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Amino acid $\delta^{13}C$ and $\delta^{15}N$ from sclerotized beaks: A new tool to investigate the trophic ecology of cephalopods, including giant and colossal squids

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ABSTRACT: Combining the use of predators as biological samplers together with measurements of the stable isotopic ratios ($\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$) of their sclerotized beaks help investigate foraging ecology of poorly known oceanic cephalopods. However, high chitin content (an amino-sugar macromolecule) lowers beak $\delta^{15}N_{Bulk}$ values, thus precluding direct isotopic comparison with other tissues and organisms. To overcome the chitin effect, compound-specific isotopic analysis of amino acids (CSIA-AA) was performed on squid beaks. The method was applied on beaks and muscle, and the resulting $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values compared between tissues. The usefulness of CSIA was tested by defining the habitat and trophic position (TP_{CSIA}) of squids using their $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values. Beak $\delta^{13}C_{AA}$ values were reliably measured on 12 AA that included 5 essential and 7 non-essential AA, and $\delta^{15}N_{AA}$ values were quantified on at least 7 AA that included 2 source and 4 trophic AA. Importantly, $\delta^{13}C_{AA}$ and $\delta15N_{AA}$ varied little between muscle and lower and upper beaks, and TP_{CSIA} estimates were identical regardless of the tissue considered. Tissue $\delta^{13}C_{AA}$ values of both essential and non-essential AA reflected the latitudinal baseline $\delta^{13}C$ gradient that occurs in the Southern Indian Ocean, while beak $\delta^{15}N_{AA}$ from source and trophic AA allowed the disentangling of the baseline effect from the trophic effect, and thus better calculations of squid TP estimates than from $\delta15N_{Bulk}$ values. Beak $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ defined isotopic niches of colossal and giant squids, the 2 largest living invertebrates. In subantarctic waters, they segregate by having species-specific foraging habitats (using $\delta^{13}C_{Gly}$ or $\delta^{15}N_{Phe}$) and TP_{CSIA} (using $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$). TP_{CSIA} is higher in colossal (4.7) than giant (4.3) squids, and both values compare well with those of myctophid-eaters, suggesting very large squids prey primarily upon small zoo planktivorous fishes. As expected, CSIA-AA overcomes the chitin effect on beaks and it is a powerful tool to investigate trophic interactions of cephalopods. The method has a great potential with arthropods, because chitin is a main component of their exoskeleton but the deleterious effect of chitin is overlooked in isotopic studies focusing on crustaceans and insects.

Keywords: Arthropod · Carbon · Chitin · CSIA · Habitat · Nitrogen · Stable isotope · Trophic position

Introduction

Cephalopods play a major role in marine trophic webs, as underlined by their global biomass and annual consumption of resources (Clarke 1996, Coll et al. 2013). Determining and quantifying their trophic relationships is therefore key to understanding the structure and functioning of marine ecosystems. The role of cephalopods as prey is demonstrated by their importance in the diet of predators, but knowledge of their food is limited by lack of data (Clarke 1996). A new approach to investigate cephalopod feeding ecology was developed by combining the use of their predators as biological samplers together with measurements of the stable isotopic values of their beaks (Cherel & Hobson 2005, Cherel et al. 2009b). Predators as samplers have 2 main advantages: (1) they catch larger specimens and a greater diversity of cephalopods than fisheries sampling gear (Rodhouse 1990), and (2) they accumulate hundreds to thousands of beaks in their stomachs, because beaks are hard sclerotized structures that resist digestion (Clarke 1980, Cherel et al. 2017).

The basic concept of the stable isotope method is that an animal's isotopic composition is directly influenced by the food it assimilates. The 2 main elements used in isotopic ecology are carbon and nitrogen, whose isotopic ratios are measured on bulk tissue (mainly muscle) that contains primarily proteins. Consumer proteins are enriched in ^{15}N relative to dietary proteins, and consequently δ^{15}NBulk measurements serve as indicators of a consumer's diet and trophic position (TP_{Bulk}) (Vanderklift & Ponsard 2003). By contrast, $\delta^{13}C_{Bulk}$ varies little along the food web and is mainly used to determine primary sources in a trophic network (Kelly 2000). Measuring $\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$ on beaks gave new insights in cephalopod biology, such as latitudinal feeding habitats, migration patterns, TPBulk, trophic structure of the communities, and ontogenic dietary changes (Cherel & Hobson 2005, Cherel et al. 2009a,b, Navarro et al. 2013, Golikov et al. 2018). However, while $\delta^{13}C_{Bulk}$ values of beaks and soft tissues are similar, beaks have consistently lower $\delta^{15}N_{Bulk}$ values (Hobson & Cherel 2006, Ruiz-Cooley et al. 2006,

Cherel et al. 2009a). This major limitation precludes comparing raw (uncorrected) beak $\delta^{15}N_{Bulk}$ values with those of other tissues and organisms to trace trophic pathways in marine ecosystems (Hobson & Cherel 2006, Cherel et al. 2009a).

Beaks and soft tissues have different biochemical compositions. Beaks contain chitin (Hunt & Nixon 1981, Rubin et al. 2010), a modified polysaccharide that is impoverished in ^{15}N compared to consumer diet (Schimmelmann 2011). The presence of chitin explains why beaks have lower $\delta^{15}N_{Bulk}$ values than soft tissues. Moreover, the ratio of chitin to protein varies within beaks and between beaks, because the undarkened, darkening and darkened parts of beaks contain decreasing relative amounts of chitin over protein (Rubin et al. 2010). Hence, overcoming the chitin effect on beaks is of primary importance, and 4 different approaches can be used theoretically (Xavier et al. 2015). (1) Quantification of isotopic correction factors allows the comparison of $\delta^{15}N_{Bulk}$ between beaks and soft tissues, with the drawback that corrected values are estimates (Hobson & Cherel 2006, Cherel et al. 2009a). (2) The same limitation applies to chitin normalization models using C:N mass ratios as a proxy for chitin content, following similar methods applied to correct for variable lipid content in bulk $\delta^{13}C$ analyses (as lipids, chitin has a higher C:N value than proteins; Webb et al. 1998). (3) Measurements of $\delta^{15}N$ on purified proteins is not feasible because classical extraction protocols for soft tissues are not effective for beaks (the majority of proteins remain insoluble even under the most aggressive extraction procedures; Rubin et al. 2010). (4) Measurements of $\delta^{15}N$ on amino acids (AA) from protein is a promising tool, but no studies used the compound-specific isotopic analysis of amino acids (CSIA-AA) (McMahon & McCarthy 2016) on cephalopod beaks.

CSIA-AA has emerged in the last decade as a powerful approach for tracing the origins and fate of carbon and nitrogen in ecological and biogeochemical studies (McMahon et al. 2013,

Ohkouchi et al. 2017). The method has a broad range of applications, including the identification of baseline isoscapes, the assessment of the source and transformation of detrital organic matter, and tracing of animal migration. While comparatively few investigations are based on $\delta^{13}C_{AA}$ measurements, $\delta^{15}N_{AA}$ values are increasingly used to calculate accurate trophic position estimates (TP_{CSIA}) of a broad range of terrestrial and aquatic consumers (Chikaraishi et al. 2014). To our knowledge, no study has measured $\delta^{13}C_{AA}$ in cephalopods, and only limited information is available on their $\delta^{15}N_{AA}$ values. Indeed, a preliminary study shows incidentally a chromatogram from nitrogen analysis of a single squid beak (Walsh et al. 2014), but most previous $\delta^{15}N_{AA}$ measurements have been restricted to the mantle of a few specimens of ommastrephid squids (Ruiz-Cooley et al. 2013, Madigan et al. 2016, Hetherington et al. 2017).

The main goal of the present study was to use CSIA-AA on cephalopod beaks primarily to bypass the chitin effect. We focused on 3 points: (1) we validated how many and which AA can be isolated from beaks to reliably measure their $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values. (2) Using the same specimens, we compared $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values of lower and upper beaks with those of muscle, the canonical tissue for isotopic investigations. We hypothesized that AA isotopic values are identical in muscle (a metabolically active tissue) and in the more recently built parts of beaks (a metabolically inactive tissue). (3) We investigated the biological usefulness of CSIA-AA on beaks by testing (i) if beak $\delta^{13}C_{AA}$ reflects the latitudinal baseline $\delta^{13}C$ gradient occurring in the Southern Indian Ocean; the gradient allows defining the latitudinal habitat of consumers using either $\delta^{13}C_{Bulk}$ (Jaeger et al. 2010) or $\delta^{13}C_{AA}$ (Lorrain et al. 2009); and (ii) if beak $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ help to define the isotopic niche (feeding habitat and TP_{CSIA}) and thus the mechanisms allowing co-existence of 4 sympatric cephalopods that include the 2 largest

living invertebrates, the colossal squid *Mesonychoteuthis hamiltoni* and giant squid *Architeuthis dux*.

Materials and methods

STUDY SITES, DIETARY SAMPLING AND ANALYSIS

Fieldwork was carried out in the Southern Indian Ocean by fishery observers during commercial cruises. The fishery targeted Patagonian toothfish (*Dissostichus eleginoides*), with sleeper sharks (*Somniosus antarcticus*) being occasionally by caught. Cephalopod items were sorted from fish stomach and kept in 70% ethanol until analysis. Squid lower beaks were identified from their morphological features by comparison with material held in our own collection and by reference to the available literature (Xavier & Cherel 2009). Lower rostral length of beaks were measured to 0.1 mm with a vernier caliper and allometric equations were used to estimate dorsal mantle length (ML) of squids (Table 1).

