Cephalopod fauna of the Pacific Southern Ocean using Antarctic toothfish (*Dissostichus mawsoni*) as biological samplers and fisheries bycatch specimens

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Cephalopods are an important component of Southern Ocean food webs but studies analysing their habitat and trophic ecology are scarce. Here, we use the Antarctic toothfish *Dissostichus mawsoni* as a biological sampler of the Southern Ocean’s cephalopods in the Ross, Amundsen, and D’Urville Seas. Ten cephalopod taxa were identified in the diet of the Antarctic toothfish, with *Pareledone turqueti* and *Moroteuthopsis longimana* being the only species present in all the three studied areas. DNA analysis conducted on squid flesh samples allowed identification of eight and two specimens of *Mesonychoteuthis hamiltoni* and *M. longimana*, respectively, proving this technique as a useful tool to improve the knowledge of cephalopods biodiversity and biogeography in the Southern Ocean. Stable isotopes were used to compare the habitat ($\delta^{13}$C) and trophic ecology ($\delta^{15}$N) between two life-stages of the two most abundant squid species (*M. longimana* and *Psychroteuthis glacialis*) from the D’Urville Sea (both squid species) and Amundsen Sea (only *P. glacialis*). Higher $\delta^{13}$C values in *M. longimana* suggest that this species inhabits waters near the Antarctic Polar Front, with incursions into sub-Antarctic waters, whilst *P. glacialis* spends its entire life in Antarctic waters. The most recently deposited part of the beak is enriched in $^{15}$N suggesting an increase in trophic level during squid growth. These results give us the first insights into the bathyal distribution of cephalopods in the Amundsen and D’Urville Seas, as well as into the ontogenetic changes of two of the most consumed squid species by top predators in this region. Such results are an important step towards improving the biogeography of Antarctic cephalopods, being of utmost importance to understand the biodiversity, food web structure, and functioning of this region.
Keywords: DNA barcoding, Amundsen Sea, D’Urville Sea, Cephalopoda, Stable isotopes, Trophic ecology.
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1 Introduction

Cephalopods play an important role in the marine ecosystems worldwide, being a major prey of fish, seabirds and marine mammals, but also supporting some fisheries worldwide (Boyle and Rodhouse, 2005; Caddy and Rodhouse, 1998; Clarke, 1996; Piatkowski et al., 2001; Santos et al., 2001). More than 800 cephalopod species have been described, including demersal and pelagic species, and distributed in all types of environments, from coastal waters to deep-sea regions (Boyle and Rodhouse, 2005; Clarke, 1996; Hoving et al., 2014). Despite this importance, a lot remains to be discovered in terms of the distribution and the ontogenetic dietary shifts of cephalopods, especially for oceanic and deep-sea species (Clarke, 1996; Hoving et al., 2014). This mainly occurs because cephalopods have the ability to avoid scientific nets, with this sampling method being highly inefficient, catching few and mainly small specimens (Boyle and Rodhouse, 2005; Clarke, 1996; Rodhouse, 1990). To overcome this, the stomach content analysis of cephalopod predators as biological samplers has been used throughout the world to study the diversity, distribution and estimate abundances of these organisms (Boyle and Rodhouse, 2005; Cherel, 2021, 2020; Clarke et al., 1993; Dede et al., 2016; Kousteni et al., 2018; Lefkaditou and Poulopoulos, 1998; Potier et al., 2007; Romeo et al., 2012; Santos et al., 2001; Xavier et al., 2014).

The important role of cephalopods in the marine ecosystems has also been recognized in the Southern Ocean, where cephalopods are the main link between lower and higher trophic levels (Cherel, 2020; Xavier et al., 2018). In the Southern Ocean, cephalopods are consumed by top predators such as the elephant seal *Mirounga leonine* (Rodhouse et al., 1992), the Antarctic toothfish *Dissostichus mawsoni* (Stevens et al., 2014), the wandering albatross *Diomedea exulans* (Xavier et al., 2014) and the sperm whale *Physeter macrocephalus* (Clarke, 1980). Annually, it is estimated that
approximately 12.5 to 24 million tonnes of cephalopods are consumed by these predators (Santos et al., 2001). Nevertheless, information on the biogeography and ontogeny of Southern Ocean’s cephalopods remains scarce. Previous studies showed that most Southern Ocean squid occupy the pelagic zone whereas most octopuses inhabit benthic environments (Collins and Rodhouse, 2006). Geographically squid distribution can be determined, and sometimes limited, by oceanic fronts (Cherel, 2020). However, several studies confirm the presence of the same species in different water masses (Cherel, 2020; Rodhouse et al., 2014), suggesting that oceanic fronts are not absolute barrier for some squids. Concerning octopuses, the knowledge on their distribution is limited with most of the sampling targeting this group around Antarctic Peninsula, while the octopod fauna of several regions (e.g. Adélie Land in the D’Urville Sea) remains unknown (Allcock, 2014).

The use of cephalopod predators as biological samplers such as toothfish, sharks, albatrosses, penguins and seals, has been used to provide valuable information on distribution of cephalopods of the Southern Ocean (Reviewed in Cherel, 2020). However, some remote areas, such as the Ross, Amundsen and D’Urville Seas remain particularly poorly studied especially because scientific cruises targeting these organisms in these seas are even more scarce (Griffiths, 2010), and few studies looked to the cephalopod component of predators’ diet (Cherel and Kooyman, 1998; Fenaughty et al., 2003; Offredo and Ridoux, 1985; Stevens et al., 2014). One of the cephalopod predators in these regions is the Antarctic toothfish D. mawsoni (hereafter Antarctic toothfish). Previous studies using different methodologies such as stomach contents, fatty acids and metabarcoding, showed that Antarctic toothfish is a demersal top predator that feeds primarily on fish, but it also preys upon several squids, e.g. Moroteuthopsis longimana and Psychroteuthis glacialis, and octopuses, e.g. Pareledone
turqueti and Stauroteuthis gilchristi (Collins et al., 2007; Fenaughty et al., 2003; Jo et al., 2013; Remeslo et al., 2015; Roberts et al., 2011; Stevens et al., 2014; Yoon et al., 2017). The Antarctic toothfish, like its close relative the Patagonian toothfish D. eleginoides, can be found down to depths of c. 2,200 m and it is a sedentary species with individuals not moving between areas in a short time period (Hanchet et al., 2015, 2008). Due to such characteristics, both species are useful biological samplers of the areal and depth distributions of bathyal cephalopods (Cherel et al., 2004; Seco et al., 2016; Xavier et al., 2002).

