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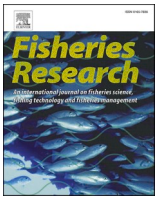

Unexpected diversity in the diet of *Doryteuthis sanpaulensis* (Brakoniecki, 1984) (Mollusca: Cephalopoda) from the southern Brazilian sardine fishery identified by metabarcoding

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ABSTRACT

Growing fishing pressure worldwide has led to an increase in the exploitation of cephalopod products in fish markets where these taxa were not traditionally consumed. Squid catches have surged to meet that demand, yet for many species little is known about their role in food webs. *Doryteuthis sanpaulensis* is an important squid species in southeastern Brazilian fisheries. Despite many previous efforts at morphological analysis of its diet, few demersal and benthic species of fishes, crustaceans and mollusks have been identified to species level because the food is consumed in small digestible fragments. Here we used metabarcoding to analyze the diet of adult *D. sanpaulensis* caught as bycatch in the southern Brazilian sardine fishery. MOTUs generated from COI amplicons were assigned to taxa by matching against the NCBI nt database. Fishes constitute the majority of the diet of the analyzed samples. Considerable variability in the relative read abundance and frequency of occurrence of prey items across samples indicates the importance of increased sample sizes in analyses investigating ontogenetic, spatial, or temporal variation in diet. The results elucidate the rich diet of *D. sanpaulensis* off the Brazilian coast, and specifically that its varied diet includes more neritic diversity than previous studies have indicated.

1. Introduction

Knowledge of food webs is important for fisheries management and to answer biological questions about the adaptability of species in marine ecosystems (Roslin and Majaneva, 2016). Some cephalopods represent key links in food webs acting as intermediaries in the energetic flux between many trophic levels and are normally subdominant predators as well as important preys and competitors of many marine fishes (Gasalla et al., 2010; Migliavacca and Simone, 2020).

In common with other loliginid squids, the genus *Doryteuthis* Naef, 1912 is generally considered to be demersal and limited to the continental shelf (to around 200 m depth) because of a dependence on specific marine substrates for egg mass deposition (Boyle and Rodhouse, 2005). They are important in the diet of various top predators (Santos and Haimovici, 2001; Cherel and Duhamel, 2004; Zeidberg, 2013) and are voracious predators of crustaceans, mollusks and fishes (Santos and Haimovici, 2001; Gasalla et al., 2010). Cannibalism occurs in cephalopods both at high population sizes and during the mating season (both intra-cohort and inter-cohort cannibalism) and the frequency of cannibalism and the main prey type varies according to species, size of predator and season (Rosas-Luis et al., 2014).

Cephalopods are frequently caught as bycatch in sardine purse-seine and shrimp trawl fisheries in Brazil. Although always considered a valuable bycatch, both gear modifications and greater availability have

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gradually increased the incidental cephalopod capture over recent decades and now there are also some small-scale targeted squid fisheries in periods of the year when other resource catch rates are low (Perez et al., 2002). Traditional trawl fishery products (e.g. pink shrimp and sciaenid fish) have reduced considerably since the 1980s (Haimovici et al., 2006) and cephalopod catches have increasedto meet the growing demand for non-fish fishery products (Vidal et al., 2013). In southeastern Brazil commercial fisheries target the loliginid species *Doryteuthis plei* (Blainville, 1823) and the Sao Paulo squid, *Doryteuthis sanpaulensis* (Brackoniecki, 1984) (Gasalla et al., 2005). The latter is found on the continental shelf of Uruguay, Argentina and southern Brazil (20°–46°S) associated with cold water (Jereb et al., 2010).

Studies that attempted to describe the diet of *D. sanpaulensis* indicated predominantly demersal and benthic species of Actinopterygii, Malacostraca and Mollusca (Andriguetto and Haimovici, 1997). However, taxonomic resolution of the prey items was limited as digestion and the chopping of prey into small pieces by cephalopod beaks (Boyle and Rodhouse, 2005) makes accurate morphological identification challenging. Molecular analyses can be effective to improve dietary identification and DNA metabarcoding has proven to be a powerful tool in the identification of diet in many organisms belonging to a variety of taxa (Bessey et al., 2019), including early life stage cephalopods (Olmos-Pérez et al., 2017; Roura et al., 2017; Fernández-Álvarez et al., 2018), but never on adult cephalopods.