Two sets of samples were analyzed. (1) Ten whole buccal masses of the giant warty squid *Kondakovia longimana* were collected from toothfish stomachs in Kerguelen (n = 7) and Crozet waters (n = 3) in 2014-2015. They were dissected in order to sort lower and upper beaks from buccal masses. Three tissues were sampled for isotopic analysis: muscle tissue, wings of lower beaks, and small pieces of lateral walls of upper beaks. (2) Accumulated beaks from stomachs of toofish and sharks caught in Kerguelen waters in 1997-2001 (Cherel & Duhamel 2004, Cherel et al. 2004) were used to compare the isotopic values of *M. hamiltoni*, *A. dux*, *K. longimana*, and Dana octopus squid *Taningia danae*. Wings or free lateral corners of lateral walls from lower beaks were cut with scissors. Importantly, all the different sampled parts of lower and upper beaks referred to newly built material, to minimize potential trophic

ontogenetic changes (Cherel & Hobson 2005, Queiros et al 2018) and different tissue-related time integration periods between beaks and the metabolically active muscle tissue.

STABLE ISOTOPE ANALYSIS

Beaks were cleaned before isotopic analysis to remove any remains of soft tissue and mucus. Samples were freeze-dried and ground to a fine powder. Bulk and AA $\delta^{13}C$ and $\delta^{15}N$ values were determined on the same samples.

Bulk isotopic measurements. Lipids of muscle tissue were removed using cyclohexane. Subsamples of the homogenates of beaks and of lipid-extracted muscle tissue were weighed with a microbalance and packed in tin cups. An elemental analyser (Thermo Scientific Flash 2000) was coupled to a continuous flow mass spectrometer (Thermo Scientific Delta V Plus) to measure carbon and nitrogen contents, and $\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$ values, respectively. Stable isotope ratios are expressed using standard δ notation relative to carbonate Vienna PeeDee Belemnite and atmospheric nitrogen. Two internal standards of caffeine (USGS 61 and USGS 62) were used for drift assessment and data normalization. Observed analytical errors on internal standards were < 0.10 ‰ for both $\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$.

CSIA-AA. Beak and and non-lipid extracted muscle samples (1− 2mg) were hydrolyzed under nitrogen (0.5 ml 6 M HCl, 110°C, 20 h). Norleucine (20 µl, 25 mM) was added to each sample as an internal standard prior to hydrolysis. The resultant AA were purified and derivatized to N-acetyl-isopropyl esters (Styring et al. 2012). Caffeine (IAEA-600) was added as an internal standard to the derivatized AA before dilution in ethyl acetate for carbon and nitrogen analyses by continuous-flow gas chromatography combustion-isotope ratio mass spectrometry (GC-C-IRMS). $\delta^{13}C$ and $\delta^{15}N$ values were measured using a Thermo Trace GC Ultra gas chromatograph coupled to a Delta V Plus isotope-ratio mass spectrometer via a GC IsoLink II interface (Thermo Scientific). The combustion/reduction reactor was maintained at

1000°C, and a liquid nitrogen cold trap was used after the reactor to remove CO_2 during nitrogen analyses. AA were separated on a VF-35MS column (30 m, 0.32mm ID, 1 μm film thickness; Agilent Technologies). Analyses were done with a splitless injection at 270°C, and a helium flow set at 1.4 ml min^{-1}. Samples were analyzed either in duplicate or triplicate. A mixture of 16 AAs and Norleucine, thoroughly calibrated by EA-IRMS and derivatized along with the samples, was injected after every 4 samples to evaluate drift and accuracy. Raw data were corrected (Docherty et al. 2001) and normalized using internal standard values (norleucine: $\delta^{13}C$: −28.77 ± 0.05‰, $\delta^{15}N$: 19.19 ± 0.08‰; caffeine: $\delta^{13}C$: −27.77 ± 0.04‰, $\delta^{15}N$: 1.00 ± 0.20‰). Depending on AA, measurement precision for $\delta^{13}C$ and $\delta^{15}N$ of the standard mixture ranged from 0.2 to 1.0‰ (mean 0.4‰), and from 0.2 to 1.2‰ (mean 0.5‰), respectively.

GENERAL COMMENTS ABOUT AA

Twenty AAs form the essential building blocks of proteins. Acid hydrolysis destroys tryptophan and precludes determining cysteine directly (Fountoulakis & Lahm 1998). It also converts asparagine (Asn) and glutamine (Gln) into aspartic acid (Asp) and glutamic acid (Glu), respectively, resulting in the measurements of combined Asn + Asp (Asx) and Gln + Glu (Glx). Since arginine is not derivatized, the analytical procedure allows the quantification of $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ for 15 standard AAs at best, because some AAs (Met, Ser, Thr, Tyr; see abbreviations in Table 2) are partially destroyed by hydrolysis (Fountoulakis & Lahm 1998). In animals, standard AAs are classified into 2 categories (essential or non-essential) with regard to carbon metabolism. Eight of the 15 measured AAs (His, Ile, Leu, Lys, Met, Phe, Thr and Val) are essential AAs, with the remaining 7 AAs being non-essential (Ala, Asx, Glx, Gly, Pro, Ser and Tyr) (Lehninger 1982). In terms of δ15N, the 15 AAs group into 5 source AAs (His, Lys, Phe, Met and Tyr) and 7 trophic AAs (Ala, Asx, Glx, Ile, Leu, Pro and Val). Trophic AAs

undergo significant ^{15}N enrichment between food and consumers, while source AAs do not, thus reflecting $\delta^{15}N$ baseline. Gly and Ser are 2 challenging AAs to classify into the source and trophic framework, and were clustered into a source/trophic group. Finally, Thr is considered as a metabolic AA because it shows ^{15}N depletion relative to dietary Thr (McMahon & McCarthy 2016).

DATA ANALYSES

Lower beak $\delta^{15}N_{AA}$ values of the two canonical source AA Phe and trophic AA Glx (McMahon & McCarthy 2016) were used to estimate TP_{CSIA} and calculate the relative trophic position (RTP) of oceanic squids. The first formulation is based on equations from Chikaraishi et al. (2010) and McMahon & McCarthy (2016), as:

$$TP_{Glx\text{-}Phe} = [(\delta^{15}N_{Glx} - \delta^{15}N_{Phe} - TDF_1 - \beta) / TDF_2] + 2$$

where TDF_1 represents the trophic discrimination factor ($TDF_{Glx\text{-}Phe}$) between food and consumers typical of lower trophic-level organisms (7.6 ‰; Chikaraishi et al. 2010), β is the difference in $\delta^{15}N$ between Glx and Phe in primary producers at the base of the food web (2.9 ‰; Nielsen et al. 2015) and TDF_2 reflects $TDF_{Glx\text{-}Phe}$ for cephalopods (5.0 ‰; McMahon & McCarthy 2016). The second formulation is a proxy for TP, as $RTP = \delta^{15}N_{Glx} - \delta^{15}N_{Phe}$ of consumers, it is expressed in ‰, thus differing from TP that is a rational number from 1 to up to 6 (with no unit). RTP calculation requires no *a priori* assumptions about the β and TDF values used to estimate $TP_{Glx\text{-}Phe}$ other than the assumption that these values remain constant

among the samples. RTP essentially removes the isotopic effect of food web baseline, focusing on relative differences in food web position (Choy et al. 2015).

Estimated TP of squids was also calculated using $\delta^{15}N_{Bulk}$ values of their lower beaks (modified from Cherel et al. 2008), as:

$$TP_{Bulk} = [(\delta^{15}N_{Bulk} + 0.10) / TDF] + 2$$

where 0.10 is the difference between 3.46 ‰ (the isotopic factor to correct the chitin effect between wings of lower beaks and muscle tissue; Cherel et al. 2009a) and 3.36‰ (the average $\delta^{15}N_{Bulk}$ value of the herbivorous salp *Salpa thompsoni* in Kerguelen waters with an assumed TP of 2.0; Cherel et al. 2008, 2010), and TDF is the $\delta^{15}N$ difference between muscle of cephalopods and their food (3.3‰; Hobson & Cherel 2006).

Estimating TP is challenging, with every method showing limitations. Three major issues are (1) quantification of TDF for TP_{Bulk} and TP_{CSIA}, (2) b for TP_{CSIA}, and (3) baseline value for TP_{Bulk}. In aquatic ecosystems, particulate organic matter (POM) is often used as a proxy for phytoplankton and as a food-web baseline for TP_{Bulk} calculations (Post 2002). However, the use of POM is not ideal, because (1) it represents an unknown mixture of phytoplankton together with detritus, bacteria and microzooplankton, and (2) its turnover is high, thus promoting large $\delta^{15}N_{Bulk}$ variations at small temporal scales that are buffered in higher TP organisms (Pakhomov et al. 2019). An alternative to POM is to consider longer-lived primary consumers (herbivorous copepods or pelagic tunicates) that are assumed to be representative of TP_{Bulk} = 2.0. However, crustacean exoskeleton contains chitin that is likely to lower their $\delta^{15}N_{Bulk}$ values (see Section 4.3). Hence, we used salps as control organisms in the present

investigation, even though salps themselves are not always appropriate due to their selective feeding habits (Kruse et al. 2015, Pakhomov et al. 2019).

Data were statistically analyzed using SYSTAT 13. Values are means ± SD.

