV ratio</td>
<td>Nematocyst surface area / Nematocyst volume</td>
<td>micrometers</td>
</tr>
<tr>
<td>Total haplooneure volume</td>
<td>Haplooneure volume * Haplooneure row number / (Cristallaria free length / Haplooneure width)</td>
<td>micrometers cubed</td>
</tr>
<tr>
<td>Total heteroneure volume</td>
<td>Heteroneure volume * Heteroneure number</td>
<td>micrometers cubed</td>
</tr>
<tr>
<td>Total nematocyst volume</td>
<td>Total haplooneure volume + Total heteroneure volume</td>
<td>micrometers cubed</td>
</tr>
</tbody>
</table>
CHAPTER 3

Prey Selectivity and Dietary Specialization in Midwater Siphonophores

Based on ROV observations

Alexandra Lapides¹‡, Alejandro Damian-Serrano²‡, Steven H.D. Haddock¹, Elizabeth D. Hetherington³, Casey W. Dunn², C. Anela Choy³

¹ Monterey Bay Aquarium Research Institute, 7700 Sandholdt Rd., Moss Landing, CA 95039, USA

² Yale University, Department of Ecology and Evolutionary Biology, 165 Prospect St., New Haven, CT 06520, USA

³ Scripps Institution of Oceanography, University of California San Diego, Integrative Oceanography Division, La Jolla, CA, 92037, USA

‡ Co-first authors.

Abstract

Siphonophores are abundant and diverse predators in open-ocean ecosystems. Early studies on the diets of epipelagic siphonophores compared their gut contents to the local prey field, finding that many species are selective, specialized predators. With the advent of submersible technologies, it has become increasingly feasible to observe deep-sea siphonophores alive and feeding in their native habitats. Recent studies based on submersible observations have shown that siphonophores are important mid-trophic predators
in deep midwater food webs. However, the extent to which their diets are determined by their local prey fields and their intrinsic biological differences as predators remains to be assessed. In a recent literature review, it was hypothesized that deep midwater siphonophores are more specialized than their epipelagic counterparts. We set out to test whether this hypothesis holds at a finer depth scale in the midwater. Here we (1) estimate the relative overlap between siphonophores and prey based on their spatiotemporal distributions, (2) assess prey selectivity and dietary specialization across different midwater species, (3) compare their dietary specialization to their prey selectivity and to their average depth, and (4) generate hypotheses on niche segregation by comparing the spatiotemporal overlap between siphonophore species to their trophic overlap. We find that most deep-sea siphonophore diets are specialized (on a narrow set of prey types) and strongly selective (deviating from environmental prey ratios) for specific prey, but this specialization bears no relationship to depth nor depth range. Our results show that trophic overlap is independent of spatiotemporal overlap, indicating no evidence of competitive trophic niche segregation. As climate change, deep-sea mining, and other anthropogenic forces threaten open-ocean ecosystems, understanding how siphonophores fit into this changing food web allows us to make predictions on how trophic links may be affected by perturbations.

**Keywords**

ROV, siphonophore, midwater, predation, selectivity, specialization, food web
Introduction

The open-ocean midwater is the largest animal habitat on Earth (Harbison, 1992), harboring complex communities and food webs (Robison, 2004). These food webs sustain many commercial fisheries, endangered megafauna, and maintain the biological carbon pump which traps carbon fixed by phytoplankton into deeper layers (Falowski et al. 1998). The open ocean is facing anthropogenic threats from overfishing, deep-sea mining, acidification, warming, and oxygen depletion (Robison, 2009). These threats are changing open-ocean communities, an effect magnified by the ecological dependencies between species (Rezende et al. 2007). Understanding the role of predators in the food web, especially their specializations and selectivities, allows us to make predictions on how trophic links may be affected by perturbations (Hambright & Hall 1992). Gelatinous animals are central players in these food webs (Choy et al. 2017), comprising a large module named the ‘jelly web’ (Robison, 2004; Chi et al. 2020). Among them, siphonophores are important mid-trophic predators consuming a wide range of prey (Fig. 3.1) such as jellyfish, salps, crustaceans, molluscs, and fish (Hetherington et al. 2021).

Early studies on species-level siphonophore predation have focused primarily on epipelagic siphonophores (reviewed in Hetherington et al. 2021). A couple of these studies (Purcell, 1981; Purcell, 1984a) have assessed their diets in the context of the local planktonic prey community composition, revealing that many of these species are strongly selective, specialized predators. Siphonophores capture prey using specialized and complex nematocyst (stinging
capsule) batteries on their tentacles or tentilla (tentacle side branches). Morphological studies of their prey capture apparatus have shown clear associations between these phenotypic traits and their dietary specializations (Purcell, 1984b; Damian-Serrano et al. 2021).

A large fraction of siphonophore species dwell far beyond the reach of SCUBA divers in the deep midwater. Their diets cannot be assessed from trawl-collected specimens, since the process typically destroys these fragile animals, and induces artificial ingestions in the cod-end of the nets. Due to these challenges, the ecology and natural history of fragile midwater taxa have been overlooked for centuries (Haddock, 2004). With the advent of remotely operated vehicles (ROVs), the abundance, diversity, and fascinating biology of many gelatinous animals were brought into the spotlight. ROV-based studies of the midwater food web revealed the diets of deep-dwelling siphonophores and their importance as predators and prey (Choy et al. 2017; Hetherington et al. 2021). However, the extent to which the diets of these species are determined by their local prey fields and their specific biological differences remains to be assessed. To do this, we require a long-term detailed assessment of the co-distribution of siphonophores and their prey in depth and time. Fortunately, the MBARI video annotation and reference system (VARS) provides a >33 year-long curated database of ROV observations of midwater animals with their associated hydrographic parameters (Schlining & Stout, 2006), including observations of siphonophore-prey interactions (Choy et al. 2017). This resource provides a
unique opportunity to explore the diets of midwater siphonophores in the context of their expected encounter proportions with different prey types.

Understanding how the diets of predators are determined by intrinsic interspecific differences in prey type enrichment and exclusion (prey selectivity) allows us to reconstruct trophic interactions from community composition alone. In addition, this knowledge can generate hypotheses about the organismal (morphological and behavioral) drivers of the feeding habits of these understudied animals. Ecological theory predicts that specialization should correlate with selectivity (Schoener, 1971), since specialists tend to strongly enrich their diets with their favored prey type and exclude all other prey. However, there are scenarios in which specialization can result from unselective predators occupying habitats with few prey types, and generalists can be simultaneously selective for multiple prey types. We set out to investigate siphonophore selectivity and its relationship to their specialization.

Hetherington et al. (2021) reviewed and summarized the extant literature and public datasets on siphonophore feeding interactions. Their bipartite network analyses showed that deep midwater siphonophores appear to be more specialized than epipelagic ones. This may be due to meaningful ecological differences between these species and environments, but it may also be due to the different biases associated with gut content inspection methods (used only in shallow-water species) and ROV-based methods (used only in deep-water species). We aim to test the hypothesized relationship between siphonophore
specialization and depth by comparing the specialization to vertical fine-scale species distribution data.

Multiple siphonophore species often coexist in similar depth ranges, exposed to similar prey field compositions. Ecological theory explains how coexisting species tend to compete for resources unless they specialize on a narrower subset of prey (niche partitioning) to avoid trophic overlap (MacArthur & Levins, 1964; Chesson 2000; Patterson et al. 2003). However, trophic niche partitioning can be avoided when prey is not the limiting factor for population growth, or when environmental disturbances reset competitive interactions (Pianka, 1974; Fox 2011). Given the scarcity of prey resources and the lack of abiotic disturbances in deep pelagic environments (Robison, 2004), we hypothesize that spatiotemporally overlapping siphonophore species are partitioning their trophic niche in response to competition, which would lead to a potential mechanism driving specialization. Alternative hypotheses would be that all siphonophores are partitioning their trophic niche regardless of spatiotemporal overlap due to the migratory behavior of the prey, or that spatiotemporally overlapping species share similar diets due to similar prey availabilities in the absence of competitive strain. To test these hypotheses, we aim to compare spatiotemporal overlap to trophic overlap across pairs of siphonophore species.

In this study, we aim to (1) estimate the relative overlap between siphonophores and prey taxa based on their spatiotemporal distributions, (2) assess prey selectivity and dietary specialization across midwater siphonophore
species, (3) compare their specialization to their prey selectivity and to their depth distribution, and (4) test hypotheses on niche partitioning by comparing the spatiotemporal overlap between siphonophore species to their trophic overlap.

Methods

Data sources

All data in this study came from the Monterey Bay Aquarium Research Institute's VARS, which contains video footage of dives extending back to 1989. Each organism seen in the dive is manually annotated to lowest taxonomic resolution by a team of in-house experts. These annotations are coupled with geographical, temporal, and CTD data to form a working record of marine life observed on MBARI dives (Schlining & Stout, 2006). All data was constrained to Monterey Bay prior to analysis.

