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ORIGINAL PAPER

Ontogenic changes in habitat and trophic ecology in the Antarctic squid *Kondakovia longimana* derived from isotopic analysis on beaks

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Abstract

The life histories of cephalopods are still not well described. Stable isotopic analysis of cephalopod beaks is an effective method to study the habitat and trophic ecology of this group of organisms. As beaks grow continuously throughout squid's life without replacement, we hypothesised that analysing different sections along the beak will provide information on the ontogenetic shifts during the individual's lifetime. Here we used the Southern Ocean squid *Kondakovia longimana* as a model species to test the reliability of this method along the beaks of Antarctic species. Growing patterns show that beaks grow throughout the squid lifetime by a continuous deposition of material. This new material can influence the results of the stable isotopic analysis. δ^{13}C and δ^{15}N values (from −26.3 to −20.6‰ and from +3.2 to +8.2‰, respectively) from different beak regions indicated that *K. longimana* inhabits regions spanning a wide latitudinal range, and the trophic level at which it feeds increases throughout its lifetime. Stable isotopic analysis of different sections of the cephalopod beak is a reliable technique to study habitat and trophic ecology throughout Antarctic squid's lifetime. Stable isotopic results showed an increase in δ^{15}N values from the tip of the rostrum to the end of the hood and crest, in the upper beak, and to the free corner of lateral wall and wing in the lower beak. Our results also suggested that the upper beak is the best beak to study ontogenetic shifts, mainly in initial stages of the cephalopods' life, presenting lower values of δ^{15}N than the lower beak.

Keywords Cephalopoda · Southern Ocean · Onychoteuthidae · *Kondakovia longimana* · Antarctica

Introduction

Cephalopod beaks are hard structures composed of chitin–protein complexes (Miserez et al. 2010; Tan et al. 2015). They are resistant to digestion, and thus accumulate in the stomach of predators (Clarke 1986; Xavier and Cherel 2009). Beaks have been used for many years in the identification of cephalopods from stomach contents (Clarke 1986; Lu and Ickeringill 2002; Xavier and Cherel 2009). However, due to the continuous growth of the beak, throughout the cephalopod's lifetime (Guerra et al. 2010; Perales-Raya et al. 2014), these structures can also be a powerful tool to investigate different life stages, from birth to death (Cherel and Hobson 2005; Hobson and Cherel 2006; Cherel et al. 2009; Guerra et al. 2010).

The stable isotopic analysis technique (mainly of δ^{13}C and δ^{15}N) is commonly used in ecological studies of terrestrial and aquatic (freshwater and marine) animals, including reptiles, arthropods, birds and mammals, across a range of body structures (e.g. carapaces, hair, teeth and feathers (Barquete et al. 2013; Ceia et al. 2014; Lowther et al. 2017;

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Micheli-Campbell et al. 2017). The application of this technique to some tissues is dependent on the tissue formation time and/or turnover rates (Ceia et al. 2014), providing information on a specific period an individuals' life, e.g. different feathers to study different life periods (Paiva et al. 2017). In some species, tissues which grow continuously can provide information on the movements, foraging and feeding ecology of the individual's lifetime (Mendes et al. 2007; Guerra et al. 2010; Ramos and González-Solís 2012; Lowther et al. 2017).

This technique is being applied to several cephalopod structures, including the gladii (entire or sectioned) or beak, mostly using the entire beak (Takai et al. 2000; Ruiz-Cooley et al. 2010; Alvito et al. 2015; Rosas-Luis et al. 2017), to determine species' distribution and trophic relationships (Takai et al. 2000; Cherel and Hobson 2005; Cherel et al. 2011). Values of $\delta^{13}C$ are a good indicator of the primary source of the food chain (Hobson and Welch 1992; Cherel and Hobson 2005; Jaeger et al. 2010), and it relates with some oceanic features. This presents a gradient in the Southern Ocean (here defined as South of the Subtropical Front—STF) (Trull and Armand 2001; Cherel and Hobson 2007; Jaeger et al. 2010; Brault et al. 2018). Therefore, $\delta^{13}C$ values can be used as a proxy for the latitudinal distribution of marine organisms (Takai et al. 2000; McCutchan et al. 2003; Cherel and Hobson 2005; Cherel et al. 2011; but see Ceia et al. 2015). Values of $\delta^{15}N$ are not stable along the food chain, with consumers typically enriched in ^{15}N (~3‰ in marine systems) relative to their food source being used to study trophic positions and shifts (Minagawa and Wada 1984; Peterson and Fry 1987; McCutchan et al. 2003). Despite being very useful to study cephalopods' ecology, when applied to the entire beak, precludes the study of the entire life cycle of one individual, i.e. it is not possible to study individual's trophic shift and/or migrations. Following the pioneering work of Guerra et al. (2010) with *Architeuthis dux* in temperate regions, the main goal of this work is to validate and establish, when applied along the beak, the stable isotopic analysis of $\delta^{13}C$ and $\delta^{15}N$ as a powerful tool to study Antarctic cephalopods' life cycle, from the individual perspective.

Cephalopods are ecologically important members of the Southern Ocean, particularly in food web dynamics (Nemoto et al. 1985; Collins and Rodhouse 2006; Xavier and Cherel 2009), as they have a major role in linking lower trophic levels (e.g. macrozooplankton) with top predators including fish, seabirds and marine mammals (e.g. toothfish, albatrosses and sperm whales; Croxall and Prince 1994; Collins and Rodhouse 2006; Xavier and Peck 2015).

Here, the onychoteuthid squid *Kondakovia longimana* (Filippova 1972) was used as model species since several entire specimens were found and several studies targeted the distribution and trophic ecology of this species (Filippova 1972; Lynnes and Rodhouse 2002; Collins and Rodhouse 2006), allowing the comparison of results. Also, it is one of the most important cephalopod prey in the Southern Ocean, being reported from the diet of a wide range of predators (Collins and Rodhouse 2006; Xavier and Cherel 2009; Xavier et al. 2007). *Kondakovia longimana* is an endemic squid of the Southern Ocean that occurs south and north of the Antarctic Polar Front (APF) (Cherel and Weimerskirch 1999; Rodhouse et al. 2014; Xavier et al. 2016b). This species has a circumpolar distribution, inhabiting the mesopelagic and bathypelagic zones (Xavier et al. 1999; Collins and Rodhouse 2006), and reaching large sizes [up to 1 m of mantle length (Lynnes and Rodhouse 2002)]. *Kondakovia longimana* is known to present a shift in the diet from juvenile to adult, feeding in crustaceans (e.g. zooplankton) in the early life and mainly in fish, e.g. myctophids, as adults (Collins and Rodhouse 2006). Cannibalism is also present in this species as shown by Nemoto et al. (1985), suggesting that this species also feeds on squid.