Results

COMPARING $\delta^{13}C_{AA}$ AND $\delta^{15}N_{AA}$ IN MUSCLE AND BEAKS

Bulk $\delta^{13}C$ and $\delta^{15}N$ values. Bulk $\delta^{13}C$ values of muscle, lower beak and upper beak of warty squids were not statistically significant (Table 2). As expected, a chitin effect was found in $\delta^{15}N_{Bulk}$ values, which were lower in beaks than in muscle tissue (Table 3).

Amino acid $\delta^{13}C$ values. The analytical method quantified $\delta^{13}C_{AA}$ of 16 AA in muscle and 12 in beaks (Table 2). Too low amounts of 4 AA (Hyp, Ile, Lys and Met) precluded reliable isotopic measurements in both lower and upper beaks. Individual $\delta^{13}C_{AA}$ values ranged widely, from -32 to -5 ‰ for $\delta^{13}C_{Leu}$ and $\delta^{13}C_{Gly}$, respectively (Fig. 1, upper panel). There were no significant isotopic differences amongst the three tissues, except $\delta^{13}C_{Thr}$ value that was marginally lower in muscle that in beaks (Table 2).

Amino acid $\delta^{15}N$ values. The method quantified $\delta^{15}N_{AA}$ of 14 AA in muscle and 11 in beaks (Table 3). $\delta^{15}N_{Hyp}$ and $\delta^{15}N_{Met}$ cannot be reproducibly measured in muscle, as were $\delta^{15}N_{AA}$ values of Hyp, Ile, Lys, Met and Thr in beaks. In some cases, $\delta^{15}N_{AA}$ values of Pro, Ser, Tyr and Val were difficult to quantify in beaks. Individual $\delta^{15}N_{AA}$ values ranged widely, from -28 to 23 ‰ for $\delta^{15}N_{AA}$ of the metabolic AA Thr and the trophic AA Ile, respectively. Source

AA (His, Lys, Phe and Tyr) had much lower $\delta^{15}N_{AA}$ values than trophic AA (Ala, Asx, Glx, Ile, Leu and Val) (Fig. 1, lower panel).

Individual $\delta^{15}N_{AA}$ values were consistent amongst tissues, with no differences in 7 AA, marginally significant differences in 3 AA and a highly significant difference for Tyr. In the 4 later cases, $\delta^{15}N_{AA}$ values were identical in lower and upper beaks but they differed from muscle values. Importantly, $\delta^{15}N_{AA}$ of the 2 canonical source AA Phe and trophic AA Glx were not significantly different amongst tissues and, consequently, RTP and $TP_{Glx\text{-}Phe}$ were identical when they were calculated using $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ values from either muscle, lower or upper beaks (Table 3).

LATITUDINAL EFFECT ON $\delta^{13}C_{AA}$ IN MUSCLE AND BEAKS

Tissue $\delta^{13}C_{Bulk}$ values values grouped giant warty squids in 2 clusters of 5 individuals (muscle: −21.3 ± 0.5 versus −24.2 ± 0.3‰; lower beak: −20.9 ± 0.4 versus −24.5 ± 0.8‰; upper beak: −20.7 ± 0.4 versus −24.3 ± 0.6‰; Mann-Whitney U-tests, all U = 0.0, all p = 0.009). According to the latitudinal $\delta^{13}C_{Bulk}$ gradient occurring in the southern Indian Ocean (Jaeger et al. 2010), clusters with the higher and lower $\delta^{13}C_{Bulk}$ values corresponded to individuals that grew in subantarctic and Antarctic waters, respectively.

Whatever the tissue and individual AA, $\delta^{13}C_{AA}$ values were always lower in the Antarctic than in the subantarctic group. The difference was significant ($p < 0.05$) in all cases, except for $\delta^{13}C_{Thr}$ in lower beaks ($U = 5.0$, $p = 0.221$) (Fig. 2, upper panel). The isotopic difference between AA of the two groups ranged from 1.9 ($\delta^{13}C_{Thr}$) to 7.3 ‰ ($\delta^{13}C_{Gly}$) in muscle, from 1.6 ($\delta^{13}C_{Thr}$) to 7.5 ‰ ($\delta^{13}C_{Gly}$) in lower beak and from 2.9 ($\delta^{13}C_{Thr}$) to 8.3 ‰ ($\delta^{13}C_{Ser}$) in upper beak. Isotopic differences between Antarctic and subantarctic specimens were consistent across

tissues, as illustrated by the positive linear relationship between $\delta^{13}C_{AA}$ differences in 12 AA from muscle and lower beak (Fig. 2, lower panel).

HABITAT AND TROPHIC POSITION OF SQUIDS

Darkening or darkened beaks indicated that individuals of the 4 species were species were either large juvenile or adult squids. Estimated ML ranged from 45 to 239 cm and mean ML values increased in the order giant warty < Dana octopus < giant = colossal squids (Table 1).

Bulk $\delta^{13}C$ and $\delta^{15}N$ values. Squids were segregated by both $\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$ values of lower beaks. Overall, bulk isotopic values defined 4 distinct isotopic niches (Fig. 3, upper panel). Giant warty squids had significantly lower $\delta^{13}C_{Bulk}$ values than Dana octopus and giant and colossal squids. Bulk $\delta^{15}N$ values increased in the following order: giant warty = giant < Dana octopus < colossal squids (Fig. 1, upper panel), and, accordingly, TP_{Bulk} followed the same increasing order from 3.9 to 5.9, a 2.0 difference (Table 4).

AA $\delta^{13}C$ values. Squids were segregated by $\delta^{13}C_{AA}$ of 10 individual AA, with only $\delta^{13}C_{Ser}$ and $\delta^{13}C_{Tyr}$ being not significantly different amongst species. In most cases, $\delta^{13}C_{AA}$ values were lower in giant warty squid than in other species. Only $\delta^{13}C_{Gly}$ showed species-specific values, ranging from −6.4 (giant warty squid) to 5.0‰ (colossal squid) (Table 4).

AA $\delta^{15}N$ values. Squids were segregated by $\delta^{15}N_{AA}$ of 10 individual AAs, with only $\delta^{15}N_{Val}$ being not significantly different amongst species. In many cases, individual $\delta^{15}N_{AA}$ values defined 2 groups, with giant and giant warty squids having lower values than colossal and Dana octopus squids. $\delta^{15}N_{Glx}$ values increased in the order giant = giant warty < Dana octopus < colossal squids, and $\delta^{15}N_{Phe}$ values also increased in a similar order: giant = giant warty ≤ Dana octopus < colossal squids (Fig. 4). RTP, and hence $TP_{Glx\text{-}Phe}$, clustered into 2 groups, with giant and giant warty squids having lower values than the colossal and Dana

octopus squids. Average $TP_{Glx\text{-}Phe}$ ranged from 4.2 to 4.8, thus encompassing a 0.6 difference (Table 4).

Comparing TP_{Bulk} and $TP_{Glx\text{-}Phe}$. When pooling all individual squids, TP_{Bulk} estimates did not fit well with $TP_{Glx\text{-}Phe}$ (Fig. 5A). At the species level, TP_{Bulk} and $TP_{Glx\text{-}Phe}$ values were not different for Dana octopus and giant squids, but they differed significantly for colossal and giant warty squids. The TP_{Bulk} estimate of the latter was lower than its $TP_{Glx\text{-}Phe}$ value, while TP_{Bulk} of the colossal squid was noticeably higher than its $TP_{Glx\text{-}Phe}$ (Table 4). Interestingly, TP_{Bulk} values were more positively related to baseline $\delta^{15}N_{Phe}$ values ($y = 0.36x + 4.63$, $r^2 = 0.615$, $F_{1,29} = 46.4$, $p < 0.0001$) (Fig. 5B) than to trophic $\delta^{15}N_{CSIA}$ values (RTP; $y = 0.27x - 1.20$, $r^2 = 0.310$, $F_{1,29} = 13.0$, $p = 0.001$).

TP_{Bulk} and $TP_{Glx\text{-}Phe}$ were not correlated with ML within each squid species (data not shown), thus suggesting no ontogenetic dietary shift within the investigated size ranges. Estimated ML of giant warty squids collected in Kerguelen waters in 2014−2015 (n = 7, first data set) and 1997−2001 (n = 5, second data set) were identical, as were their $\delta^{15}N_{Bulk}$, TP_{Bulk}, $\delta^{15}N_{Phe}$, $\delta^{15}N_{Glx}$, RTP and $TP_{Glx\text{-}Phe}$ (Mann-Whitney *U* tests, all $p \geq 0.329$).

Discussion

This study presents an innovative method to investigate the trophic ecology of cephalopods that complements the use of bulk isotopic values of their beaks (Cherel & Hobson 2005). To the best of our knowledge, it is the first to validate and test CSIA-AA on cephalopods by measuring their beak $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values. Since the method focuses on AA from proteins, it eliminates problems due to the presence of other organic compounds that may affect bulk

isotopic values. For $\delta^{15}N$, CSIA-AA discards chitin during the analytical procedure, thus removing *the chitin effect* that lowers beak $\delta^{15}N_{Bulk}$ values (Cherel et al. 2009a). Additionally, the most important applications of $\delta^{15}N$ CSIA-AA to date is to disentangle the $\delta^{15}N$ baseline effect from the $\delta^{15}N$ trophic effect by simultaneously measuring $\delta^{15}N_{Source\ AA}$ and $\delta^{15}N_{Trophic\ AA}$ in the consumer itself, thus allowing better TP_{CSIA} estimates than using $\delta^{15}N_{Bulk}$ values (McMahon & McCarthy 2016).