Feeding observations

To obtain siphonophore feeding records, we queried the VARS database for every observation of a siphonophore eating another organism. The images (and occasionally videos) associated with these records were manually double-checked to ensure the correct taxonomic ID for both predator and prey. The siphonophore taxa included in this study were: Apolemia sp., Bargmannia sp., Bargmannia elongata, Marrus sp., undescribed physonect G, undescribed physonect Z, Erenna richardi, Agalma sp., Nanomia bijuga, Forskalia sp.,
Resomia ornicephala, Lychnagalma utricularia, Praya dubia, Desmophyes haematogaster, and Lensia conoidea. Analyses were carried out both on the individual prey concepts (operational taxonomic units) at the lowest possible taxonomic identification, as well as on prey concepts grouped into the larger taxonomic bins. The bin concepts used in our analyses were: Ctenophora, Medusae, Siphonophorae, Salpida, Larvacea, Polychaeta, Cephalopoda, Chaetognatha, Actinopterygii, Copepoda, Decapoda, Lophogastrida, and Euphausiacea.

Spatiotemporal overlap

To estimate the spatiotemporal overlap between predators and prey, we queried the VARS database for every occurrence of each siphonophore predator and each prey item from the feeding records associated with the Haddock Lab cruises. For prey taxa that could only be identified to broad taxonomic categories, we included all observations of organisms under that category except those that were already representing a different prey item (for example, for prey only identified as Cydippida, we included all observations of ctenophores that fell under that category except for Pleurobrachia sp. as they were already identified in a separate feeding observation). Two prey concepts were re-binned to higher taxonomic groups due to rarity in VARS: Careproctus melanurus became Liparidae and Stenobrachius leucopsarus became Myctophidae. The occurrence data per species all had some amount of outliers by depth, indicating incorrect annotations. Using a z-score or other standard outlier method is impractical.
given that most depth distributions are non-normal and skewed towards shallower regions. To address these issues, we built a customized outlier removal method that removes annotations that are significantly far (95th percentile) from other annotations. This method allows both shallow and deep outliers to be flagged, regardless of the shape of the bulk of the annotations.

All data was normalized to 50m depth bins and 1 month time bins per seasonal cycle to account for variation in recording effort and control for seasonal differences in species abundances and depth distributions. A loess smoothing curve was fit to all depth distribution data, and a two-part model was used for all species with sufficient numbers of observations. The two part model was used to account for zero-inflation in all occurrence data by modeling the zeroes as a separate process. Both parts of the two-part model were mixed-effects generalized additive models (GAMs). In both cases, depth was considered a fixed effect, temperature and oxygen considered random effects, and month of the year was fit to a cyclic spline to ensure no discontinuity between years. The first model was a presence-absence logit model to determine where along depth and month of year gradients an organism existed. The second model was a standard GAM with the parameters noted above. We multiplied the responses of these models together to obtain a final distribution prediction for each species. To obtain a final overlap parameter, we multiplied the Loess and two-part model curves for each siphonophore predator against each prey item to achieve a matrix of spatiotemporal overlap values. (SM Figure 3.1b).

*Estimating prey selectivity*
In order to estimate predators’ selectivity of prey types, we used Strauss’ (1979) linear index (LI) described by:

\[ LI = r_i - p_i \]

This index shows the difference between the proportions of each prey type in the environment \((p_i)\) and the observed proportions in the diet \((r_i)\). To estimate \(p_i\) for each predator, we divided the overlap metric with each prey type by the sum of overlaps with all prey. Similarly, we estimated \(r_i\) for each predator by dividing each prey type’s number of feeding observations by the total number of feeding observations. In order to test the explanatory power of prey availability on diet, we ran Mantel permutations tests \((n=999)\) at the individual and grouped concept levels.

*Estimating predator specialization and trophic overlap*

In order to estimate the degree of specialization of different siphonophore species, we used the Shannon-Wiener information variable ‘H’ (Lehner, 1979) implemented through the `diversity` function in the R package vegan (Oksanen et al. 2007) on the feeding observation data. To calculate the trophic overlap between pairs of siphonophore species, we computed the Bray-Curtis distance between predators using the relative proportions of prey found in the feeding observations \((r_i)\), implemented through the `vegdist` function in vegan. We then contrasted these pairwise trophic overlaps with the log-transformed pairwise spatiotemporal overlap metrics between siphonophore species using a linear correlation. We also applied a \(X^2\) test using the number of siphonophore species
pairs that lie on each side of the midrange of spatiotemporal and trophic overlaps against the null expectation of each quadrant containing the same number of points. Scripts and data are available in our BitBucket repository (https://bitbucket.org/ALapides/siphweb_vars/).

Results

Natural history lessons — Our feeding observations and selectivity estimates derived from relative spatiotemporal overlaps with prey shed new light on the natural history of midwater siphonophore feeding. Our findings indicate that Apolemia primarily feeds on gelatinous animals, occasionally consuming fishes and crustaceans, with little selectivity for specific prey types. We find that different Bargmannia species feed selectively on very different prey types, such as squids and krill. Among the large, deep-dwelling “Clade B” physonects (Erenna richardi, Marrus sp., undescribed physonects G and Z; as defined in Munro et al. 2018), we find high affinity for large soft-bodied prey such as gelatinous animals, cephalopods, and fish. The lophogastrid prey found in Erenna richardi challenges the fish specialization hypothesized in Damian-Serrano et al. (2021). These results suggest that Clade B physonects might be specialists in large prey regardless of taxonomy. All five “Clade A” physonects included in this study (Agalma sp., Nanomia bijuga, Forskalia sp., Resomia ornicephala, and Lychnagalma utricularia; as defined in Munro et al. 2018) appear to be highly specialized and selective for crustacean prey (copepods in Agalma, large crustaceans in the rest). L. utricularia feeds on both sergestid shrimps and krill, but N. bijuga, R. ornicephala, and Forskalia sp. appear to feed
exclusively on krill. Krill specialization in *N. bijuga* was originally reported by Choy et al. (2017), while krill specialization in *R. ornicephala* was hypothesized by Pugh and Haddock (2010). Calycophorans (*Praya dubia, Desmophyes haematogaster*, and *Lensia conoidea*) were observed feeding on a broad variety of prey items. Among the prayids, *P. dubia* non-selectively consumed gelatinous animals as well as krill, while *D. haematogaster* was observed selectively consuming a copepod. The small diphyomorph *L. conoidea* was observed selectively consuming a chaetognath.

*Prey selectivity and specialization* — We found that *Agalma* sp., *Bargmannia* sp., *B. elongata, D. haematogaster, Marrus* sp., *Forskalia* sp., undescribed physonect G, *L. conoidea, L. utricularia, N. bijuga*, and *R. ornicephala* are strongly selective (L.I. > 0.5) and specialized on singular prey types. On the other hand, *Apolemia* sp. and *P. dubia* appear to be generalists with relatively weak selectivity signals across all prey types (Fig. 3.3C). The deep-sea physonects *E. richardi* and undescribed physonect Z appear to be moderately selective of two prey types each. Prey selectivity and specialization are strongly correlated (using individual prey concepts $R^2 = 0.77$, $p = 0.0007$; and grouped prey concepts $R^2 = 0.86$, $p = 0.00004$) across these siphonophores species (Fig. 3.4). Four species (*B. elongata, Forskalia* sp., *N. bijuga*, and *R. ornicephala*) are specialized on euphausiacean prey (krill). The predator-prey feeding matrix was poorly explained by the spatiotemporal overlap matrix alone both using individual (Mantel test $p = 0.08$) and grouped (Mantel test $p = 0.12$) concepts.
Depth and specialization — To test the hypothesis that deeper siphonophores are more specialized, we compared the median distribution depth to the degree of specialization of each siphonophore species (Fig. 3.5). We found no significant correlation (using individual prey concepts $R^2 = 0.14$, $p = 0.63$; using grouped prey concepts $R^2 = 0.13$, $p = 0.64$) between median depth and predatory specialization. We then hypothesized that dietary specialization may be inversely related to habitat size since broader depth ranges would warrant access to a broader variety of prey. To test this hypothesis, we compared siphonophore specialization to their depth range (SM Figure 3.2) and again found no significant correlation (using individual prey concepts $R^2 = 0.08$, $p = 0.77$; using grouped prey concepts $R^2 = 0.17$, $p = 0.54$).

Niche overlap and segregation — In order to evaluate how siphonophore species partition their spatiotemporal and trophic niches, we compared the degree of spatiotemporal overlap to the degree of trophic overlap across all pairs of siphonophore species (Fig. 3.6). In the presence of competitive niche segregation, we would expect dietary overlap to be negatively correlated with habitat overlap, and an exclusion of cases with both high spatiotemporal and trophic overlap. We found a significant positive linear correlation between these two types of overlap ($R^2 = 0.22$, $p = 0.02$). In addition, we found a significant ($X^2$, $p < 0.000001$) quadrant exclusion signal, with many more siphonophore species pairs with low (<0.5) trophic niche overlaps (Fig. 3.6). We did not find significant relationships between the total specialization of the species in each dyad and trophic overlap (using individual prey concepts $R^2 = 0.12$, $p = 0.20$; using
Discussion

In this study we estimated the spatiotemporal overlaps between midwater siphonophores and their potential prey and examined the prey selectivity and specialization of these siphonophore species. Moreover, we set out to test whether siphonophore specialization scales with prey selectivity, and whether it increases with habitat depth. After finding a high degree of selectivity and specialization, we assessed the degree of niche overlap finding no significant signal for niche segregation.

