



# Stomach content characterisation of the marine range-shifting *Octopus tetricus* using DNA metabarcoding

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**ABSTRACT:** The common Sydney octopus *Octopus tetricus* has undergone range extension along the east coast of Australia, associated with regional warming and the strengthening and southward extension of the East Australian Current (EAC). Its historical range of distribution is from southern Queensland to southern New South Wales, but it is now also found off north-east Tasmania, where it may affect local ecosystem dynamics due to changes in trophic interactions. This study aims to identify the prey and trophic level of *O. tetricus* from specimens collected off Tasmania to anticipate potential ecological, economic, and conservation effects in the range-extended area. The stomach contents of 18 *O. tetricus* individuals captured off north-east Tasmania in 2011 were characterised using DNA metabarcoding. Sixteen families of prey were identified; crustaceans (Alpheidae, Calcinidae, Diogenidae, and Galatheidae) were the most frequently detected prey, followed by fishes and bivalves. Prey species of commercial importance included blue-throated wrasse *Notolabrus tetricus* and the scallop *Pecten fumatus*. *O. tetricus* was found to have an intermediate trophic level of 3.66, participating in the transfer of energy from lower to upper trophic levels. Predation by *O. tetricus* could present competition to local fishers for resources and additional pressure on local fishery stocks, and may alter estimates of natural mortality used by fisheries management. The present study may be useful to scientists, fishery managers, and conservationists because it provides a preliminary assessment of the diet of *O. tetricus*, with potential ecological, economic, and conservation implications in the range-extended area.

**KEY WORDS:** Molecular prey identification · Diet · Trophic level · Common Sydney octopus · Gloomy octopus · Range extension · East Australian Current

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## 1. INTRODUCTION

The global redistribution of marine species is one of the most frequently documented responses to cli-

mate change, with species reportedly moving polewards as they track their preferred temperatures (Sunday et al. 2012, Poloczanska et al. 2013). Redistribution of marine species can result in the reorgan-

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isation of marine communities via reshuffling of existing species and the arrival of 'new' species, with consequences on ecological networks (Edwards & Richardson 2004, Alheit 2009, Walther 2010). Challenges will not only arise for scientists investigating effects on ecosystem processes associated with the reorganisation of marine communities (Sorte et al. 2010), but also for resource managers and policy-makers, as impacts can also extend to important socio-economic activities such as fisheries (Madin et al. 2012).

South-east Australia's marine environment is a global 'hotspot' of oceanic warming (Hobday & Pecl 2014), and marine range shifts have been documented extensively in this region (Johnson et al. 2011, Range Extension Database and Mapping Project 2013). The East Australian Current (EAC) is the major western boundary current that flows southward along the east coast of Australia, from the southern Coral Sea in Queensland to southern New South Wales (Ridgway & Dunn 2003). Over the past 60 yr, the EAC has intensified and has extended approximately 350 km further south from southern New South Wales to the east coast of Tasmania (Ridgway 2007, Hill et al. 2008, Phillips et al. 2020). The strengthening of the EAC contributes to a warming rate in the southern Tasman Sea of almost 4 times the global average (Hobday & Pecl 2014), with marine heatwaves that have reached 2.9°C above the long-term average (Oliver et al. 2018). The strengthening of the EAC and the associated warming is enabling the southwards range extension of several species of cephalopods, gastropods, bivalves, echinoderms, arthropods (Pitt et al. 2010, Gervais et al. 2021), fishes (Last et al. 2011, Gervais et al. 2021), and algae (Gervais et al. 2021) down to the east coast of Tasmania.

The common Sydney octopus *Octopus tetricus* has extended its distribution from southern Queensland and New South Wales to Victoria (Villanueva & Norman 2008, Edgar & Stuart-Smith 2014) and to north-east Tasmania, where it was first detected in 2006 (Tasmanian Department of Primary Industries and Water 2009, Johnson et al. 2011, Range Extension Database and Mapping Project 2013, Edgar & Stuart-Smith 2014). This species can be found at 0–60 m depth, where it inhabits shallow coastal waters in subtidal areas and in rocky reefs (Norman et al. 2016). At the core of its historical distribution off eastern mainland Australia (32.5° S, 152.5° E), average sea surface temperature (SST) ranged between 22.7°C in the warm season (October to March) and 20.6°C in the cold season (April to September), and

between 17.1°C in the warm season and 14.9°C in the cold season at the range-extended area off north-east Tasmania (39.5° S, 148.5° E) (average SST was calculated from the Copernicus Marine Environment Monitoring Service; <https://doi.org/10.48670/moi-00024>).

*O. tetricus* may be able to establish and persist in the range-extended area because of its rapid population turnover (Ramos et al. 2014), high reproductive capacity (Ramos et al. 2015), and constant gene flow from a diversity of source areas along the east coast of Australia that may promote relatively high genetic diversity (Amor et al. 2014, Ramos et al. 2018). Indeed, a decrease in habitat suitability for *O. tetricus* over its northern distribution off eastern mainland Australia and an extension in habitat suitability off Tasmania were predicted between the years 2050 and 2100 under the Representative Concentration Pathways 2.6, 4.5, 6.0, and 8.5 scenarios (Borges et al. 2022). Within its historical distribution in Australian and New Zealand waters, *O. tetricus* feeds on crustaceans and molluscs, such as gastropods, bivalves, and other octopuses including conspecifics (Anderson 1997, Norman & Reid 2000), but the nature and diversity of its prey have not been described within the recently extended areas. Food availability and temperature can influence growth in paralarvae and juveniles, while gender, sexual maturity, and reproductive investment can also affect growth in adults (Semmens et al. 2004). Having shown different growth rates in warm and cold seasons (Ramos et al. 2014), the diversity and quantities of prey in *O. tetricus* diets may also differ between seasons in the range-extended area.

Characterisation of *O. tetricus* prey can be used to inform potential ecological, economic, and conservation impacts in these new areas. The range shift of *O. tetricus* has already been associated with possible negative impacts on the Tasmanian temperate reef by predation upon the southern rock lobster *Jasus edwardsii* (Marzloff et al. 2016), a natural predator of the invasive long-spined sea urchin *Centrostephanus rodgersii* that has contributed to the depletion of the Tasmanian kelp forests (Ling et al. 2015). *O. tetricus* may compete with local predators in the extended areas (Villanueva & Norman 2008) and increase top-down pressure (Borges et al. 2022), possibly leading to ecological and economic impacts if they prey on local species of ecological or commercial importance, or upon the prey of these species. For instance, a Western Australian octopus species, *O. djinda* (Amor et al. 2014, 2017, Amor & Hart 2021), preys on trap-caught spiny lobsters *Panulirus cyg-*

*nus* (Joll 1977). The East Asian common octopus *O. sinensis* preys on the commercially important Pacific abalone *Haliotis discus hannai* in the Northwest Pacific (Okei 1999). In Tasmanian waters, the Maori octopus *Macroctopus maorum* preys on queen scallop *Equichlamys bifrons* beds (Wolf & White 1997) and trap-caught *J. edwardsii* (Harrington et al. 2006). The predation by *M. maorum* on trap-caught *J. edwardsii* results in losses of 4% of the annual total catch in South Australia (Brock et al. 2003) and up to 10% losses of the annual total catch in some areas of New Zealand (Brock et al. 2006 and references therein). The incursion of another predator, such as *O. tetricus*, into Tasmanian waters may represent an additional source of mortality for commercially important fisheries species with implications for the structure and function of the marine ecosystem and for sustainable fisheries management in the range-extended area.

