**Ontogenic changes in habitat and trophic ecology in Antarctic squid from stable isotopic analysis on beaks**

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# Abstract

Cephalopods beaks are indigestible chitinous structures that grow continuously along the individuals’ life without being replaced. Beaks have been used to study a wide range of ecological issues in cephalopods, including their habitat and trophic ecology using stable isotopic analysis. 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 along the squid life through a continuous deposition of material that can influence the results of the stable isotopic analysis. *δ*13C and *δ*15N values (from –26.3 to –20.6 ‰ and from +3.2 to +8.2 ‰, respectively) from different beak regions suggest that *K. longimana* inhabits in a wide latitudinal range and increase the trophic level throughout its life. Stable isotopic analysis on cephalopod beaks is a reliable technique to study habitat and trophic ecology of Antarctic squid. Applied into different beak parts, stable isotopic analysis also allows the study of ontogenic shifts along the life cycle, from juvenile to the time of the death.

# 1. Introduction

Cephalopod beaks are hard structures composed of chitin–protein complexes ([Miserez et al., 2010](#_ENREF_30); [Tan et al., 2015](#_ENREF_42)), which resist to digestion, and can therefore be accumulated in the stomachs of predators ([Xavier and Cherel, 2009](#_ENREF_44)). Beaks have been used for many years in the identification of cephalopods from stomach contents ([Clarke, 1986](#_ENREF_11); [Lu and Ickeringill, 2002](#_ENREF_25); [Xavier and Cherel, 2009](#_ENREF_44)). However, as beaks grow throughout the individuals’ life without being replaced, these structures can also be a powerful tool to investigate different life stages, from birth to death ([Cherel and Hobson, 2005](#_ENREF_8); [Hobson and Cherel, 2006](#_ENREF_18); [Cherel et al., 2009](#_ENREF_6); [Guerra et al., 2010](#_ENREF_16)).

Stable isotopic analysis applied to cephalopod beaks allows to determine species’ distribution and trophic relationships ([McCutchan et al., 2003](#_ENREF_27); [Cherel and Hobson, 2005](#_ENREF_8); [Cherel et al., 2011](#_ENREF_7)). Values of *δ*13C are a good indicator of the primary source in the food chains ([Cherel and Hobson, 2005](#_ENREF_8); [Jaeger et al., 2010](#_ENREF_20); [Ceia et al., 2015](#_ENREF_5)), being also related with some oceanic features, presenting a gradient in the Southern Ocean (here defined as South of the Subtropical Front - STF) ([Cherel and Hobson, 2007](#_ENREF_9); [Jaeger et al., 2010](#_ENREF_20)). Therefore, *δ*13C values can be used as a proxy for the latitudinal distribution of marine organisms in different water masses ([McCutchan et al., 2003](#_ENREF_27); [Cherel and Hobson, 2005](#_ENREF_8); [Cherel et al., 2011](#_ENREF_7); [but see Ceia et al., 2015](#_ENREF_5)). Moreover, trophic shifts can be determined by values of *δ*15N, as consumers are typically enriched in 15N (~3‰ in marine systems) relative to their food source ([Peterson and Fry, 1987](#_ENREF_32); [McCutchan et al., 2003](#_ENREF_27)). This technique, when applied to entire-life-growing structures, such as cephalopod beaks, can be a powerful tool to study individuals’ migration and ontogenic shifts in a large time-scale (e.g. mammal whiskers and teeth; ([Mendes et al., 2007](#_ENREF_28); [Lowther et al., 2017](#_ENREF_24)). The main goal of this work is to validate and establish, when applied along the beak, the stable isotopic analysis of *δ*13C and *δ*15N 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 ([Collins and Rodhouse, 2006](#_ENREF_12); [Xavier and Cherel, 2009](#_ENREF_44); [Young et al., 2013](#_ENREF_51)), 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](#_ENREF_13); [Collins and Rodhouse, 2006](#_ENREF_12); [Xavier and Peck, 2015](#_ENREF_46)).