We found that most midwater siphonophore species are strongly selective of one or two prey types, with a high degree of specialization. This result indicates that midwater siphonophore diets are not explained by prey availability alone, and that intrinsic biological differences (such as tentilla morphology and nematocyst complement) may be responsible for their distinct roles in the food web (Damian-Serrano et al. 2021). However, some of these species are rarely encountered by ROVs, therefore some of these apparent specializations may be artifacts of their scarcity of feeding observations. In addition, ROV observations of feeding events are biased towards large prey (Hetherington et al. 2021), potentially overlooking the importance of small prey taxa such as copepods, ostracods, or larvaceans. Furthermore, some of the

grouped prey concepts $R^2 = 0.14$, $p = 0.15$) nor spatiotemporal overlap (using individual prey concepts $R^2 = 0.07$, $p = 0.46$; using grouped prey concepts $R^2 = 0.17$, $p = 0.08$).
species included in this study have one or few feeding observations, thus our estimated specializations should be interpreted with care. The two taxa (*Apolemia* sp. and *P. dubia*) that show generalized diets are among the longest siphonophores casting the largest tentacle ‘net’ in the water. We hypothesize that a large prey-capture surface area leads to more frequent and more diverse prey captures for each individual colony, which increases the probability of ROV observation of rare prey types in any given colony. This may lead to lower apparent specialization in large frequent feeders and higher apparent specialization in smaller and rare feeders.

The prey types that midwater siphonophore species specialize on are not among the most abundantly encountered, which explains the necessary correlation between high selectivity and specialization. Nonetheless, the object-size bias of ROV observations is also relevant here, since the most frequently annotated concepts are slow, large, gelatinous animals (Choy et al. 2017). This contrasts with the typical pelagic community composition dominated by small crustaceans followed by fishes in the mesopelagic region (Steinberg et al. 2008). With a more accurate representation of the water column community composition, such as that provided by a combination of MOCNESS trawls and ROVs, we may find that the degree of medusan-prey specialization of siphonophores like *Apolemia* is much higher than what we find here.

The results in Hetherington et al. (2021) suggest that in siphonophores, feeding specialization may increase with depth. Their analyses compared the
diets of epipelagic species to those of deep water species (including meso- and bathypelagic species). Our results are congruent with this hypothesis but we do not find significant support for it across midwater (meso- and bathypelagic) species. In light of this result, we hypothesize that the differences between epipelagic and deep water species found in Hetherington et al. (2021) are due to unique ecological characteristics of epipelagic siphonophores, or to the differences in diet-assessment methods employed (gut content analysis for epipelagic species v.s. ROV observations for deep-dwelling species). Additionally, we investigated whether specialization could be related to the species’ depth range, since broader ranges may overlap with a higher number of potential prey. We did not find any evidence of such a relationship, indicating that the observed specializations are likely a product of the species’ high prey selectivity driven by their intrinsic biological differences. These differences are likely related to colony size and morphology, swimming behavior, tentacle-deploying behavior (Hetherington et al. 2021), and tentilla morphology (Damian-Serrano et al. 2021). Research on other sit-and-wait venomous predators like spiders (Jackson, 1992; Pekar & Toft, 2015) suggests that an aggressive-mimicry strategy is associated with more specialized diets. Some siphonophore species bear lures on their tentilla to attract specific prey through aggressive mimicry (Mapstone, 2014). Among the taxa included in this study, *E. richardi* has a bioluminescent lure (Pugh, 2001; Pugh & Haddock, 2016), *R. ornicephala* has a fluorescent lure (Pugh & Haddock, 2010), and *L. utricularia* has a buoyant medusa-shaped lure (Pugh & Harbison, 1986). We found that these three
species were all strongly selective for specific prey such as fish, krill, and shrimps respectively, thus supporting the idea that aggressive mimicry drives prey-type specialization.

The high proportion of specialists among siphonophores may be due to their ambush strategy, which is heavily reliant on the functional morphology of their tentilla. Thus in order to be efficient at capturing prey, they must fine-tune their tentilla to subdue the scarce prey that pass by their tentacles. This efficiency is heavily influenced by specific morphological features of the prey beyond its size, such as hardness or swimming behavior (Damian-Serrano et al. 2021). Active prey-pursuing predators in midwater food webs (such as fishes, squids, or cetaceans) rely on their great speed, size, and complex behaviors to pursue and capture prey. Their strategy is far less constrained to specific prey types but more constrained by prey size (Eggers, 1982; Gill, 2003), leading to the idea of a size-structured food web (Ward et al., 2012). Our results show that siphonophores, like other ambush predators in the midwater, may contribute to a more phylogenetically-structured (rather than size-structured) food web.

The correlation between selectivity and specialization suggests that specialization is not a consequence of species inhabiting depth ranges with fewer prey type options. If our high trophic specialization estimates are accurate, they may be driven by competitive niche segregation where spatiotemporally co-occurring species evolved to specialize on distinct prey types to avoid competition. Our results are partially congruent with this hypothesis, since the
large majority of pairs of species do not have overlapping diets. The fact that we found a positive (instead of the expected negative) correlation between spatiotemporal and trophic niche overlap contradicts this hypothesis, and suggests that similarities in prey availability found among similar spatiotemporal niches may be responsible for high trophic niche overlaps. In addition, we found no significant correlation between specialization and niche overlap (neither trophic nor spatiotemporal), which indicates that niche partitioning is not likely driving the interspecific differences in specialization we observe. It seems plausible that prey resources such as copepods or krill are not limiting enough to elicit competitive niche partitioning among midwater siphonophores. The siphonophore species dyads with high trophic and spatiotemporal overlap correspond to the krill specialists and copepod specialists. Krill are abundant within a relatively narrow depth range (100-500m) in the Monterey Bay, and can form large swarms which may lead to an underestimation of their abundance from ROV observations. We hypothesize that krill specialists must overlap in space and time to match the narrow distribution of this prey type, and likely avoid competition due to the large surplus of their prey resource. Copepods are among the most abundant animals in the pelagic realm, potentially abundant enough to also prevent displacing competition among specialists.

Oceanic food webs are complex systems structured by stochastic processes driving community compositions and predator-prey encounters. However, the complexity of these networks is constrained by organismal traits that circumscribe which encounters are more likely to yield a feeding event.
Siphonophores are a robust natural system to evaluate the role of these drivers since they are non-visual ambush predators distributed across the whole water column, with discrete interspecific variation in their vertical distributions, prey-capture apparatus, and diets (Damian-Serrano et al. 2021). Spatiotemporal overlap between planktonic predators and their prey can be assessed using underwater imaging technology, and is commonly used as a proxy for predator-prey interactions (Greer et al. 2014; Greer & Woodson, 2016; Axler et al. 2020). Our work takes this approach one step further, incorporating and comparing direct feeding observations to the expected prey encounters estimated from overlaps. Our approach presents a new generalizable way to utilize underwater imaging data to characterize the feeding habits of midwater organisms. The scarcity of feeding observations for important deep-sea predators such as siphonophores highlights the importance of continuing and expanding midwater exploration programs. Damian-Serrano et al. (2021) hypothesized that siphonophores may be able to evolve between different feeding specializations and generalism circumventing typical constraints thanks to their modular prey capture apparatus. Our results indicate that Apolemia sp. and P. dubia could play a generalist role in the midwater food web. Given the phylogenetic position of these taxa relative to other siphonophore generalists, these findings (if correctly interpreted) add two more independently-evolved instances of generalism in siphonophores, thus supporting the evolutionary patterns hypothesized in Damian-Serrano et al. (2021).
In conclusion, we find that midwater siphonophores are highly specialized and selective of their specific prey type, and that their diets are likely driven by intrinsic biological differences in their morphology and feeding behavior. Our results suggest that these distinct feeding specializations are not driven by competitive niche segregation, and appear to be unrelated to ecological factors associated with depth or depth range.

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References


Figure 3.1. Midwater siphonophores capturing and digesting prey. (A) *Apolemia rubriversa* eating *Solmissus* hydromedusa. (B) Undescribed physonect G eating *Cyclothone* fish. (C) *Bargmannia* c.f. *elongata* eating krill. (D) *Lychnagalma utricularia* eating a *Eusergestes similis* decapod shrimp. (E) Undescribed physonect G eating *Stenobranchius* sp. fish.
Figure 3.2. Conceptual diagram of the methodological pipeline generating the different types of data used in this study.
Figure 3.3 - Heatmaps of siphonophores and broad prey groups showing (A) relative feeding interactions with insets representing the number of interactions, (B) relative spatiotemporal overlap, and (C) Strauss’ L.I. index of prey selectivity.