Since Antarctic squid are rarely caught by nets (Collins and Rodhouse 2006; Rodhouse 2013; Rodhouse et al. 2014), most of the information about their ecology is obtained by using their indigestible beaks (Clarke 1986; Jackson et al. 2007; Xavier et al. 2015, 2016a). In this study, values of $\delta^{13}C$ and $\delta^{15}N$ were measured in several sections of the upper and lower beaks of *K. longimana,* collected from the diet of predators, to (1) investigate growing patterns of these chitinous structures (expecting a decrease of beak thickness from the tip of the rostrum to the end of each studied section), (2) confirm that this technique can be used to investigate ontogenetic shifts in Antarctic cephalopods (we expect the $\delta^{13}C$ values to remain stable from the tip of the rostrum to the end of the sections; and the $\delta^{15}N$ values to increase from the tip of the rostrum to the end of the sections), and (3) to assess the habitat and trophic changes of *K. longimana* throughout their life cycle (we predict that squid do not perform huge movements during their life cycle, that they inhabit the Antarctic and Subantarctic waters and show an increase in trophic level throughout their lifetime).

Materials and methods

Sample collection and processing

Beaks of *K. longimana* were collected from stomachs of Patagonian toothfish (*Dissostichus eleginoides*) captured in 2009, South of the South Sandwich Islands (from 55.7°S to 59.9°S (Fig. 1 (Roberts et al. 2011; Seco et al. 2016)). Patagonian toothfish were caught on board of the FV *San Aspiring* with an auto-line system set between 917 and 1720 m deep, using arrow squid (*Nototodarus sloanii*) as bait. Beaks were recovered using the method

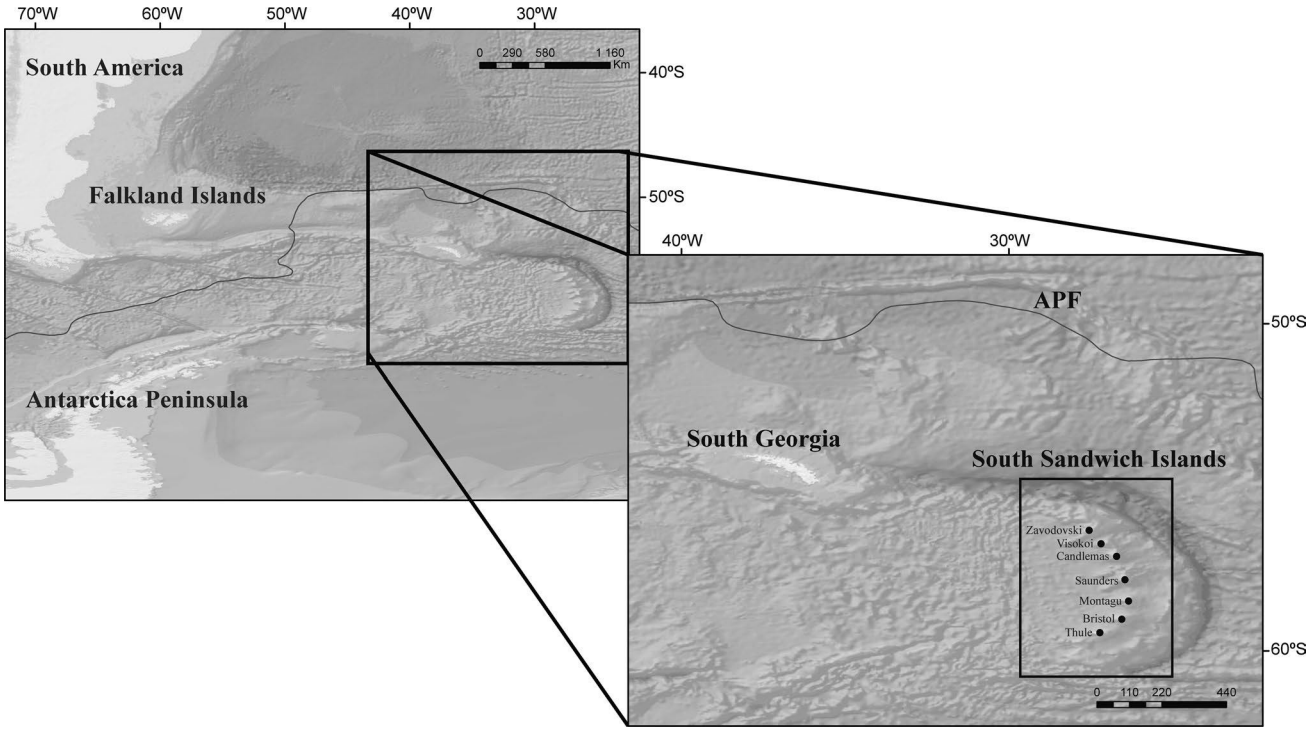

Fig. 1 Map showing the study region. Square represents the South Sandwich Islands where the Patagonian toothfish (*Dissostichus eleginoides*) were caught. *APF* Antarctic Polar Front. Basemap shows the bathymetry of the region

of Roberts et al. (2011), preserved in 90% ethanol, identified following Xavier and Cherel (2009) and transferred to 70% ethanol.

The 10 largest upper beaks were chosen to analyse older and bigger individuals, and the upper hood length (UHL) was measured using a digital calliper nearest to 0.01 mm. Using scissors, a section (U1) along the hood was cut to the junction between the hood and the crest (Fig. 2). This section was measured using a ruler (±0.5 mm) and divided into 4 equal subsections (IV being the earliest life stage, and I the oldest life stage). Due to the size of the beak, this number of sections shows a good perspective of squid life cycle. The tip of the rostrum (R—first to be formed during squids' life) and a section (U2) along the crest to the same junction were also cut (Fig. 2). The thickness of each subsection (U1 and U2 subsections) was measured at the nearest side relative to the tip of the rostrum using a digital calliper (±0.01 mm).

Similarly, the 10 largest lower beaks with complete wings were selected (these lower beaks do not correspond to the upper beaks selected above). The lower rostral length (LRL) was measured using a digital calliper (±0.01 mm). Mantle length (ML in mm) and mass (M in g) were estimated using allometric equations following Brown and Klages (1987). The wing (W), tip of rostrum (R) and a section along the lateral wall (L1), from the free corner to the junction between hood and crest (Fig. 2), were cut using scissors. L1 was measured with a ruler (±0.5 mm) and divided into 4 subsections of equal length. The thickness of each subsection was measured as described above.

Stable Isotopic Analysis

All pieces were cleaned with 80% ethanol, stored in separated microtubes and dried at 60 °C. After drying, the pieces were milled using a mixer mill Retsch® MM400 for 10 min with a frequency of 30 s^{-1}. Harder pieces were milled during 20 min and the hardest were smashed with a mortar and pestle and posteriorly milled in the mixer. Approximately 0.35 mg (0.34 ± 0.04 mg) of each piece was weighted in a tin capsule, using a Mettler Toledo® UMX2 ultra-microbalance. $\delta^{13}C$ and $\delta^{15}N$ values were determined using a Continuous Flow Isotope Ratio Mass Spectrometer (Delta V™ Advantage—Thermo Scientific®) with an organic elemental analyser (Flash™ EA 1112—Thermo Scientific®) at MARE—Figueira da Foz, following Seco et al. (2016).