Here, the onychoteuthid squid *Kondakovia longimana* ([Filippova, 1972](#_ENREF_15))was used as model species. *K. longimana* is an endemic squid of the Southern Ocean that occurs south and north of the Antarctic polar front (APF) ([Cherel and Weimerskirch, 1999](#_ENREF_10); [Rodhouse et al., 2014](#_ENREF_37); [Xavier et al., 2016](#_ENREF_48)). This species has a circumpolar distribution and inhabits the mesopelagic and bathypelagic zones ([Xavier et al., 1999](#_ENREF_49); [Collins and Rodhouse, 2006](#_ENREF_12)). It reaches large sizes (up to 1 m of mantle length; ([Lynnes and Rodhouse, 2002](#_ENREF_26))) and 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](#_ENREF_12); [Xavier et al., 2007](#_ENREF_50); [Xavier and Cherel, 2009](#_ENREF_44)).

Since oceanic squid are rarely caught by nets ([Collins and Rodhouse, 2006](#_ENREF_12); [Rodhouse, 2013](#_ENREF_36); [Rodhouse et al., 2014](#_ENREF_37)), most of the information about their ecology is obtained by using their indigestible beaks ([Clarke, 1986](#_ENREF_11); [Xavier et al., 2005](#_ENREF_45); [Xavier et al., 2011](#_ENREF_47)). In this study, values of *δ*13C and *δ*15N 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, (2) confirm that this technique can be used to investigate ontogenic shifts in Antarctic cephalopods and (3) to assess the habitat and trophic shift of *K. longimana* throughout its life cycle.

# 2. Material and Methods

2.1 Sample collection and processing

Beaks of *K. longimana* were collected from stomachs of patagonian toothfish (*Dissostichus eleginoides*) captured in 2009, South of the Sandwich Islands (from 55.7ºS to 59.9ºS – Figure 1 (create a map with south sandwich islands and with predicted K.longimana distribution); ([Roberts et al., 2011](#_ENREF_33); [Seco et al., 2016](#_ENREF_40))). 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 of [Roberts et al. (2011)](#_ENREF_33), preserved in 90% ethanol, identified following [Xavier and Cherel (2009)](#_ENREF_44) and transferred to 70% ethanol.

The 10 larger upper beaks were chosen and the upper hood length (UHL) 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 (Figure 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). The tip of the rostrum (R) and a section (U2) along the crest to the same junction were also cut (Figure 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. Mantle length (ML in mm) and mass (M in g) were estimated using allometric equations following [Xavier and Cherel (2009)](#_ENREF_44). 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 (Figure 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.

2.2 Stable isotopic analysis

All pieces were cleaned with 80% ethanol, stored in separated microtubes and dried at 60ºC. 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. *δ*13C and *δ*15N 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)](#_ENREF_40).

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

*δ* X = [(HX/LX sample - HX/LX standard -1) x 1000],

where X represents C or N and HX/LX ratios 13C/12C or 15N/14N. Standard values were obtained using Vienna-Pee Dee Belemnite Limestone and Atmospheric N2 for C and N, respectively. Reference material (acetanilide – Thermo®) was measured during the analyses to determine internal measurement errors (<0.1 ‰ and <0.3 ‰ for *δ*13C and *δ*15N, respectively).

2.3 Data analyses

Statistical analyses and graphs were made using GraphPad Prism® v6.01. All tests were performed using α = 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.

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

# 3. 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 decrease gradually from subsection IV (oldest) to subsection I (youngest) in both upper and lower beaks (Figure 3). There are significant differences between all subsections’ thickness of U1 and U2 (ANOVA, U1: F3,36=4.55, *p* < 0.0001; U2: F3,36=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, *p* = 0.99). Lower beaks show statistically significant differences between subsection thickness (ANOVA, F3,36=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) are positively correlated with UHL (Pearson’s correlations, *p* = 0.016, *r* = 0.73 and *p* = 0.006, *r* = 0.80 for hood and crest, respectively - Figure 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, *p* = 0.005, *r*= 0.84), when an outlier value was removed (Figure 4b). However, a positive correlation was also found between LRL and thickness of all other subsections in L1.