Figure 3.4 - Relationship between siphonophore prey selectivity and specialization (-Shannon-Wiener’s H). Point size scales with the number of feeding events recorded for each species. A. Indices generated using individual prey species concepts. B. Indices generated using grouped prey species concepts. The blue lines represent linear regressions and the grey zones indicate the 95% confidence margins of the models.
Figure 3.5 - Relationship between median depth and specialization (Shannon-Wiener’s H). Point size scales with the number of feeding events recorded for each species. A. Indices generated using individual prey species concepts. B. Indices generated using grouped prey species concepts. The blue lines represent linear regressions and the grey zones indicate the 95% confidence margins of the models.
Figure 3.6 - Pairwise species contrasts of siphonophore trophic niche overlap (dietary Bray-Curtis distance) and log-transformed spatiotemporal niche overlap. The number of species dyads in each quadrant is labeled on the center of each quadrant.
Supplementary Information

SM-Figure 3.1 - Heatmaps of siphonophores and individual prey groups showing (A) relative feeding interactions with insets representing the number of interactions, (B) relative spatiotemporal overlap, and (C) Strauss’ L.I. index of prey selectivity.
SM-Figure 3.2 - Relationship between depth range and specialization (-Shannon-Wiener’s H). Point size scales with the number of feeding events recorded for each species. Indices generated using individual prey species concepts. The blue line represents a linear regression and the grey zone indicates the 95% confidence margins of the model.
CHAPTER 4

Characterizing the Secret Diets of Siphonophores Using DNA Metabarcoding

Alejandro Damian-Serrano, Elizabeth D. Hetherington, C. Anela Choy, Steven H.D. Haddock, Alexandra Lapides, Casey W. Dunn

1 Yale University, Department of Ecology and Evolutionary Biology, 165 Prospect St., New Haven, CT 06520, USA
2 Scripps Institution of Oceanography, University of California San Diego, Integrative Oceanography Division, La Jolla, CA, 92037, USA
3 Monterey Bay Aquarium Research Institute, 7700 Sandholdt Rd., Moss Landing, CA 95039, USA

Abstract

Siphonophores (Cnidaria: Hydrozoa) are abundant and diverse predators in open-ocean ecosystems. Due to limited access to the deep midwater environment, little is known about the diets of most deep-dwelling species. Visual gut content inspection is a powerful approach but can rarely identify soft-bodied prey that digest quickly and do not leave recognizable parts behind. Observations of feeding from submersibles through video recordings are useful for deeper species but do not account for small prey items (e.g., copepods, ostracods, and larval fish). Recently, the application of DNA metabarcoding in marine predators has revealed the importance of prey taxa that were overlooked.
by visual methods. Metabarcoding can also detect prey that were ingested several hours before sample processing and thus is well suited for the study of deep-sea ambush predators with long intervals between prey captures and between specimen collection and sample processing. We performed DNA metabarcoding analyses on the gut contents of several siphonophore species across depths and described their diets. We collected siphonophores using blue-water dives and ROV dives in open-ocean waters. We extracted DNA from the feeding zooids of 159 siphonophore specimens from 41 species, then amplified and sequenced six regions along the 18S gene. Taxonomic identifications were assigned to prey OTUs using SILVA databases combined with local zooplankton sequences. We found prey DNA in 47 specimens across 24 species. Most of these species appear to be specialized and strongly selective predators. We reported the first insights into the diets of nine siphonophore species, and revealed 29 novel predator-prey interactions. Many of the feeding interactions are congruent with predictions based on tentilla morphology. Our analyses were able to detect small prey and gelatinous prey taxa underrepresented by visual methods. Our results reveal hidden links between siphonophores and filter-feeding urochordates near the base of the oceanic 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 species diversity for nutrient flow and ecosystem functioning.

Keywords
Gelatinous zooplankton, trophic ecology, predator-prey interactions, prey selectivity, pelagic food webs

**Introduction**

The open-ocean midwater is the largest volume of the biosphere habitable by animals (Harbison 1992). This environment hosts diverse communities and complex food webs (Robison 2004). Midwater food webs sustain manifold fisheries, charismatic megafauna, and sustain the biological carbon pump. Gelatinous animals play fundamental roles in these food webs (Choy et al. 2017), acting as herbivores, predators, and prey in the ‘jelly web’ (Robison 2004, Chi et al. 2020). Among the most abundant (O’Brien 2007, Grossman et al. 2015) and trophically-connected (Choy et al. 2017) gelatinous predators are siphonophores — mid-trophic organisms that feed on a broad variety of prey such as medusae, salps, crustaceans, molluscs, and fishes (Purcell 1981a, Hetherington et al. 2021). Siphonophores are sit-and-wait, non-visual, ambush predators that rely on prey coming into contact with their tentacles and tentilla (Mackie et al. 1988). They are abundant and locally diverse colonial cnidarians in open-ocean communities, present in every ocean of the world, with species ranging from the surface (like the Portuguese man-o-war) to the abyssopelagic region (>4000m deep) (Mapstone 2014). In addition, siphonophore aggregations can have significant predatory impacts on larval fish stocks (Purcell 1981b).

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

The diets of some epipelagic siphonophores have been examined through gut content analyses of SCUBA-collected colonies (Biggs 1977, Purcell 1981a, reviewed in Hetherington et al. 2021). Recent studies based on ROV observations have shed some light on the diets of deep midwater siphonophores (Choy et al. 2017, Hetherington et al. 2021, Lapides et al. 2021). However, these approaches have been limited by their biases. Visual gut content inspection favors hard-bodied prey that digest slowly, leaving behind diagnostic body parts (i.e. exoskeleton, shell, eyes, etc.). Therefore, soft-bodied, rapidly-digested taxa, such as gelatinous zooplankton are often underrepresented in dietary assessments. ROVs are able to observe feeding on gelatinous prey before they become digested. However, ROV observations are skewed towards large prey items that can be easily identified from the camera screen (such as large medusae, ctenophores, crustaceans, or fishes), and can overlook important prey items such as copepods and larvae (Hetherington et al. 2021). In addition, prey are relatively scarce in the open ocean, especially in the deeper regions.
(Robison 2004), thus it is infrequent to find specimens capturing prey or carrying visually-identifiable prey in their guts (Purcell, 1981a).

With the advent of DNA metabarcoding, the diets of many marine predators have been established from gut content DNA (Leray et al. 2013, Harms-Tuohy et al. 2016, Fernández-Álvarez et al. 2018, Reis et al. 2018). These high-throughput amplicon sequencing technologies have extremely high detection sensitivity and bypass the biases posed by visual methods. Recently, the application of DNA metabarcoding to marine predator gut contents has demonstrated the capacity of these methods to detect gelatinous prey (Connell et al. 2014, McInnes et al. 2017, Clarke et al. 2018, Jensen et al. 2018, Marques et al. 2019). However, this technology has not yet been applied to study the diets of gelatinous animals.

In Hetherington et al. (2021), we reviewed and summarized the literature on siphonophore diets, and observed significant differences between the diets of epipelagic and deep-dwelling siphonophore species. Gelatinous prey appeared to be more prevalent in deep-sea observations while small crustaceans appeared to be the predominant prey in shallow gut content samples. Since epipelagic species’ diets were exclusively assessed through microscopic gut content inspection and deep-sea species’ diets through ROV observations, it is not possible to determine whether these differences are due to ecological or methodological reasons. In order to disentangle these confounding factors, it is critical to assess both shallow and deep species’ diets under the same
methodological framework. In this case, DNA metabarcoding is an ideal choice, since it can detect both small and gelatinous prey, thus being able to bridge across the methodological shortcomings of visual methods.

Siphonophore tentillum and nematocyst morphology are directly linked to feeding guild (Damian Serrano et al. 2021a). Damian-Serrano et al. (2021b) used these relationships to generate feeding guild predictions for 45 siphonophore species using their tentillum and nematocyst morphology as predictors. The feeding guild categories comprise fish specialists (which feed primarily on teleost fish prey), large crustacean specialists (which feed primarily on krill, decapod shrimps, mysids, lophogastrids, amphipods, and other macro-planktonic crustaceans larger than 1cm), small crustacean specialists (which feed primarily on copepods, ostracods, cladocerans, larvae, and other meso-planktonic crustaceans smaller than 1cm), gelatinous specialists (which are able to feed on large gelatinous animals such as salps, ctenophores, or medusae in addition to other zooplankton), and generalists (which feed on a variety of small and large, soft- and hard-bodied prey not including gelatinous animals). These predictions were cast on siphonophore species for which no dietary information was available, and thus remained to be tested with new data on siphonophore diets.

Here we use DNA metabarcoding to identify the gut contents of several siphonophore species to obtain more comprehensive insights into their diets. Our primary aims are: (1) Expand the existing knowledge on the diets of open-ocean siphonophores using DNA metabarcoding, (2) qualitatively compare the prey
detected by visual and molecular methods to evaluate their technical biases, (3) compare the prey found in the gut contents to the local planktonic community composition to identify instances of selectivity and specialization, and (4) evaluate the morphology-based predictions of feeding guilds.

Methods

*Siphonophore collections* — In order to sample a representative set of taxa across the siphonophore phylogeny, we targeted a set of 41 species (aiming for 10 specimens per species) including cystonects, apolemiids, pyrostephids, euphysonects, and calycophorans from shallow and deep waters. Most species were sampled from the Offshore California Current Ecosystem (OCCE) except for the Portuguese man-o-war *Physalia physalis*, which was collected off Bermuda in the Sargasso Sea; *Sulculeolaria chuni* and some *Nanomia* spp. (labeled as “Atlantic”) which were collected off Rhode Island in the Block Island sound. While all the *Nanomia* populations sampled in this study have been referred to as *N. bijuga*, we suspect that there may be undescribed cryptic *Nanomia* species among the specimens sampled. Therefore, we decided to have them labeled at the genus level. The pleustonic (surface floating) *Physalia physalis* samples were collected manually using a bucket from a small boat. Species found between the 0-20m deep were collected using blue water diving techniques following the guidelines in Haddock & Heine (2005). Species from 200-4000m were collected using ROVs. All animals were collected live and brought back to the ship (or field station in Bermuda for *P. physalis*) for dissection.
Live colonies were photographed (sometimes recorded on video), and zooids of diagnostic value (nectophores, bracts, tentacles) were dissected, fixed in 4% formalin, and stored as vouchers at the Yale Peabody Museum of Natural History.