The results are presented with δ notation in ‰, following the equation:

$$\delta X = \left[\left({}^{H}X/{}^{L}X_{\text{sample}} - {}^{H}X/{}^{L}X_{\text{standard}} - 1\right) \times 1000\right],$$

where X represents C or N and ${}^{H}X/{}^{L}X$ ratios $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Standard values were obtained using Vienna-Pee Dee Belemnite Limestone and Atmospheric N_2 for C and N, respectively. Reference material (acetanilide—Thermo®)

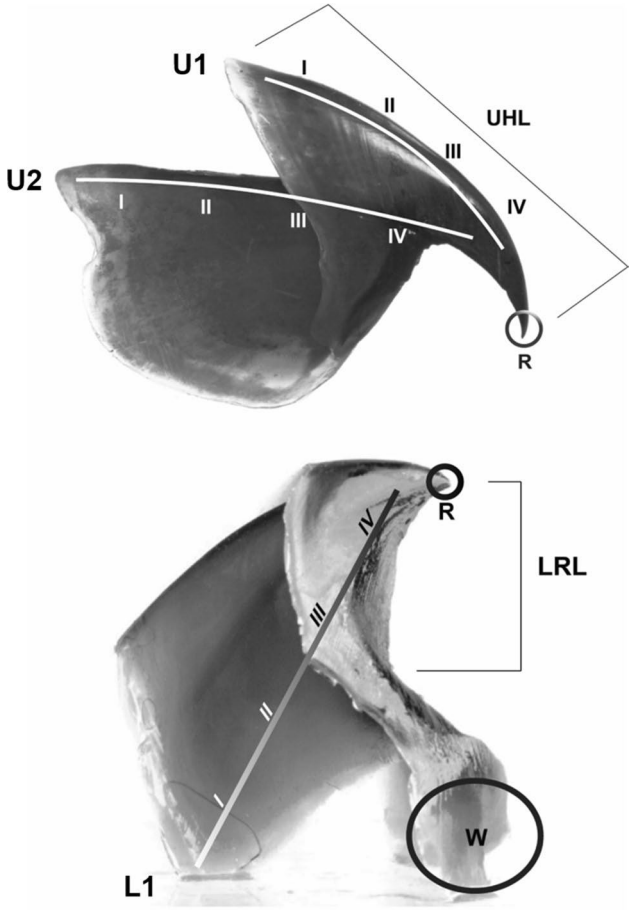

Fig. 2 Analysed sections. Upper beak (upper panel) and lower beak (lower panel) were cut along the lines. *UHL* upper hood length, *U1* hood section, *U2* crest section, *LRL* lower rostral length (that is measured in the inside), *L1* lateral wall section, *R* tip of the rostrum, *W* wing

was measured during the analyses to determine internal measurement errors (< 0.1 and < 0.3‰ for δ^{13}C and δ^{15}N, respectively).

Data analyses

All the beaks showed the same pattern along the sections (δ^{13}C values stable from R to section I (U1, U2 and L1) and W; δ^{15}N values increased from R to I (U1, U2 and L1) and W). For this reason, the statistical tests are made using the arithmetic mean values and standard deviations of the sections. Since both upper and lower beaks were collected in the toothfish stomachs, not attached to themselves (i.e. it is not possible to confirm which upper beak corresponds to their lower beak), analyses of upper and lower beaks were performed independently. All the comparisons between upper and lower beaks were realised using the entire sample, as all the beaks came from the same population around the South Sandwich Islands, expecting to have the same distribution, migration patterns, trophic changes and diet.

Statistical analyses and graphs were made using GraphPad Prism® v6.01. All tests were performed using $\alpha = 0.05$ and preceded by a Shappiro–Wilk normality test. If data followed a normal distribution, a Bartlett's test was performed to test the homogeneity. Means were compared using *t* test and ANOVA for parametric data, and Mann–Whitney and Kruskal–Wallis tests for non-parametric data. Multiple comparisons were performed using Tukey's multiple comparisons test and Dunn's multiple comparisons test in parametric and non-parametric data, respectively.

Values of δ^{13}C were analysed in relation to the isoscape of this ratio in the Southern Ocean (Jaeger et al. 2010; Brault et al. 2018), with lower values being considered waters closer to the continent and higher values to waters further North. These values were also considered in relation to a value of − 22.9‰ to APF (lowest value obtained for this front by Cherel and Hobson (2007) and Jaeger et al. (2010) in penguin blood and albatross plasma), with values smaller than − 22.9‰ being considered as those inhabiting Antarctic waters and values higher than this inhabiting Subantarctic waters. Significant differences between beak sections' δ^{13}C values reflected significant movements of the individual during the life cycle, yet always considered in relation to APF. Significant differences in δ^{15}N values reflected a change in the diet of the individual. Nevertheless, the analysis took into account the enrichment of ~ 3‰ of ^{15}N by trophic level in marine systems (McCutchan et al. 2003), considering and calculating the trophic level change with consideration of this value.

Images were prepared using ArcGis® ArcMap™, Adobe Photoshop CC 2015® and Adobe Illustrator CC 2015®.

Results

All the beaks used in this study belong to adult individuals, with UHL ranging from 40 to 57 mm and LRL from 15 to 18 mm (Table 1).

Thickness of subsections decreased gradually from subsection IV (first to be formed and older than the other sections) to subsection I (last to be formed and recently than the other sections) in both upper and lower beaks (Fig. 3). There are significant differences between all subsections' thickness of U1 and U2 (ANOVA, U1: $F_{3,36} = 4.55$, $p < 0.0001$; U2: $F_{3,36} = 2.25$, $p < 0.0001$). Significant differences between adjacent subsections in both sections were found, except for subsections II and I of U2 (Tukey's multiple comparisons test—Table 2). No differences were found between the thickness of the hood and the crest (Mann–Whitney test, $U = 798.5$, $p = 0.99$). Lower beaks showed statistically significant differences