Values of *δ*13C 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 (Figure 5, Table 4). Values of *δ*13C obtained in the hood and the crest, which grow simultaneously, were also not significantly different (Mann-Whitney, *p* = 0.13) (Figure 5a). In lower beaks, values of *δ*13C did not differ between the different regions of the beak (Table 4), not presenting high variations throughout the beak growth (Figure 5b).

Values of *δ*15N increase along the upper beak from the tip of the rostrum to subsection I in both sections (U1 and U2, Figure 5c). Similarly, in lower beaks the values of *δ*15N rise from the tip of the rostrum to subsection I of the lateral wall (Figure 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 (Figure 5 and Table 4). An identical result was obtained for the lower beak, with the values of *δ*15N from the tip of the rostrum being significantly different than those from both the lateral wall and the wing (Table 5).

In upper beaks no significant differences were found between the *δ*15N values of the hood and the crest (t-test, *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 multiple comparisons test – table 2). In the lower beaks, neither significant differences were found 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*15N values of tip of the rostrum and the wing (t-test, *p* < 0.0001), with wing *δ*15N values being higher than values of the tip of the rostrum.

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

Mass ratios of C/N were statistically different within upper beaks’ sections and between the 3 sections (Table 2 and 4). Values decrease along the hood with the tip of the rostrum presenting the highest values (Table 2). Crest’s 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, tip of the rostrum values were non-statistically different from the rest of the beak (Table 3). 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 are significantly different (Mann-Whitney test, *U* = 7, *p* = 0.0005) (Table 2 and 3).

The isotopic analyses of all pieces presented a wide range of *δ*13C values, varying from –26.3 to –20.6 ‰. Beak *δ*15N values ranged from +3.2 to +8.2 ‰ (Figure 7), with the tip of the rostrum always showing the lowest *δ*15N values in both upper and lower beaks.

# 4. Discussion

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

 The stable isotopic analysis technique is commonly used in terrestrial and aquatic (freshwater and marine) animals, including reptiles, arthropods, birds, mammals, in different body structures (e.g. carapaces, hair, teeth and feathers) ([Barquete et al., 2013](#_ENREF_3); [Ceia et al., 2014](#_ENREF_4); [Divine et al., 2017](#_ENREF_14); [Lowther et al., 2017](#_ENREF_24); [Micheli-Campbell et al., 2017](#_ENREF_29)) ([Kuitems et al., 2012](#_ENREF_21)). The usage of tissues is dependent on moult (on birds) and/or tissues turnover rates ([Ceia et al., 2014](#_ENREF_4)) and can provide particular information, such as movements, foraging and feeding ecology along individuals’ life ([Mendes et al., 2007](#_ENREF_28); [Lowther et al., 2017](#_ENREF_24)).

 The same can now be applied to cephalopod beaks giving us the chance of study individuals’ life. Our study used the stable isotopic composition of upper and lower beaks to assess ontogenic shifts of a Southern Ocean cephalopod all along its life. It follows previous works using giant squid beaks (*Architeuthis dux*; ([Guerra et al., 2010](#_ENREF_16)), Southern Ocean octopod (*Bentoctopus thielei*) and squid beaks(*K. longimana* waspreviously studied using different individuals of different lower beaksizes; ([Cherel and Hobson, 2005](#_ENREF_8)). Throughout the squid life, beak material (i.e. chitin–protein complexes) is synthetized and is retained relatively unchanged (progressive beak darkening is induced by a small reduction of the amount of chitin and increase of proteins ([Miserez et al., 2008](#_ENREF_31))) ([Cherel and Hobson, 2005](#_ENREF_8); [Cherel et al., 2009](#_ENREF_6); [Guerra et al., 2010](#_ENREF_16)). Stable isotopic signatures of the beak may change intrinsically with this accumulation (chitin versus proteins), but also and most importantly, with habitat and trophic shifts along the species life ([Cherel and Hobson, 2005](#_ENREF_8); [Guerra et al., 2010](#_ENREF_16); [Seco et al., 2016](#_ENREF_40)). Our results show that older beak subsection (IV) contain layers of old and recent beak material, since there is a continuous deposition of material throughout squid life, 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, suggest a horizontal deposition. The similar thicknesses found between the hood and the crest of the upper beaks suggests that beaks grow uniformly. Although adjacent subsections did not always present statistically significant differences in their thickness, this is likely due to the beak morphology, such as the presence of folds and ridges ([Xavier and Cherel, 2009](#_ENREF_44)). The horizontal deposition 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*15N values, must be interpreted with caution, as they may be influenced by recent material, increasing these values.