**Gut content metabarcoding** — Shortly after collection of the live specimens, we dissected and pooled several gastrozooids, prioritizing those with visible gut contents, in addition to any visible egested food pellets at the bottom of the sampling container. Samples were frozen at -80°C until DNA extraction. Further details on the DNA extraction, quality control, PCR, amplicon purification, and amplicon pooling are fully described in the online protocol (Damian-Serrano 2020). All molecular bench work was carried out at the Yale DNA Analysis Facility. We used a set of six primer pairs that amplify six regions within the 18S gene (and part of the ITS1) named after their expected amplicon length (‘134’, ‘152’, ‘166’, ‘179’, ‘261’, and ‘272’). The primers were designed using Geneious v.x.x.x. (Kearse et al. 2012), seeking short (>300bp) amplicon products with a high chance of remaining uncleaved after digestion in the gastrozooid, flanked by priming sites conserved (to a maximum mismatch of 3bp) across metazoans. The search for conserved priming sites was conducted on an alignment of 18S genes from 975 species across all metazoan phyla downloaded from GenBank. The primer search was optimized to only retrieve primer pairs with compatible annealing temperatures and without problematic dimerization and hairpin temperatures. Primer sequences and properties can be found in Table T1 in Damian-Serrano (2020). Amplicon pools were sequenced using Illumina MiSeq.
250bp paired-end technology (except samples in run 0 which was sequenced using MiSeq 150bp) at the Yale Center for Genomic Analysis.

Prey reference database — In order to enhance the accuracy of the taxonomic assignments of reads, we also built an 18S gene barcoding database. To do this, we collected 60 specimens of 30 species of zooplankton and micronekton from the OCCE using a Tucker trawl. We targeted plausible prey species from motile oceanic taxa that cohabitate with siphonophores and are underrepresented in SILVA databases, including fishes, crustaceans, jellyfishes, urochordates, chaetognaths, polychaetes, and mollusks. Specimens were photographed live, tissue was sampled and frozen, and the rest of the animal was fixed in formalin as a voucher to be identified and preserved at the Yale Peabody Museum of Natural History. DNA extraction, quality control, PCR, and amplicon cleanup was carried out in a similar fashion as the metabarcoding protocol in Damian-Serrano (2020), except that only one PCR program (Damian-Serrano 2020, Table T5A), and only one pair of primers were used (166F and 134R), spanning the full extent of the sequence containing all barcode regions used in the gut content metabarcoding (~1800bp). Purified amplicons were sent in plates with the forward and reverse primer separately for Sanger sequencing from both ends at the Yale DNA Analysis Facility. These sequences were then assembled and trimmed at a 95% quality cutoff in Geneious and concatenated with the latest SILVA database (SILVA_138_SSURef_NR99 pruned to remove bacterial sequences) downloaded on February 23, 2021 to generate our custom-built database.
Bioinformatic pipeline — Amplicon libraries were demultiplexed by primer sequence using custom bash code. Primer sequences were removed using cutadapt (Martin 2011). The forward and reverse reads were matched and repaired using bbtools (Bushnell et al. 2017), then denoised and de-replicated using the DADA2 (Callahan et al. 2016) plugin in QIIME2 (Bolyen et al. 2019) with a truncation quality threshold of 28. We de novo clustered the unique features into OTUs using the VSEARCH (Rognes et al. 2016) plugin in QIIME2 with a similarity threshold of 95%. Using QIIME2, we computed sample composition and diversity metrics and aligned the feature sequences with MAFFT (Katoh et al. 2009) to build a phylogenetic tree with Fasttree (Price et al. 2009). To reduce computational load, only the top 100 most abundant features among the clustered OTUs were selected for taxonomic assignment. Taxonomic identifications were assigned using the assignment software METAXA2 (Bengtsson-Palme et al. 2015) with a 70% reliability cutoff, comparing the sequences against the standard GenBank reference library, the SILVA123.1 reference library (Quast et al. 2012), and our custom-built library (based on SILVA138). All bioinformatics analyses were carried out in the Yale High Performance Computing Cluster. The taxonomic assignments and read count data were merged, then parsed to match the sample of origin and the DNA sequence they derived from. Sequence post-processing scripts can be found in the GitHub repository (https://github.com/dunnlab/siphweb_metabarcoding).

Assignment interpretation — Taxonomic assignments were manually inspected and annotated with the interpreted consensus taxon and interpreted
source (predator, prey, secondary predation, parasite, environmental eukaryote, unrecognized sequence, contamination, or cross contamination). A combination of annotation database consensus, barcode region consensus, number of reads, manual BLAST checks, and natural history informed priors were used to assign these interpretations. Amplification experiments on negative controls indicated that the human, mite, and insect contaminants originated from specimen manipulation in the field and not from the lab bench. Cross-contamination at the lab bench was suspected for some samples in runs 0 and 5 due to simultaneous DNA extractions of reference prey samples. 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 labelled as such. Crustacean, gastropod, and larvacean sequences in Physalia samples were interpreted as secondary predation (prey of their fish prey) given our knowledge on the prey-capture limitations of these animals and the feeding habits of their fish prey. When all barcode regions except ‘152’ indicate mysid prey but ‘152’ identifies a similar number of reads as stomatopod prey, we interpreted those reads as mysid prey. Assignments of shark identities by barcode region ‘152’ in one of the Physalia samples (extraction 169) 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 ‘152’ (in extractions 111, 218, and 225) 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.

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

\[ LI = r_i - p_i \]

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

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

Pipeline performance — We extracted, amplified, and sequenced the gut contents of 159 specimens from 41 siphonophore species. We obtained a total of 4148 unique sequences, including 1502 sequences from region “134”, 614 from region “152”, 758 from region “166”, 497 from region “179”, and 341 from region “261”, and 442 from region “272”. A total of 337 unique sequences were interpreted as prey items, 36 as secondary predation, 292 as contamination from extrinsic sources, 2857 as natural environmental DNA sources, 791 as siphonophore sequences, 85 as parasites (myxozoans, trematodes, and other helminths), and 14 unrecognizable sequences. We identified prey items in 47 specimens from 24 siphonophore species (SM-Figure 4.4). We identified 55 unique prey items, 42 of which were crustaceans (25 of which were copepods), three of them were fishes, four of them were thaliaceans, five corresponded to other gelatinous predators (ctenophores and a medusa), and one matching to a bivalve mollusc (SM-Figure 4.1). Most (112 out of 159) siphonophore specimens collected did not yield any putative prey taxaconcepts. Among the 47 specimens with prey, 40 of them had DNA from a single prey item, while only six had two prey items, and one Apolemia sp. specimen had three prey items (SM-Figure 4.1). This is consistent with the feeding habits of sit-and-wait ambush predators.
in oligotrophic environments, with scarce feeding events separated by periods of starvation. This is also consistent with the feeding habits of other macrophagous predators that feed on relatively large prey (i.e. snakes, piscivorous fish) (Griffiths 1975).

**Results**

*Dietary findings by taxon*

*Physalia physalis* — In our gut content samples of the Portuguese man-o-war from Bermuda, we found three specimens with ray-finned fish sequences, some of which had visually recognizable fish in the gastrozooids when collected. Fish prey is congruent with published visual inspections of their gut contents (Purcell 1984, Bardi & Marques 2007). In all three specimens with fish prey we also found benthic and hard-bodied taxa (mysid, alpheid shrimp, spider crab, copepod, benthic gastropod, and a sipunculid worm), as well as larvacean prey sequences, which were interpreted as the gut contents of the fish prey. In addition, we also detected ctenophore prey in one specimen. Comparisons with their surrounding prey field show these specimens were strongly selective for fish and strongly exclusive of copepods (Figure 4.4.2).

*Apolemia* spp. — All species of *Apolemia* analyzed here had consumed copepods, the *A. rubriversa* specimen had also consumed a salp, and the undescribed *Apolemia* species also had ctenophore, larvacean, mysid, and euphausiid prey sequences. The high selectivity of *A. rubriversa* for salp prey is congruent with its characterization as a gelatinous specialist in Damian-Serrano
et al. (2021a). While the morphology-based predictions derived from Damian-Serrano et al. (2021b) indicate that *A. lanosa* is a gelatinous prey specialist, we only found copepod prey in our sample. However, it is possible that the doliolid and hydromedusa reads we conservatively labelled as potential cross-contamination could correspond to real prey.