Table 1 Beak general characteristics

Beak	Upper beaks						Lower beaks							C/N mass ratio
	UHL (mm)	U1 (mm)	U2 (mm)	δ^{13}C (‰)	δ^{15}N (‰)	C/N mass ratio	Beak	LRL (mm)	L1 (mm)	ML (mm)	M (g)	δ^{13}C (‰)	δ^{15}N (‰)	
KU1	40	40	48	−24.9 ± 0.6	+6.1 ± 1.0	3.4 ± 0.3	KL1	15	34	538	3640	−24.6 ± 0.3	+6.6 ± 0.9	3.2 ± 0.1
KU2	41	40	47	−25.2 ± 0.6	+6.5 ± 1.4	3.5 ± 0.3	KL2	15	39	546	3802	−24.3 ± 0.9	+6.6 ± 0.8	3.3 ± 0.2
KU3	42	41	48	−23.7 ± 0.4	+6.2 ± 0.9	3.6 ± 0.8	KL3	16	45	588	4775	−22.8 ± 0.4	+6.3 ± 0.5	3.3 ± 0.3
KU4	43	40	51	−24.5 ± 0.3	+5.8 ± 1.1	3.4 ± 0.2	KL4	17	44	615	5459	−24.5 ± 1.1	+6.0 ± 0.4	3.3 ± 0.2
KU5	46	45	52	−24.7 ± 0.6	+6.5 ± 1.1	3.4 ± 0.3	KL5	17	43	616	5499	−24.0 ± 0.5	+6.3 ± 0.8	3.2 ± 0.1
KU6	49	46	53	−21.3 ± 0.8	+5.7 ± 1.0	3.4 ± 0.2	KL6	17	49	622	5663	−24.4 ± 0.3	+7.1 ± 0.7	3.3 ± 0.1
KU7	51	49	63	−24.8 ± 0.5	+6.8 ± 1.1	3.7 ± 0.8	KL7	18	51	636	6065	−24.1 ± 0.4	+6.9 ± 0.4	3.3 ± 0.3
KU8	52	49	67	−24.9 ± 0.6	+6.6 ± 0.9	3.5 ± 0.3	KL8	18	59	652	6543	−24.3 ± 0.5	+7.3 ± 0.8	3.2 ± 0.2
KU9	53	51	61	−22.8 ± 0.5	+6.4 ± 0.8	3.4 ± 0.2	KL9	18	55	658	6727	−24.3 ± 0.4	+7.1 ± 1.1	3.2 ± 0.2
KU10	57	54	69	−24.5 ± 0.8	+7.0 ± 1.2	3.4 ± 0.2	KL10	18	53	664	6903	−24.5 ± 0.5	+7.1 ± 1.0	3.3 ± 0.2
Mean ± SD	48 ± 6	46 ± 5	56 ± 8	−24.1 ± 1.3	+6.4 ± 1.1	3.5 ± 0.4		17 ± 1	47 ± 1	614 ± 44	5508 ± 1144	−24.2 ± 0.7	+6.7 ± 0.8	3.3 ± 0.2
Statistics (KW)				$H_{10} = 61.50$ $p < 0.001$	$H_{10} = 17.74$ $p = 0.0383$	$H_{10} = 1.876$ $p = 0.9933$						$H_{10} = 21.99$ $p = 0.0089$	$H_{10} = 16.35$ $p = 0.0599$	$H_{10} = 9.819$ $p = 0.3645$

Upper and lower beaks are not related (they do not belong to the same 10 individuals) and isotopic values are the mean of all the subsection measurements

UHL upper hood length, *U1* and *U2* size of sections from upper beaks, *LRL* lower rostral length, *L1* size of section from lower beaks, *ML* estimated mantle length, *M* estimated mass

Values are mean ± SD

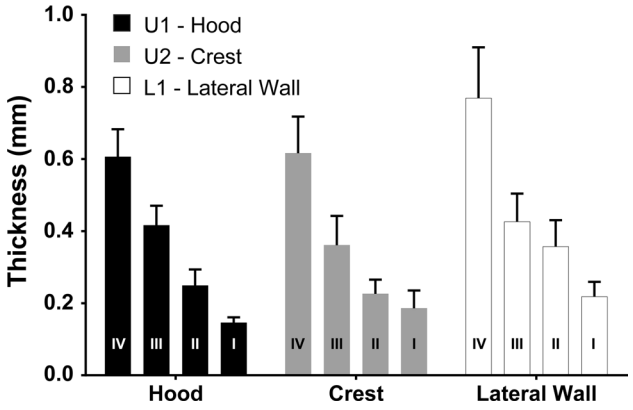

Fig. 3 Thickness along the beak sections ($n = 10$ for each section). Subsection IV is nearest to the tip of the rostrum and I the farthest. Hood (black bars) and crest (grey bars) belong to the upper beak and lateral wall (white bars) to the lower beak. Letters inside the bars are the subsections. Values are mean ± SD

between subsections' thickness (ANOVA, $F_{3,36} = 9.50$, $p < 0.0001$) except between subsections III and II (Tukey's multiple comparisons test—Table 3). Upper beaks' subsections IV thicknesses (U1.IV and U2.IV) were positively correlated with UHL (Pearson's correlations, $r = 0.73$, $n = 10$, $p = 0.016$, and $r = 0.80$, $n = 10$, $p = 0.006$, for hood and crest, respectively—Fig. 4a). A positive correlation was also found between UHL and U2.II, but not between UHL and any other subsections. In the lower beaks, a similar correlation was found between subsection IV and LRL (Pearson's correlation, $r = 0.84$, $n = 10$, $p = 0.005$), when an outlier value was removed (Fig. 4b). A positive correlation was also found between LRL and thickness of all other subsections in L1.

The isotopic analyses of all pieces presented a wide range of $\delta^{13}C$ values, varying from −26.3 to −20.6‰ (Table 4). Values of $\delta^{13}C$ were not statistically different between all sections and subsections of the upper and the lower beaks, except for subsections of the lateral wall of the lower beaks (Fig. 5, Table 4). Values of $\delta^{13}C$ obtained in the hood and the crest, which grow simultaneously, were also not significantly different (Mann–Whitney test, $U = 641.0$, $p = 0.13$) (Fig. 5a). In lower beaks, values of $\delta^{13}C$ did not differ between the different regions of the beak (Table 4), not presenting high variation throughout the beak growth (Fig. 5b).

Beak $\delta^{15}N$ values ranged from +3.2 to +8.2‰, with the tip of the rostrum always showing the lowest $\delta^{15}N$ values in both upper and lower beaks (Table 4). Values of $\delta^{15}N$ increased along the upper beak from the tip of the rostrum to subsection I in both sections (U1 and U2, Fig. 5c). Similarly, in lower beaks the values of $\delta^{15}N$ rose from the tip of the rostrum to subsection I of the lateral wall (Fig. 5d). Statistically significant differences were found between subsections of the hood and between the tip of the rostrum of the upper beak and both the hood and crest (Fig. 5, Table 4). An identical result was obtained for the lower beak, with the values of $\delta^{15}N$ from the tip of the rostrum being significantly different than those from both the lateral wall and the wing (Table 4). In upper beaks, no significant differences were found between the $\delta^{15}N$ values of the hood and the crest (t test, $t_{39} = 0.04$, $p = 0.97$). Between adjacent subsections, significant differences were only found between subsections R and IV in the hood and crest of the upper beaks (Tukey's