Here, we used a metabarcoding approach to investigate the diet of adult cephalopods for the first time, specifically focusing on *D. sanpaulensis*, an important bycatch in the Brazilian sardine fisheries.

2. Material and methods

Samples were collected at 26°38′47.29″S, 48°34′48.04″W in September of 2016 during fisheries monitoring of the sardine fishery of southeastern Brazil. Amongst general sampling of all species morphologically identified in the bycatch, we obtained nine adult individuals of *D. sanpaulensis* (Appendix A). Although samples were obtained by purse seining (that, as far as marine net sampling methods go, is the method that leaves least time for potential predator prey interactions), all co-collected species (squids and fishes) were recorded to infer possible predation events in the net (Appendix A). Samples were frozen until dissection at the University of São Paulo. Stomach contents were removed using external decontamination protocols (washing of squid and unopened stomachs using low concentration bleach and then ultrapure water to remove environmental contaminants) and decontaminated equipment to reduce the contamination risk of the stomach contents (Zinger et al., 2019). Only six of the stomachs sampled were full and their contents were preserved in pure ethanol with external labelling and transferred to the Federal University of Pará - UFPA, for further processing. Samples were collected under SISBIO license number 53022-2 from the "Instituto Chico Mendes de Conservação da Biodiversidade". Work was performed under approval of the UFPA Ethics Committee (CEUA-UFPA - Permit 68/2015).

All procedures were performed using bleach and/or UV light exposure to decontaminate all material. Individual stomach contents were separated from preservative by three cycles of centrifugation and washing with ultrapure and UV sterilized water, before being homogenized, and subsample replicates stored in separate microcentrifuge tubes (2–4 subsamples depending on the sample volume available). DNA was extracted using a CTAB/phenol/chloroform protocol (Doyle and Doyle, 1987). Within the overall project, 16 subsamples representing the six samples *D. sanpaulensis* were available for sequencing as well as multiple negative controls for sample processing (tubes opened and filled only with ethanol during dissection), DNA extraction and amplicon production steps.

A portion of the Cytochrome c oxidase subunit I marker (130bp) was amplified using the primers Minibar-Mod-F and Minibar-Mod-R (Berry et al., 2015), including a 12 bp upstream index (Fadrosh et al., 2014), using polymerase chain reactions with the following conditions: 1X Q5 High-Fidelity master mix (New England Biolabs), 1X Q5 enhancer (New England Biolabs), 0.5 μM of each primer and 2−3 ng of template DNA in a final volume of 25 μl. Unique dual-index primer combinations were used for each subsample. The thermal profile was as recommended by the supplier using 45 °C as the annealing temperature.

Amplicons were visualized on agarose gels and quantified using the ImageLab Software v6.0 (Bio-Rad Laboratory). Based on the quantification, uniform amounts of each amplicon were merged using a Biomek 4000 liquid handling robot (Beckman Coulter). The DNA library was cleaned using 1.0X AMpure beads (Beckman Coulter), and sequence adapters ligated to the dual-indexed amplicons using the NEBNext Fast DNA Library Prep Set for Ion Torrent (New England Biolabs). The amplified libraries were size selected using BluePippin (Sage Science). The final libraries were quantified on a Fragment Analyzer (Agilent) using the High Sensitivity Genomic DNA Kit (Agilent). The libraries were sequenced on two 530 chips on an Ion GeneStudio S5 system (Thermo Fisher).