VALIDATING THE USE OF $\delta^{13}C_{AA}$ AND $\delta^{15}N_{AA}$ IN CEPHALOPOD BEAKS

As expected, $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values were measured on 14-15 standard AA isolated from muscle of the giant warty squid, thus validating the analytical procedure on cephalopod tissues. Smaller numbers of AAs were measured in beaks, which can be explained by tissue-specific AA composition (Hunt & Nixon 1981, Miserez et al. 2007). Data from giant warty squids may be generalized to other cephalopods, because beaks of Dana octopus, and colossal and giant squids gave similar results, which were therefore consistent across species and across beaks with different sclerotized levels that reflect different amounts of chitin relative to proteins (Rubin et al. 2010). To sum up, beak $\delta^{13}C_{AA}$ can be confidently measured on 12 AA that include 5 essential (His, Leu, Phe, Thr, Val) and 7 non-essential (Ala, Asx, Glx, Gly, Pro, Ser, Tyr) AAS, and beak $\delta^{15}N_{AA}$ can be quantified on at least 7 AAS that include 2 source (His, Phe), four trophic (Ala, Asx, Glx, Leu) AAS.

Another major finding was the similarity of $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values amongst tissues. Lower and upper beaks showed no AA isotopic differences, and beaks and muscle had either identical or slightly different AA isotopic values. The only exception is $\delta^{15}N_{Tyr}$ that was much higher in muscle than in beaks and we have no explanation for that difference. Three consequences of the inter-tissue isotopic comparison are notable. (i) Since CSIA-AA overcomes the chitin effect

of beaks, the method may be successfully applied to the gladius (Ruiz-Cooley et al. 2013), another chitin-containing hard structure (Hunt & Nixon 1981, Cortizo et al. 2008), whose morphology allows serial sampling to isotopically reconstruct the past individual trophic history of squids (Cherel et al. 2009a, Ruiz-Cooley et al. 2010, Lorrain et al. 2011). (ii) Beak (hard tissue) is as representative as muscle (soft tissue) to investigate the isotopic ecology of cephalopods. This paves the way to use the numerous accumulated beaks sorted from predator stomachs to gather useful biological information on rarely investigated cephalopod species. (iii) Since $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ did not vary amongst tissues with a similar time integration period, RTP and $TP_{Glx\text{-}Phe}$ were not tissue-specific and accurate $TP_{Glx\text{-}Phe}$ estimates can be calculated either from beak or muscle values.

TESTING THE USEFULNESS OF BEAK $\delta^{13}C_{AA}$ AND $\delta^{15}N_{AA}$ OF CEPHALOPODS

Foraging habitat and beak $\delta^{13}C_{AA}$. The most important application of $\delta^{13}C_{Bulk}$ of marine consumers is to define their isotopic habitats as a proxy of their foraging habitats. Tissue $\delta^{13}C_{Bulk}$ clustered giant warty squids into an Antarctic and a subantarctic group, thus allowing us to test the ability of $\delta^{13}C_{AA}$ to discriminate the squid feeding grounds. All $\delta^{13}C_{AA}$ values from the 3 tissues were lower in the Antarctic than subantarctic group. This confirms—for muscle and beaks of cephalopods—the results obtained on blood and feathers of penguins at different spatial oceanographic scales (Lorrain et al. 2009, Polito et al. 2017), and emphasizes the usefulness of $\delta^{13}C_{AA}$ to depict foraging latitudes and migration patterns of consumers from the Southern Ocean. Thus, $\delta^{13}C_{AA}$ of accumulated beaks can also help with re constructing the

foraging habitats of cephalopod eaters (e.g. albatrosses, sharks) over the weeks/ months preceding sampling.

Not all AAs were equally efficient to differentiate feeding grounds. Surprisingly, essential AAs discriminated less well the 2 latitudinal habitats than non-essential AAs, with Gly being the most discriminant AAs. Leu was the single exception, because it grouped with the non-essential AAs (Fig. 2 lower panel). The data compare well with a previous investigation on penguins showing that $\delta^{13}C_{AA}$ of all measured AAs vary with $\delta^{13}C_{Bulk}$ and latitudes (Lorrain et al. 2009). Some previous studies focused on essential AAs (Polito et al. 2017), the rationale being that essential AAs tracked $\delta^{13}C$ baseline levels, because many non-essential AAs are trophically ^{13}C enriched (McMahon et al. 2013, 2015). Here, there was no differential trophic effect between the 2 groups of giant warty squids, since RTP and $TP_{Glx\text{-}Phe}$ of Antarctic and subantarctic specimens were not significantly different, whatever the tissue considered (Mann-Whitney U-tests, all p ³ 0.175). Consequently, the usefulness of the non-essential Gly was tested in the second set of samples. Amongst the 4 squid species, lower beak d13CGly was positively and linearly related to $\delta^{15}N_{Phe}$ ($y = 1.63x - 2.38$, $r^2 = 0.417$, $F_{1,29} = 20.7$, $p < 0.0001$), but it did not correlate with RTP, meaning that $\delta^{13}C_{Gly}$ was more linked to isotopic baseline than to trophic enrichment. Consequently, our data highlight that $\delta^{13}C_{AA}$ of non-essential AAs can be helpful to delineate foraging habitats of consumers, and this merits further investigations on other animal models living in different ecosystems.

Estimating TP_{CSIA} using beak $\delta^{15}N_{AA}$. RTP and $TP_{Glx\text{-}Phe}$ estimates grouped the 4 oceanic squids into 2 clusters, with giant and giant warty squids having lower values than colossal and Dana octopus squids. This contrasts with $\delta^{15}N_{Bulk}$ values depicting 3 different TP_{Bulk} estimates. Comparing RTP and $TP_{Glx\text{-}Phe}$ to $\delta^{15}N_{Bulk}$ and TP_{Bulk} emphasizes the ability of CSIA-AA to quantify and disentangle isotopic $\delta^{15}N$ baseline from trophic ^{15}N enrichment (McMahon et al.

2013, Ohkouchi et al. 2017). This is well illustrated by the positive correlation between squid TP_{Bulk} and $\delta^{15}N_{Phe}$ that indicates a consistent baseline effect on $\delta^{15}N_{Bulk}$ and TP_{Bulk} values. $TP_{Glx\text{-}Phe}$ and TP_{Bulk} were identical for Dana octopus and giant squids, slightly different for giant warty squid, but strongly differed for colossal squid. TP_{Bulk} of the latter species was previously estimated at 6.1 (Cherel et al. 2008), a value close to 5.9 reported here. By contrast, its $TP_{Glx\text{-}Phe}$ was 4.7, thus lowering TP by ~1.3, which is a highly relevant difference to assess the role of colossal squid within the oceanic ecosystem both as a predator and prey. Overestimation of $\delta^{15}N_{Bulk}$ and TP_{Bulk} due to a baseline effect is also the likely explanation of some high values recorded in the literature for deep-sea oceanic squids (e.g. gonatids, histioteuthids), which include small and delicate forms (e.g. chiroteuthids, mastigoteuthids) (Cherel & Hobson 2005, Cherel et al. 2008, 2009b, Guerreiro et al. 2015, Golikov et al. 2018). Clearly, the issue merits further investigations using CSIA-AA to compare thoroughly TP_{Bulk} with TP_{CSIA} estimates.

Knowledge on the food of colossal, Dana octopus, giant and giant warty squids remains very limited. The few dietary information collected opportunistically from cephalopods eaten by sperm whales showed that both colossal and giant warty squids prey primarily upon mesopelagic fish (Lubimova 1985). Accordingly, TP_{Bulk} estimates of most Kerguelen myctophids, the main mesopelagic fish biomass of the Southern Ocean, are lower (3.3−3.9) than squid $TP_{Glx\text{-}Phe}$ (4.2−4.8), and the latter compares well with TP_{Bulk} estimates (4.3−4.8) of predators that are known to feed primarily on myctophids (blue petrel, king penguin, southern elephant seal and Antarctic fur seal) (Cherel et al. 2008, 2010, 2017). Hence, the largest invertebrates living on Earth are not apex predators, but, instead, they exploit mesopelagic fish that constitutes the highest oceanic micronektonic biomass available in the Southern Ocean and worldwide (Kozlov 1995, Irigoien et al. 2014).

Comparing the isotopic niche using bulk and AA $\delta^{13}C$ and $\delta^{15}N$. In Kerguelen waters, beak $\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$ values depict species-specific isotopic niches, with each sympatric

squid using a unique combination amongst 2 isotopic habitats and 3 TP_{Bulk} (Fig. 3A). CSIA-AA challenged this traditional approach. RTP and $TP_{Gly\text{-}Phe}$ specified that trophic segregation operates at 2 different TP, and $\delta^{15}N_{Phe}$ depicted different baseline levels corresponding to 3 foraging habitats. The use of Gly (the most discriminant AA for habitat; see above) further detailed spatial segregation with 4 different $\delta^{13}C_{Gly}$ values. Consequently, a contrasted and more precise picture emerged when using CSIA-AA. Again, iso topic niches were species-specific, but along 4 isotopic habitats and 2 $TP_{Gly\text{-}Phe}$ (Fig. 3, lower panel).