Table 2 Upper beak subsection thickness and isotopic values

	Thickness (mm)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C/N mass ratio
U1				
IV	0.61 ± 0.08^{a}	-24.6 ± 1.2	$+6.0 \pm 0.6$	$3.6 \pm 0.1^{a,c}$
III	$0.42 \pm 0.06^{a,b,e}$	-24.5 ± 1.1	$+6.5 \pm 0.5$	$3.4 \pm 0.1^{a,b,c}$
II	$0.25 \pm 0.05^{b,c}$	-24.1 ± 1.3	$+6.9 \pm 0.8$	3.3 ± 0.1^{b}
I	0.15 ± 0.02^{c}	-23.8 ± 1.3	$+7.2 \pm 0.6$	$3.3 \pm 0.1^{a,b}$
Statistics (ANOVA and Kruskal–Wallis)	$F_{(3, 36)} = 119.4$ $p < 0.0001$	$H_4 = 3.590$ $p = 0.3092$	$F_{(3, 36)} = 6.899$ $p = 0.0009$	$F_{(3, 36)} = 20.04$ $p < 0.0001$
U2				
IV	0.62 ± 0.11^{a}	-24.4 ± 1.3	$+6.4 \pm 0.6$	$3.4 \pm 0.1^{a,b,c}$
III	$0.36 \pm 0.09^{a,d,e}$	-23.9 ± 1.3	$+6.7 \pm 0.6$	3.2 ± 0.1^{b}
II	$0.23 \pm 0.04^{b,c,d}$	-23.6 ± 1.2	$+6.8 \pm 0.7$	3.3 ± 0.1^{b}
I	$0.19 \pm 0.05^{c,e}$	-23.7 ± 1.2	$+6.6 \pm 1.7$	$3.5 \pm 0.3^{a,b,c}$
Statistics (ANOVA and Kruskal–Wallis)	$H_4 = 32.15$ $p < 0.0001$	$H_4 = 6.549$ $p = 0.0878$	$F_{(3, 36)} = 0.3663$ $p = 0.7777$	$H_4 = 14.90$ $p = 0.0019$
R		-24.4 ± 1.4	$+4.2 \pm 0.5$	4.2 ± 0.8^{c}
Statistics (ANOVA and Kruskal–Wallis)		$H_9 = 11.44$ $p = 0.1779$	$F_{(8, 81)} = 1504$ $p < 0.0001$	$H_9 = 56.18$ $p < 0.0001$

Values of the same column with a different superscript letter are significantly different ($p < 0.05$). Values are mean ± SD

Table 3 Lower beak subsection thickness and isotopic values

	Thickness (mm)	δ^{13}C (‰)	δ^{15}N (‰)	C/N mass ratio
L1				
IV	0.77 ± 0.15^{a}	-24.0 ± 0.9	$+6.6 \pm 0.3$	3.2 ± 0.2^{a}
III	$0.43 \pm 0.08^{a,b}$	-24.6 ± 0.8	$+6.7 \pm 0.6$	3.2 ± 0.1^{a}
II	0.36 ± 0.08^{a}	-24.3 ± 0.7	$+7.2 \pm 0.5$	3.1 ± 0.1^{a}
I	0.22 ± 0.04^{b}	-24.1 ± 0.4	$+7.1 \pm 0.9$	$3.3 \pm 0.2^{a,b}$
Statistics (Kruskal–Wallis)	$H_4 = 32.12$ $p < 0.0001$	$H_4 = 10.18$ $p = 0.0171$	$H_4 = 7.676$ $p = 0.0532$	$H_4 = 4.264$ $p = 0.2343$
R		-23.9 ± 1.0	$+5.6 \pm 0.4$	$3.5 \pm 0.2^{a,b}$
W		-24.2 ± 0.6	$+7.2 \pm 0.7^{a}$	3.3 ± 0.2^{b}
Statistics (Kruskal–Wallis)		$H_6 = 9.565$ $p = 0.0885$	$H_6 = 27.02$ $p < 0.0001$	$H_6 = 22.99$ $p = 0.0003$

Values of the same column with a different superscript letter are significantly different ($p < 0.05$). Values are mean ± SD

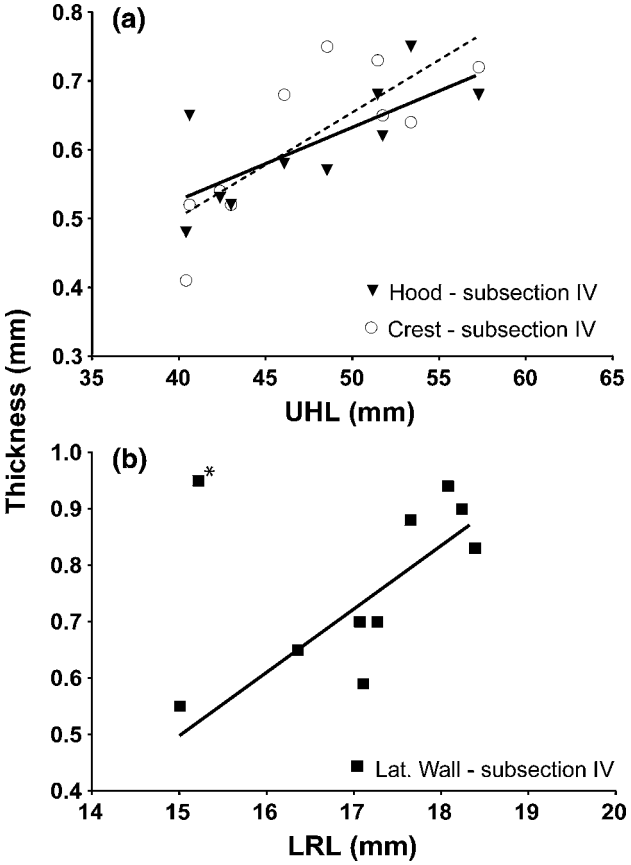

Fig. 4 Relationship between beak size (UHL and LRL) and subsection IV thickness. **a** Relationship between UHL and thickness of subsections IV from hood and crest (hood—straight line: $y = 0.01x + 0.10$; $r = 0.73$, $F = 9.23$, $p = 0.016$; crest—dashed line: $y = 0.02x - 0.11$; $r = 0.79$, $F = 13.82$, $p = 0.006$) ($n = 10$ for each section); **b** relationship between LRL and thickness of subsections IV from lateral wall ($y = 0.11x - 1.20$; $r = 0.84$, $F = 16.94$, $p = 0.005$) (correlation with $n = 9$). Square with * is the outlier value

multiple comparisons test—Table 2). In the lower beaks, significant differences were found neither between adjacent subsections nor between subsection I and the wing (Dunn's multiple comparisons test—Table 3). A statistically significant difference was found between the δ^{15}N values of the tip of the rostrum and the wing (t test, $t_9 = 6.226$, $p < 0.0001$), with wing δ^{15}N values being higher than values of the tip of the rostrum.

A comparison between the tip of the rostrum values from both beaks showed similar δ^{13}C values (t test, $t_9 = 0.919$ $p = 0.37$) but significantly different δ^{15}N values (t test, $t_9 = 6.853$ $p < 0.0001$), with lower beaks presenting higher values (mean difference: 1.4 ± 0.2‰; Fig. 6).

Mass ratios of C/N were statistically different within upper beaks' sections and between the 3 sections (Tables 2, 4). Values decreased along the hood, with the tip of the rostrum presenting the highest values (Table 2). Crest values presented the same tendency except in subsection I that presented similar values to the tip of the rostrum (Table 2). In the lower beaks, the tip of the rostrum values were non-statistically different from the rest of the beak (Table 3). The wing presented the most different values from most of the L1 subsections (Table 3). C/N ratios of upper and lower beaks' tip of the rostrum were significantly different (Mann–Whitney test, $U_9 = 7$, $p = 0.0005$) (Table 2, 3).