First, raw sequencing data were demultiplexed with an in-house script that uses the dual-index barcodes. All data for each sample was deposited in GenBank (Appendix A). The demultiplexed FASTQ files were cleaned to remove low-quality bases (PHRED < 20) using PRINSEQ (Schmieder and Edwards, 2011). Then, we used QIIME (Caporaso et al., 2010) and VSEARCH (Rognes et al., 2016) pipelines to perform dereplication, discard singletons, trim sequences to 130 bases, and cluster molecular operational taxonomic units (MOTUs) using similarity thresholds of 97 % and 99 %, removing chimeras. MOTUs were blasted against the NCBI nt reference database for taxonomic assignment, using LULU (Frøslev et al., 2017) to remove erroneous MOTUs (MOTUs representing high within-sample intraspecific diversity or PCR and sequencing errors that are detectable based on sequence similarity and co-occurrence patterns) and an in-house script to check for and exclude MOTUs representing nuclear mitochondrial pseudogenes (NUMTs) based on descriptors considering the 10 most similar sequences identified by BLAST. Assignment was evaluated using minimum thresholds of 97 % similarity and the % similarity to the most similar taxon was recorded.

Rarefaction curves for each replicate were performed to assess whether adequate sequencing depth had been achieved, using random sampling of 999 sequences without replacement in the "rarecurve" function from vegan (Oksanen et al., 2007) in R3.5 (R Core Team, 2018).

To remove false positives and possible contaminants or sequencing errors we applied the following rules: i) the maximum number of reads detected in the controls was removed for each MOTU from all samples; ii) MOTUs containing less than 10 reads overall were discarded; iii) obvious non-target species or MOTUs likely originating from carry-over contaminations were removed from the dataset (Li et al., 2018; Ushio et al., 2018). Reads were then averaged across subsamples (to avoid inflation of read abundance in samples with more subsamples e.g. 4 vs. 2 subsamples). The final assignment of taxonomic identification of dietary items was confirmed based on similarity values and knowledge on taxonomy and geographic distributions in the literature that were accessed through FishBase (Froese and Pauly, 2020) and SeaLifeBase (Palomares and Pauly, 2020). No blocking primers were used in this study, so the predator DNA (squid) was co-amplified alongside dietary items. Although cannibalism is recorded for *D. sanpaulensis*, for MOTU relative read abundance (RRA) analyses (Deagle et al., 2019) these data were excluded. Secondary consumption of items was evaluated using both RRA within samples (excluded when RRA < 1%, and evaluated in combination with other data/literature when RRA < 3% following suggestions in Deagle et al., 2019) and comparative data from the literature and ongoing metabarcoding analysis of dietary items of other samples from the fishery (unpublished data). High RRA and frequent occurrence of items in the diet of multiple individuals of the prey identified here would suggest that those dietary items could easily be ingested by *D. sanpaulensis* secondarily even though squid do not usually

ingest whole prey. Items were visualized across samples using bubble plots of RRA of prey items in each sample using ggplot2 (Wickham, 2009) in R3.5 (R Core Team, 2018).

3. Results

Clustering MOTUs at 97 % similarity was better than at 99 % as the higher similarity threshold provided no new taxon assignments, significantly increased the number of MOTUs (1000s more) and only resulted in duplicated MOTU assignments to the same taxa. The *D. sanpaulensis* diet dataset clustered at 97 % similarity comprised 176711 reads, representing 763 MOTUs. While some subsamples showed low sequencing depth (Appendix A), pooling subsamples from the same stomach sample results in good sequencing depth per sample (Appendix A). Most MOTUs were represented by <100 reads (Appendix A). Negative controls presented very few contaminant MOTUs and very few reads. These included <10 reads in any given sample for human DNA and species being worked on by the research groups involved, indicating that decontamination procedures were successful at reducing contamination throughout the experiment.