The use of predators as biological samplers is commonly used to study the biogeography of Southern Ocean cephalopods (Cherel, 2020). It mostly relies on the use of cephalopod beaks, which accumulate in the predators’ stomachs (Clarke, 1962) and allow the species’ identification, as well as the estimation of the cephalopod mass and mantle length using allometric equations (Clarke, 1986; Lu and Ickeringill, 2002; Xavier and Cherel, 2009). Stable isotopic analysis of such beaks are commonly used to study their habitat (δ¹³C) and trophic ecology (δ¹⁵N) (Cherel and Hobson, 2005). When applied to different parts of the cephalopod beak allows investigating ontogenetic changes, with the tip of the rostrum being a proxy for early life-stages and the end of the hood in upper beaks for the later period of the squid life (Guerra et al., 2010; Queirós et al., 2018). Combining the cephalopod beak identification with the genetic analysis of unidentifiable flesh fragments from food samples has also the potential to gather useful information on the repartition and trophic relationships of cephalopods (Carreon-Martinez et al., 2011).

The aim of the present study was to describe the geographic and bathymetric distribution of Antarctic cephalopods by using Antarctic toothfish as biological sampler and bycatch specimens from three locations, i.e. Ross, Amundsen and D’Urville Seas.
The identification of the cephalopods found in the stomachs of Antarctic toothfish was achieved by the taxonomic identification of cephalopod beaks and DNA barcoding of cephalopod muscle tissue. Furthermore, using stable isotopic analysis of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in two sections of the beak, we assessed the ontogenetic changes in habitat and trophic ecology of the two most common cephalopod species, *Moroteuthopsis longimana* and *Psychroteuthis glacialis*, in the Amundsen and D’Urville Seas.

**2 Material and Methods**

**2.1 Data collection**

Loose cephalopod beaks, partially digested specimens and pieces of cephalopod flesh were collected from 1,350 stomachs of the Antarctic toothfish caught between December 2016 and February 2017 on board the *FV Antarctic Discovery* within the CCAMLR (Commission for the Conservation of Antarctic Marine Living Resources) convention area in the Small Scale Research Units: 88.1C (Ross Sea), 88.2H, 88.2F (Amundsen Sea) and 58.4.1G (D’Urville Sea). These capture sites include oceanic seamount (88.1C and 88.2H) and slope (88.2F and 58.4.1G) areas at depths between ~650 m and ~1,900 m. Antarctic toothfish were captured using a longline system baited with the squid *Illex argentinus*. After hauled the fish were beheaded and gutted by professional fisherman and the stomach was separated from the other viscera. Due to limitations onboard during fishing operations the fish could not be measure before sampling the stomachs. Stomachs were dissected onboard and cephalopod prey items were sorted. All beaks identified from stomachs belonging to Antarctic toothfish captured in the same fishing set were stored together in the same jar. To complement the information on cephalopod species obtained from the stomach contents of Antarctic toothfish, three octopuses accidentally caught in the fishing hooks (bycatch specimens)
were also included in the study. Cephalopod beaks, partially digested specimens, pieces of cephalopod flesh and bycatch specimens were frozen at -30 °C in plastic jars and plastic bags, respectively, until laboratory analysis.

2.2 Cephalopod beak identification

At the laboratory, cephalopod beaks, partially digested specimens, pieces of cephalopod flesh and bycatch samples were defrosted, and loose beaks preserved in 70% ethanol. Squid loose beaks were separated into upper and lower beaks and identified to the lowest taxonomic level using a cephalopod beak guide (Xavier and Cherel, 2009) and the NIWA (National Institute for Water and Atmospheric Research, New Zealand) reference collection. Partially digested and bycatch octopuses were identified using specimens from the NIWA invertebrate collection, and their beaks posteriorly removed and preserved in 70% ethanol. Loose beaks from Antarctic octopuses were not identified because detailed guides are unavailable for these organisms. The lower hood length (LHL) in octopuses, lower rostral length (LRL) and upper hood length (UHL in beaks analysed for stable isotopes) in squid were measured using a digital calliper (± 0.01 mm; Figure 1). Estimated mass (in g) and mantle length (in mm) were calculated using allometric equations (Lu and Ickeringill, 2002; Xavier

![Figure 1. Lower beak of Pareleledone turqueti (left) and lower (middle) and upper (right) beaks of Moroteuthopsis longimana. Upper beak shows the sections analysed for δ¹³C and δ¹⁵N values. LHL: Lower Hood Length; LRL: Lower Rostral Length (measured internally); UHL: Upper Hood Length; R: tip of the rostrum; H: end of the hood. Photos adapted from Xavier and Cherel (2009).]
2.3 Genetic analyses

Fifty-four cephalopod muscle tissue samples were stored in 99% ethanol and subsequently used for species identification through sequencing of the mitochondrial cytochrome c oxidase subunit I gene (COI) as elective DNA barcoding marker (Hebert et al., 2003). The universal metazoan primer pair LCO1490 and HCO2198, already described as DNA markers for cephalopods (Gebhardt and Knebelsberger, 2015; Queirós et al., 2020; Xavier et al., 2016a), was used as described by Folmer et al., (1994).