Common methods to determine diet composition may be invasive, such as direct observation of the interaction between predator and prey or by stomach content visual analysis, or indirect such as biochemical and molecular faecal analyses (Horswill et al. 2018). However, trophic interactions are not easy to track when observing elusive animals (Sheppard & Harwood 2005), such as octopuses. Stomach or faecal visual analysis is only possible with components of the diet resistant to digestion, e.g. otoliths and scales of fish, skeletal parts, beaks and sucker rings of cephalopods, etc. Moreover, cephalopods have a sharp parrot-like chitinous beak that enables a strong bite (Uyeno & Kier 2007) and breaks the prey in small pieces. The mechanical action of the beak and rapid digestion rates of about 6.5 h in octopuses (Bastos et al. 2020) complicates the visual identification and quantification of stomach contents (Symondson 2002).

Molecular approaches such as PCR-based techniques using DNA cloning and sequencing can be useful to analyse the diet of marine generalists (Jarman et al. 2004, Deagle et al. 2005a, Braley et al. 2010), especially when visual identification is not possible (Casper et al. 2007). DNA analyses provide high-resolution information on prey species diversity (Symondson 2002, Jarman et al. 2013). In these cases, the digested prey can be identified using amplified DNA sequences from diagnostic gene regions (Sheppard & Harwood 2005). The complementary use of universal and group-specific primers, which target these gene regions, may facilitate the detection of prey of interest (e.g. commercial or threatened species), as well as the detection of unknown prey (Jar-

man et al. 2004, Blankenship & Yayanos 2005). The development and continuous improvement of next-generation sequencing methods allow for the screening of multiple prey items simultaneously (DNA metabarcoding) and with a high taxonomic resolution by characterising thousands of sequences per PCR product (Pompanon et al. 2012) regardless of the degree of maceration.

DNA-based approaches have been successfully used to characterise the prey of a wide variety of predators of different trophic levels, such as the Adélie penguin *Pygoscelis adeliae* (Jarman et al. 2013), fur seals *Arctocephalus forsteri* and *A. tropicalis* (Casper et al. 2007), and sevengill shark *Notorynchus cepedianus* (Barnett et al. 2010). DNA-based approaches have also been used to examine the diet of invasive marine species such as the lionfish *Pterois volitans* (Harms-Tuohy et al. 2016), and of cephalopods in the adult stage such as the arrow squid *Nototodarus gouldi* (Braley et al. 2010) and the giant squid *Architeuthis* sp. (Deagle et al. 2005b), as well as early life history stages, including *O. vulgaris* (Roura et al. 2012, 2023) and *Alloteuthis media* paralarvae (Olmos-Pérez et al. 2017). These approaches can allow for the characterisation of the prey of range-shifting marine species. Therefore, this study aims to qualitatively and quantitatively characterise the prey contained in the stomachs of female and male *O. tetricus* in its range-extended area off Tasmania during the warm and cold seasons by using DNA metabarcoding, and to assess its trophic level. This study provides an early indication to scientists, fishery managers, and conservationists on the potential ecological, economic, and conservation implications of this range-shifting species in Tasmanian waters.

## 2. MATERIALS AND METHODS

### 2.1. Sample collection and processing

*Octopus tetricus* were collected at around 40° S, 147° E, off the east coast of Flinders Island, north-east Tasmania, Australia, within its range-extended area (Fig. 1) during 2011. Octopuses were collected by commercial fishing aboard the FV 'Farquharson' using unbaited black plastic pots (30 cm long × 10 cm high × 10 cm wide) laid on the seafloor at 35–46 m depth. The pots were left to soak for 20–30 d, and the date of retrieval of the pots was recorded. Since octopuses have rapid digestion rates of about 6.5 h (Bastos et al. 2020), stomach content was expected to represent the prey consumed over the past few hours be-

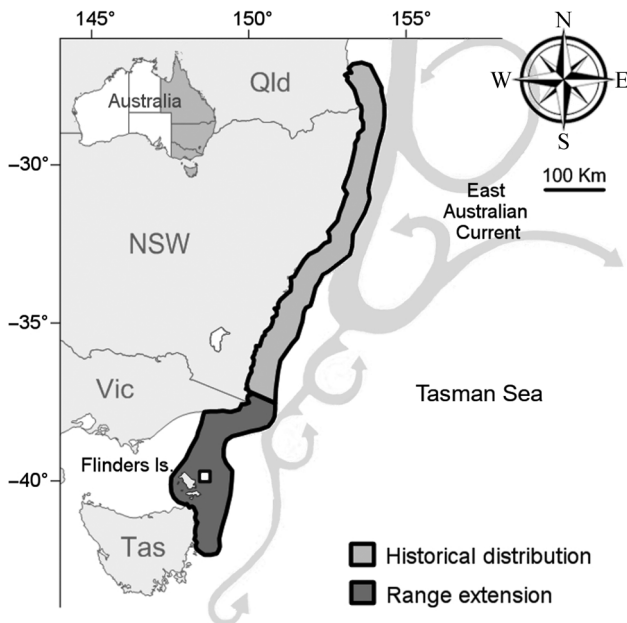


Fig. 1. Historical distribution and range-extended areas of *Octopus tetricus* along the east coast of Australia. The collection site off the east coast of Flinders Island, north-east Tasmania, Australia, during 2011 is indicated with the white box. Qld: Queensland; NSW: New South Wales; Vic: Victoria; Tas: Tasmania

fore the pots were retrieved. Octopuses use the pots as shelter and can leave them at any time; 1 octopus was generally found per pot. Whole octopus carcasses were provided by the fishers. Uncut stomachs were examined from the outside, visually and through palpation, to determine if there were prey contents. Empty stomachs were discarded; only stomachs that contained gastric liquid or remnants of prey were used ( $n = 48$ ), stored individually in plastic bags, and frozen on board at  $-20^{\circ}\text{C}$ . Sex and maturity of octopuses were recorded following Ramos et al. (2015).

## 2.2. DNA extraction

Stomachs were thawed at room temperature and dissected. Hard structures, soft tissue, and gastric

juices were collected and homogenised by shaking in 50 ml sterile plastic bottles for each stomach. A subsample of 500 mg of hard and soft structures was used for DNA isolation. Three kits were tested following the manufacturers' procedures: (1) FastDNA SPIN Kit for Soil (MP Biomedicals), (2) DNeasy Blood & Tissue Kit (Qiagen), and (3) Ultraclean Fecal DNA Isolation Kit (MO BIO). Fluorescence curves confirmed that the FastDNA SPIN Kit for Soil yielded higher DNA concentrations compared with the other 2 kits. The FastDNA SPIN Kit for Soil is designed to isolate bacterial, fungal, plant, and animal genomic DNA from soil and other environmental samples (MP Biomedicals), and has yielded larger amounts of DNA from human faeces and at reasonable cost compared with other kits (Ariefdjohan et al. 2010). Therefore, this cost-effective kit was used for the subsequent DNA extractions. Extraction blanks containing no DNA were included to check for contamination.

## 2.3. Primer selection and semi-nested polymerase chain reactions (PCR)

Universal and group-specific primers were used to amplify regions of DNA from the mitochondrial 16S ribosomal RNA (16S rRNA) gene of prey contents remaining in the stomachs of *O. tetricus* (Table 1). This set of universal and group-specific primers proved useful for the identification of prey in the stomach of the arrow squid *Nototodarus gouldi* (Bralley et al. 2010) and in the gut of *O. vulgaris* paralarvae (Roura et al. 2012), and was therefore selected for this study.