Three consequences of the comparison between bulk and AA isotopic values of squid beaks are notable. (i) A principle in isotopic ecology is that isotopic differences carry relevant biological information, but a lack of isotopic difference does not always correspond to identical ecological features. This latter issue is exemplified by the squid foraging habitats. While $\delta^{13}C_{Bulk}$ values of colossal, Dana octopus and giant squids suggested identical foraging grounds, both d13CGly and $\delta^{15}N_{Phe}$ depicted 3 contrasting feeding habitats, thus underlining how difficult the biological interpretation of isotopic data can be. (ii) Within that context, it is notable that most animal isotopic studies are based on bulk analysis, a few on $\delta^{15}N$ CSIA-AA, a very few on $\delta^{13}C$ CSIA-AA and almost none on both $\delta^{13}C$ and $\delta^{15}N$ CSIA-AA (but see Petzke et al. 2005, Jarman et al. 2017, Pomerleau et al. 2017). The present work underlines that the isotopic method is at its best when including concomitant measurements of bulk and AA $\delta^{13}C$ and $\delta^{15}N$ analyses on the same samples. (iii) A recurrent limitation of the bulk isotopic method is the availability (or not) of marine isoscapes to help interpret isotopic data in terms of meaningful biological information (Graham et al. 2010, McMahon et al. 2013). The problem is even more difficult when using CSIA-AA, due to the complete lack of information. We recommend that future studies aim at constructing maps of the geographical distribution of $\delta^{13}C_{AA}$ values of essential and non-

essential AAs, and for $\delta^{15}N_{AA}$ values of source AAs at spatial scales that are ecologically relevant to the studied animals.

PERSPECTIVES

The presence of chitin is not restricted to cephalopod beaks. It is one of the most abundant macromolecules in the biosphere, being a main component of arthropods, the most diverse and successful animals on Earth. Arthropod exoskeleton is made of cuticle, which consists of varying amounts of protein and chitin, with the latter representing up to 40% of its dry mass (Merzendorfer & Zimoch 2003). Many isotopic investigations include arthropods, and, owing to their small size, isotopic measurements were generally made on whole organisms that include exoskeleton. Arthropod isotopic studies overlook the deleterious effect of chitin, which applies to both crustaceans and insects (Søreide & Nygard 2012, Perkins et al. 2013). Consequently, arthropod TP_{Bulk} values are likely to be systematically under-estimated (e.g. Chikaraishi et al. 2011, Steffan et al. 2013), which can alter the description and functioning of trophic relationships. Calculation of consumer TP_{Bulk} requires the use of $\delta^{15}N_{Bulk}$ value of a food web baseline that is, in many cases, a primary consumer. Herbivorous copepods and euphausiids are the mostly commonly used marine organisms (e.g. Marsh et al. 2017, McClain-Counts et al. 2017), thus propagating the isotopic error associated with low $\delta^{15}N_{Bulk}$ values to consumer TP_{Bulk} estimates through the food web. Finally, some maps of marine $\delta^{15}N_{Bulk}$ isoscapes are modeled using zooplankton data sets (Graham et al. 2010, McMahon et al. 2013), with the direct potential biological outcome of erroneous interpretation of animal movements.

To conclude, CSIA-AA on sclerotized beaks is a powerful tool to investigate trophic interactions of cephalopods. It has a great potential due to the high number of beaks that accumulate in predator stomachs. CSIA-AA is intrinsically effective to bypass the deleterious

effect of non-proteinaceous compounds (e.g. chitin on d15NBulk, lipids on d13CBulk), which hamper the biological interpretation of bulk isotopic values. Due to the high chitin content of exoskeleton, CSIAA-AA merits further consideration in studies focusing on the ecological role of arthropods in both marine and terrestrial ecosystems.

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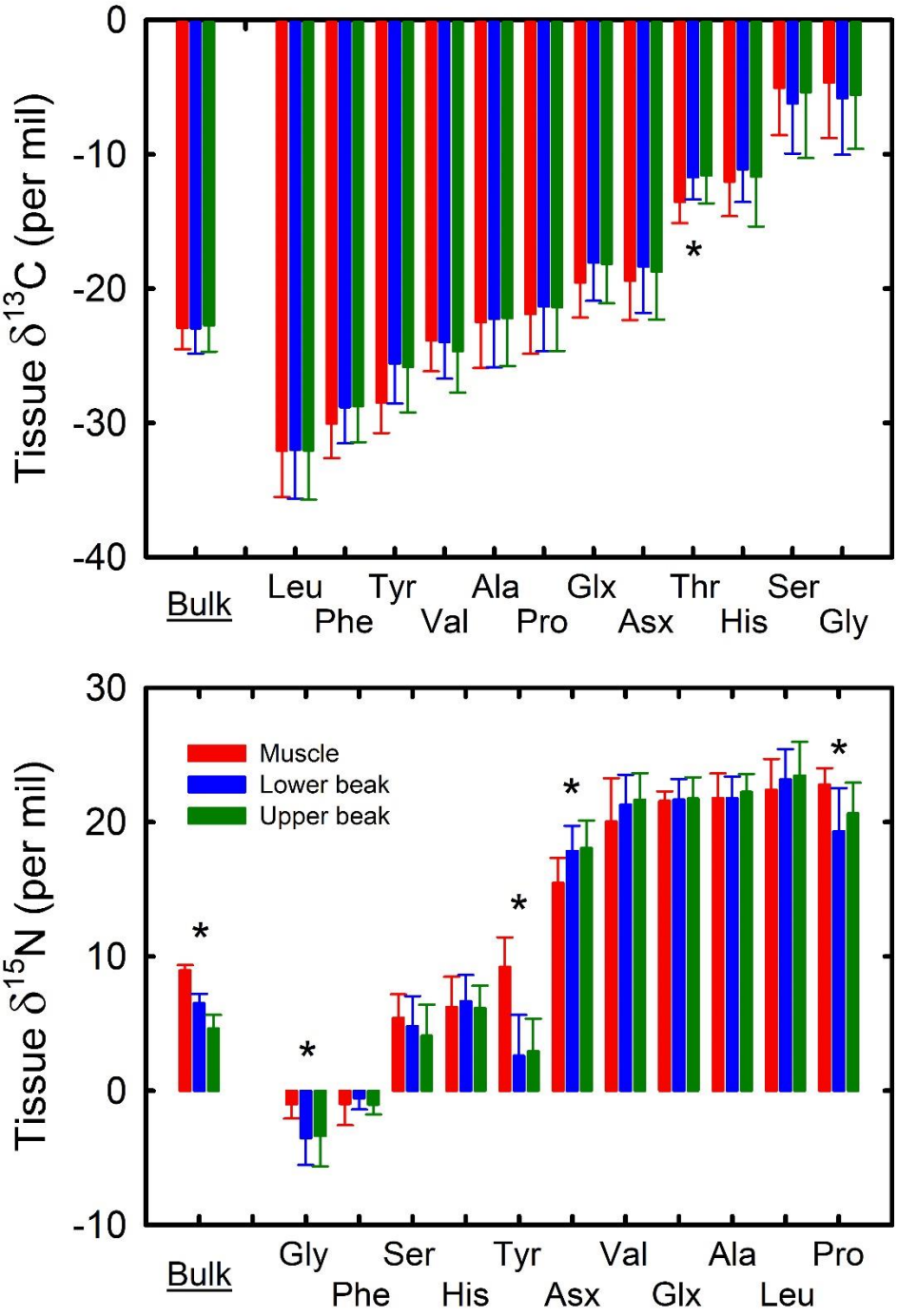

Figure 1. Bulk and individual amino acid $\delta^{13}C$ (upper panel) and $\delta^{15}N$ (lower panel) values of muscle, lower beak and upper beak from buccal masses of giant warty squids *Kondakovia longimana*. Values are means ± SD. * indicates significant differences at $p < 0.05$ (Kruskal-Wallis *H* tests, details in Tables 1 and 2).