Discussion

Stable isotopic analysis on sequential samples from parts of organisms, such as beaks

Our study used the stable isotopic composition of upper and lower beaks to assess the ontogenetic shifts of a Southern Ocean cephalopod, throughout its lifetime. Our results show that older beak subsection (IV) contains layers of old and recent beak material, since there is a continuous deposition

Table 4 Isotopic values of different sections of the beak

Tissue	δ^{13}C (‰)	δ^{15}N (‰)	C/N mass ratio
Upper beak			
Tip of rostrum	-24.4 ± 1.4^{a}	$+4.2 \pm 0.5^{a}$	4.2 ± 0.8^{a}
Hood	-24.2 ± 1.2^{a}	$+6.6 \pm 0.8^{b}$	3.4 ± 0.2^{b}
Crest	-23.9 ± 1.3^{a}	$+6.6 \pm 0.8^{b}$	3.3 ± 0.2^{b}
Statistics (ANOVA and Kruskal–Wallis)	$H_3 = 2.702$ $p = 0.2589$	$F_{(2, 87)} = 48.80$ $p < 0.0001$	$H_3 = 28.59$ $p < 0.0001$
Lower beak			
Tip of rostrum	-23.9 ± 0.9^{b}	$+5.6 \pm 0.4^{c}$	3.5 ± 0.2^{c}
Lateral wall	-24.2 ± 0.7^{b}	$+6.9 \pm 0.7^{d}$	3.2 ± 0.1^{d}
Wing	-24.2 ± 0.6^{b}	$+7.2 \pm 0.7^{d}$	$3.3 \pm 0.2^{c,d}$
Statistics (ANOVA and Kruskal–Wallis)	$H_3 = 1.485$ $p = 0.4759$	$F_{(2, 57)} = 21.05$ $p < 0.0001$	$H_3 = 18.80$ $p < 0.0001$

Values of the same column with a different superscript letter are significantly different ($p < 0.05$). Values are mean ± SD

of material throughout a squid's lifetime, reflected by the higher thickness near the tip of the rostrum. The thickness decreases gradually from subsections IV to I, and the positive correlation between UHL and LRL and the thickness of subsection IV suggests a horizontal deposition (Fig. 7), as shown by microscopic images obtained by Perales-Raya et al. (2014) for *Octopus vulgaris*. The similar thicknesses found between the hood and the crest of the upper beaks suggest that beaks grow uniformly. Although adjacent subsections did not always present statistically significant differences between their thickness, this is likely due to the presence of folds or ridges in the lateral wall of *K. longimana* beaks (Xavier and Cherel 2009). The horizontal deposition (Fig. 7) of beak material from subsection IV to subsection I implies that the isotopic signatures of the "early" subsections are influenced by later life stages. Therefore, stable isotopic signatures in the context of a specific life stage, especially δ^{15}N values, must be interpreted with caution, as they may be influenced by recent material, increasing these values. For example, if the squid presents a δ^{15}N value around 3‰ in early life, with the deposition of new beak material it will present values around 4–5‰, because the beak material deposited in adult life and enriched in ^{15}N will thus influence the juvenile isotopic signature.

Kondakovia longimana is known to occur in Antarctic and Subantarctic waters (Rodhouse et al. 2014; Alvito et al. 2015; Guerreiro et al. 2015). Our results (δ^{13}C values < -20.6‰) corroborate the overall distribution of *K. longimana* and support the suggestion that analysis of δ^{13}C in beaks allows determination of the latitudinal squid distributions. This is because all values are below the ^{13}C isotopic value for the Subtropical front (-19.5‰ = the maximum value obtained for this front by Cherel and Hobson (2007) and Jaeger et al. (2010) using penguin blood and albatross plasma, respectively), showing that this species inhabits areas South of this front. Since no significant differences in δ^{13}C values were found between the hood and the crest of the upper beak, we suggest that future studies can use any of these regions to study the habitat of squid species.

Regarding the δ^{15}N values, our results show that *K. longimana,* as with other oceanic squid species, increases its trophic position over time (Cherel and Hobson 2005; Guerra et al. 2010; Seco et al. 2016). This is the first time that ontogenetic changes in the trophic ecology of a Southern Ocean squid have been assessed at the individual level. Our results show an increase of δ^{15}N values along the beak, from the tip of the rostrum (early squid life) to subsection I (shortly before death), reflecting the entire life of the individual. Similar results were found by Cherel and Hobson (2005) for a Southern Ocean octopod, an increase in δ^{15}N values from the tip of the rostrum to the rest of the beak. However, the difference between the lateral wall and the wing value is higher in the Southern Ocean octopod than in our study. This difference could be due to a methodological issue (i.e. Cherel and Hobson (2005) uses the entire lateral wall instead of 4 different sections of the lateral wall (our study)), or to differences in the feeding ecology (i.e. *Benthoctopus thielei* may have a different ontogenetic shift than *K. longimana*).

The larger difference between the δ^{15}N values from the tip of the rostrum and crest's subsection IV in comparison to the hood may be related to abrasive processes. The anterior end of the hood protrudes out of the buccal mass and is consequently more exposed. Therefore, we suggest that future studies investigating the trophic ecology of Antarctic squid should analyse the isotopic composition of the tip of the rostrum and along the hood, as this gives values closer to the initial life stages of the individual.

In all individuals, the lowest δ^{15}N values was found in the tip of the rostrum, clearly showing that the isotopic composition of this region of the beak reflects an earlier stage of squid life, relative to the rest of the beak. Furthermore,