Two semi-nested PCR amplifications were performed independently on the DNA obtained from the stomach of each of the *O. tetricus* individuals following Bybee et al. (2011). To generate an amplicon pool, the first PCR was carried out with the universal primer 16Sar and the reverse group-specific primer 16Scrur (designed for crustaceans, gastropods, and bivalves). The prey-specific primers

Table 1. Primers used to detect prey in the stomachs of *Octopus tetricus* in its extended range off north-east Tasmania. Annealing temperature was  $54^{\circ}\text{C}$  for all PCRs

Target taxon	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product size (bp)
Universal	16Sar CGC CTG TTT ATC AAA AAC AT	16Scrur CGC TGT TAT CCC TAA AGT AA	550–620
Crustaceans/fishes	16Scruf1 GAC GAT AAG ACC CTA TAA	16Scrur CGC TGT TAT CCC TAA AGT AA	194–204
Gastropods/bivalves	16Scruf2 GAC GAT AAG ACC CTG TGA	16Scrur CGC TGT TAT CCC TAA AGT AA	194–204

16Scruf1 (designed for crustaceans and fishes) and 16Scruf2 (designed for gastropods and bivalves), and the reverse group-specific primer 16Scruf were modified by adding the adapter GGA AGG TGA CCA AGT TCA TGC T to the 5' end of each oligonucleotide. The modified primers did not allow for the amplification of *O. tetricus* itself. The prey sample enriched amplicons were labeled with Illumina-specific adapters and an index using a fusion primer approach in a 10-cycle PCR. The forward primer was constructed with the 5'-3' Illumina i5 adapter AAT GAT ACG GCG ACC ACC GAG ATC TAC AC, an 8–10 bp barcode, a primer pad, and the adapter GGA AGG TGA CCA AGT TCA TGC T (Bybee et al. 2011). The reverse fusion primer was constructed with the 5'-3' Illumina i7 adapter CAA GCA GAA GAC GGC ATA CGA GAT, an 8–10 bp barcode, a primer pad, and the adapter GGA AGG TGA CCA AGT TCA TGC T (Bybee et al. 2011). Primer pads were designed to ensure the primer pad/primer combination had a melting temperature of 63–66°C (Kozich & Schloss 2014). Amplifications were performed in 25 µl reactions with Qiagen HotStar Taq master mix, 1 µl of each 5 µM primer, and 1 µl of DNA template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems) under the following thermal profile: 95°C for 5 min; followed by 35 cycles of 94°C for 30 s, 54°C for 40 s and 72°C for 1 min; followed by 1 cycle of 72°C for 10 min and 4°C hold.

Amplification products were visualized with eGels (Life Technologies). Products were then pooled equimolar, and each pool was size selected in 2 rounds using Agencourt AMPure XP (Beckman Coulter) in a 0.7 ratio for both rounds. Size-selected pools were then quantified using the Qubit 2.0 fluorometer (Life Technologies) and loaded on an Illumina MiSeq 2×300 flow cell at 10 pM. DNA extractions and PCR were conducted at the Molecular Genetics Laboratory of the University of Tasmania (Hobart). Sequencing was conducted at the Research and Testing Laboratory (Lubbock, Texas, USA).

#### 2.4. Sequencing data processing

Paired-end reads in FASTQ format were analysed using QIIME2 v2021.2 (Bolyen et al. 2019). A total of 1759316 raw reads were obtained (from 13 to 179032 reads per individual). Only 257922 reads (14.7% of the original reads) were retained after quality filtering, denoising, chimera removal, joining of denoised paired-end reads, and final derepli-

cation of the sequences using the DADA2 pipeline. DADA2 uses the sequence quality scores to filter out reads with the lowest quality, and the q-scores to predict errors and perform sequence correction. Representative sequences returned by DADA2 were further filtered by length (>50 bp), leaving 20 specimens with sequences for dietary analysis (from 723 to 39441 reads). Two out of the 20 octopuses analysed had no prey DNA in their guts; instead, these individuals had 723 and 2716 reads that corresponded to contamination, respectively (Fig. A1 in the Appendix). The remaining 18 octopuses had  $13\,647 \pm 10\,398$  prey reads (average  $\pm$  SD) belonging to the following groups: crustaceans (124 amplicon sequence variants [ASVs] corresponding to 21 taxa), fishes (30 ASVs corresponding to 6 taxa), and bivalves (13 ASVs corresponding to 2 taxa). Only 5 out of the 18 octopuses with prey also had reads that were considered contamination (between 93 and 6537 reads, corresponding to 2.3 and 20.9% of the reads retrieved from their stomachs). This procedure produced a final list of 197 ASVs, with a minimum count of 2 reads assigned to an ASV.

#### 2.5. Taxonomic identification and phylogenetic analysis

Taxonomic assignment was performed to each ASV sequence according to its best match against the non-redundant (nr) database from the National Center for Biotechnology Information (NCBI), using the local BLAST+ (v2.2.31) toolkit (Camacho et al. 2009).

Out of the 197 ASVs, 29 were assigned to inconsistent taxonomic groups (i.e. humans, insects, plants, and parasitic protozoans), accounting for 12294 reads (4.8% of the total filtered reads). Additionally, 5 out of 197 ASVs did not return any result using BLAST+ against the NCBI nr database or their unique match was different from the mitochondrial 16S rRNA gene. Altogether, 33 ASVs (accounting for 10515 reads, 4.13% of the total filtered reads) were discarded, leaving 164 out of the 197 initial ASVs that were suitable for further analysis.

The final set of 164 ASVs was used to build a phylogenetic tree; 26 reference sequences were taken from their best match using BLAST+, and 1 additional sequence was used as an outgroup (corresponding to *Hydra canadensis*, accession number: GU722798.1). Hence, a total of 191 sequences were included in the construction of the phylogenetic tree. First, sequences were aligned using MAFFT v7.310



(Katoh & Standley 2013), and multiple alignment (MA) was trimmed using UGENE v33.0 (Rose et al. 2019), producing a final MA of 550 positions. Secondly, the phylogenetic tree was calculated by maximum likelihood with bootstrapping of 1000 replicates. Graphical development was performed using the package 'ape' (Paradis & Schliep 2019) in R programming language (R Core Team 2021).

## 2.6. Data analysis

Only 18 out of the 48 stomachs collected in this study contained useful reads ( $n = 12$  males, 6 females), resulting in a smaller than intended sample size. Sequencing of the males resulted in 164 735 reads, while sequencing of the females resulted in 80 902 reads. Sequencing of the 15 immature octopus resulted in 215 454 reads, while sequencing of the 3 mature octopus resulted in 30 183 reads. Examination per season showed 6508 reads from a single stomach in spring, a total of 61 583 reads from 7 stomachs in summer, a total of 164 336 reads from 9 stomachs in autumn, and 13 210 reads from 1 stomach in winter. The unbalanced number of samples did not allow comparison between immature and mature individuals, or between seasons of the year. Therefore, data were described only for females and males, and for warm (October to March) and cold (April to September) seasons of the year.