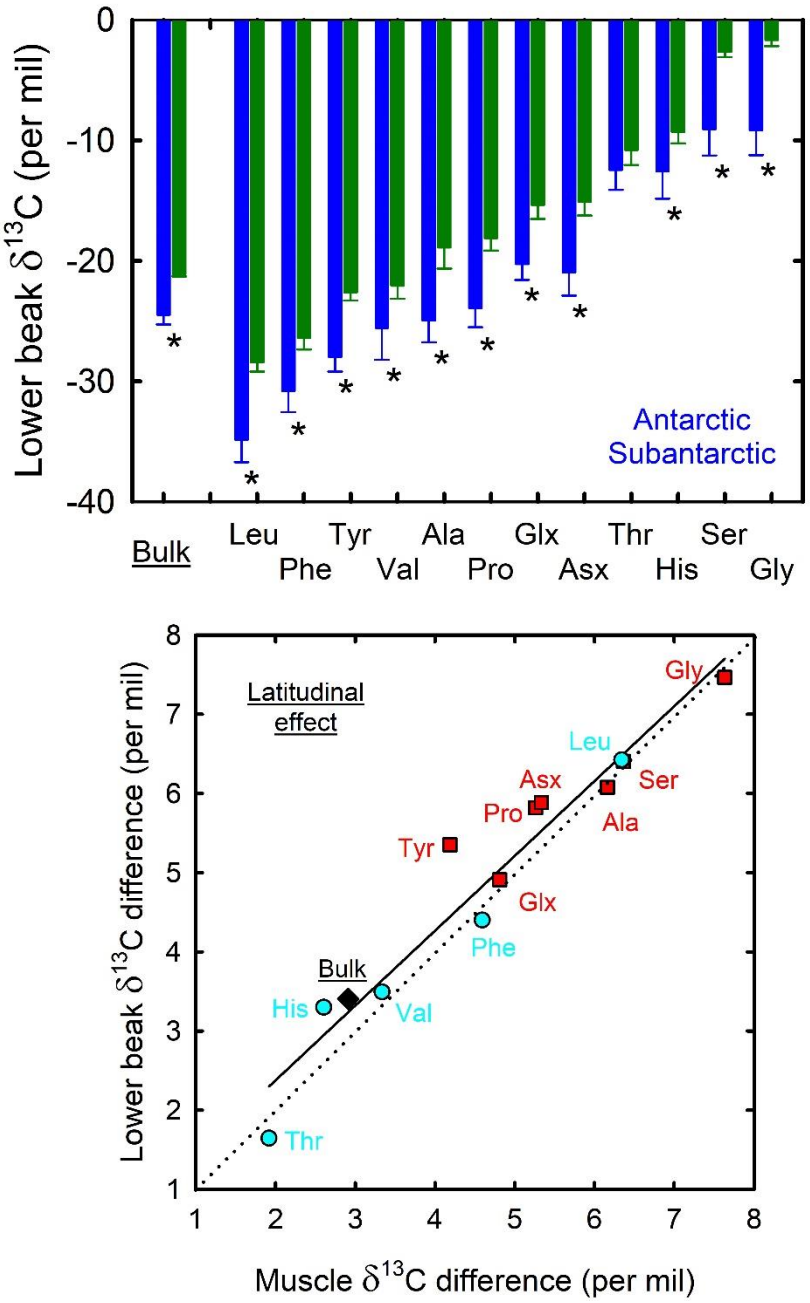

Figure 2. Upper panel: bulk and individual amino acid $\delta^{13}C$ values of lower beaks from Antarctic and subantarctic *Kondakovia longimana*. Values are means ± SD. * indicates significant differences at $p < 0.05$ (Mann-Whitney *U* tests). Lower panel: linear regression between individual $\delta^{13}C_{AA}$ differences between the Antarctic and subantarctic groups in lower beak and muscle of *K. longimana* (y = 0.945x + 0.485, $r^2 = 0.933$, $F_{1,10} = 139.5$, $p < 0.0001$). Bulk value in black (diamond), essential AA in cyan (circles) and non-essential AA in red (squares)

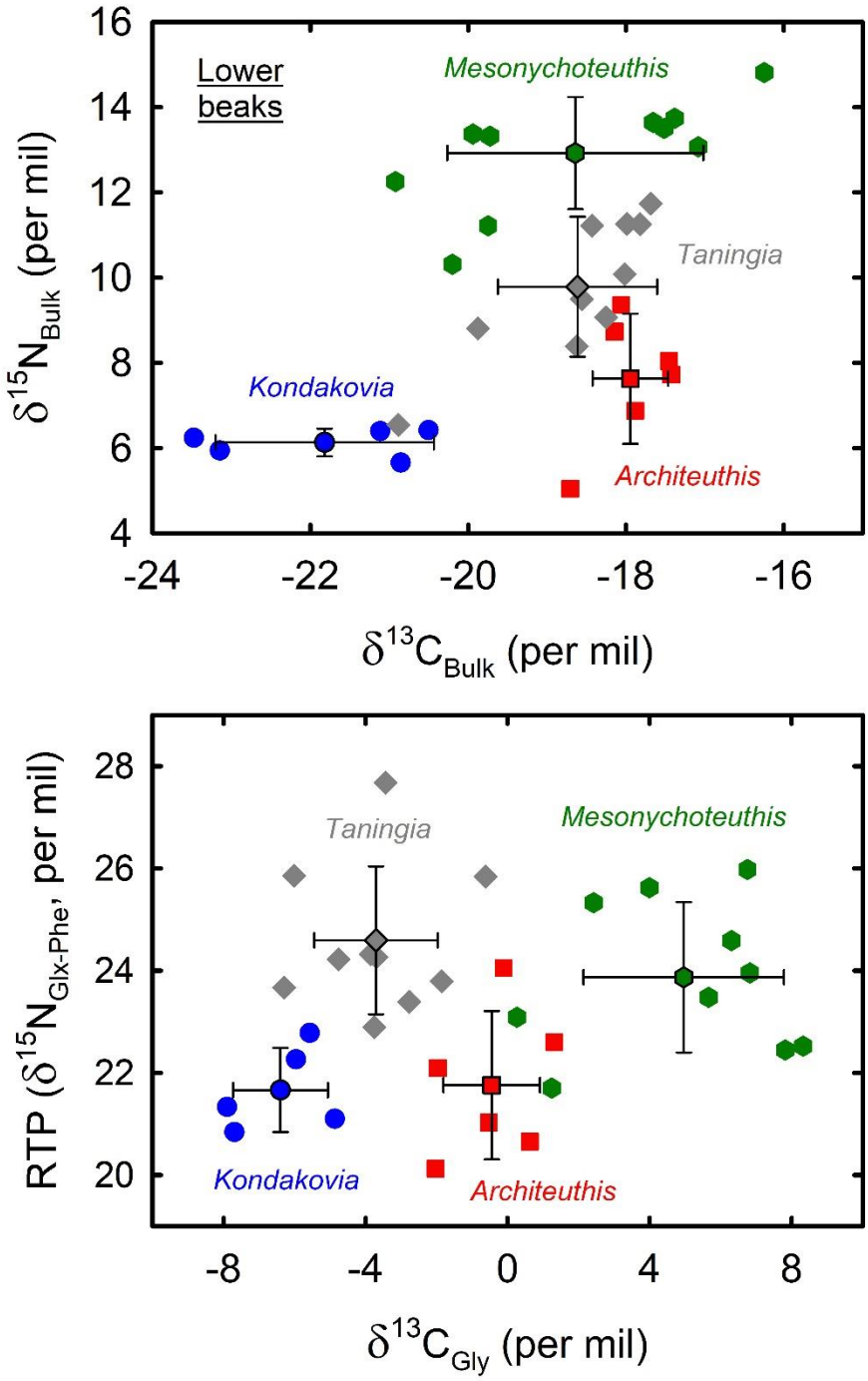

Figure 3. Isotopic niches of oceanic squids using $\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$ values (upper panel) and representative $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values (lower panel) of their lower beaks. Both squid individual values and species mean values (± SD) are shown. Abbreviation: RTP, relative trophic position.

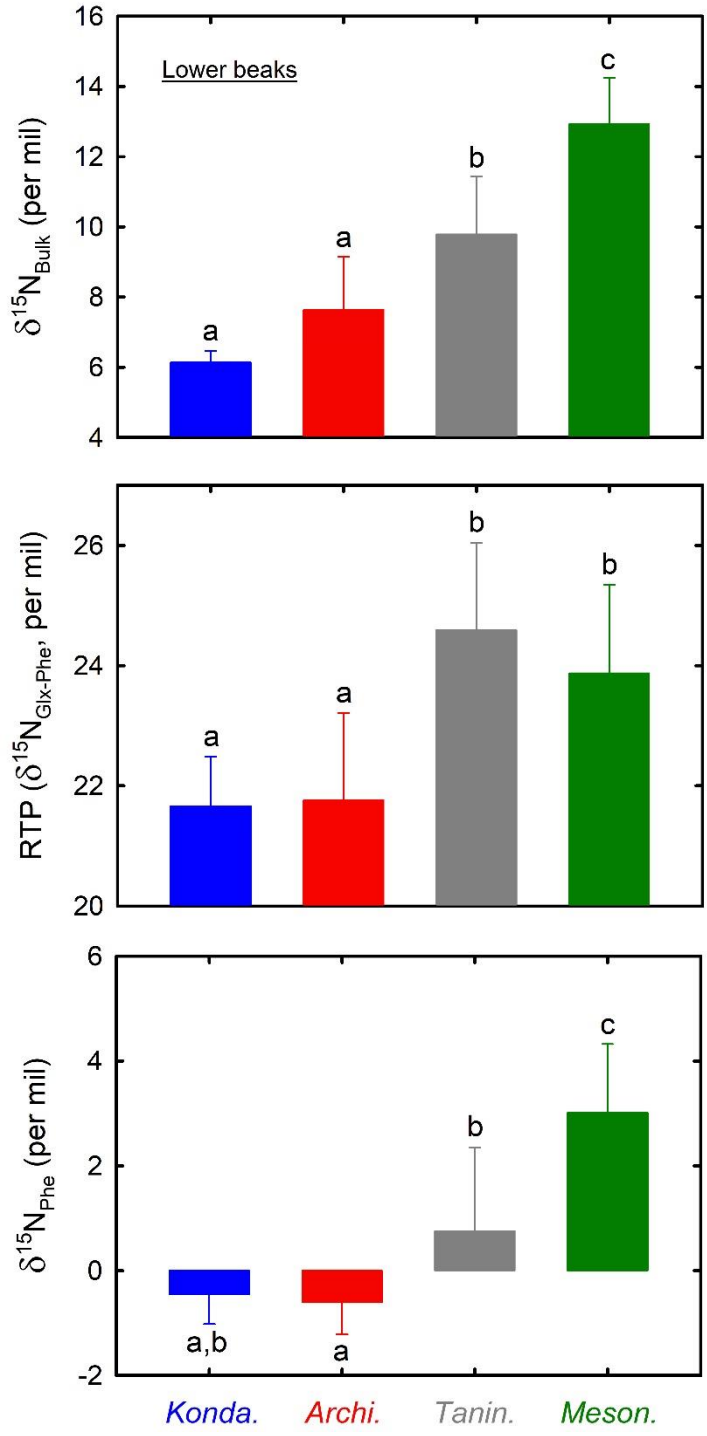

Figure 4. Bulk $\delta^{15}N$ (upper panel), RTP (middle panel) and $\delta^{15}N_{Phe}$ (lower panel) values of lower beaks from oceanic squids from Kerguelen waters. Values are means ± SD. Kruskal-Wallis *H* tests and Conover-Inman tests for pairwise comparisons were performed to compare values from the four squids; values not sharing the same superscript letter are statistically different at $p < 0.05$ (details in Table 3). Abbreviation: RTP, relative trophic position.