About 20 mg of muscle tissue of each specimen was used for total genomic DNA (gDNA) extraction using the CTAB protocol (Borges et al., 2009) with minor modifications. Briefly, tissues were minced with a sterile blade, manually grinded with a plastic mortar in a 1.5 mL microtube and incubated 30 min at 60 °C with agitation in 750 μl of pre-heated CTAB extraction buffer (20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB; and 2% β-mercaptoethanol added just before use) until the sample was dissolved. An equal volume of chloroform-isooamylalcohol (24:1) was added (v/v), and the samples were vortexed and centrifuged for ten minutes at 18 000 x g. Then, the organic phase was recovered and resuspended in 0.6 volume of cold isopropanol and left to precipitate at -20 °C for two hours. Samples were then centrifuged for ten minutes at 18 000 x g and the resulting pellet was washed once (EtOH 70%; 10 mM ammonium acetate) and centrifuged at 18 000 x g for ten minutes. Pellets were resuspended in 50 μl of milli-Q H₂O. DNA integrity was visualized through gel electrophoresis, while DNA quantity and quality were assessed using ND-1000 UV Spectrophotometer NanoDrop (Thermo Fisher Scientific Inc, USA).
The DNA barcode region—648 bp from the 5′ end of cytochrome c oxidase subunit 1 (COI)—was amplified in 25 μl-reactions with the following conditions: 5X PCR buffer (GoTaq® Flexi DNA Polymerase, Promega), 2 mM MgCl₂, 200 mM of each primer, 200 mM dNTPs, 1.25 U of GoTaq® Flexi DNA Polymerase (Promega), between 25-100 ng of gDNA per sample was used as template. The PCR reaction profile was set as follows: ten minutes at 94 °C, 30 cycles of one minute at 94 °C, one minute at 50 °C, three minutes at 72 °C and a final extension step of seven minutes at 72 °C.

PCR amplified products were purified using the Sureclean plus kit (Bioline) following the manufacturer’s instructions. Sanger sequencing of the purified PCR products using the forward and reverse primers was conducted at STAB Vida (Investigaçao e Servicos em Ciencias Biologicas, Lda, Monte da Caparica, Portugal). DNA sequences were then manually edited using the program BioEdit version 7.2.2. (Hall, 1999) and matched against those held in Barcode of Life Data Systems BOLD (http://www.boldsystems.org/index.php/IDS_OpenIdEngine, (Ratnasingham and Hebert, 2007)) and in NCBI BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=blastsearch h&LINK_LOC=blasthome, GenBank, (Benson et al., 2013)) for species-level identification.

2.4 Stable Isotope Analysis

Ten upper beaks, with similar size, per species and location of the two most common species, *M. longimana* (D’Urville Sea) and *P. glacialis* (Amundsen and D’Urville Seas) were randomly selected. Using stainless scissors, two different regions were sectioned from each beak following Queirós et al., (2019): the tip of the rostrum (R) and end of the hood (H) (Figure 1). These sections were cleaned with 80% ethanol
and stored in separate microtubes. Beak samples were dried overnight in an oven at 60°C and milled using a mixer mill Retsch® MM400.

Stable isotopic analyses of δ\(^{13}\)C and δ\(^{15}\)N were performed in both beak sections (R and H). Approximately 0.35 mg of each sample was weighed in a tin capsule using a Mettler Toledo® UMX2 ultra-microbalance. Stable isotope ratios of carbon and nitrogen were measured with an elemental analyser connected on-line to a continuous flow isotope ratio mass spectrometer. Results are presented using δ notation in parts per mil (‰) as deviation from standards using the equation

\[
\delta X = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1
\]

where X represents \(^{13}\)C or \(^{15}\)N and \(R_{\text{sample}}\) the ratios \(^{13}\)C/\(^{12}\)C or \(^{15}\)N/\(^{14}\)N. \(R_{\text{standard}}\) is the reference standard value of Vienna PeeDee Belemnite (V-PDB) and Atmospheric Nitrogen (AIR) for carbon and nitrogen, respectively. As chitin is depleted in \(^{15}\)N, but not in \(^{13}\)C, in relation to proteins (Cherel et al., 2009), and the ratio between chitin and protein concentrations vary along the beak (Miserez et al., 2008), δ\(^{15}\)N values can be lowered in beak regions with higher concentrations of chitin (Cherel et al., 2009). To compare the relative chitin content between beak regions of the same species, C:N mass ratios were calculated. Internal machine errors were systematically measured using acetanilide (STD: Thermo Scientific®; PN 338 36700), indicating an error of <0.3 ‰ for δ\(^{13}\)C and <0.1 ‰ for δ\(^{15}\)N.

2.5 Statistical Analyses

The \(\chi^2\)-test was used to analyse the proportion of identified species in each sampling location, with the \textit{p-value} being calculated using a statistical online software (http://www.socscistatistics.com/pvalues/chidistribution.aspx). To test if larger individuals are found in deeper waters, Spearman correlation tests were performed
between the average depth of the longlines and the lower rostral length of squid (*P. glacialis* and *M. longimana*) or the lower hood length of octopuses (*Megaleledone setebos* and *Stauroteuthis gilchristi*). These correlations were made on species for which more than ten lower beaks were identified and preceded by the Shapiro-Wilk normality test. The normality and homogeneity of δ¹³C and δ¹⁵N values and C:N mass ratios of each population in both beak sections was tested using the Shapiro-Wilk test and the Bartletts’ test, respectively. Statistically significant differences between the δ¹³C and δ¹⁵N values and C:N mass ratios of the sections of each beak were tested using the Wilcoxon-test (*P. glacialis* in the Amundsen Sea) and the paired t-test (*M. longimana* and *P. glacialis* in the D’Urville Sea). Using δ¹⁵N as a proxy of cephalopod trophic position, changes in the trophic position between life stages were calculated considering an increase of δ¹⁵N values of 3.3 ‰ per trophic level (Hobson and Cherel, 2006). These tests were performed in GraphPad Prism® v6.01 using α=0.05 and images were prepared using Adobe Illustrator® CC 2015. Given values are Mean ± SD.