The number of prey reads and the number of contamination reads were described for each octopus with stomach content. The relative frequency (%) of each prey family in relation to the total number of prey reads, and also the relative frequency of the presence of each prey family in the 18 stomachs with prey reads were calculated. Total number and average  $\pm$  SD of families of prey are presented for females and males. Cumulative rarefaction prey read curves were constructed for all samples pooled, for females, for males, and for both warm and cold seasons using the prey reads identified in the stomachs to determine if a sufficient number of prey reads accurately describe the diet of *O. tetricus*. When the curve reaches the asymptote, it is considered that a sufficient number of prey reads describe the diet. The Shannon diversity index ( $H$ ; Eq. 1) and the Shannon equitability index ( $E_H$ ; Eq. 2) were calculated for females, for males, and for both warm and cold seasons using the package 'vegan' (Oksanen et al. 2022) in R programming language (R Core Team 2021), using the following equations:

$$H = -\sum p_i \times \ln(p_i) \quad (1)$$

$$E_H = \frac{H}{\ln(S)} \quad (2)$$

where  $p_i$  is the proportion of the entire prey reads detected made up of species  $i$ , and  $S$  is the total number of unique species. The Shannon diversity index was used to measure the diversity of species in the stomachs, and the Shannon equitability index was used to measure the evenness of species, i.e. how similar the abundances of different species are in the stomachs.

Identified prey families were clustered in 4 general prey categories, and the following equation (Ebert & Bizzarro 2007) was used to calculate the trophic level (TL; Eq. 3) for females, for males, and for both warm and cold seasons:

$$TL_k = 1 + \left( \sum_{j=1}^n P_j \times TL_j \right) \quad (3)$$

where  $TL_k$  = TL of species  $k$ ,  $P_j$  = average proportion of prey category  $j$  in the diet of species  $k$ ,  $n$  = total number of prey categories, and  $TL_j$  = TL of prey category  $j$ .  $TL_j$  was calculated as the mean of the TL values listed in Ebert & Bizzarro (2007), Vinagre et al. (2012), and Du et al. (2020), where isopods TL = 3.18, molluscs TL = 2.30, decapods TL = 2.52, and fishes TL = 2.94.

## 3. RESULTS

Rarefaction prey read curves calculated for all samples pooled, females, males, and warm and cold seasons of the year were in all cases close to the asymptote, indicating that the sequencing results from the 18 stomachs sampled are representative of the prey diversity of *Octopus tetricus* (Fig. A2 in the Appendix).

Overall, 29 different taxa, including crustaceans, fishes, and bivalves, were detected in the stomach contents of *O. tetricus* (Table 2). The most abundant prey reads in relation to the total number of prey reads identified in the stomachs of the 18 octopuses belonged to the phylum Arthropoda, class Malacostraca (81.2%), followed by the phylum Chordata, class Teleostei (15.0%), and the phylum Mollusca, class Bivalvia (3.7%) (Fig. 2). Sixteen different prey families were detected. The most frequent families were Alpheidae and Galatheidae (44.4% each; 8/18 stomachs each) (Fig. 3), with pistol shrimps (Alpheidae) having the greatest proportion (27.8%; 5/18 stomachs). The families Calcinidae and Pilumnidae had the second greatest proportions (22.2%; 4/18

Table 2. Number of reads detected in the stomach contents of *Octopus tetricus* individuals caught off north-east Tasmania. F: Female; M: Male; Im: Immature; Ma: Mature; Sp: Spring; S: Summer; A: Autumn; W: Winter; Wa: Warm; Co: Cold

Phylum	Family	Prey	Reads																						
			1	2	7	8	9	10	12	13	15	16	17	19	20	21	23	24	32	46	18	45			
Arthropoda	Alpheidae	Alpheidae 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
		Alpheidae 2	0	0	0	0	241	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		Alpheoidea 1	2	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		Alpheoidea 2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		Alpheoidea 3	15520	0	0	0	15040	2760	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		Alpheoidea 4	0	0	0	0	0	0	0	0	0	0	18	0	0	0	26	0	0	0	0	0	0	0	
		Alpheoidea 5	472	0	0	0	0	0	0	0	0	0	6988	0	196	0	11387	0	0	0	0	0	0	0	
		Alpheoidea 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36	0	0	0	0	0	0	0	
		Calcimidae	<i>Dardanus arrosor</i>	0	0	0	0	0	2	0	0	0	0	26448	0	0	0	0	27776	9	0	0	0	0	0
		Diogenidae	<i>Strigopagurus</i>	0	14058	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Diogenidae 1	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Diogenidae 2	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	666	0	0	0	0	0
		Chirostylidae	Chirostylidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	43	0	0	0	0	0
		Galatheiidae	Galatheiidae	0	0	0	0	38	0	0	0	0	0	37	0	44	6809	35	0	46	10420	0	0	0	0
		Munidopsidae	Munidopsidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2736	0	0	0	0	0
Polybiidae	<i>Liocarcinus corrugatus</i>	303	0	0	0	1169	0	0	0	0	0	0	0	192	0	0	2599	0	0	0	0	0	0		
Pilumnidae	<i>Heteropanope</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2230	0	0	0	0	0		
	Pilumnidae 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0		
	Pilumnidae 2	30	0	0	0	0	0	0	0	0	17	0	0	0	0	0	0	16480	0	0	0	0	0		
	Pilumnidae 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	0	0	0	0	0		
Sphaeromatidae	<i>Cymodoce</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6508	0	0	0	0		
Chordata	Congridae	<i>Conger</i>	0	0	2231	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	Tripterygiidae	<i>Forsterygion</i>	0	0	361	0	0	0	0	113	0	0	0	0	0	0	0	0	0	0	0	0	0		
	Carangidae	<i>Trachurus murphyi</i>	0	0	0	0	0	0	0	0	7	0	0	0	0	21420	0	0	0	0	0	0	0		
	Labridae	<i>Notolabrus tetricus</i>	0	0	0	0	0	0	0	1297	0	7089	0	2757	0	0	0	0	0	0	0	0	0		
	Odocidae	<i>Neodax balteatus</i>	0	0	369	0	0	0	0	79	0	0	0	160	0	0	0	0	0	0	0	0	0		
	Trachichthyidae	<i>Hoplostethus atlanticus</i>	0	0	1057	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
Mollusca	Pectinidae	<i>Pecten fumatus</i>	0	0	0	0	0	0	6371	0	0	0	0	0	0	0	0	0	2790	0	0	0	0		
	Pectinida	Pectinida	0	0	0	0	0	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	Contamination	Contamination	0	0	93	0	0	0	0	0	430	0	773	1013	0	6537	0	0	0	0	0	723	2716		