 *Kondakovia longimana* is known to occur in Antarctic and Subantarctic waters ([Rodhouse et al., 2014](#_ENREF_37); [Alvito et al., 2015](#_ENREF_1); [Guerreiro et al., 2015](#_ENREF_17)). Our results (*δ*13C values < –20.6‰) corroborate the overall distribution of *K. longimana* and support that analysis of *δ*13C in beaks allow determining latitudinal squid distributions (see oceanic fronts *δ*13C values in [Guerreiro et al. (2015)](#_ENREF_17). Since no significant differences in *δ*13C 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*15N values, our results show that *K. longimana,* as other oceanic squid species, rises its trophic position with time ([Cherel and Hobson, 2005](#_ENREF_8); [Guerra et al., 2010](#_ENREF_16); [Seco et al., 2016](#_ENREF_40)). This is the first time that ontogenic changes in the trophic ecology of a Southern Ocean squid is assessed at the individual level. Our results show an increase of *δ*15N values along the beak, from the tip of the rostrum (beginning of squid life) to subsection I (previous to death), reflecting the entire life of the individual. Similar results were found by [Cherel and Hobson (2005)](#_ENREF_8) for a Southern Ocean octopod, i.e. an increase of *δ*15N values from the tip of the rostrum to the rest of the beak. However, the difference between the lateral wall and the wing values is higher in the Southern Ocean octopod than in our study. This difference can be due to the use of the entire lateral wall by [Cherel and Hobson (2005)](#_ENREF_8) or by differences in the feeding ecology between this octopod species and *K. longimana*, such as a slower increase through the trophic web by the octopod.

The higher difference found between the *δ*15N values of the tip of the rostrum and crest’s subsection IV in comparison to the hood may be related to abrasive processes, as the anterior end of the hood is out of the buccal mass and consequently more exposed. Therefore, we suggest that future studies that want to investigate the trophic ecology of Antarctic squid 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*15N 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, 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, in the last period of the individual’s life. For these reasons, we suggest that further studies on cephalopods ontogenic 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 due to the easier identification of the lower beaks from predators’ diet ([Xavier and Cherel, 2009](#_ENREF_44)), 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 = 1.5 ± 0.3 mm; ([Cherel and Hobson, 2005](#_ENREF_8)) have lower values of *δ*15N than those from the tip of the rostrum obtained in this study. This difference may be due to an accumulation of new beak material in adult life time to the tip of the rostrum and/or due to the higher chitin content in small undarkened beaks in comparison with large darkened beak ([Miserez et al., 2008](#_ENREF_31)), which influences the *δ*15N values ([Cherel et al., 2009](#_ENREF_6)). 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*15N 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](#_ENREF_44)); the hood of lower beaks are shorter, suggesting that it grows slower. Consequently, the tip of the rostrum of the lower beaks has more recent beak material than the tip of the rostrum of the upper beaks. 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 happen in the rest of the hood.

 Beak colour is due to different beak composition. Darkened beaks present lower chitin content than untanned beak ([Miserez et al., 2008](#_ENREF_31)). Since chitin is impoverished in 15N, highly chitinized beak present higher C/N ratio and lower *δ*15N values ([Cherel et al., 2009](#_ENREF_6)). Differences within upper beak sections may be due to the lower chitin amount in the oldest subsections (old beak is the darker). Relative to the lower beaks, the non-statistically differences within the lateral wall suggest a homogeneous composition along the beak. Despite darkness, highest C/N ratios in the tip of the rostrum of both beaks suggest higher chitin content in this region ([Miserez et al., 2008](#_ENREF_31); [Cherel et al., 2009](#_ENREF_6)). This may suggest that o ther beak components or the time spent in predators stomachs (with the time in the stomach, beaks tend to stay darker) can influence C/N ratios, something that must be studied in the future. However, oscillations in C/N ratios along the beak, compared with the expected results of *δ*15N values suggest that differences in chitin does not influences the results when we study ontogenic shifts in Antarctic squid.