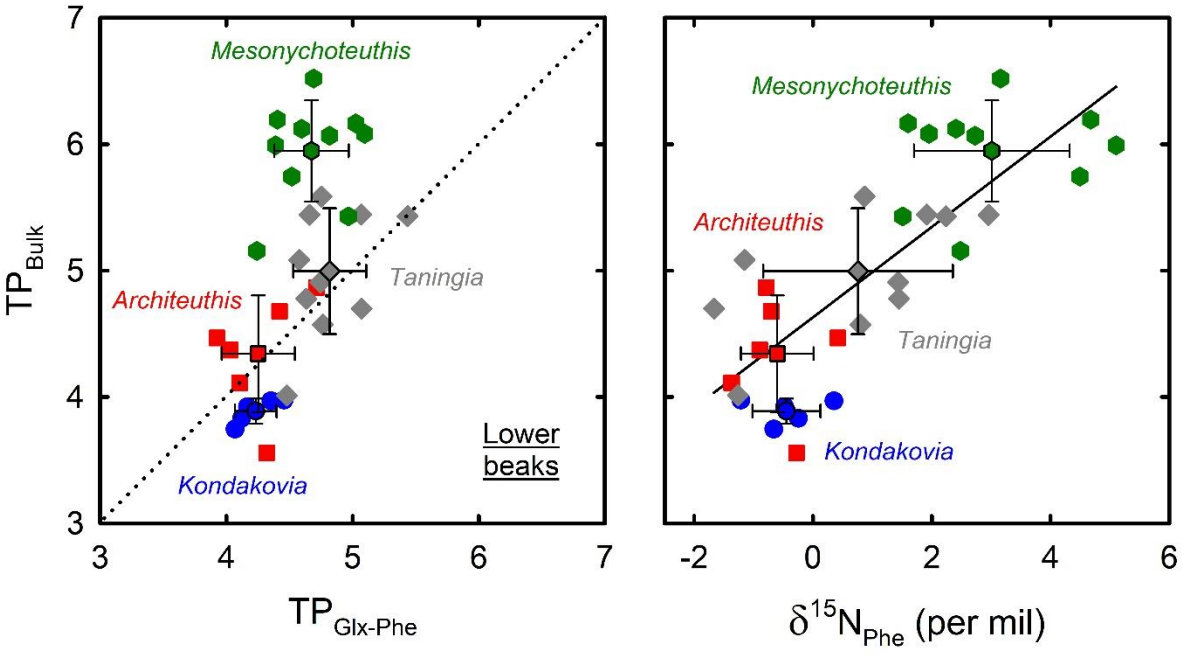

Figure 5. Relationships between TP_{Bulk} and $TP_{Glx\text{-}Phe}$ (left panel), and between TP_{Bulk} and $\delta^{15}N_{Phe}$ values (right panel) of lower beaks from oceanic squids from Kerguelen waters. Values are means ± SD. Abbreviation: TP, trophic position.

Table 1. Measured lower rostral length (LRL) and estimated mantle length (ML) of oceanic squids from Kerguelen waters. Predator: species from which the beaks were extracted. ML was calculated using species-specific allometric equations from the corresponding references. Values are means ± SD (parentheses: range). Kruskal-Wallis H-tests and Conover-Inman tests for pairwise comparisons were performed to compare values amongst the 4 species (values in the same column with differing superscript letters are statistically different). Significant differences ($p < 0.05$) are highlighted in bold

Squid species		Predator	n	LRL (mm)	ML (cm)	References
Architeuthis dux		*Somniosus antarcticus*	6	15.3 ± 3.0^{a} (11.3-18.0)	141 ± 72^{a} (54-215)	Roeleveld (2000)
Kondakovia longimana		*Dissostichus eleginoides*	15	13.9 ± 1.7^{a} (10.5-17.8)	50 ± 6^{b} (37-64)	Adams & Klages (1987)
Mesonychoteuthis hamiltoni		*Somniosus antarcticus*	10	29.2 ± 5.8^{b} (23.2-39.0)	178 ± 35^{a} (141-239)	Clarke (1986)
Taningia danae		*Somniosus antarcticus*	10	18.7 ± 1.8^{c} (15.5-21.0)	85 ± 13^{c} (61-102)	Clarke (1986)
Kruskal-Wallis *H*-tests	*H*			**31.4**	**31.6**	
	p			**<0.0001**	**<0.0001**	

Table 2. Bulk and individual amino acid $\delta^{13}C$ values (‰) of muscle, lower beak and upper beak from buccal masses of giant warty squids. Values are means ± SD. Kruskal-Wallis H-tests and Conover-Inman tests for pairwise comparisons were performed to compare $\delta^{13}C$ values from the 3 tissues (values in the same row with differing superscript letters are statistically different). Significant differences ($p < 0.05$) are highlighted in bold. (–) No data

Amino acids	abbreviation	Group	Muscle	Lower beak	Upper beak	Kruskal-Wallis *H*-tests	
						H	*p*
Bulk			-22.9 ± 1.6[a]	-23.0 ± 1.9[a]	-22.7 ± 1.9[a]	0.32	0.852
Alanine	Ala	non essential	-22.5 ± 3.4[a]	-22.2 ± 3.6[a]	-22.2 ± 3.6[a]	0.07	0.967
Aspartic acid	Asx=Asn+Asp	non essential	-19.4 ± 2.9[a]	-18.4 ± 3.5[a]	-18.7 ± 3.6[a]	0.88	0.644
Glutamic acid	Glx=Gln+Glu	non essential	-19.6 ± 2.6[a]	-18.1 ± 2.8[a]	-18.2 ± 2.9[a]	1.79	0.409
Glycine	Gly	non essential	-4.6 ± 4.2[a]	-5.8 ± 4.2[a]	-5.6 ± 4.0[a]	0.87	0.647
Histidine	His	essential	-12.0 ± 2.6[a]	-11.1 ± 2.4[a]	-11.7 ± 3.7[a]	0.10	0.952
Hydroxyproline	Hyp	non essential	-20.2 ± 2.3				
Isoleucine	Ile	essential	-24.3 ± 2.6				
Leucine	Leu	essential	-32.1 ± 3.4 [a]	-32.0 ± 3.7[a]	-32.0 ± 3.7[a]	0.07	0.967
Lysine	Lys	essential	-21.9 ± 2.1				
Methionine	Met	essential	-28.1 ± 2.5				
Phenylalanine	Phe	essential	-30.1 ± 2.6[a]	-28.8 ± 2.7[a]	-28.7 ± 2.7[a]	1.68	0.433
Proline	Pro	non essential	-21.9 ± 2.4[a]	-21.3 ± 3.3[a]	-21.4 ± 3.2[a]	0.35	0.839
Serine	Ser	non essential	-5.1 ± 3.5[a]	-6.2 ± 3.7[a]	-5.4 ± 4.9[a]	0.47	0.791
Threonine	Thr	essential	-13.6 ± 1.6[a]	-11.7 ± 1.6[b]	-11.6 ± 2.1[b]	**6.13**	**0.047**
Tyrosine	Tyr	non essential	-28.5 ± 2.3[a]	-25.6 ± 3.0[a]	-25.8 ± 3.4[a]	4.07	0.131
Valine	Val	essential	-23.9 ± 2.3[a]	-24.0 ± 2.7[a]	-24.7 ± 3.1[a]	0.73	0.693

Table 3. Bulk and individual amino acid $\delta^{15}N$ values (‰) of muscle, lower beak and upper beak from buccal masses of giant warty squids. Values are means ± SD. Kruskal-Wallis H-tests and Conover-Inman tests for pairwise comparisons were performed to compare d15N values from the 3 tissues (values in the same row with differing superscript letters are statistically different). Significant differences ($p < 0.05$) are highlighted in bold. TP: trophic position; RTP: relative trophic position (‰); (–) no data