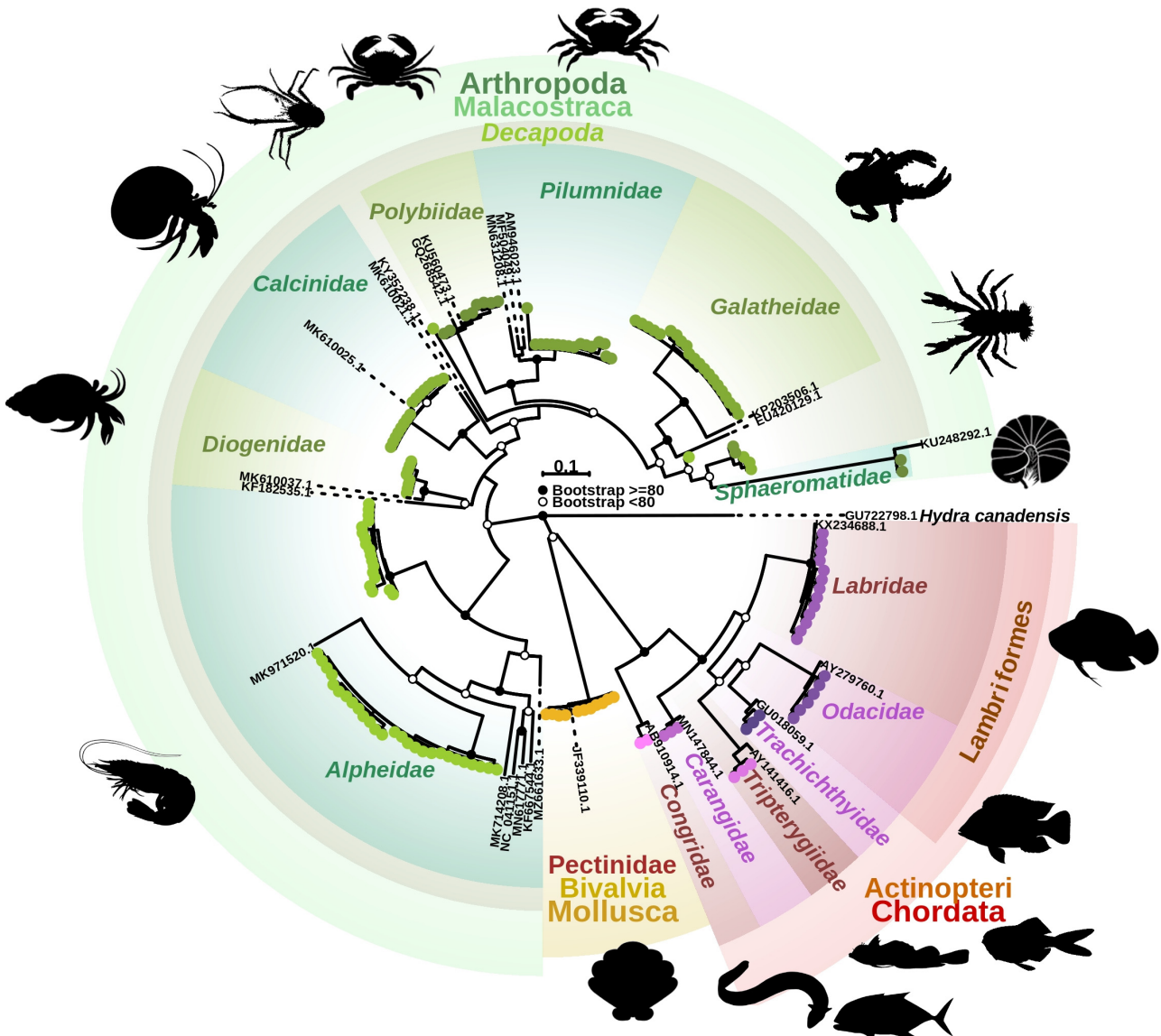


Fig. 2. Phylogenetic tree of the DNA metabarcoding amplicon sequence variants ( $n = 164$ ) of prey in the stomachs of *Octopus tetricus*. Families Munidopsidae and Chirostyliidae (labels not shown) are represented by sequences EU420129.1 and KY352238.1, respectively. The *Hydra canadensis* mitochondrial 16S rRNA gene was used as an outgroup (GU722798.1). The tree was built with a total of 191 sequences using the maximum likelihood method with a bootstrap of 1000 replicates

stomachs each) (Fig. 3). The striated hermit crab *Dardanus arrosor* (Calcinidae) and the wrinkled swimming crab *Liocarcinus corrugatus* (Polybiidae) were present in similar proportions (22.2%; 4/18 stomachs each). Other taxa that were present in 16.7% of the stomachs (3/18 stomachs) were an undetermined crab of the family Pilumnidae, the blue-throated wrasse *Notolabrus tetricus* (Labridae), and the little weed whiting *Neodax balteatus* (Odacidae) (Table 2).

Prey reads grouped into families show the important contribution of the family Alpheidae (30% of prey reads) and the family Calcinidae (22% of prey

reads), both accounting for 52% of the prey reads (Fig. 3). The rest of the prey contributed <10% of total prey reads each, i.e. Galatheidae (9.9%), Carangidae (8.7%), and Pilumnidae (7.7%). The taxa with the greatest number of reads were the calcinid *D. arrosor* (22.1% of total prey reads; 54 235 out of 245 637 reads), an unspecified snapping shrimp of the family Alpheidae (15.1%), an unspecified galatheid (9.9%), and the carangid Chilean jack mackerel *Trachurus murphyi* (8.7%). The rest of the species of prey contributed <8% to the total number of prey reads within the stomachs (Table 2).



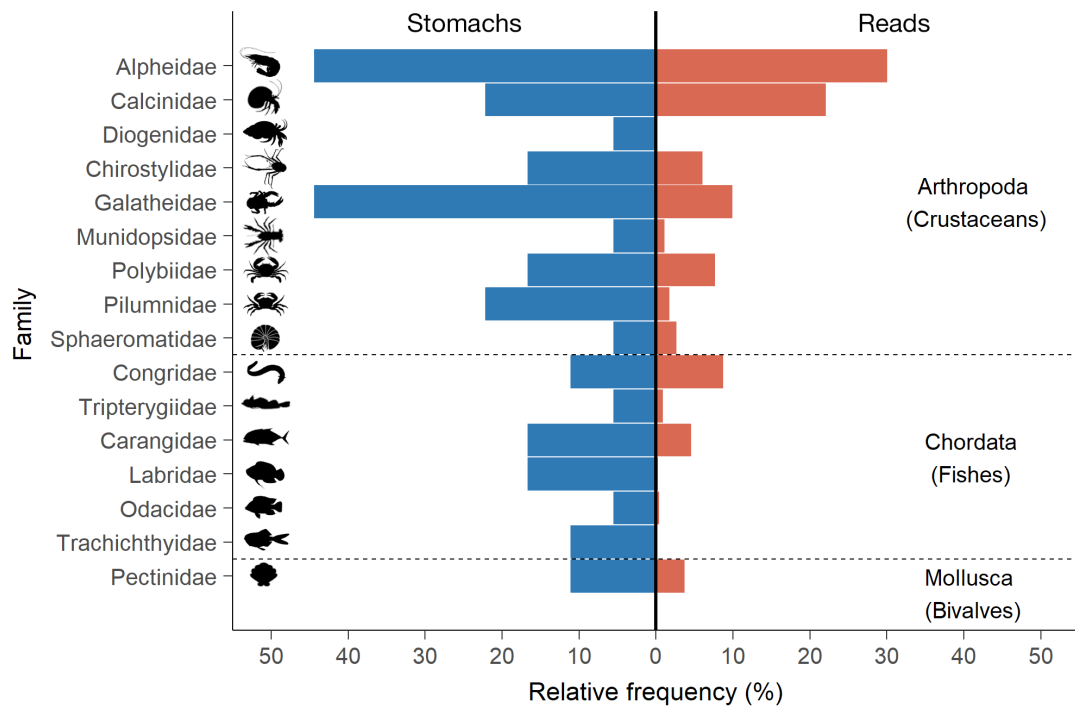


Fig. 3. Contribution of prey at the family level defined as the presence in stomachs (blue;  $n = 18$ ) and relative to the total of prey reads (red;  $n = 245637$ ) across all *Octopus tetricus* stomachs pooled from specimens collected off north-east Tasmania

A total of 9 prey families were detected in females (80 902 reads in 6 stomachs), with  $2.0 \pm 1.4$  prey families detected per female. Crustaceans were the most frequent prey (87.6% of the total reads) in females, with the families Calcinidae (32.7%), Alpheidae (28.5%), and Diogenidae (17.4%) being the greatest contributors. Only 12.4% of the total reads were from fishes; no bivalves were detected (Fig. 4). Males (164 735 reads in 12 stomachs) ingested up to 16 different families, with  $2.5 \pm 1.5$  prey families detected per male. In males, crustacean families accounted for 78.1%, fishes 16.3%, and bivalves 5.6%; the main crustacean families were Alpheidae (30.8%), Calcinidae (16.9%), Pilmunidae (11.4%), and Galatheidae (10.6%) (Fig. 4).

Individuals collected in the warm season were characterised by 68 091 reads from 13 prey families detected in 8 stomachs. Crustaceans were the most frequent prey, with the families Alpheidae (49.9%), Diogenidae (20.7%), and Sphaeromatidae (9.6%) contributing most of the total number of reads. The mollusc family Pecti-

nidae was the fourth main prey and contributed 9.4% of the total number of reads (Fig. 5). The cold season of the year was characterised by 177 546 reads that belong to 12 families detected in 10 stomachs. Crustaceans were the most frequent prey in the

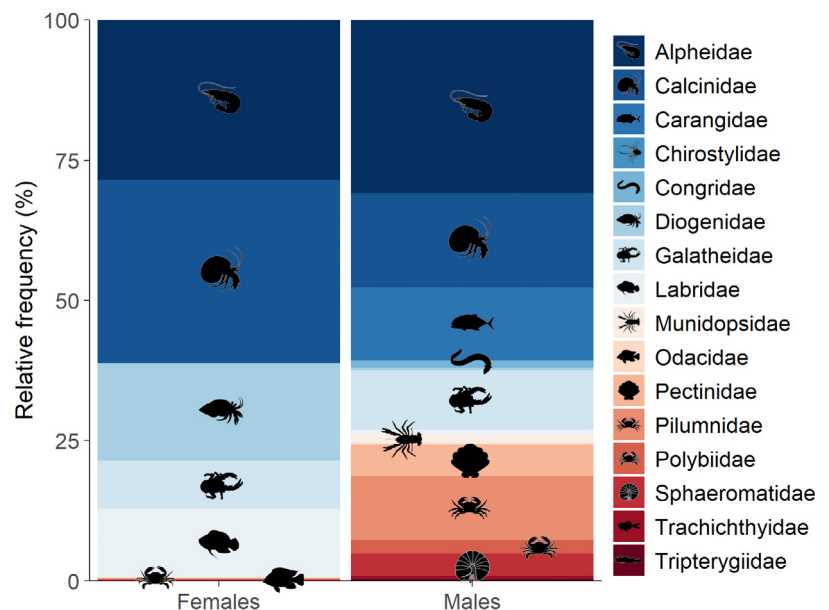


Fig. 4. Relative frequency of prey at the family level in the stomach contents of female ( $n = 80902$  reads in 6 stomachs) and male ( $n = 164735$  reads in 12 stomachs) *Octopus tetricus* caught off north-east Tasmania

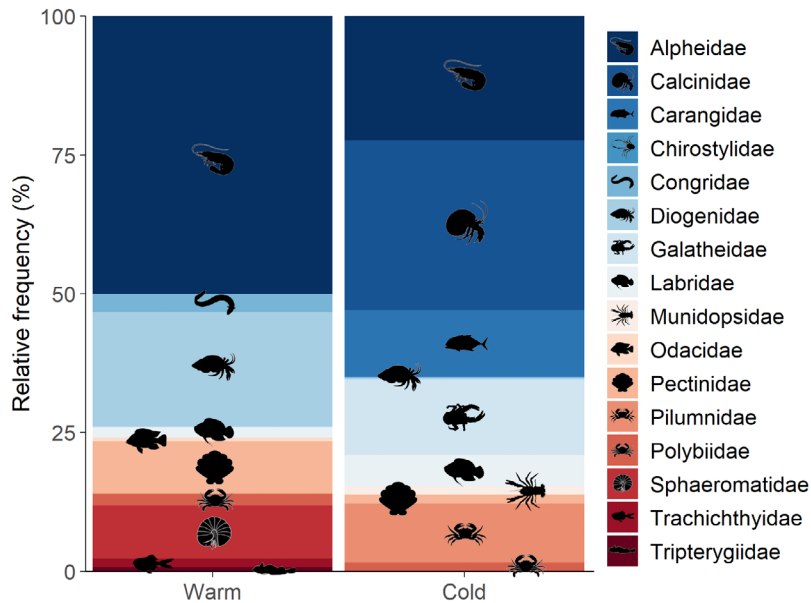


Fig. 5. Relative frequency of prey at the family level in the stomach contents of *Octopus tetricus* caught off north-east Tasmania during the warm ( $n = 68091$  reads in 8 stomachs) and cold ( $n = 177546$  reads in 10 stomachs) seasons of the year

cold season of the year, similar to the warm season. The main crustacean prey families were Calcinidae (30.5%), Alpheidae (22.4%), Galatheidae (13.7%), and Pilumnidae (10.6%). The main fish prey families were Carangidae (12.1%) and Labridae (5.6%) (Fig. 5).

The Shannon diversity index ( $H$ ) was 4.23 for all samples pooled; species diversity was higher for males ( $H = 3.8$ ) compared with females ( $H = 3.2$ ), and for the cold season ( $H = 3.76$ ) compared with the warm season ( $H = 3.41$ ). The Shannon equitability index ( $E_H$ ) showed high similarity in species abundance for all samples pooled ( $E_H = 0.83$ ). Evenness of species abundance was higher in males ( $E_H = 0.74$ ) compared with females ( $E_H = 0.63$ ), and in the cold season ( $E_H = 0.74$ ) compared with the warm season ( $E_H = 0.67$ ). The overall TL of *O. tetricus* was calculated to be 3.66, with TL = 3.69 for females and TL = 3.64 for males. TL was calculated to be 3.69 in the warm season and 3.63 in the cold season.

#### 4. DISCUSSION

In the present study, the use of DNA metabarcoding allowed the identification of a wide variety of prey of *Octopus tetricus* in its range-extended area off north-east Tasmania, i.e. 29 taxa were identified in the stomachs of 18 *O. tetricus* individuals. This approach allowed detection of a greater diversity of

prey in males compared with females, and in the cold season compared with the warm season. Crustaceans ( $n = 9$  families) were the most frequent prey, followed by fishes ( $n = 6$  families) and bivalves ( $n = 1$  family). Overall, the prey found in the range-extended area are consistent with the prey described in parts of the historical range of distribution off eastern Australia (Norman & Reid 2000) and off northern New Zealand (Anderson 1997).

The TL of *O. tetricus* off north-east Tasmania (TL = 3.66) is consistent with the trophic position (TP) or TL reported for *O. hubbsorum* in the central Mexican Pacific (TP = 3.65, Serrano-Tadeo et al. 2021), and for cephalopods in general (TL = 3.6, Field et al. 2006; TL = 3.2, Ebert & Bizzarro 2007; TL = 3.3, Du et al. 2020). These values are higher than the TL calculated for *O. djinda* in an abalone *Haliotis laevis* sea ranch located at Flinders Bay in Western Australia (TL = 2.52, Greenwell et al. 2019), and may be more representative of the natural environment. The TL of cephalopods is comparable with that of teleosts in the natural environment (Ebert & Bizzarro 2007, de la Chesnais et al. 2019), where octopuses can play a key role in the transfer of energy from lower to upper TLs (Urrutia-Olvera et al. 2021).

Redistribution of marine species can have important consequences for marine communities via changes in marine food webs due to new trophic interactions (Edwards & Richardson 2004, Alheit 2009, Walther 2010), and with effects on socio-economic activities (Madin et al. 2012). The most frequent families detected in the stomach contents of *O. tetricus* are macrozoobenthic groups with an important role in the marine trophic chain, i.e. shrimp, hermit crabs, and squat lobsters (Fransozo et al. 1998, Fierro-Rengifo et al. 2008). These groups have also been identified as prey of other cephalopods and also at different life stages, i.e. *O. vulgaris* paralarvae (Olmos-Pérez et al. 2017). Shrimp are gregarious and important prey for echinoderms, crustaceans, fishes, marine mammals, and even seabirds (Fierro-Rengifo et al. 2008). Hermit crabs are key components in the benthic sublittoral and intertidal habitats, where they often attain large populations and are an important link in the food chain, acting as food for other crustaceans and fishes (Fransozo et

al. 1998). Competition for hermit crabs is anticipated, as predation by octopuses has been reported to affect hermit crab population density (Scheel et al. 2014). Squat lobsters are gregarious and can reach high densities (Fierro-Rengifo et al. 2008), which make them a target for predators and key prey for cephalopods including those of commercial importance such as the jumbo squid *Dosidicus gigas* (Bazzino et al. 2010). The ecological importance of squat lobsters extends to different TLs, and the decrease in their abundance has been associated with the decrease in biomass of fishes and whales off Chile (Haro et al. 2023).

The family Pectinidae represented one of the smaller components of the *O. tetricus* prey spectrum, as it was present only in 2 of the 18 stomachs (11%) with detected prey, and contributed only 3.7% of the total number of reads. Nevertheless, the predation of this group must be monitored given its commercial importance and the current 'depleted' status of scallop stocks off Tasmania (Semmens & Woodhams 2020). Commercial landings of scallops were highly variable in Tasmanian waters from the years 2003 to 2015, with average annual landings of  $1649 \pm 1729$  t (SD) (Semmens et al. 2018) and annual profits of up to AU\$6 million (~US\$4 million) (Australian Government 2005). Overfishing, low recruitment, and low abundance caused the closure of the scallop fishery in Tasmanian waters in 2000–2002, 2009–2010, and since 2016 (Semmens & Woodhams 2020). Other prey species of commercial value in Tasmanian waters are blue-throated wrasse *Notolabrus tetricus* and Chilean jack mackerel *Trachurus murphyi* (André et al. 2014, Lyle et al. 2014a,b; <https://fishing.tas.gov.au/>). The stock status of *N. tetricus* in Tasmanian waters was classified as 'sustainable' in 2021 (<https://tasfisheriesresearch.org/btwrasse/>), with combined wrasse species annual landings of  $78 \pm 15$  t and annual value of AU\$906 000  $\pm$  189 000 (~US\$712 000  $\pm$  148 000) from 2001 to 2020 (<https://www.agriculture.gov.au/abares/research-topics/fisheries/fisheries-data#australian-fisheries-and-aquaculture-statistics-2020>). Predation of *O. tetricus* on *P. fumatus* and *N. tetricus* is an additional source of natural mortality for these commercially important species, which would need to be taken in consideration for their management if *O. tetricus* abundance increases as its habitat also expands in the range-extended area (Borges et al. 2022). Juvenile and adult *O. tetricus* are benthic (Ramos et al. 2014, 2015), and predation on pelagic *T. murphyi* (Parada et al. 2017) is

unlikely. Nevertheless, foraging behaviour has been observed in other octopuses (i.e. big blue octopus *O. cyanea*; Forsythe & Hanlon 1997) and it is plausible that *O. tetricus* forage on carcasses of *T. murphyi*.

New interactions can result between range-shifting species and local species (Walther 2010) that in turn have the potential to regulate the range shift process. Off Tasmania, the resident Tasmanian southern rock lobster *Jasus edwardsii* is considered a possible 'biotic barrier' for the range-shifting eastern rock lobster *Sagmariasus verreauxi*, with common distribution in New South Wales (Twiname et al. 2022). *O. tetricus* and *Macroctopus maorum* are the largest species of octopuses in Tasmanian waters, with maximum total weights of nearly 2.3 and 15 kg, respectively (Doubleday et al. 2011, Ramos et al. 2014). Both species of octopus prey on crustaceans, fishes, and bivalves (Norman & Reid 2000, Finn & Norman 2010). Competition for prey and habitat takes place in areas where they co-exist, e.g. soft-sediment and hard-reef or rocky areas off New Zealand, and predation of *M. maorum* on *O. tetricus* is known to occur (Anderson 1999). Competition with *M. maorum* may have an effect on the range shift of *O. tetricus* in Tasmanian waters.

Using qualitative modelling of system feedback, Marzloff et al. (2016) detected likely negative effects on the Tasmanian temperate reef associated with the range shift of *O. tetricus*, in part via predation upon a natural predator (*J. edwardsii*) of the invasive long-spined sea urchin *Centrostephanus rodgersii*, an ecosystem engineer that has contributed extensively to the depletion of Tasmanian kelp forests (Ling et al. 2015). This finding highlights the importance of empirical studies to examine the trophic effects of range-shifting species in range-extended areas. *O. djinda*, a sister species of *O. tetricus* (Amor et al. 2014, 2017, Amor & Hart 2021), can consume up to 35% of its body weight each day at 17°C (Joll 1977). The north-east coast of Tasmania has a relatively similar annual SST (average of 16°C). Assuming increasing abundance of the *O. tetricus* population in the range-extended area, and comparable daily food intake, changes in trophic interactions associated with this range-shifting species can be expected to have ecological, economic, and conservation-related impacts on the marine biodiversity in the range-extended area (Madin et al. 2012).

Several factors may have affected the scope of this study, such as the final and limited number of samples that prevented comparisons between maturity stages and between seasons of the year. However, comparisons were made where the data allowed, and

this study provides a general and preliminary, yet valuable, description of the prey of *O. tetricus* in the range-extended area. The rapid digestion rates in octopuses (Bastos et al. 2020) and the type of tissue that was ingested (i.e. hard structures or soft tissue) can affect the quality and quantity of the remaining prey tissue in the stomach (Symondson 2002, Harper et al. 2005), and therefore its detection. Collecting the samples via soaking lines with shelter pots by the commercial Tasmanian octopus fishery was the only viable option at the time given the location of the fishing areas, and the limited knowledge on the geographic distribution of this recent range-shifting octopus off Tasmania. Therefore, it was not possible to control the time between feeding and stomach collection. Similar logistic limitations were encountered in other studies. For instance, prey in gut contents of the elusive giant squid *Architeuthis* sp. were identified from 1 individual caught accidentally by a trawler fishing vessel (Deagle et al. 2005b). The time that elapsed since the squid was caught and frozen, and the time that elapsed since the squid was thawed, and the stomach was removed and stored in 95% ethanol were not reported. The quality and quantity of prey items will decline with residence time in the stomach of cephalopods, therefore brevity between these steps is a priority in this type of study.

Secondary predation is the detection of the prey of a primary predator that was itself eaten by another predator (Symondson 2002). Next-generation sequencing may have detected small amounts of DNA from secondary predation in the stomachs of *O. tetricus*, as suggested in a study on the diet of *O. vulgaris* and *Alloteuthis media* paralarvae (Olmos-Pérez et al. 2017). Given that *O. tetricus* were caught in the natural environment, our study did not aim to differentiate secondary predation from primary predation. However, detection of secondary predation can be achieved in a controlled environment, with known prey and feeding times. For instance, Sheppard et al. (2005) were able to detect secondary predation for up to 8 h in the carabid *Pterostichus melanarius*.

To avoid amplification of *O. tetricus* itself within the stomachs sampled, the modified primers did not allow for the detection of cannibalism. Still, the possibility of cannibalism cannot be neglected. Cannibalism is common in cephalopods (Ibañez & Keyl 2010) and has been reported for *O. vulgaris* (Hernández-Urcera et al. 2014, 2019) and for *O. hubbsorum* (Serrano-Tadeo et al. 2021). In Tasmanian waters, *M. maorum* will prey infrequently but in large amounts upon smaller conspecifics and upon *M. maorum* egg clusters (Grubert et al. 1999). Indeed, cannibalism

has been reported for *O. tetricus* within its historical distribution (Norman & Reid 2000). Cannibalism may play an important role for *O. tetricus* at an advanced phase of its range-shifting process, when it has increased in numbers and when conspecific interactions are more likely, contributing to shaping the structure, dynamics, and size of the population (Claessen et al. 2004) in the range-extended area. The modified primers also may not have detected local species of cephalopods of commercial importance. The Tasmanian octopus fishery is sustained by the pale octopus *O. pallidus* around northern Tasmania (Leporati et al. 2008) but also includes *M. maorum* and more recently *O. tetricus*. This fishery is currently classified as 'depleting' (Krueck et al. 2021), with combined octopus species annual landings in the range of  $93 \pm 26$  t and with annual values of AU\$736 000  $\pm$  307 000 (~US\$580 000  $\pm$  241 000) from 2001 to 2020 (<https://www.agriculture.gov.au/abares/research-topics/fisheries/fisheries-data#australian-fisheries-and-aquaculture-statistics-2020>). Predation of *O. tetricus* on local cephalopods and on octopus species that sustain the octopus fishery in the range-extended area should be examined.

Only 18 out of 48 stomachs originally collected resulted in useful reads. The smaller than intended sample size did not allow a more robust assessment of the differences in prey contents between immature and mature individuals and between seasons of the year. This study would benefit from increasing the number of samples to allow for prey comparisons between areas, seasons, and immature and mature octopuses. The inclusion of additional group-specific primers would allow the detection of a wider range of prey. Finer resolution in prey identification would facilitate the calculation of trophic niche breadth to better understand the role of *O. tetricus* in the range-extended area. These improvements would provide more robust information for the examination of ecological, economic, and conservation-related impacts associated with the range shift of *O. tetricus*. Future research could also be directed toward monitoring the abundance of *O. tetricus* in the range-extended area, in addition to the citizen science project established to detect range-shifting species (Redmap Australia, Range Extension Database and Mapping; Pecl et al. 2019). Species distribution models such as those implemented by Borges et al. (2022) could be used to predict the overlap in the distribution of this species with its prey, and trophic models would be useful to further understand the trophic role of this range-shifting species in the range-extended area (de la Chesnais et al. 2019).



## 5. CONCLUSIONS

The range-shifting *Octopus tetricus* preys upon a wide range of species as evidenced by DNA metabarcoding, whereby 29 taxa were identified from the stomachs of 18 specimens. Crustaceans (n = 9 families) were the most frequent prey, followed by fishes (n = 6 families) and bivalves (n = 1 family). Some of those prey are commercially fished and are currently classified as depleted in Tasmanian waters. *O. tetricus*, with an intermediate TL of 3.66, likely plays an important role in the transfer of energy from lower to upper TLs off north-east Tasmania. The impact of a growing population of *O. tetricus* on the Tasmanian marine ecosystem could translate into changes in abundance and trophic interactions of local species, with potential implications for species of ecological or commercial importance. Research efforts could be directed to project the future abundance and distribution of *O. tetricus* in relation to its prey, to identify and quantify possible trophic interactions in Tasmanian waters, and to generate trophic models to identify the trophic role of this species in the range-extended area. The present study provides a preliminary description of the diet of *O. tetricus* in its range-extended area; this information can be useful to scientists, fishery managers, and conservationists to anticipate ecological, economic, and conservation impacts of this range-shifting species in Tasmanian waters.

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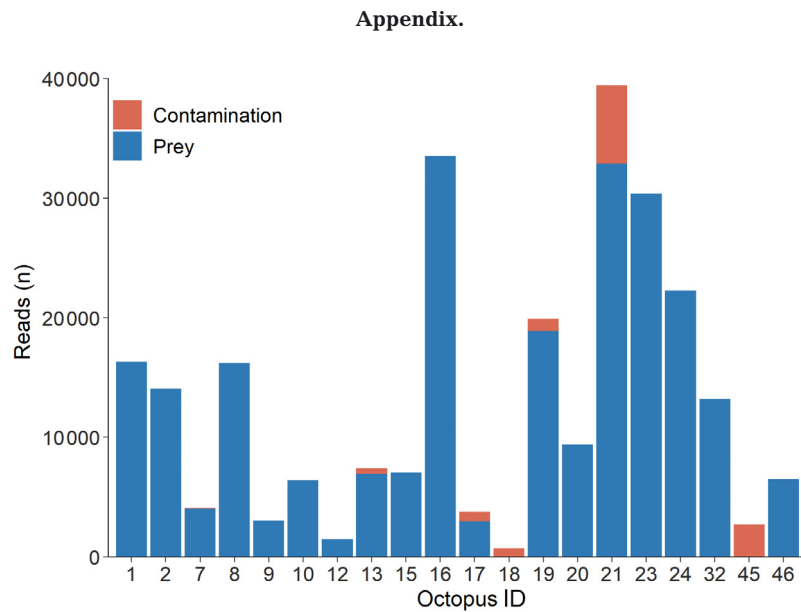


Fig. A1. Final number of prey and contamination reads for each *Octopus tetricus* individual caught off north-east Tasmania

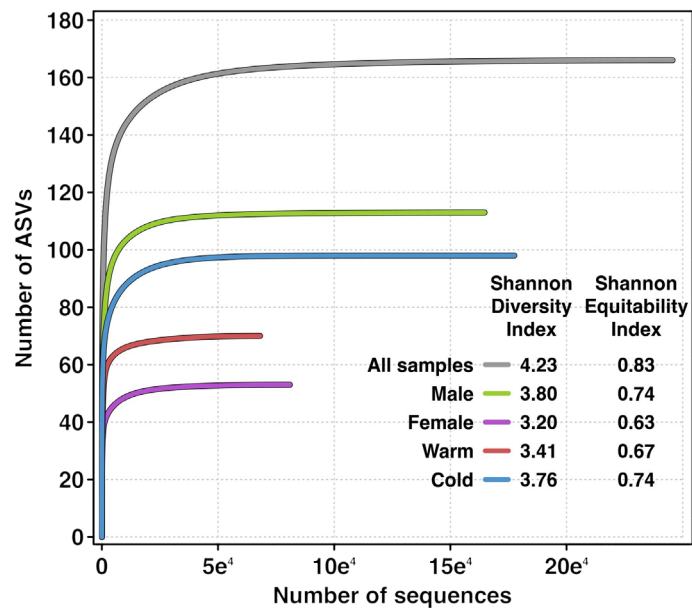


Fig. A2. Rarefaction prey read curves from stomachs of *Octopus tetricus* ( $n = 18$ ) collected off north-east Tasmania. ASV: amplicon sequence variant