**3 Results**

**3.1 Cephalopod component of the diet of Antarctic toothfish**

A total of 232 cephalopod beaks were sorted from 1,350 stomach contents (including 86 beaks from partially digested cephalopod specimens and from three octopuses caught on fishing hooks). The cephalopods beaks were divided into 157 lower (67.7%) and 75 upper (32.3%) beaks corresponding to ten cephalopod taxa (Table 1). Based on macroscopic examination of cephalopod beaks and DNA barcoding analysis, a total of eight, six and four species of cephalopods were identified in the D’Urville, the Amundsen and the Ross Sea, respectively. The species identified by DNA barcoding were already identified by macroscopic examination of the beaks.
Ten out of 54 muscle tissue samples were successfully sequenced for the COI gene region and identified. Two specimens of *M. longimana* and three out of eight specimens of *M. hamiltoni* were identified genetically in the stomach contents of the Antarctic toothfish captured in the Amundsen Sea. (Table 2). The remaining five specimens of *M. hamiltoni* were obtained from stomachs of Antarctic toothfish caught in the D’Urville Sea (Table 2). Nucleotide sequences were submitted to GenBank and have accession numbers MT358595 - MT358604.

The oceanic squid *P. glacialis* was the most abundant species in the Antarctic toothfish stomachs in Amundsen (58.5%; \(\chi^2 = 157.3, p < 0.001\)) and D’Urville (59.9%; \(\chi^2 = 414.4, p < 0.001\)) Seas, whereas the octopus, *S. gilchristi* was the most abundant species in the Ross Sea (35.7%; \(\chi^2 = 8.10, p = 0.044\)) (Table 1).
Table 1. Cephalopod taxa identified from food samples of Antarctic toothfish caught in the Ross, Amundsen and D’Urville Seas. nLB is the number of lower beaks. LHL/LRL corresponds to the lower hood length of octopuses and to the lower rostral length of squids. Body mass was estimated using allometric equations. Values are mean ± SD.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ross Sea</th>
<th></th>
<th>Amundsen Sea</th>
<th></th>
<th>D’Urville Sea</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>nLB LHL/LRL (mm)</td>
<td>Estimated mass (g)</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Octopoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cirroctopus mawsoni</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>4</td>
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<td>10</td>
<td>12.2 ± 4.2</td>
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<td>1.5</td>
</tr>
<tr>
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<td>5.1 ± 0.4</td>
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<td>0</td>
<td>0</td>
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<td>19.0 ± 9.2</td>
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<td>21.4</td>
<td>2</td>
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<td>82</td>
<td>58</td>
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<td>100</td>
<td>1</td>
<td>197</td>
<td>199</td>
<td>168</td>
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</table>

*Both species were identified using beaks and DNA barcoding. a- no allometric equation; b- one beak was broken, precluding calculations; c- calculated using Muusoctopus thielei’s specific equation; d- LHL based on the measurement of both captured individuals and without the transparent part from the fresh beak; e- both specimens were caught as bycatch and their body mass determined using a balance.
3.2 Bathymetric distribution of cephalopods based on Antarctic toothfish diet

Cephalopods were recovered from Antarctic toothfish that were caught between 700 and 1,900 m, with the highest number of beaks found between 1,300 and 1,500 m of depth (Figure 2). *P. glacialis* was the only cephalopod found in the stomach of the Antarctic toothfish at all depths, and the only found deeper than 1,800 m (Appendix 1). *M. setebos* was the only species presenting a discontinuous depth distribution (from 700 to 900 m and from 1,300 to 1,800 m) with two beaks identified between 700 and 900 m (two bycatch individuals) and the other ten beaks from Antarctic toothfish captured between 1,300 and 1,800 m deep (Figure 2, Appendix 1). Significant correlations between depth and lower rostral length were found for both *P. glacialis* (n= 80, r= 0.420, p< 0.001) and *M. longimana* (n= 28, r= 0.399, p= 0.036). No significant correlations were obtained between the depth and lower hood length in the octopuses *M. setebos* (n= 12, p= 0.058) and *S. gilchristi* (n= 14, p= 0.398).

![Figure 2. Total number of beaks of each cephalopod species (bars) and of Antarctic toothfish non-empty stomachs (symbols and line) in relation to bathymetry in the Ross, Amundsen and D’Urville Seas combined. The legend includes the known vertical distribution of the species.](image-url)
Table 2. List of DNA-barcoded specimens and their corresponding GenBank accession numbers.

<table>
<thead>
<tr>
<th>Seq. No.</th>
<th>Tissue</th>
<th>Location</th>
<th>BLAST annotation</th>
<th>Database</th>
<th>e-value/Similarity</th>
<th>GenBank Acc. No.</th>
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<td>D'Urville Sea</td>
<td>Mesonychoteuthis hamiltoni</td>
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<td>(58.4.1G)</td>
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<td>NCBIn</td>
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<tr>
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<td>Tentacle</td>
<td>Amundsen Sea</td>
<td>Moroteuthopsis longimana</td>
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<td></td>
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3.3 Upper beak $\delta^{13}C$ and $\delta^{15}N$ values of *M. longimana* and *P. glacialis*

The smallest $\delta^{13}C$ value (-26.0 ‰) was measured in the end of the hood of *P. glacialis*, whereas the highest $\delta^{13}C$ value (-21.5 ‰) was in the end of the hood of *M. longimana*, both from the D’Urville Sea (Table 1). Significant differences in $\delta^{13}C$ values were found between the tip of the rostrum and the end of the hood for *P. glacialis* from the Amundsen Sea (Wilcoxon test, $W= 21.0, p= 0.031$) (Figure 3). Conversely, no significant differences in $\delta^{13}C$ values were found between beak sections in *M. longimana* (paired t-test, $t_6= 0.28, p= 0.788$) and *P. glacialis* (paired t-test, $t_7= 0.514, p= 0.6232$) from the D’Urville Sea (Table 3, Figure 3).