*Bargmannia* spp. — ROVs have recorded *Bargmannia elongata* consuming crustaceans and cephalopods, and during specimen collection we observed a mysid prey in a specimen *Bargmannia amoena*. Nothing was previously known, however, about the diet of *Bargmannnia lata*. DNA metabarcoding confirmed the identity of the mysid in *B. amoena* as *Boreomysis californica*, and found a copepod in another specimen. One *B. elongata* specimen had euphausiid and ostracod prey, in agreement with the DAPC prediction for *B. elongata* to feed mainly on large crustaceans, but also marginally on small crustaceans. The two *B. lata* specimens consumed a ctenophore and a copepod, respectively. These results are not congruent with the morphology-based prediction for *B. lata* to be a large-crustacean specialist (Figure 4.4.3).

Other deep-sea physonects — Undescribed physonect sp. L was predicted to be a fish specialist with a secondary affinity for large crustacean prey. However, we found this specimen consuming a ctenophore. *Resomia dunni* was predicted to be a generalist (consumer of all types of prey except gelatinous taxa), which is not incongruent with the copepod prey we found in its gut.
contents. *Forskalia* species have been observed to consume various crustaceans, molluscs, worms and fish (Purcell 1981a). Morphology predicts *Forskalia* species to be large crustacean specialists. We found three midwater *Forskalia* specimens with copepod prey in the guts, one of them also had consumed a sergestid shrimp. These results are fully congruent with those derived from visual methods, and partly congruent with the morphological predictions. *Lychnagalma utricularia* is unique among the physonects for bearing a medusa-shaped floating vesicle at the end of their large, coiled tentilla. They have been observed through ROVs consuming sergestid shrimp. We found two specimens both with sergestid shrimp prey (for which they are strongly selective), yet one of them was also digesting a euphausiid. This is consistent with their large-crustacean specialization. *Halistemma rubrum* tentilla closely resemble those of *Forskalia*, and thus they are also predicted to be large-crustacean specialists. This prediction is congruent with our identification of a lophogastrid in the gut contents for which it was strongly selective.

*Nanomia* spp. — Midwater ROV observations of deep-dwelling *Nanomia* have predominantly reported interactions with krill prey, as well as with the occasional chaetognath or sergestid shrimp (Choy et al. 2017). We identified one specimen of mesopelagic *Nanomia* with krill and stomatopod DNA in its gut contents, in agreement with its large-crustacean specialist characterization. Epipelagic *Nanomia* might not be as specialized on large crustacean prey, since the literature reports a combination of copepod, decapod, mysid, and chaetognath prey. In the North Pacific Ocean, our metabarcoding identified
copepod prey in an epipelagic *Nanomia* off California, a hyperiid amphipod prey in an epipelagic *Nanomia* off Hawaii. In the North Atlantic Ocean, we sampled 14 specimens of epipelagic *Nanomia*, seven of which contained copepod prey. Upon visual inspection of the sampled gastrozooids we could identify *Temora*, *Centropages*, and *Acartia* copepods, the most abundant genera in the plankton sample, whose identity was also validated by the metabarcoding results. The corresponding environmental plankton samples showed that these waters were dominated by cladocerans, and thus these *Nanomia* were positively selecting for copepod prey and selecting against cladoceran prey (not detected in the guts).

Calycophorans — We provided the first insights into the diets of two highly abundant deep-sea calycophorans, *Lensia conoidea* and *Chuniphyes multidentata*, which morphology predicted as small-crustacean specialists. Both sequenced specimens contained copepod DNA, supporting these predictions. Gelatinous prey has been reported for *Desmophyes annectens* from ROV observations, however we found only copepod prey sequences. We report the first instances of gelatinous prey in *Diphyes dispar* (salp prey), *Muggiaea atlantica* (larvacean), and *Sphaeronectes christiansonae* (naustheid medusa). The far more common epipelagic *Sphaeronectes* species, *S. koellikeri*, appears to be a copepod specialist according to visual gut content analysis (Purcell 1981a). We sequenced the gut contents of two specimens of this species, one of them indeed was consuming a copepod, yet the other was consuming a crab larva. Another validated expectation occurred with *Sulculeolaria chuni*, a visually-assessed copepod specialist in Purcell (1981a), for which we detected copepod
prey in an Atlantic specimen. Our DNA metabarcoding on *Vogtia serrata* has revealed one specimen feeding on an ostracod (with high selectivity), and a specimen feeding on a sergestid shrimp and a bivalve. These results are consistent with the generalist morphological prediction, and congruent with the single visual finding of an ostracod in a congener from Pugh (1986).

**Comparisons with visual methods**

We report the first insights into the diets of nine siphonophore species and reveal 29 novel predator-prey interactions (Figure 4.4.3). When comparing our metabarcoding findings with the published visual observations from gut content inspections and submersible dives, we found five interactions congruent with ROV observations, and eight interactions (six of them involving copepods) congruent with visual gut content inspections of SCUBA-collected colonies (Figure 4.4.3). In mesopelagic species, we suspect that submersible observations may have missed copepod prey in *D. annectens*, *Forskalia* sp., and *Apolemia* sp.; ostracod prey in *B. elongata*; and larvacean prey in *Apolemia* sp.

**Comparisons with prey field and selectivity estimates**

We found both positive and negative selectivity when comparing identified siphonophore prey to co-localized prey fields. We found strong negative (<-0.5) selectivity for copepods in *P. physalis* specimens and in one specimen of *V. serrata*. However, in 19 specimens from 12 species (out of the 15 species assessed), we found strong positive selectivity (>0.5) for a specific prey type.
These cases include: selectivity for fish in *P. physalis*; selectivity for copepods in *L. conoidea, C. multidentata, D. annectens, S. chuni,* and Atlantic *Nanomia* sp.; selectivity for ostracods in *V. serrata*; selectivity for decapods in *L. utricularia*, for lophogastrids in *H. rubrum*, and for mysids in *Apolemia* sp.; and selectivity for salps in *A. rubriversa* and *D. dispar* (Figure 4.4.2).

*Comparisons with morphology predictions*

Comparing our metabarcoding findings with the morphology-based predictions from Damian-Serrano et al. (2021b), we found support for 10 of the predicted interactions between siphonophores and prey. Among the physonects, our results supported the predictions of *B. elongata* eating krill and ostracods, *R. dunni* eating copepods, *Forskalia* sp. eating decapods, and *H. rubrum* eating lophogastrids. Among the calycophorans, we found support for the predictions of *V. serrata* eating decapods, ostracods, and molluscs; also *C. multidentata* and *L. conoidea* eating copepods. Among the species studied there were 70 predicted interactions that were not found among the metabarcoding results (Figure 4.4.3). Out of the 10 taxa with both morphology-based predictions and metabarcoding results, six had all prey congruent with the predictions, three had all prey incongruent with the predictions, and *Forskalia* sp. presented both cases.

**Discussion**

This study constitutes the first use of DNA metabarcoding technology to investigate the diets 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, thaliaceans, molluscs, and gelatinous predators (Figure 4.4.2). Our results expand the existing knowledge on siphonophore diets, detecting prey types previously missed by visual methods, and providing the first insights into the diets of several understudied siphonophore species. 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 siphonophore species are strongly selective specialists on distinct components of zooplankton and micronekton communities (Figure 4.4.2). Moreover, we find that many of the tentilla morphology-based dietary predictions for these species were supported by the metabarcoding results (Figure 4.4.3).

Dietary findings by taxon

*Physalia physalis* — The Portuguese man-o-war is the only pleustonic (surface floating) member of the siphonophores, and the most commonly encountered by beachgoers. Man-o-wars are well-known to feed exclusively on relatively large and motile soft-bodied prey such as fish, chaetognaths, or pelagic gastropods (Purcell 1984). Their nematocysts are not able to subdue crustacean prey, and their feeding reflex would not trigger with a prey as small as a larvacean (Purcell 1984). Therefore, we interpreted the presence of these taxa in the gut contents as secondary predation (prey of the fish prey). The ctenophore prey item found in one specimen could be also a case of secondary predation,
but we suspect a ctenophore could be large enough to be prey of the man-o-war. If that is the case, this would be the first record of *P. physalis* consuming gelatinous zooplankton, which would place the man-o-war as a central species in the epipelagic ‘jelly-web’ (Chi et al. 2020).

*Apolemia* spp. — These physonects are among the longest siphonophores, with colonies reaching as long as 30m of length (Mackie et al. 1988). Their tentacles are different from other siphonophores since they have no tentilla and carry birhopaloid heteroneme nematocysts directly on their tentacles (Damian-Serrano et al. 2021b). *Apoloemia* species are known to consume diverse prey including crustaceans, molluscs, polychaetes, chaetognaths, fish, and gelatinous zooplankton (Purcell 1981a, Choy et al. 2017). While this may suggest these species are generalists, Damian-Serrano et al. (2021a) hypothesized that they may be gelatinous zooplankton specialists, since they consume a larger proportion of this prey type than other siphonophores. In addition, the nematocysts of *Apoloemia* have similar traits to those in other gelativore cnidarians (Purcell & Mills 1988), and their apparent generality could be explained by the sheer number of fine tentacles deployed for prey capture per colony, which would inevitably entangle almost anything that swims by. Considering the differences we found between species, it seems possible that these coexisting species of midwater *Apoloemia* are partitioning their trophic niche by varying the proportion of crustacean versus gelatinous prey they consume. Moreover, the high selectivity for salp prey in *A. rubriversa* indicates a direct connection between phytoplankton consumers and siphonophores.
**Bargmannia** spp. — The three species considered here are frequently-observed siphonophore species in the midwaters off Monterey Bay. These physnoects have relatively simple tentilla with large stenotele nematocysts and an undifferentiated terminal filament (Damian-Serrano et al. 2021b). The diets of these three closely related, coexisting species appear to be non-overlapping, which could be a consequence of competitive trophic niche partitioning. The lack of congruence between the DAPC prediction and our metabarcoding results for *B. lata* suggests that the lack of taxon sampling among the pyrostephids in Damian-Serrano et al. (2021a) could have biased the DAPC model. We found ctenophore prey in *B. lata* and in the undescribed physonect L, indicating further involvement of deep-sea siphonophores in the midwater ‘jelly web’. Deep-sea undescribed physonects with close morphological affinity to our species have been observed consuming fish and squid prey (Choy et al. 2017), thus it is possible that they are specialized in capturing and digesting soft-bodied prey more generally.