After automated data cleaning and manual curation in post-processing, MOTUs were assigned to 19 potential prey taxa and *D. sanpaulensis* itself. All were assigned with similarities of >98 %. Of these, three MOTUs were considered to represent likely secondary consumption (*Pleoticus muelleri* (Bate, 1888) and *Dactylopterus volitans* (Linnaeus, 1758) based on the rule of RRA < 1%, *Trichiurus lepturus* Linnaeus, 1758 based on the rule of RRA < 3% plus additional data, Appendix A) and removed from further analysis leaving 16 principal prey items belonging to two phyla, two classes, seven orders, eight families, 15 genera and 16 species (Fig. 1; Table 1; Appendix A). High read counts for the MOTU assigned to *D. sanpaulensis* confirmed that all stomachs originated from the Sao Paulo squid (and not the co-occurring *D. plei* to which <10 reads were assigned in the whole dataset).

Considering the dataset for principal prey items, teleost fishes were the most common prey with over 99 % of reads with one crustacean, the redspotted shrimp *Penaeus brasiliensis* Latreille, 1817 (Malacostraca: Decapoda), represented by <1% of all reads but present in two samples (Appendix A; Fig. 1). Members of the fish family Carangidae were the most commonly identified MOTUs in the diet of *D. sanpaulensis*, found in five of the six samples (all except DSA306). *Chloroscombrus chrysurus* (Linnaeus, 1766) was caught in the same net as the sampled squid and was the main item identified in sample DSA310 (it was also found in three of the six samples - DSA307, DSA310 and DSA313), while *Selene setapinnis* (Mitchill, 1815) was not caught in the same net and was found in five samples (all samples except DSA306). Members of the family Sciaenidae were also common, found in five of the six samples (all except DSA306), with *Cynoscion guatucupa* (Cuvier, 1830) (not collected in the same net) dominant in samples DSA311 and DSA312. *Aluterus monoceros* (Linnaeus, 1758) (Monacanthidae) was not collected in the same net and was the main item in the diet of sample DSA313 and showed moderately high RRA in DSA307 and DSA310 and, although with lower RRA, it was the second most represented item in sample DSA312. *Porichthys porosissimus* (Cuvier, 1829) (Batrachoididae) was not caught in the same net and was found in four of the six samples, being one of the dominant items in samples DSA306 and DSA307. The

Table 1
Summary of habitat use of the 16 prey species identified in stomach samples of *D. sanpaulensis* collected offshore from Barra Velha, Santa Catarina state, Brazil, ordered by frequency in the six samples analyzed.

Phylum	Class	Species	Frequency in Samples	Habitat Use
Chordata	Actinopterygii	*Selene setapinnis*	5/6	Benthopelagic
Chordata	Actinopterygii	*Aluterus monoceros*	4/6	Benthopelagic
Chordata	Actinopterygii	*Porichthys porosissimus*	4/6	Demersal
Chordata	Actinopterygii	*Chloroscombrus chrysurus*	3/6	Pelagic/ Neritic
Chordata	Actinopterygii	*Hemicaranx amblyrhynchus*	3/6	Pelagic/ Neritic
Chordata	Actinopterygii	*Paralonchurus brasiliensis*	3/6	Demersal
Chordata	Actinopterygii	*Caranx crysos*	2/6	Pelagic
Chordata	Actinopterygii	*Cynoscion guatucupa*	2/6	Demersal
Chordata	Actinopterygii	*Cynoscion jamaicensis*	2/6	Demersal
Chordata	Actinopterygii	*Micropogonias furnieri*	2/6	Demersal
Chordata	Actinopterygii	*Orthopristis ruber*	2/6	Demersal
Chordata	Actinopterygii	*Sardinella brasiliensis*	2/6	Pelagic/ Neritic
Arthropoda	Malacostraca	*Penaeus brasiliensis*	2/6	Demersal
Chordata	Actinopterygii	*Engraulis anchoita*	1/6	Pelagic/ Neritic
Chordata	Actinopterygii	*Macrodon ancylodon*	1/6	Demersal
Chordata	Actinopterygii	*Oligoplites saliens*	1/6	Benthopelagic