Regarding $\delta^{15}N$ values, the tip of the rostrum of *M. longimana* from the D’Urville Sea (+3.0 ‰) and the end of the hood of *P. glacialis* from Amundsen Sea (+11.3 ‰) had the lowest and highest values, respectively (Table 1). Significant differences in $\delta^{15}N$ values were found between the tip of rostrum and the end of hood of the two species in both studied seas (paired t-test: *M. longimana* in D’Urville Sea, $t_6= 8.20, p< 0.001$; *P. glacialis* in D’Urville Sea, $t_7= 2.65, p= 0.033$; Wilcoxon test: *P. glacialis* in Amundsen Sea, $W= 21.0, p= 0.031$; Table 3, Figure 3).

Concerning C:N mass ratios, both squid species in the Amundsen and D’Urville Seas presented significant differences between beak sections (paired t-test: *M. longimana* in D’Urville Sea, $t_6= 14.2, p< 0.001$; *P. glacialis* in D’Urville Sea, $t_7= 3.53, p= 0.009$; Wilcoxon test: *P. glacialis* in Amundsen Sea, $W= -21.0, p= 0.031$), with the tip of the rostrum showing higher C:N mass ratios than the end of the hood (Table 3).
Figure 3. Stable isotope values in each section of upper beaks of *Moroteuthopsis longimana* and *Psychroteuthis glacialis*. Grey dashed line is the estimated isotopic value for the Antarctic Polar Front based on wandering albatross *Diomedea exulans* plasma. Values are mean ± SD (n=10). *p*-value <0.05; ****p*-value < 0.001.
Table 3. Stable isotope values of upper beaks of *Moroteuthopsis longimana* and *Psychroteuthis glacialis* from the Amundsen and D’Urville Seas. UHL: Upper Hood Length; R: tip of the rostrum; H: end of the hood. Values are mean ± SD.

<table>
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<th>Sea</th>
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<th>Beak section</th>
<th>Stable Isotopic Analysis</th>
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<td></td>
<td></td>
<td></td>
<td>n</td>
<td>δ¹³C values (‰)</td>
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<td><em>Moroteuthopsis longimana</em></td>
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<td>45.2 ± 7.4</td>
<td>R</td>
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<td>H</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td><em>Psychroteuthis glacialis</em></td>
<td>10</td>
<td>21.6 ± 2.2</td>
<td>R</td>
<td>8</td>
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<td>H</td>
<td>8</td>
</tr>
<tr>
<td>Amundsen Sea</td>
<td><em>Psychroteuthis glacialis</em></td>
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<td>21.2 ± 1.7</td>
<td>R</td>
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</table>
4 Discussion

Ten cephalopod species, four demersal/benthonic octopuses (Cirroctopus mawsoni, Pareledone turqueti, M. setebos, Muusoctopus (formerly Benthoctopus) sp.), one pelagic octopus (S. gilchristi) and four pelagic squids (Galiteuthis glacialis, Filippovia knipovitchi, M. hamiltoni, M. longimana and P. glacialis) were identified in this study by macroscopically identifying beaks and applying DNA barcoding of cephalopod muscle tissue found in the stomachs of the Antarctic toothfish. Using the depth of capture of Antarctic toothfish we were able to determine that four species (C. mawsoni, Muusoctopus sp., M. setebos, P. turqueti, and P. glacialis) inhabit deeper waters than previously known. Stable isotopic analysis of $\delta^{15}$N and $\delta^{13}$C of two beak sections of P. glacialis from Amundsen and D’Urville Seas and M. longimana from the D’Urville Sea confirmed also that both species increase the trophic position with growth, but do not overlap in their foraging habitat.

4.1 Cephalopod taxa in the diet of Antarctic toothfish in the Ross, Amundsen and D’Urville Seas

Our study provides new information on the biodiversity and biogeography of cephalopods of the Ross, Amundsen and D’Urville Seas. These regions are poorly documented with few studies describing the cephalopod species present in the diet of predators, e.g. Emperor penguins Aptenodytes forsteri and Antarctic toothfish (Cherel and Kooyman, 1998; Fenaughty et al., 2003; Ridoux and Offredo, 1989; Stevens et al., 2014). However, Garcia (2010) and Thompson et al. (2012) made the first detailed studies of cephalopods in the Ross Sea by combining data from research voyages (both studies) and analysis of the diet of predators (Thompson et al., 2012). The ten cephalopod species identified in the present work have already been reported in the diet...
of Antarctic toothfish from the Ross Sea (Stevens et al., 2014; Thompson et al., 2012). These data complements information from biological research surveys (e.g. M. *hamiltoni*: reviewed in Rosa et al. (2017); P. *tursiops*: Strugnell et al. (2012)) and from other predators (e.g. Emperor penguin: Cherel and Looymans (1998)). To our knowledge, this is the first study on the cephalopods from the Amundsen and D’Urville Seas, using the Antarctic toothfish as a biological sampler.