**Nanomia** spp. — These are among the most common siphonophores in both Atlantic and Pacific waters, both in epipelagic and midwater environments. We have observed that epipelagic *Nanomia* tend to have smaller tentilla than their mesopelagic counterparts, which may account for their specialization on smaller crustaceans (such as copepods) instead of larger crustaceans (such as krill). The exclusion of the overabundant cladocerans from the diet of Atlantic *Nanomia* suggests that their specialization could be copepod-specific. The hyperiid amphipod we found in the guts of a Pacific epipelagic specimen could
have been a commensal or parasite on the *Nanomia* instead of prey, though this is unlikely since only the gastrozooids were dissected while amphipods tend to colonize the nectophores or bracts.

Calycophorans — These siphonophores are characterized by their lack of a pneumatophore (gas-filled apical vesicle) and their structurally-homogeneous tentilla Damian-Serrano et al. (2021b). However, these tentilla present a great variation in nematocyst number and size, which may translate into dietary differences (Damian-Serrano et al. 2021a). Our finding of a crab larva in *S. koellikeri* constitutes a novel prey type for this species, yet still within the expected range of a small-crustacean specialist. *Vogtia* is the closest relative to *Hippopodius*, the only siphonophore known to be an ostracod specialist (Purcell 1981a). Like many other hard-to-access mesopelagic taxa, the diet of *Vogtia* has remained unknown, though tentilla morphology predicted them to be generalists (Damian-Serrano et al. 2021b). Pugh (1986) found spatial correlations between ostracods and *Vogtia* species, and even mentions a *Vogtia* sp. specimen which had the exoskeleton of an ostracod in its gut contents. The presence in the gut contents of one of our specimens of an ostracod and a bivalve (likely a pediveliger larva), which has a very similar shape to an ostracod (with two hard valves), indicates phylogenetic conservatism of prey traits within Hippopodiidae. This is also supported by the fact that the ostracod capture appears to be highly selective given the corresponding community composition. ROVs have recorded prayids such as *Praya dubia* and *D. annectens* consuming gelatinous prey. We did not find sequences from any gelatinous taxa in our prayid samples, which
suggests that either our sample sizes were not large enough, or ROVs had observed accidental entanglement of jellies on their tentacle nets which did not end in ingestion. However, we did find gelatinous prey in other calycophorans, including salp prey in *D. dispar*, and nausithoid prey in *S. christiansonae*. The latter constitutes the first record of *S. christiansonae* feeding. While these medusae can be very small, the minute size of this siphonophore may render this interaction dubious.

Our findings are congruent with the idea that most epipelagic and mesopelagic siphonophore species are strongly selective and specialized predators. Different species were found consuming prey across low (salps, larvaceans, copepods, ostracods) and high (fish, ctenophores, medusae) trophic levels. In addition, finding larvaceans and salps in the diets of four species should shift our perspective on the role of siphonophores in open ocean foodwebs. Our results indicate that they span multiple trophic levels, with some species feeding on filter-feeding prey at lower trophic levels, potentially placing some species as close as one trophic level away from phytoplankton and microbial loop processes.

*Comparisons with prey field and selectivity estimates*

In this study, we assessed the feeding selectivity of siphonophore species by comparing their gut content composition to the environmental abundances of different prey types. Overall, crustaceans (especially copepods) were identified as the most frequent prey type among siphonophore diets. Copepods are
typically the most abundant prey type in planktonic communities, thus being able to feed on them is likely an advantageous strategy for any planktivorous predator.

Fish prey were detected only in the Portuguese man-o-war samples, in agreement with published observations of man-o-war feeding. For most siphonophore species assessed herein, their prey belonged to less-abundant components of the planktonic community, demonstrating high prey selectivity. However, the selectivity index values presented in this study should be interpreted with care, since the prey field data is quantitative (abundance-based) but the gut content values are only binary at the specimen level, and frequency-based at the species level.

**Comparisons with visual methods**

We have detected prey types previously missed by visual methods (microscopic gut content observations and submersible observations) such as larvaceans, ctenophores, bivalves, and ostracods. Our novel findings suggest that visual inspections of gut contents may have missed gelatinous prey in *P. physalis*, and potentially missed larvacean prey in *Apolemia* and *M. atlantica*. These results are consistent with the hypothesis that small prey is underestimated in submersible observations and rapidly-digested prey is underestimated by gut content inspections. DNA metabarcoding was able to detect prey both small and large, gelatinous and hard-bodied, for both deep and shallow-dwelling species. We show that whole gastrozoooids can be utilized for DNA metabarcoding of diets without need for further dissection nor the use of
predator blocking primers. We identified representatives from diverse animals (SM-Figures 4.6-4.9), which demonstrates the phylogenetic range of taxa that can be amplified with our primer pairs.

Comparisons with morphology predictions

In Damian-Serrano et al. (2021b) we predicted the diets of understudied siphonophore species based on the morphology of their tentilla and nematocysts. We were able to test these predictions for ten species and found that most of the prey items found were congruent with these predictions, indicating that tentilla morphology is a strong predictor of siphonophore diets. Siphonophores are hypothesized to easily evolve between feeding specializations and into a generalist diet due to their modular body plan and their functionally-specialized tentilla (Damian-Serrano et al. 2021a). Our results show that closely-related species such as those within the genera Bargmannia, Apolemia, and Nanomia may have evolved distinct feeding specializations. These results are congruent with the conclusions from Damian-Serrano et al. (2021a), further indicating that siphonophore dietary evolution can drive rapid shifts even within the same genus. Moreover, we find that Apolemia sp., as well as V. serrata, could be generalists feeding on a variety of crustacean and soft-bodied prey. These results suggest that a generalist diet may have evolved not just three but up to five times independently, thus reinforcing the conclusions from Damian-Serrano et al. (2021a) on the evolution of feeding guilds.

Methodological considerations
While DNA-based tools can detect prey unrecognized by visual methods, they are not free of shortcomings. Since all life stages of an animal have the same genetic signature, metabarcoding tools are unable to distinguish between larval, juvenile, or adult prey. These ontogenetic stages can have vastly different ecological implications and pose different challenges during prey capture. In addition, the application of metabarcoding to predator diets is usually not quantitative, since too many sources of variation may lead to differences in read abundance. For example, different animal clades have different sizes, cell densities (due to variable acellular mesoglea content), digestion rates, number of copies of the target gene, or primer affinities during the PCR (Deagle & Tollit 2007, Troedsson et al. 2009, Valentini et al. 2009). Due to the difficulties inherent to locating and sampling the species examined in this study, frequency-based quantitative comparisons were not possible for most species either. In addition, the sample size limitations of this study may have biased the results towards higher apparent specialization, and may have missed some important components of the diets of some target species. This caveat is also common in submersible observation data and limits the reliability of comparisons across these methods.

Siphonophores differ from other consumers in several ways which impose further limitations to the value of gut content metabarcoding. The most important difference is the feeding mode and feeding rate, especially for deep-sea species, which typically consume one prey at a time and do not get a chance to capture another until far after the former has been digested (Mackie et al. 1988).
Therefore, most siphonophores are found with empty guts or digesting one or few prey items at a time. Thus the sample size required for frequency-based analyses is much higher than for other consumers which feed more frequently. Our prey frequency results are consistent with this idea. Moreover, except for a couple species such as *Rhizophysa* and *Rosacea* which are diurnal feeders (Purcell 1981a), most species also feed during the night. In the open ocean, diel vertical migration drastically changes the prey field composition for siphonophores at night. Given the fieldwork limitations in this study, we were only able to collect siphonophore gut contents during the day, thus likely biasing their diet towards their diurnal prey captures.

**Conclusions**

Overall, we provide novel insights into the ecology and natural history of several siphonophore species, revealing that siphonophores 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 ‘jelly web’, highlight suspected biases from visual methods, and support the hypothesized value of tentilla morphology to predict their diets. This study also demonstrates the suitability and effectiveness of DNA metabarcoding to identify the prey consumed by gelatinous predators.

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Figure 4.1. Gut content metabarcoding workflow used in this study. Siphonophore colony illustrated by Freya Goetz. Silhouettes in the plankton net downloaded from phylopic.org. Solid arrows indicate physical material transfer and processing, dashed lines indicate information transfers and processing. Yellow islands indicate elements processed in the laboratory bench, green islands represent bioinformatic datasets processed in the high-performance computing cluster, and red islands represent curated data products.
Figure 4.2. Species-wise grid with the frequency of the major prey types identified from the metabarcoding data (left) and the average prey-type selectivity estimated in comparison with the local planktonic community composition (right). Gut content cells in white indicate absence, and cells in grey indicate presence in one specimen, or more than one specimen if labeled with a number. Selectivity colors mapped to Strauss’ L.I. values. Phylogenetic cladogram based on the tree published in Damian-Serrano et al. (2021a).
Figure 4.3. Feeding interactions between siphonophore species and their prey identified by our metabarcoding results (red), published submersible observations (blue), published visual gut content analyses (green), and predicted by the morphology-based DAPC model in Damian-Serrano et al. (2021b).
SM-Figure 4.1. Species-wise grid with the frequency of the major prey types identified from the metabarcoding data (left) and the average prey-type selectivity estimated in comparison with the local planktonic community composition (right). Gut content cells in white indicate absence, and cells in grey indicate presence in one specimen, or more than one specimen if labeled with a number. Selectivity colors mapped to Strauss’ L.I. values.
SM-Figure 4.2. Relative read log-abundance colored by OTU source interpretation for each species.

SM-Figure 4.3. Relative read log-abundance colored by OTU source interpretation for each species and barcode region.
SM-Figure 4.4. Relative read log-abundance colored by OTU source interpretation for each specimen.
SM-Figure 4.5. Relative read log-abundance colored by OTU source interpretation for each specimen and barcode region.
SM-Figure 4.6. Relative read log-abundance colored by OTU taxon for each species.

SM-Figure 4.7. Relative read log-abundance colored by OTU taxon for each species and barcode region.
SM-Figure 4.8. Relative read log-abundance colored by OTU taxon for each specimen.
SM-Figure 4.9. Relative read log-abundance colored by OTU taxon for each specimen and barcode.
GENERAL DISCUSSION

The primary questions motivating this thesis are: (1) how do trophic interactions and specializations evolve, (2) how does trait evolution shape those evolutionary outcomes, and (3) what are the evolutionary histories and relationships between siphonophore diets and their tentilla morphology? These questions were prompted by several open ends in the literature. First of all, advances in siphonophore natural history have shown that some shallow-water siphonophores are highly specialized in particular prey types, and such specializations are correlated with characteristics of their tentilla and nematocysts (Purcell, 1984). Also, as more deep-sea siphonophore species were described, the exuberant diversity of tentilla morphologies was revealed (examples in Dunn et al. 2005a, Pugh & Haddock 2010, Pugh & Haddock 2016). Finally, the recent application of molecular phylogenetics to siphonophores opened up the opportunity to explore the evolutionary history of their morphological features and their ecological outcomes (Dunn et al. 2005b, Munro et al. 2018). To this end, chapters 1 and 2 present the first macroevolutionary perspective on the diets and tentilla morphologies of siphonophores, revealing inherited homologies and homoplastic convergences.

At the same time, there was an active discussion in the literature about general patterns and expectations for the evolution of specialization and generalism (Futuyma & Moreno 1988, Forister et al. 2012). The most common expectation was for specialists to evolve from generalists, and subsequently get
stuck in an evolutionary ‘dead-end’, where extreme morphological adaptations create strong constraints preventing further evolution into different specializations or generalism (Simpson 1944; Kelley & Farrell 1998; Crespi & Sandoval 2000). These specialized lineages would then be expected to diversify less and eventually go extinct. However, other studies have contested this paradigm showing that exceptions to this rule abound in nature due to a myriad of ecological compensatory mechanisms (Schluter 2000, Janz et al. 2001, Stireman 2005, Nosil 2002, Winkler and Mitter 2008, Colles et al. 2009). In Chapter 1, we found that siphonophore specialists evolved into generalists at least twice independently and that specialists shifted their prey-type specialization at least five times (Damian-Serrano et al. 2021). This result led to the hypothesis of an additional alternative mechanism to avoid ‘dead-end’: organismal modularity. Siphonophores’ uniquely modular body plan may provide sufficient functional and spatial independence of their prey-capture apparatus to circumvent evolutionary constraints.

The siphonophore zoology literature has described a myriad of tentilla shapes, sizes, and nematocyst compositions (Mapstone 2014). However, the evolutionary history of gains and losses and the evolutionary mechanisms leading to this diversity has remained purely speculative. Tentilla discharge behaviors have been generally described as a fast slinging motion of the cnidoband that traps the prey within the firing nematocysts (Mackie et al. 1988). While this described behavior is unique among cnidarians, the diversity of speeds, times, and accelerations of tentillum discharge across species had
never been measured, nor compared to the underlying morphology. In Chapter 1 we find that many of the morphological characters of siphonophore tentilla and nematocysts are highly correlated with each other. The fact that the functionality of the precisely-coordinated discharge strike is preserved across the broad variety of tentillum shapes and sizes (functional integration) leads to the hypothesis that such evolutionary correlations could be maintained by phenotypic integration (when multiple functionally-related traits evolve in correlation with each other). We also found that the evolutionary optima for these characters (and their evolutionary correlations) were significantly distinct between feeding guilds, but the differences in morphospace occupation and breadth between those guilds remained unexplored. In Chapter 2 we bridge all these gaps by describing the evolutionary history of tentillum and nematocyst novelties, the functional relationships between morphological and kinematic performance during discharge, finding a significant degree of phenotypic integration across morphological modules, and reporting significant differences between the tentillum morphospaces of siphonophores in different feeding guilds.

While the morphological diversity of siphonophore tentilla and their nematocysts presented a promising avenue for the study of their trophic ecomorphology, there still were large gaps of knowledge regarding the diets and prey selectivities of deep-sea siphonophore species. In Chapter 3 we characterize and summarize the diets and specializations of deep midwater siphonophores, and develop a novel distribution modeling method to estimate
relative prey availability and selectivities. Our results showed that similarly to their epipelagic relatives, deep-sea siphonophores are also highly specialized and selective predators, where spatially overlapping species tend to specialize on different prey. These findings validated the assumptions made in Chapter 1’s preliminary assessment on the evolution of siphonophore specialization. Hetherington et al. (2021) found higher apparent specialization in deep-dwelling siphonophore species than in epipelagic species. This could be revealing an ecological feature of open-ocean food webs where specialization increases with depth. In Chapter 3 we found no relationship between specialization and depth (nor depth range) in siphonophores. This suggests that the differences found in Hetherington et al. (2021) are likely due to the methodological differences inherent to studying shallow and deep-dwelling species.

This confounding disparity of methods used to assess the diets of shallow and deep siphonophore species has limited the reliability of ecological comparisons across species in shallow and deep environments (Hetherington et al. 2021). Published studies on shallow-dwelling siphonophore species have assessed their diets by visually inspecting their gut contents (Purcell 1981), potentially underestimating the presence of rapidly-digested, soft-bodied, and gelatinous prey. On the other hand, studies exploring the diets of deep-dwelling species have focused on observations from submersibles (Choy et al. 2017), potentially underestimating the capture and ingestion of hard-to-see small prey. In Chapter 4, I examined the diets of shallow and deep-dwelling siphonophore species using DNA metabarcoding of their gut contents and compared them to
their surrounding prey fields from plankton samples. This provided a complementary and alternative approach to characterizing the diets of siphonophores under a common methodological framework. In addition, it provided the first insights into the diets of several understudied species. When comparing the metabarcoding results to the published records of siphonophore diets derived from visual methods, I found that metabarcoding was able to confirm many of the published interactions, but also detected several instances of small gelatinous prey undetected by visual methods. In Chapter 2 I used tentilla morphology and published dietary data to generate predictions on the diets of understudied siphonophore species. The metabarcoding results of Chapter 4 were congruent with the predictions for the majority of species assessed, further confirming the robustness of the ecomorphological link between siphonophore tentilla and diet.

In addition, the results from chapters 3 & 4 identified three new instances of potential generalists that may have evolved independently from specialist ancestors further supporting the conclusions from Chapter 1. I hypothesized that siphonophores’ ability to evolve from specialists to generalists is related to their uniquely de-coupled prey-capture apparatus. This stands in contrast to integrated prey-capture apparatus such as the mouth in most vertebrate predators. In fact, when we examine the evolutionary history of specialization in a clade of fishes such as cichlids, we observe a clear evolutionary directionality from more generalized diets to more specialized ones (Arbour et al., 2020). The high prevalence of prey-type specialization observed in
chapters 3 & 4 suggests that siphonophores, as opposed to more familiar active-pursuit predators, are far less constrained by prey size but more constrained by prey type. Therefore, siphonophores may contribute to making midwater food webs less size-structured and more phylogenetically-structured.

In conclusion, this dissertation has advanced our understanding of siphonophore ecology and evolutionary history, open-ocean food web structure, and the evolution of specialization. Integrating the insights from all four chapters we find that siphonophores have evolved from and into specialized predators, often strongly selective for specific prey types driven by specialized tentilla and nematocyst morphologies. Their modularized prey-capture apparatus has released siphonophores from the morphological constraints that bind more familiar predators, which has allowed them to occupy different trophic roles in the open-ocean food web.

References