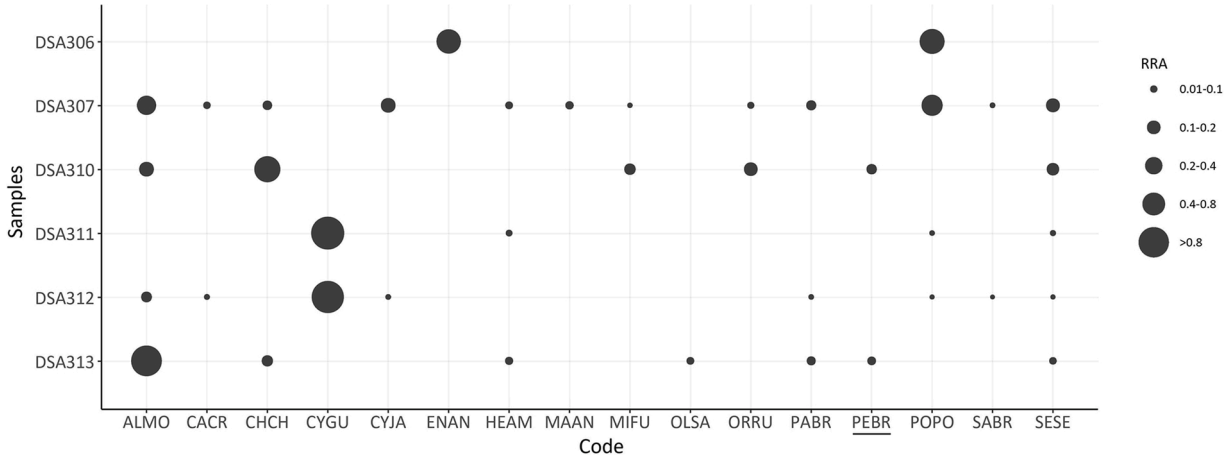

Fig. 1. Bubble graph of relative read abundance of the 16 prey items identified in stomach samples of *D. sanpaulensis* collected offshore from Barra Velha, Santa Catarina state, Brazil. RRA = Relative Read Abundance. ALMO = *Aluterus monoceros*; CACR = *Caranx crysos*; CHCH = *Chloroscombrus chrysurus*; CYGU = *Cynoscion guatucupa*; CYJA = *Cynoscion jamaicensis*; ENAN = *Engraulis anchoita*; HEAM= *Hemicaranx amblyrhynchus*; MAAN = *Macrodon ancylodon*; MIFU = *Micropogonias furnieri*; OLSA = *Oligoplites saliens*; ORRU = *Orthopristis ruber*; PABR = *Paralonchurus brasiliensis*; PEBR= *Penaeus brasiliensis*; POPO = *Porichthys porosissimus*; SABR = *Sardinella brasiliensis* and SESE = *Selene setapinnis*. All prey are fishes except PEBR, which is a crustacean.

only non-teleost, *P. brasiliensis*, was not collected in the net and was present in only two of the six samples (DSA310 and DSA313).

In one sample (DSA306) only two prey items were identified. In other samples between four (sample DSA311) and 12 (sample DSA307) prey items were identified. In DSA306 RRA was relatively evenly distributed between the two dietary items. In three of the other samples there was a very dominant single prey item, indicating that this prey item was the most recently ingested or comprised the greatest volume of stomach content, but the two remaining samples (DSA307 and DSA310) showed more evenly distributed RRA across a range of prey items (Fig. 1).