4.2 *Kondakovia longimana* ecology

Our results suggest that *K. longimana* from around the South Sandwich Islands grow up South of the APF, i.e. without large latitudinal movements, since most individuals had low *δ*13C values (<–22.2 ‰) typical of Antarctic waters throughout their life. However, some individuals presented higher *δ*13C values (e.g. KU6), similar to those of subantarctic waters. These results agree with previous studies ([Cherel and Hobson, 2005](#_ENREF_8); [Alvito et al., 2015](#_ENREF_1); [Guerreiro et al., 2015](#_ENREF_17)) and contradict the hypothesis that the APF functions as a barrier for the distribution of *K. longimana* ([Laptikhovsky et al., 2009](#_ENREF_22)). The presence of *K. longimana* north of the APF has also been predicted in models of habitat suitability ([Xavier et al., 2016](#_ENREF_48)).

 Values of *δ*15N in *K. longimana* beaks show a clear gradient from the earliest life stages to the adult. The difference (5.0 ‰) between the lowest value (+3.2 ‰) obtained in tip of the rostrum of the upper beaks and the highest (+8.2 ‰) obtained in subsection I of the lower beaks suggests an increase of more than one trophic level on this species ([Peterson and Fry, 1987](#_ENREF_32); [Hobson and Welch, 1992](#_ENREF_19); [McCutchan et al., 2003](#_ENREF_27)). Our results are similar to those obtained in other studies that compare beaks of different sizes ([Cherel and Hobson, 2005](#_ENREF_8); [Seco et al., 2016](#_ENREF_40)), which confirms ontogenic dietary shifts, possibly from zooplankton to fish and squids ([Collins and Rodhouse, 2006](#_ENREF_12)). Since the biggest differences between *δ*15N 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 time related to the formation of the posterior portion of the tip of rostrum. Dietary shifts at this period of the life-cycle was also observed in other species, including the giant squid (*Architeuthis dux;* ([Guerra et al., 2010](#_ENREF_16))and other Southern Ocean species ([Cherel et al., 2009](#_ENREF_6)). In the three analysed sections (hood, crest and lateral wall) the *δ*15N 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](#_ENREF_35)).

# 5. Conclusion

This study confirms the method of stable isotopic analysis as reliable to study habitat and trophic ecology of cephalopods. Future studies should use the tip of the rostrum and hood of the upper beak to evaluate ontogenic shifts along squid life. However, if only lower beaks are available they may be used if the tip of the rostrum is cut even 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*15N values from earliest stages. We recommend that to investigate ontogenic changes between young and adult life-stages using stable isotopic analysis on lower beaks only the tip of the rostrum and the wing, or the free corner of the lateral wall (subsection I – L1) must be used.

 Similarly to the beak, other cephalopods hard structures like gladii, statoliths and eye lenses have a continuous grow along the individual’s life ([Sivak et al., 1994](#_ENREF_41); [Ruiz-Cooley et al., 2010](#_ENREF_39); [Arkhipkin and Shcherbich, 2012](#_ENREF_2); [Rosas-Luis et al., 2017](#_ENREF_38)). Some studies have already applied stable isotopic analysis on these structures from other taxa, e.g. fish eye lenses ([Wallace et al., 2014](#_ENREF_43)) and also on the gladii of cephalopods ([Cherel et al., 2009](#_ENREF_6); [Ruiz-Cooley et al., 2010](#_ENREF_39); [Lorrain et al., 2011](#_ENREF_23); [Rosas-Luis et al., 2017](#_ENREF_38)). In the future, the use of stable isotopic analysis on these structures should be evaluated in detail. For example it should be understood if there are differences on the isotopic signatures of different structures in the same individual, as it was proposed by ([Cherel et al. (2009)](#_ENREF_6)).

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