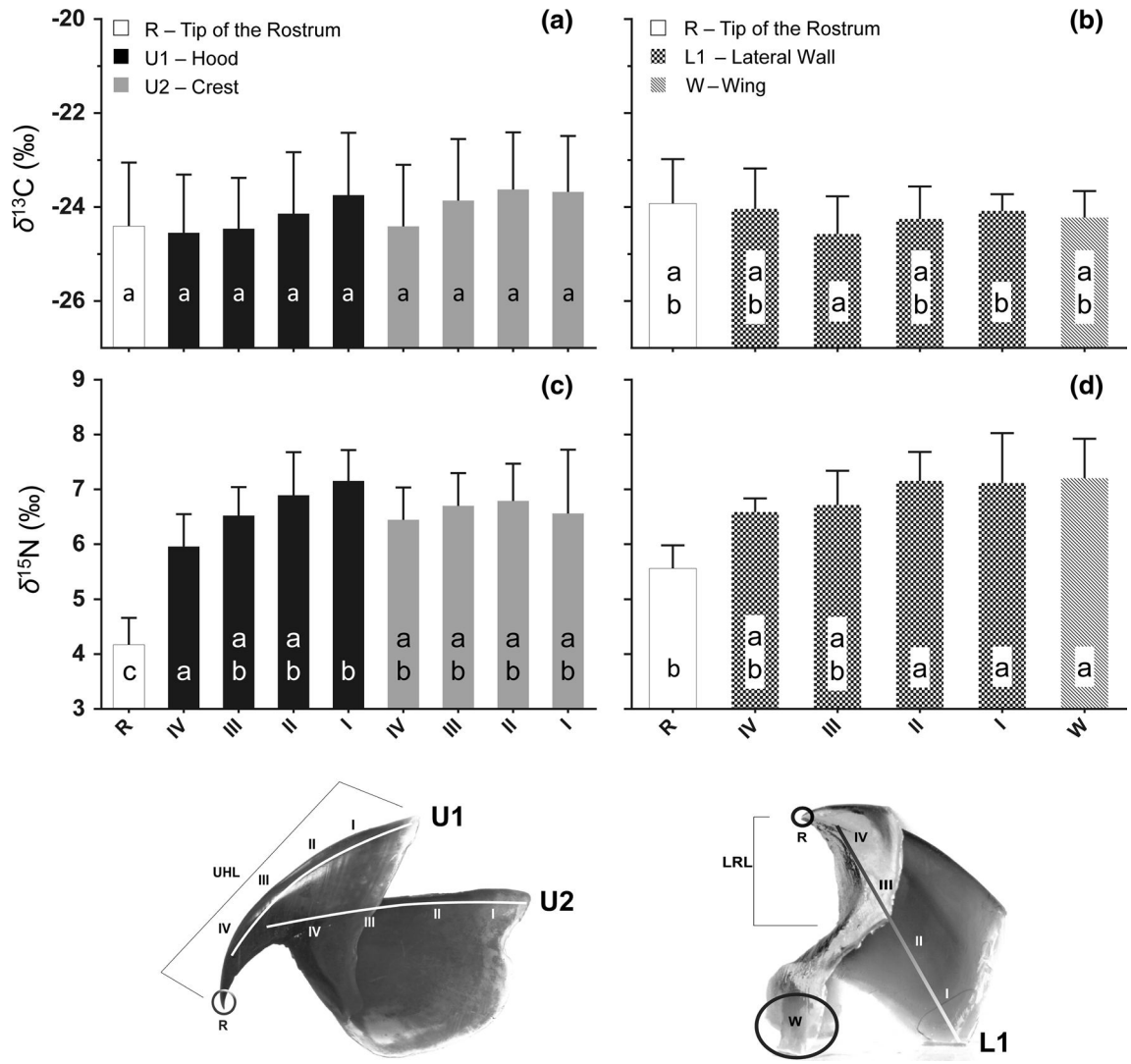

Fig. 5 Isotopic values (δ^{13}C and δ^{15}N) along the beaks ($n = 10$ for each section). Subsection IV forms earlier than I that represents the last part of squid life cycle. Different letters inside bars are statistically different ($p < 0.05$, differences analysed using Dunn's and Tukey's multiple comparison test). **a** Upper beak δ^{13}C values; **b** lower beak δ^{13}C values; **c** upper beak δ^{15}N values, **d** lower beak δ^{15}N values. Values are mean ± SD

the most posterior regions (subsection I) present the highest values, which are similar to those obtained in the wing of lower beaks, revealing that both regions form at the same time, i.e. in the last period of the individual's life. For these reasons, we suggest that further studies on cephalopods ontogenetic shifts, comparing the youngest and the latest life stages, should use the tip of rostrum and the end of hood of the upper beak. Although there is easier identification of the lower beaks from a predator's diet (Xavier and Cherel 2009), the tip of rostrum and the wing or lateral wall free corner from the lower beaks can also be used.

Isotopic signatures of entire small beaks of *K. longimana* (LRL ± SD = 1.5 ± 0.3 mm (Cherel and Hobson 2005) have lower values of δ^{15}N than those from the tip of the rostrum obtained in this study. This difference may be due to an accumulation of new beak material to the tip of the rostrum during adult life stages and/or due to the higher chitin content in small undarkened beaks in comparison with large darkened beak (Miserez et al. 2008), which influences the δ^{15}N values (Cherel et al. 2009). We suggest that to obtain isotopic values closer to the real values of young life stages, future studies should analyse the shortest possible anterior section of the tip of the rostrum rather than the entire tip of rostrum.

The observed differences in δ^{15}N values between the tip of the rostrum of upper and lower beaks may be due to morphological differences in the hood (Xavier and Cherel 2009); the hood of lower beaks is shorter, suggesting that it grows slower. This hypothesis is supported by several analyses

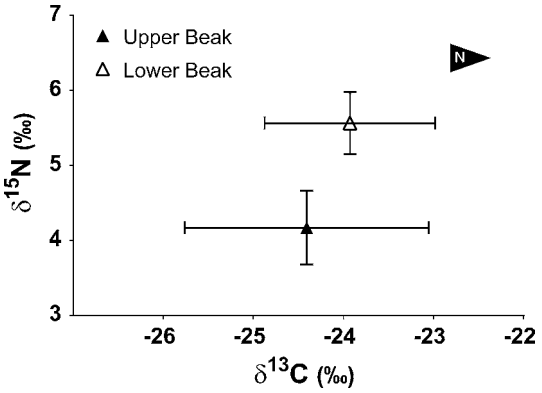

Fig. 6 Comparison of δ^{13}C and δ^{15}N values between upper beak tip of the rostrum and lower beak tip of the rostrum. $n = 10$ upper and lower beaks. Values are mean ± SD

of *O. vulgaris* beaks with Raya and Hernández-González (1998) showing that hood length of upper beaks is larger than those from lower beaks. However, both beaks present a similar number of growing micro-rings (Perales-Raya et al. 2010), suggesting that this structure in the lower beak grows slower. Consequently, the tip of the rostrum of the lower beak has more recent beak material than the tip of the rostrum of the upper beak. Due to this difference, we hypothesised that the accumulation of beak material in the tip of the rostrum, i.e. in the beginning of the individual's life, is different than in the rest of the beak. We suggest that in the tip of the rostrum, the accumulation of beak material is made in vertical layers deposited posteriorly to the paralarval beak, instead of horizontal layers as in the rest of the hood (Fig. 7). This is also confirmed by the position of micro-rings found by Perales-Raya et al. (2014) in the rostrum sagittal section in beaks of *O. vulgaris*. Due to differences found between upper and lower beaks we also advise that, when possible, authors should use both upper and lower beak analyses to compare isotopic niches of different species in the early life stages.

Differences in the beak colour are due to different beak compositions. Darkened beaks present lower chitin content than an untanned beak (Miserez et al. 2008). Since chitin is impoverished in ^{15}N, highly chitinized beaks suggest a higher C/N ratio and lower δ^{15}N values (Cherel et al. 2009). Differences amongst upper beak sections may be due to the lower chitin content in the older subsections (old beak is the darkest). Relative to the lower beaks, the non-statistically significant differences within the lateral wall suggest a homogeneous composition along the beak. Despite darkness, higher C/N ratios in the tip of the rostrum of both beaks suggest higher chitin content in this region (Miserez et al. 2008; Cherel et al. 2009). This may be influenced by differences in the beak composition or the time spent in predators' stomachs (with the time in the stomach, beaks tend to stay darker). This is something that should be studied in the future. However, oscillations in C/N ratios along the beak, compared with the expected results of δ^{15}N values, suggest that differences in chitin do not influence the results when we study ontogenetic shifts in Antarctic squid.