The identification of ten flesh samples proved that DNA barcoding can be a potential tool to identify Southern Ocean squid species from the contents of predators’ stomachs. However, a main obstacle to a successful barcode can be the advanced state of digestion of prey (Buckland et al., 2017; Rosel and Kocher, 2002), because gDNA can be too fragmented (Blankenship and Yayanos, 2005; Moran et al., 2015; Smith et al., 2005). The successful identification of ten samples from stomachs of a deep-sea fish, suggest that this technique might be a promising tool to study the bathyal, abyssal and hadal cephalopods which might not float after death (Hoving et al., 2017). Moreover, as marine fish tend to regurgitate (Bowman, 1986), especially deep-sea species during their ascent to the surface (Drazen and Sutton, 2017; Pilling et al., 2001), the identification of any type and amount of sample assumes a major importance in this research area (Gutt et al., 2020). Metabarcoding of stomach contents, regardless of their digestive stage, has been recently applied (e.g. Barbato et al., 2019), and this technology could be considered in future studies as an optional approach for an overall assessment of taxonomic composition and diversity of Southern Ocean cephalopods.

From the ten identified taxa, only the octopuses C. *mawsoni* and S. *gilchristi* are not known to have a circumpolar distribution (Allcock, 2014; Rodhouse et al., 2014). However, as C. *antarctica* (known to occur in South Shetland Islands) and C. *glacialis* (known to occur in the Weddell Sea and Antarctic Peninsula) might be synonymous of
C. mawsoni, it has been hypothesised that C. mawsoni also have a circumpolar distribution (Allcock, 2014). Regarding S. gilchristi, this species is distributed throughout the South Atlantic, with only one record from the Ross Sea (Allcock, 2014). The presence of several beaks in the stomachs of Antarctic toothfish from the Ross and Amundsen Seas in our study (Table 1) shows that S. gilchristi also inhabits the Pacific sector of the Southern Ocean.

Although most of the identified taxa in our study have a circumpolar distribution (Allcock, 2014; Cherel, 2020; Rodhouse et al., 2014), only P. turqueti and M. longimana were found in the diet of Antarctic toothfish in the Ross, Amundsen and D’Urville Seas (Table 1). In the Amundsen Sea, benthic/demersal octopuses (e.g. M. setebos), pelagic octopuses (e.g. S. gilchristi) and pelagic squids (e.g. M. longimana) were found in Antarctic toothfish stomachs, reflecting sampling from both the seamount and slope areas. This presence/absence pattern is probably due to differences between the topography of the sampling sites (i.e., slope areas in the Amundsen and D’Urville Seas and oceanic seamount areas in Ross and Amundsen Seas). A previous study, that modelled the biogeography of Southern Ocean squid, hypothesised that depth and topography might influence the distribution of these animals (Xavier et al., 2016b). Our study hypothesises that, at least topography, also influences the distribution of Antarctic octopuses.

Previous studies identified the cephalopod fauna of the different Southern Ocean regions using both Antarctic and Patagonian toothfish as biological samplers. Some of these found a higher number of taxa than those identified here; 29 and 25 species were identified in Crozet and Kerguelen Islands (Cherel et al., 2004), respectively, and 16 in South Georgia (Xavier et al., 2002) and Macquarie Islands (Goldsworthy et al., 2002). In contrast, only seven, four and three species were found in South Sandwich Islands
(Seco et al., 2016), Argentinian continental shelf/slope (García de la Rosa et al., 1997) and Falkland Islands (Troccoli et al., 2020). These differences might suggest a circumpolar regional variation in the abundance of cephalopod species in the diet of toothfish species but also a latitudinal variability, with Subantarctic archipelagos presenting a larger number of cephalopod species than slope and oceanic waters south of Antarctic Polar Front (this study; Cherel, 2020). Yet, toothfish inhabiting Antarctic waters present a higher number of cephalopod species in their diet than those from areas in the Patagonian shelf (García de la Rosa et al., 1997; Troccoli et al., 2020). However, we cannot exclude the possibility that such differences are related to the sampling effort, i.e., the number of stomachs analysed. Furthermore, it can also be related with the capture depth of toothfish, the topography of the region (shelf, slope or seamount), and to specific ecological differences between toothfish populations.

4.2 Bathymetric distribution of cephalopods in the Ross, Amundsen and D’Urville Seas

This study provided new insights into the bathymetric distribution of Southern Ocean cephalopod species. Our results suggest that *C. mawsoni*, *Muusoctopus* sp., *M. setebos*, *P. turqueti*, and *P. glacialis* can inhabit deeper waters than previously known, yet within expected depth range of the species, i.e. in the meso-bathypelagic zone (Table 4). Several cephalopod species tend to present an ontogenetic descent to deeper waters (Hunt and Seibel, 2000), including *P. glacialis* and *M. longimana* (reviewed in Xavier and Croxall, 2007) and *M. setebos* (Queirós et al., 2020). As Antarctic toothfish tend to feed in larger cephalopods than other predators and those captured by scientific nets (Cherel, 2020), the presence of these species in deeper waters than previous known is not surprising. This ontogenetic descent is supported by the correlation found for *P. glacialis* and *M. longimana* between depth and the beak size. Nevertheless, such
correlation was not found for *M. longimana* in previous studies (Seco et al., 2016). No significant correlation between depth and beak size occurred for both *M. setebos* and *S. gilchristi*. This contrast with a previous study in *M. setebos* that found a significant correlation between the depth of capture and the mantle length of captured individuals (Queirós et al., 2020).