4. Discussion

The diet of the Sao Paulo squid, *D. sanpaulensis*, was found to include 15 fishes and one crustacean. Traditional studies of its diet had identified a variety of taxa (summarized in Vidal et al., 2013), but these only included one of the species identified here as primarily consumed (*Micropogonias furnieri* (Desmarest, 1823): Perciformes) and one species that we conservatively removed as a possible secondary consumption artefact (*Pleoticus muelleri*: Decapoda). An unidentified penaeid crustacean (Castellanos, 1967b) and carangid fishes (represented by *Trachurus lathami* Nichols, 1920, Rodrigues, 2008) have also previously been recorded. The majority of prey identified in this and previous studies are benthopelagic or demersal, but we observed the presence of pelagic-neritic carangid fishes including *C. chrysurus* and *Hemicaranx amblyrhynchus* (Cuvier, 1833) as well as the pelagic-neritic *Engraulis anchoita* Hubbs & Marini, 1935. These are usually found at depths of 0−100 m, which is consistent with the observed foraging amplitude of *D. sanpaulensis* (Rodrigues and Gasalla, 2008). Although *C. chrysurus* was caught in the same net, *H. amblyrhynchus* and *E. anchoita* were not, indicating that the pelagic-neritic signal is not just a result of within net predation. Within net predation could be resolved by sampling using artificial lures for jigging or by diving, but neither of these methods are viable cost-effective sampling methods for *D. sanpaulensis* (jigging crews are only known to catch *D. plei* - no *D. sanpaulensis* have been recorded from them). Compared to previous morphological diet analyses, generally made using trawl samples where coexistence in the net is protracted, our data is less likely to be impacted by within net consumption.

This is the first use of metabarcoding to investigate the diet of adult cephalopods. The few metabarcoding analyses of cephalopod diet to date have been in paralarvae and larvae. Paralarvae of *Octopus vulgaris* Cuvier, 1797 and *Alloteuthis media* (Linnaeus, 1758), were found to consume various crustaceans, echinoderms, mollusks, and hydroids (Olmos-Pérez et al., 2017; Roura et al., 2017), while paralarval and larval flying squids (various species of Ommastrephidae) were classified as detritivores (Fernández-Álvarez et al., 2018). Diet of *D. sanpaulensis* has been recorded as shifting ontogenetically, as in other squids, with crustaceans being replaced by fish at larger mantle sizes (Martins, 2002). However, fish are important at a large range of mantle sizes (Andriguetto and Haimovici, 1997) and form a large part of the unidentified matter in many morphological studies of diet in this species (Vidal et al., 2013). Our results complement these previous morphological analyses with precise species-level identification of a larger number of prey items including many fish (Appendix A, Fig. 1).

Our data suggests that, because of their different method of feeding, the relative signal of secondary consumption in cephalopods may be lower than that found in organisms that swallow prey whole. Secondarily consumed items can be useful for providing a snapshot of regional biodiversity (Siegenthaler et al., 2019). Many sequences from our samples were unmatched and these could represent new taxa (e.g. Sales et al., 2019).

Cannibalism is common in cephalopods, and squids are often identified in the diet of *D. sanpaulensis*, though not as a significant percentage of consumption (Vidal et al., 2013). Cannibalism normally occurs in dense aggregations, that are not common for *D. sanpaulensis* (Santos and Haimovici, 1998). Food and mate competition and stressful situations are other reported causes of cannibalism in squid (Ibánez and Keyl, 2010).

The variability in the individual diet of squids identified here highlights the importance of bigger sample sizes if diet is to be compared seasonally (e.g. associated to reproductive periods) or across fishing regions. Inclusion of other molecular markers may also help recover the identity of more taxa (Berry et al., 2015), as should improvements in the taxonomic coverage of reference databases. While a significant contribution to species level identification of more prey items than in morphological analyses was made, increased sampling will help define trophic relationships for these ecologically and commercially important species.

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CRediT authorship contribution statement

Talita Fernanda Augusto Ribas: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing. **João Bráullio de Luna Sales:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing. **Hugo de Boer:** Resources, Supervision, Writing - review & editing, Funding acquisition. **Jarl Andreas Anmarkrud:** Methodology, Investigation, Data curation, Writing - review & editing. **Renato Renison Moreira Oliveira:** Software, Formal analysis, Resources, Data curation, Writing - review & editing. **Marcele Laux:** Software, Formal analysis, Resources, Data curation, Writing - review & editing. **Fabricio dos Anjos Santa Rosa:** Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Guilherme Corrêa Oliveira:** Writing - review & editing, Supervision, Funding acquisition. **Felippe A. Postuma:** Investigation, Resources, Data curation, Writing - review & editing. **Maria A. Gasalla:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Jonathan Stuart Ready:** Conceptualization, Methodology, Data curation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that the research for the paper below was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

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