Amino acids	Group	Muscle	Lower beak	Upper beak	Kruskal-Wallis *H*-tests	
					H	*p*
Bulk		9.0 ± 0.4[a]	6.5 ± 0.7[b]	4.6 ± 1.0[c]	**24.5**	**<0.0001**
Alanine	trophic	21.7 ± 2.0[a]	21.8 ± 1.6[a]	22.3 ± 1.3[a]	0.11	0.945
Aspartic acid	trophic	15.5 ± 1.9[a]	17.9 ± 1.9[b]	18.1 ± 2.0[b]	**7.44**	**0.024**
Glutamic acid	trophic	21.6 ± 0.7[a]	21.7 ± 1.5[a]	21.8 ± 1.6[a]	0.56	0.756
Glycine	source/trophic	-1.0 ± 1.1[a]	-3.5 ± 2.0[b]	-3.4 ± 2.3[b]	**9.25**	**0.010**
Histidine	source	6.2 ± 2.2[a]	6.7 ± 1.9[a]	6.2 ± 1.7[a]	0.49	0.784
Isoleucine	trophic	23.0 ± 1.2				
Leucine	trophic	22.4 ± 2.3[a]	23.2 ± 2.3[a]	23.5 ± 2.5[a]	1.24	0.537
Lysine	source	2.6 ± 0.6				
Phenylalanine	source	-1.0 ± 1.6[a]	-0.5 ± 0.8[a]	-1.0 ± 0.7[a]	2.51	0.285
Proline	trophic	22.8 ± 1.2[a]	19.3 ± 3.2[b]	20.7 ± 2.3[b]	**8.47**	**0.014**
Serine	source/trophic	5.4 ± 1.7[a]	4.8 ± 2.2[a]	4.1 ± 2.3[a]	2.11	0.348
Threonine	metabolic	-28.2 ± 3.8				
Tyrosine	source	9.2 ± 2.2[a]	2.6 ± 3.0[b]	2.9 ± 2.4[b]	**16.4**	**<0.0001**
Valine	trophic	20.0 ± 3.2[a]	21.3 ± 2.2[a]	21.7 ± 2.0[a]	0.56	0.756
RTP ($\delta^{15}N_{Glx}$ - $\delta^{15}N_{Phe}$)		22.6 ± 1.8[a]	22.2 ± 1.5[a]	22.8 ± 1.6[a]	1.58	0.455
$TP_{Glx\text{-}Phe}$		4.4 ± 0.4[a]	4.3 ± 0.3[a]	4.5 ± 0.3[a]	1.32	0.516

Table 4. Bulk and individual amino acid $\delta^{13}C$ and $\delta^{15}N$ values of lower beaks, and calculated relative trophic position (RTP) and estimated trophic position (TP) of *Architeuthis dux* (giant squid), *Kondakovia longimana* (giant warty squid), *Mesonychoteuthis hamiltoni* (colossal squid) and *Taningia danae* (Dana octopus squid) from subantarctic Kerguelen waters. Values are means ± SD. Kruskal-Wallis H-tests and Conover-Inman tests for pairwise comparisons were performed to compare $\delta^{13}C$ and $\delta^{15}N$ values from the 4 squids (values not sharing the same superscript letter are statistically different), and paired t-tests were performed to compare $TP_{Glx\text{-}Phe}$ and TP_{Bulk} of each squid species. Significant differences ($p < 0.05$) are highlighted in bold. (–) No data

Amino acids	Isotopic values (‰)	*Architeuthis dux* (n = 6)	*Kondakovia longimana* (n = 5)	*Mesonychoteuthis hamiltoni* (n = 10)	*Taningia danae* (n = 10)	Kruskal-Wallis *H*-tests	
						H	*p*
Bulk	$\delta^{13}C$	-17.9 ± 0.5^{a}	-21.8 ± 1.4^{b}	-18.6 ± 1.6^{a}	-18.6 ± 1.0^{a}	**11.7**	**0.008**
	$\delta^{15}N$	7.6 ± 0.5^{a}	6.1 ± 0.3^{a}	12.9 ± 1.3^{b}	9.8 ± 1.6^{c}	**23.6**	**<0.0001**
Alanine	$\delta^{13}C$	-16.5 ± 1.0^{a}	-23.4 ± 2.2^{b}	-17.2 ± 1.4^{a}	-17.2 ± 2.6^{a}	**12.6**	**0.006**
	$\delta^{15}N$	21.1 ± 1.1^{a}	19.6 ± 0.8^{a}	26.2 ± 0.8^{b}	27.2 ± 2.1^{c}	**22.8**	**<0.0001**
Aspartic acid	$\delta^{13}C$	-14.4 ± 0.6^{a,b}	-17.2 ± 1.9^{a}	-13.4 ± 1.7^{b}	-13.4 ± 3.1^{b}	**9.57**	**0.023**
	$\delta^{15}N$	17.3 ± 1.2^{a}	17.4 ± 0.6^{a}	21.0 ± 0.8^{b}	20.3 ± 2.3^{b}	**16.2**	**0.001**
Glutamic acid	$\delta^{13}C$	-16.3 ± 0.3^{a}	-19.8 ± 1.8^{b}	-19.1 ± 1.3^{b}	-18.5 ± 0.7^{b}	**15.4**	**0.002**
	$\delta^{15}N$	21.2 ± 1.4^{a}	21.2 ± 0.9^{a}	26.9 ± 1.1^{b}	25.4 ± 2.4^{c}	**20.3**	**<0.0001**
Glycine	$\delta^{13}C$	-0.4 ± 1.4^{a}	-6.4 ± 1.3^{b}	5.0 ± 2.8^{c}	-3.7 ± 1.7^{d}	**25.3**	**<0.0001**
	$\delta^{15}N$	-3.0 ± 1.3^{a}	-8.0 ± 1.9^{b}	9.3 ± 0.7^{c}	0.6 ± 1.5^{d}	**27.3**	**<0.0001**
Histidine	$\delta^{13}C$	-7.7 ± 1.7^{a}	-11.6 ± 2.8^{b}	-14.8 ± 3.8^{b}	-6.3 ± 2.8^{a}	**20.6**	**<0.0001**
	$\delta^{15}N$	4.7 ± 0.8^{a,b}	5.0 ± 1.2^{a,b}	3.4 ± 1.6^{a}	5.6 ± 2.0^{b}	**7.85**	**0.049**
Leucine	$\delta^{13}C$	-26.4 ± 0.6^{a}	-32.5 ± 1.5^{b}	-28.7 ± 1.8^{c}	-25.4 ± 1.6^{a}	**20.8**	**<0.0001**
	$\delta^{15}N$	22.8 ± 2.4^{a}	22.2 ± 0.9^{a}	24.6 ± 1.2^{b}	25.9 ± 1.8^{b}	**13.4**	**0.004**
Phenylalanine	$\delta^{13}C$	-25.2 ± 0.9^{a}	-29.4 ± 1.9^{b}	-28.7 ± 1.6^{b}	-25.0 ± 2.0^{a}	**21.2**	**<0.0001**
	$\delta^{15}N$	-0.6 ± 0.6^{a}	-0.4 ± 0.6^{a,b}	3.0 ± 1.3^{c}	0.8 ± 1.6^{b}	**18.0**	**<0.0001**
Proline	$\delta^{13}C$	-17.6 ± 0.8^{a}	-21.5 ± 2.0^{b}	-17.1 ± 1.8^{a}	-18.5 ± 1.8^{a}	**11.6**	**0.009**
	$\delta^{15}N$	20.5 ± 2.2^{a,b}	20.2 ± 1.2^{a}	28.0 ± 2.6^{c}	22.7 ± 2.4^{b}	**18.0**	**<0.0001**
Serine	$\delta^{13}C$	-3.7 ± 2.3^{a}	-6.1 ± 2.6^{a}	-5.3 ± 3.1^{a}	-4.2 ± 2.5^{a}	3.58	0.310
	$\delta^{15}N$	-3.2 ± 1.7^{a}	4.0 ± 1.6^{b}	3.9 ± 2.6^{b}	3.5 ± 3.0^{b}	**13.3**	**0.004**
Threonine	$\delta^{13}C$	-5.2 ± 1.4^{a,b}	-10.8 ± 1.6^{c}	-4.1 ± 3.3^{a}	-7.2 ± 3.2^{b}	**12.4**	**0.006**
	$\delta^{15}N$						
Tyrosine	$\delta^{13}C$	-23.4 ± 0.6^{a}	-24.1 ± 1.7^{a}	-24.6 ± 1.7^{a}	-22.9 ± 2.8^{a}	2.51	0.473
	$\delta^{15}N$	5.0 ± 1.4^{a,b}	3.5 ± 0.4^{a}	5.3 ± 1.9^{a,b}	6.3 ± 2.0^{b}	**7.88**	**0.048**
Valine	$\delta^{13}C$	-20.6 ± 1.0^{a}	-27.0 ± 2.9^{b}	-16.0 ± 2.7^{c}	-20.0 ± 1.8^{a}	**20.7**	**<0.0001**
	$\delta^{15}N$	18.0 ± 1.9^{a}	19.4 ± 1.4^{a}	19.0 ± 3.8^{a}	18.4 ± 4.1^{a}	1.19	0.755
RTP ($\delta^{15}N_{Glx}$ - $\delta^{15}N_{Phe}$)	$\delta^{15}N$	21.8 ± 1.5^{a}	21.7 ± 0.8^{a}	23.9 ± 1.5^{b}	24.6 ± 1.4^{b}	**15.2**	**0.002**
$TP_{Glx\text{-}Phe}$		4.3 ± 0.3^{a}	4.2 ± 0.2^{a}	4.7 ± 0.3^{b}	4.8 ± 0.3^{b}	**15.2**	**0.002**
TP_{Bulk}		4.3 ± 0.5^{a}	3.9 ± 0.1^{a}	5.9 ± 0.4^{b}	5.0 ± 0.5^{c}	**23.6**	**<0.0001**
Paired *t*-tests *t*		0.49	**-8.69**	**9.44**	1.24		
p		0.643	**0.001**	**<0.0001**	0.246		
Difference (TP_{Bulk} - $TP_{Glx\text{-}Phe}$)		0.1 ± 0.5^{a}	-0.3 ± 0.1^{b}	1.3 ± 0.4^{c}	0.2 ± 0.5^{a}	**20.9**	**<0.0001**