The highest number of cephalopods identified between 1,300 and 1,500 m is similar to a previous study performed at South Sandwich Islands using both Antarctic and Patagonian toothfish as biological samplers (Seco et al., 2016). However, it contrasts with the results obtained by Xavier et al. (2002) in South Georgia. This suggests that cephalopods in our study area are an important prey to the Antarctic toothfish in the bathyal area (from 1000 to 4000 m deep) of the ocean. Furthermore, it supports the current knowledge in the vertical distribution of Southern Ocean squids (Cherel, 2020) and octopuses (Allcock, 2014; Strugnell et al., 2011). However, due to the relationship between the number of beaks and non-empty stomachs, the higher number of beaks identified at these depths might not be related to a higher abundance of cephalopods, but to a larger sampling effort.
<table>
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<th>Known Vertical Depth Distribution</th>
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<td><em>Cirroctopus mawsoni</em></td>
<td>900 to 1600</td>
<td>333 to 940</td>
<td>†</td>
<td>(Allcock, 2014; Collins and Villanueva, 2006; Vecchione et al., 1998)</td>
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<tr>
<td><em>Muusoctopus sp.</em></td>
<td>900 to 1700</td>
<td>250 to 1491</td>
<td>†</td>
<td>(Strugnell et al., 2011; Vecchione et al., 2009)</td>
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<td>700 to 1800</td>
<td>0 to 930</td>
<td>†</td>
<td>(Allcock, 2014; Allcock et al., 2003; Schwarz et al., 2019)</td>
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<td><em>Pareledone turqueti</em></td>
<td>1300 to 1700</td>
<td>70 to 1200</td>
<td>†</td>
<td>(Collins et al., 2004; Matias et al., 2019; Schwarz et al., 2019; Strugnell et al., 2017, 2012, 2008; Xavier et al., 2002)</td>
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<td><em>Stauroteuthis gilchristi</em></td>
<td>1300 to 1700</td>
<td>700 to 3000</td>
<td>→</td>
<td>(Allcock, 2014; Collins et al., 2004)</td>
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<tr>
<td><em>Filippovia knipovitchi</em></td>
<td>1300 to 1600</td>
<td>0 to 1700</td>
<td>→</td>
<td>(Collins et al., 2004; Rodhouse et al., 1996; Seco et al., 2016)</td>
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<tr>
<td><em>Galiteuthis glacialis</em></td>
<td>1400 to 1500</td>
<td>200 to 1700</td>
<td>→</td>
<td>(Filippova and Pakhomov, 1994; Rodhouse et al., 1996; Rodhouse and Clarke, 1986; Seco et al., 2016; Xavier et al., 2002)</td>
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<tr>
<td><em>Mesonychoteuthis hamiltoni</em></td>
<td>1300 to 1600</td>
<td>300 to ~2500</td>
<td>→</td>
<td>(Filippova and Pakhomov, 1994; Rosa et al., 2017; Seco et al., 2016; Xavier et al., 2002)</td>
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<tr>
<td><em>Moroteuthopsis longimana</em></td>
<td>700 to 1700</td>
<td>0 to 1700</td>
<td>→</td>
<td>(Collins et al., 2004; Seco et al., 2016; Xavier et al., 2002)</td>
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<tr>
<td><em>Psychroteuthis glacialis</em></td>
<td>700 to 1900</td>
<td>100 to 1500</td>
<td>†</td>
<td>(Collins et al., 2004; Filippova and Pakhomov, 1994; Seco et al., 2016)</td>
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Table 4. Comparison of the known depth distribution of cephalopods with results of this study. → the depth obtained in this study is within the previous depth limits; † this study increased the known depth distribution.
4.3 Ontogenetic changes in habitat and trophic level of *M. longimana* and *P. glacialis*

\[ \delta^{13}C \] values are relatively stable through the food web and vary with waters masses, being used as a proxy for predators’ foraging habitat (Cherel and Hobson, 2007). In the Southern Ocean these values present a latitudinal gradient with southern waters being impoverished in \[ ^{13}C \] (Brault et al., 2018; Jaeger et al., 2010). Such gradient allows the study of the distribution of *M. longimana* in the D’Urville Sea and of *P. glacialis* in the Amundsen and D’Urville Seas. The lowest \[ \delta^{13}C \] values found in *P. glacialis* from the D’Urville Sea suggest that these individuals inhabit more southern areas than both *P. glacialis* and *M. longimana* from the Amundsen and D’Urville Seas, respectively. In contrast, our study showed that *M. longimana* may migrate from the Polar Frontal Zone, with individuals having \[ \delta^{13}C \] values above -22.9‰ (the estimated value for the Antarctic Polar Front (Jaeger et al., 2010)). This confirms the presence of *M. longimana* in northern/warmer waters, as already demonstrated by its presence in the diet of predators (e.g. King penguins *A. patagonicus* and Patagonian toothfish from the sub-Antarctic Crozet and Kerguelen Islands (Cherel et al., 2004; Cherel and Weimerskirch, 1999)).

The stable isotope analysis in the two sections of upper beak unraveled habitat changes between early and more advanced life-stages (Guerra et al., 2010; Queirós et al., 2018). The significantly higher \[ \delta^{13}C \] values in the end of the hood of *P. glacialis* upper beaks from the Amundsen Sea suggest a northwards movement throughout the life of the species. In both life-periods, \[ \delta^{13}C \] values shows that *M. longimana* inhabit more northern areas than *P. glacialis*, suggesting an absence of habitat overlap between both species. *P. glacialis* in the D’Urville Sea presented similar \[ \delta^{13}C \] values for both life-stages, suggesting that both juveniles and adults remained in the same habitat. This is in agreement with *P. glacialis* of different sizes being captured in the same trawls.
Complementing the presence of juveniles of *M. longimana* in sub-Antarctic waters from the Indian Sector (Cherel and Hobson, 2005; Cherel and Weimerskirch, 1999), our results support that Antarctic waters are important areas for the growth of *M. longimana* juveniles.