Kondakovia longimana ecology

Our results suggest that *K. longimana* from around the South Sandwich Islands mature South of the APF, i.e. without large latitudinal movements. Most individuals had low δ^{13}C values typical of Antarctic waters (Antarctic waters considered to present δ^{13}C values bellow −22.9‰ which

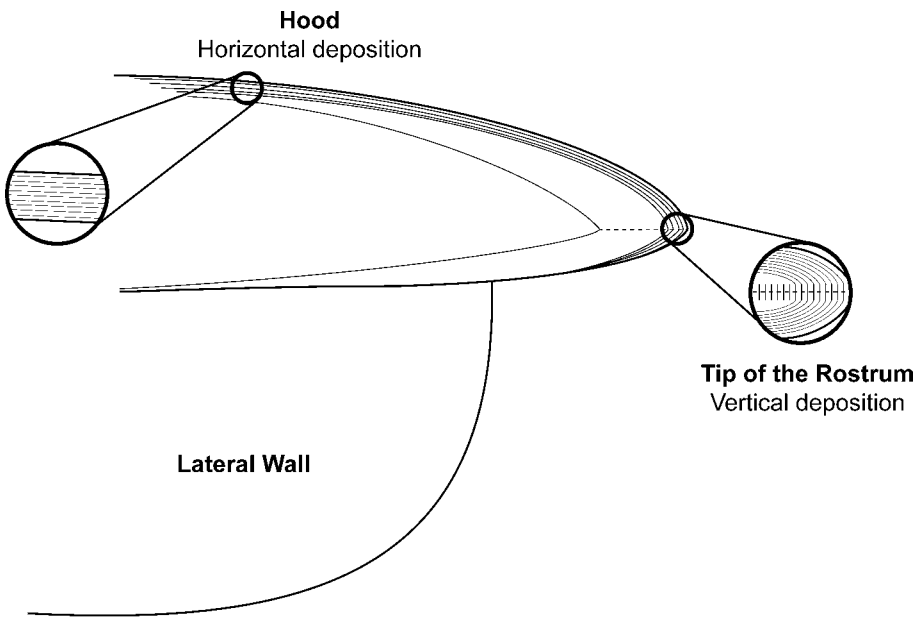

Fig. 7 Scheme about the beak material deposition along the hood and in the tip of the rostrum

is the minimum value obtained for this front by Cherel and Hobson (2007) in penguins' blood and Jaeger et al. (2010) in albatrosses' plasma) throughout their lifetime. However, some individuals presented higher δ^{13}C values (e.g. KU6), similar to those of subantarctic waters. These results agree with previous studies (Cherel and Hobson 2005; Alvito et al. 2015; Guerreiro et al. 2015) and contradict the hypothesis that the APF functions as a barrier for the distribution of *K. longimana* (Laptikhovsky et al. 2009). The presence of *K. longimana* north of the APF has also been predicted in models of habitat suitability (Xavier et al. 2016b).

Values of δ^{15}N in *K. longimana* beaks show a clear gradient from the earliest life stages to the latter. The difference (5.0‰) between the lowest value (+3.2‰—minimum value measured in all the sections and beaks), obtained from the tip of the rostrum of the upper beaks, and the highest (+8.2‰—maximum value measured in all the sections and beaks), obtained in subsection I of the lower beaks, suggests an increase of more than one trophic level for this species. Previous studies showed that in marine systems there is an enrichment of ~3‰ per trophic level (Peterson and Fry 1987; Hobson and Welch 1992; McCutchan et al. 2003). Our results are similar to those obtained in other studies that compare beaks of different sizes (Cherel and Hobson 2005; Zimmer et al. 2007; Seco et al. 2016), which confirms ontogenetic dietary shifts, possibly from zooplankton to fish and squid (Collins and Rodhouse 2006). Since the biggest differences between δ^{15}N values occur from the tip of the rostrum to subsection IV of hood, crest and lateral wall of both beaks, we suggest that the main changes in diet occur in the life stage related to the formation of the posterior portion of the tip of rostrum. Dietary shifts at this period of the life cycle were also observed in other species, including the giant squid (*Architeuthis dux;* (Guerra et al. 2010) and other Southern Ocean species (Cherel et al. 2009). In the three analysed sections (hood, crest and lateral wall), the δ^{15}N values reach a plateau in the most posterior subsections (III to I), suggesting that *K. longimana* reaches its higher trophic position half-way through its life cycle, which coincides with the maturation of the gonads (Rodhouse 1998). In the case of *K. longimana*, this does not occur at the end of the individual's lifetime, since the species present iteroparity (Laptikhovsky et al. 2013).

Conclusion

Using a stable isotopic analysis of *K. longimana* δ^{13}C and δ^{15}N values, we confirmed *K. longimana* as an inhabitant of Antarctic and Subantarctic waters, but without undertaking large latitudinal movements. We also showed that this species presents an increase in the trophic chain in the first quarter of its lifetime, reaching the highest maximum trophic position in the middle of its life. The stable isotopic analyses also suggested that *K. longimana* changes its diet from zooplankton to fish or squid.

Moreover, in this study we also confirmed the stable isotopic analysis of δ^{13}C and δ^{15}N on squid beaks as a useful method to study habitat and trophic ecology throughout cephalopods' life. Future studies should use the tip of the rostrum and hood of the upper beak to evaluate ontogenetic shifts within a squid's lifetime. However, if only lower beaks are available, they may be used if the tip of the rostrum is cut closer to the anterior part than in the upper beaks. Results from the early beak must be analysed with caution because the accumulation of beak material increases the δ^{15}N values from earlier life stages. We recommend that to investigate ontogenic changes between young and adult life stages using stable isotopic analysis on the lower beak, only the tip of the rostrum and the wing or the free corner of the lateral wall (subsection I–L1) should be used. This technique can be applied to smaller cephalopod species in the Antarctic and elsewhere, even in well sampled species whose habitat and trophic levels are poorly known. Indeed, this technique in beaks of smaller species (*Pareledone turqueti*, *Histioteuthis eltaninae*, *Illex argentinus*; our unpublished data) has already been applied successfully.

Similarly to the beak, other hard structures (gladii, statoliths and eye lenses) have a continuous grow within the cephalopod's lifetime (Lipinski 2001; Arkhipkin 2005). However, comparisons between isotopic composition of different structures within the same individual that still needs to be studied (Cherel et al. 2009).

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Compliance with ethical standards

Conflicts of interest There are no conflicts of interest to declare.

Research involving animals This research uses beaks from squid. These beaks were obtained from the stomachs of fish captured in a Fishing Vessel for human consumption. No harm was made to any animal while performing this research.

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