In contrast to δ^{13}C values, δ^{15}N values are not stable through the food web, with predators being ^15^N enriched in relation to their prey (McCutchan et al., 2003; Peterson and Fry, 1987). Therefore, δ^{15}N values are used to study the trophic ecology of the organisms (Peterson and Fry, 1987). As expected, individuals had significant higher δ^{15}N values in the end of the hood than in the rostrum, which demonstrated an increase in the trophic position throughout the life of squids. The largest increase of δ^{15}N value was found in upper beaks of *M. longimana* from the D’Urville Sea and reflects a ~1.1 trophic level increase, whereas *P. glacialis* showed an increase of ~0.9 and ~0.4 trophic level, in the Amundsen and D’Urville Seas, respectively. This trophic shift is well-documented for *P. glacialis* and it is related to a change from zooplankton and small fish (as juveniles) to larger fish and squid (as adults) (Kear, 1992; Lu and Williams, 1994a). Nevertheless, the smaller increase in *P. glacialis’* δ^{15}N values from D’Urville Sea may reflect a more varied diet in these smaller individuals or feeding on smaller prey as juveniles; or a combination of both, which is the most likely explanation. Some studies analysed stomach contents of *M. longimana* and suggested a diet change from zooplankton to fish/squid as they grow (Lu and Williams, 1994b; Nemoto et al., 1988). Previous stable isotope analyses in beaks showed similar results (Cherel and Hobson, 2005; Queirós et al., 2021, 2018; Seco et al., 2016). An ontogenetic trophic shift is also well-documented for other Southern Ocean species, e.g. *M. thielei, M. ingens* and *I. argentines* (Cherel and Hobson, 2005; Collins and Rodhouse, 2006; Queirós et al., 2016).
2019; Rodhouse, 2013), and in squids elsewhere (Guerra et al., 2010; Hoving et al., 2014; Rodhouse and Nigmatullin, 1996).

Higher C:N mass ratios in the tip of the rostrum of *M. longimana* and *P. glacialis* in both seas suggest that $\delta^{15}$N values are lowered (i.e. $\delta^{15}$N values obtained are lower than supposed due to the higher amount of chitin in this beak section (Cherel et al., 2009; Miserez et al., 2008)). However, the tip of the rostrum is composed by a mixture of old and new beak material that might increase the $\delta^{15}$N values for the early stage of squid’s life (Queirós et al., 2018). This balance between higher C:N mass ratio (decrease) and new beak material (increase) might suggest that $\delta^{15}$N values in the tip of the rostrum of all individuals is not higher nor lower than supposed. This indicate that the trophic increases observed in our results are realistic and that *M. longimana* from D’Urville Sea and *P. glacialis* from Amundsen Sea increase approximately one trophic level from juvenile to adult and *P. glacialis* from D’Urville Sea only half a trophic level.

5 Conclusions

This was the first study to describe the bathyal cephalopod fauna of the Amundsen and D’Urville Seas, using the Antarctic toothfish as a biological sampler. Furthermore, it also added new knowledge for the Ross Sea teuthofauna. Ten different octopus and squid species were identified, showing a circumpolar distribution except for *S. gilchristi*. Furthermore, we showed that *C. mawsoni*, *Muusoctopus* sp., *M. setebos*, *P. turqueti*, and *P. glacialis* inhabited deeper waters than previously known. As expected, using stable isotope analyses in two beak regions of the two most abundant species, *P. glacialis* and *M. longimana*, in the Amundsen and D’Urville Seas, showed that both species increase their trophic level between life-stages, and also that, whether
they are juveniles or adults, the foraging habitat of neither species is overlapped. The identification of some species using DNA barcoding in flesh tissue from the stomach of Antarctic toothfish suggest this technique as a promising tool to identify cephalopod species in the Southern Ocean. Given the major role of cephalopods in the Southern Ocean ecosystem, this study contributes to a better knowledge of the biodiversity and biogeography of these organisms in the Amundsen and D’Urville Seas of the Southern Ocean, and may aid future studies on the structure and functioning of Antarctic marine food webs.

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Figures

Figure 1. Lower beak of *Pareleledone turqueti* (left) and lower (middle) and upper (right) beaks of *Moroteuthopsis longimana*. Upper beak shows the sections analysed for δ13C and δ15N values. LHL: Lower Hood Length; LRL: Lower Rostral Length (measured internally); UHL: Upper Hood Length; R: tip of the rostrum; H: end of the hood. Photos adapted from Xavier and Cherel (2009).

Figure 2. Total number of beaks of each cephalopod species (bars) and of Antarctic toothfish non-empty stomachs (symbols and line) in relation to bathymetry in the Ross, Amundsen and D’Urville Seas combined. The legend includes the known vertical distribution of the species.

Figure 3. Stable isotope values in each section of upper beaks of *Moroteuthopsis longimana* and *Psychroteuthis glacialis*. Grey dashed line is the estimated isotopic value for the Antarctic Polar Front based on wandering albatross *Diomedea exulans* plasma. Values are mean ± SD (n=10). *p-value <0.05; ****p-value < 0.001.

Tables

Table 1. Cephalopod taxa identified from food samples of Antarctic toothfish caught in the Ross, Amundsen and D’Urville Seas. nLB is the number of lower beaks. LHL/LRL corresponds to the lower hood length of octopuses and to the lower rostral length of squids. Body mass was estimated using allometric equations. Values are mean ± SD.

Table 2. List of DNA-barcoded specimens and their corresponding GenBank accession numbers.
Table 3. Stable isotope values of upper beaks of *Moroteuthopsis longimana* and *Psychroteuthis glacialis* from the Amundsen and D’Urville Seas. UHL: Upper Hood Length; R: tip of the rostrum; H: end of the hood. Values are mean ± SD.

Table 4. Comparison of the known depth distribution of cephalopods with results of this study. → the depth obtained in this study is within the previous depth limits; ↑ this study increased the known depth distribution.
Supplementary Material

Appendix 1. Number of beaks of each cephalopod species in relation to bathymetry at which Antarctic toothfish were caught.
Highlights

- Ten cephalopod species were identified in the diet of the Antarctic toothfish
- Four octopod and one squid species were found in deeper waters than known to date
- The habitats of either juvenile or adult *M. longimana* and *P. glacialis* do not overlap
- *M. longimana* and *P. glacialis* increase trophic level throughout their lives
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: